Novel roles of *ARR2* in pathogen response and flowering time

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät

der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

Marko Vesić

aus Kruševac, Republik Serbien

Tübingen

2014

Tag der mündlichen Qualifikation: 28.03.2014

Dekan: Prof. Dr. Wolfgang Rosenstiel

1. Berichterstatter: Prof. Dr. Klaus Harter

2. Berichterstatter: PD Dr. Markus Schmid

Table of Contents

Summary

Two-component system (TCS) and MAPK signalling cascades play essential roles in plant signal transduction. The *Arabidopsis* Response Regulator 2 (ARR2), a member of B-type response regulators in the two-component circuit, serves as a molecular hub integrating several incoming signals. A comparable hub function is also known for the MAPK signalling cascade. Based on preliminary experiments we have started to address the hypothesis that there exists a link between ARR2-dependent TCS and MAPK signalling cascade, *i.e.* a crosstalk of those two evolutionary divergent signal transduction systems.

Potential protein-protein interactions between ARR2 and MAPK cascade members (MKKKs, MKKs, and MPKs respectively), were tested. The results revealed that ARR2 as well as other tested B-type ARRs, but not the A-type ARRs, strongly interacted with MKKs and some MPKs where none of them interacted with MKKKs. Interestingly, despite a high sequence similarity with ARR2, ARR1 did not interact with the all those MAPK members ARR2 is interacting with. ARR2 was found to interact preferentially with MKK4 and MKK5, which play an important role in the biotic stress defence specially including responses to pathogen attacks. Pathogenesis assays applied on *ARR2* and *ARR1* mutants lines, using the necrothrophic, semi biotrophic and biotrophic pathogens differing in evolution and distinct defence strategies in plants, revealed us that ARR2 mutants showed difference only in necrotrophic stress responses but not in biotrophic stresses.

The detailed analysis of *arr1* and *arr2* single loss-of-function mutants as well as an *arr1 arr2* double mutant revealed an early flowering phenotype of these plants especially visible under short day conditions (SDs) with the double mutant having an additive effect compared to the single parents. Despite very similar sequence homology of the *ARR1* and *ARR2* genes, the loss of *ARR2* effects the flowering time was quite stronger than *ARR1* but they still function in the same signalling pathway. From the mechanistic point of view the lack of *ARR1/ARR2* activity causes down-regulation of specific floral repressor genes but not of genes that act as floral activators and this miss-regulation of expression is not due developmental defects but directly linked to floral induction. ARR2 expressed either in the shoot apical meristem (SAM) or in phloem companion cells recues the early flowering phenotype of the *arr2* single and the *arr1 arr2* double mutants. The crossing of *ARR1* and *ARR2* mutants with flowering time-related mutants revealed that *ARR1* and *ARR2* function predominantly independent of *Flowering Locus C* (*FLC*). However, this approach revealed that *Flowering Locus M* (*FLM*) acts epistatic to *ARR1* and *ARR2*.

These findings are revealing and describing special features of the TCS elements and their crosstalks with other signalling pathways and contributing to a better understanding of their physiological role.

Zusammenfassung

Zweikomponentensystem (TCS) und MAPK Signalkaskaden spielen wichtige Rollen in der Signaltransduktion der Pflanze. Der *Arabidopsis* Response Regulator 2 (ARR2), der ein Mitglied der B-Typ Response Regulatoren des Zweikomponentensystems ist, dient als molekularer Knotenpunkt, welcher viele Signale integriert. Eine ähnliche Funktion wurde bereits für die MAPK Signalkaskade gezeigt. Basierend auf früheren Experimenten stellten wir die Hypothese auf, dass eine Verbindung zwischen ARR2-abhängigen TCS und der MAPK Signalkaskade besteht, beispielsweise eine Interaktion dieser evolutionär divergenten Signaltransduktionssystemen.

Protein-Protein-Interaktionen zwischen ARR2 und Proteinen der MAPK Signalkaskade (MKKKs, MKKs und MPK) wurden untersucht. Wir konnten zeigen, dass ARR2 und auch andere ARRs aus der Gruppe der B-Typ Response Regulatoren, aber nicht der A-Typ ARRs, stark mit MKKs und einigen MPKs interagieren. Dabei gab es keine Interaktion mit den MKKKs. Trotz einer hohen Sequenzänlichkeit zwischen ARR2 und ARR1, interagiert ARR1 nicht mit allen Komponenten der MAPK Signalkaskade mit denen ARR2 wechselwirkt. Wir konnten zeigen, dass ARR2 mit MKK4 und MKK5 interagiert. Beide haben eine wichtige Rolle bei der Stressantwort der Pflanze, vor allem bei der Pathogenabwehr. Versuche an *ARR2*- und *ARR1*-Knockoutlinien mit nekrotrophen, hemibiotrophen und biotrophen Pathogenen, die jeweils evolutionär unterschiedliche Abwehrstartegien in Pflanzen hervorrufen, haben gezeigt, dass *ARR2*-Mutanten keine Unterschiede bei biotrophen, dafür aber bei nekrotrophen Stressantworten hervorrufen.

Genaue Untersuchungen der *arr1*- und *arr2*-Einzelmutanten, sowie der *arr1 arr2* Doppelmutante zeigen einen Frühblüher-Phänotyp, vor allem unter Kurztagbedingungen. Trotz der großen Homologie der *ARR1* und *ARR2* Gene, wirkt sich der Knockout des *ARR2* Gens auf den Blühzeitpunkt wesentlich stärker aus als der Verlust von *ARR1*, dennoch wirken sie beide im selben Signaltransduktionsweg. Die Abwesenheit von ARR1/ARR2 führt zur negativen Regulation von spezifischen Blüh-Repressorgenen. Gene die für die Blüh-Induktion zuständig sind, werden davon nicht betroffen. Diese Unterschiede in der Genregulation haben keinen Efekt in der Entwicklung der Pflanze, sondern wirken sich nur auf den Zeitpunkt der Blühinduktion aus. ARR2 wird im Sprossapikalmeristem (SAM) oder in den Geleitzellen im Phloem expremiert, und ist für den Frühblüher-Phänotyp in der arr2 Einzelmutante und in der *arr1 arr2* Doppelmutante verantwortlich. Kreuzungen von ARR1 und ARR2 Mutanten mit Mutanten, die eine Veränderung des Blühzeitpunkts aufweisen, zeigen, dass *ARR1* und *ARR2* überwiegend unabhängig von dem *Flowering Locus C (FLC)* wirken. Die Kreuzungslinien belegen, dass der *Flowering Locus M (FLM*) epistatisch zu *ARR1* und *ARR2* ist.

Diese Daten beschreiben spezielle Elemente des TCS und deren Interaktion mit anderen Signaltransduktionswegen, welche zu einem besseren Verständnis der physiologischen Rolle führt.

1 General Introduction

1.1 Two-component systems

Two-component transduction systems (TCS or TSCT) pathways represent principal means for coordinating responses to environmental changes in bacteria and also in plants, some fungi, *archaea* and *protozoa*, but it is absent in higher eukaryotes and metazoans (Koretke *et al.* 2000; Hwang *et al.* 2002; Capra and Laub 2012). In prokaryotes these systems are mostly composed of a receptor sensor histidine kinase (HK or SK) and its cognate response regulator (Koretke *et al.* 2000; Capra and Laub 2012). Histidine kinases sense a specific signal and generally form functional homodimers allowing kinases to autophosphorylate at an internal histidine (H, His) by the γ-phosphoryl group of ATP (Koretke *et al.* 2000). The high-energy phosphoryl group is further transferred to an aspartate (D, Asp) residue of the two-component receiver domain in the response regulator protein. Response regulators (RRs) typically contain a two-component receiver domain and an effector domain allowing the protein to modify and regulate cellular behaviour in a TCS-dependent manner (Koretke *et al.* 2000; Hwang *et al.* 2002). The name "two-component" actually originates from this fact: in its simplest case only two elements are involved in TCS signal transduction, however there are exceptions which are elaborated on below. HKs share very high homology their ATP-binding domains with Hsp90, type II topoisomerases and MutL (mismatch repair protein) (Dutta *et al.* 1999; Koretke *et al.* 2000; Capra and Laub 2012). These proteins are all members of GHKL superfamily and it is suggested that HKs originated from one of these ATPases and had further evolved by series of duplications, lineage-specific expansion and divergence (Capra and Laub 2012). Generally, HKs are bifunctional, *i.e.* they can also act as phosphatases of their response regulators (Capra and Laub 2012). In order to make dimers, autophosphorylate and then successfully transfer the γ-phosphate group, histidine kinases must contain the dimerization and histidine phosphotransfer domain (DHp) and the catalytic and ATP binding (CA) domain (Koretke *et al.* 2000). Other domains most frequent in HKs are the sensory binding domains Per Arnt Sim (PAS) and cGMP-specific phosphodiesterases Adenylyl cyclases and FhlA (GAF), a conserved "linker" domain Histidine kinase Adenyl cyclases Methyl accepting proteins and Phophatases (HAMP) (Galperin *et al.* 2001). These periplasmic sensory domains are responsible for direct signal (PAS and GAF) recognition and relaying this information to the DHp and CA domains; consequently these domains more variable than the other ones as reflected by the large diversity of detectable signalling molecules (Moglich *et al.* 2009; Parkinson 2010). Besides these domains, HKs usually have transmembrane domains and other domains which specialise and define their function. As mentioned, HK autophosphorylation appears to be dependent on forming homodimers (Ashenberg *et al.* 2011). HAMP domains are likely to be involved in dimerization in a sensory dependent manner (Parkinson 2010). Formation of functional homodimers is common for almost all HKs as there is only a single observation of a physiologically functional heterodimer in cyanobacteria *Pseudomonas aeruginosa* (Goodman *et al.* 2009). Some HKs have an additional

receiver domain fused to their C-terminus; these are called hybrid histidine kinases (also abbreviated HKs) and almost 25% of all bacterial HKs belong to this group (Cock and Whitworth 2007). Hybrid HKs might have originated by fusion of upstream encoded HKs and RRs through the mutation of stop codons in operons (Qian *et al.* 2008). After a signal is perceived and autophosphorylation occurs in hybrid HKs, the high energy phosphate group is delivered from the DHp histidine to the *cis* internal receiver domain. This phosphate is then shuttled by other elements of the TCS in a typically a four-step relay via hybrid HK to HPt (a free DHp domain protein called a histidine phosphotransfer protein) to a classical RR protein, thus the ~P group moves from His to Asp to His to Asp. This multistep phospho-transfer is commonly referred to as a phosphorelay. In eukaryotes, hybrid HKs are found in the majority of systems (Koretke *et al.* 2000) although the real reason for such selective pressure is not known. It has been proposed that the spatial arrangement within the hybrid HK enforces the specificity of phosphotransfer avoiding crosstalk with other TCS pathways (Wegener-Feldbrugge and Sogaard-Andersen 2009). In support to this claim, it has been proven that HKs missing their receiver domains can phosphorylate non-cognate RRs even better than their own internal response regulator domain (Biondi *et al.* 2006; Wegener-Feldbrugge and Sogaard-Andersen 2009). Next crucial element of two-component system signalling is RRs, as up until now, HPt proteins have only been shown to be ~P shuttles and add little specificity to TCSs with the exception of YPD1, which has been shown to stabilize the phosphoryl~RR form of SSK1 (Janiak-Spens *et al.* 2000) and the *Arabidopsis* protein AHP6 which cannot receive a TCS phosphate. AHP6 has been shown regulate developmental and hormonal processes, presumably at the level of protein-protein interaction within the TCS network (Mahonen *et al.* 2006; Moreira *et al.* 2013). All RRs have an evolutionary well conserved receiver domain which pulls the phosphate group onto its Asp residue thereby causing conformal changes to the protein which activate or even inhibit it initiating an output response (Gao *et al.* 2007; Capra and Laub 2012). Numerous RRs possess DNA-binding output domains (Galperin 2006) whereby phosphorylation of the conserved Asp promotes also dimerization of receiver domains favouring the DNA binding and direct control of target gene transcription (Capra and Laub 2012). Other domains frequent within RRs are diguanylate cyclases and methyltransferases domains (Koretke *et al.* 2000). In bacteria, RRs are signal integrators as these organisms usually have, if not an equal number, more HKs than RRs (Koretke *et al.* 2000; Capra and Laub 2012). Free RRs are therefore the last direct step of TCS signalling pathways and they are the final factor responsible ascribed for causing TCS-dependent physiological changes (Capra and Laub 2012). The diversity of in bacterial RRs is a direct consequence of gene duplications, lateral gene transfer events and point mutations (Rabin and Stewart 1993). Divergent evolution of the RRs has enabled them to specifically recognize promoters and control different genes (Price *et al.* 2008). Fifty percent of all known RRs form homodimers upon phosphorylation (Gao and Stock 2010) and there are subsets of interfacial residues that enforce homo-dimerisation and prevent hetero-dimerisation which lie within the β4-α4-β5-α5 region of the conserved RR domain (Weigt *et al.* 2009; Szurmant and Hoch 2010; Capra and Laub 2012).

In both *Archea* and eukaryotes, TCS independently originated from bacteria by lateral gene transfer (Koretke *et al.* 2000; Kim and Forst 2001). In plants, TCS plays important roles in developmental processes (Ren *et al.* 2009; El-Showk *et al.* 2013) and most likely they obtained their TCS from chloroplasts after integration of chloroplast genes into their nuclear genome (Martin *et al.* 2002). As mentinoed higher eukaryotes and metazoans do not possess TCS (Hwang *et al.* 2002; Schaller *et al.* 2011) and instead, TCS (histidine/aspartal phosphorylation) has been replaced by serine/threonine/tyrosine phosphorylation perhaps due to the intrinsic liability of phosphoryl groups on Asp compared to its stability on serine, threonine or tyrosine (Capra and Laub 2012). Thus the idea is that eukaryotes as "compartmented" organisms need more stable and longer lived outputs for signal conduction from the cell membrane to the nucleus. Supporting this claim would be the direct modulation of activity MAPK kinase signalling pathway in *Saccharomyces cerevisiae* by the SLN1-YDP1-SSK1 TCS pathway (Posas *et al.* 1996). On the other hand this is not entirely the case in plants. Here TCS is directly controlling expression of many target genes as in classical TCSs (Hwang *et al.* 2012; El-Showk *et al.* 2013). One of the most described examples in plants is cytokinin signalling where the TCS plays a crucial role, and B-Type RRs directly modulate gene activity of A-Type *RRs* and other genes (Shi and Rashotte 2012; El-Showk *et al.* 2013). The shuttling of the phosphoryl group from hybrid HKs in the plasma membrane and endoplasmic reticulum by HPts to RRs in the nucleus (Shi and Rashotte 2012) could have been selected for in plants because it is strongly believed that the histidyl~P residue is more stable than an aspartyl~P residue (Koretke *et al.* 2000; Capra and Laub 2012).

1.2 References for General Introduction

Ashenberg, O, Rozen-Gagnon, K, *et al.* (2011). "Determinants of homodimerization specificity in histidine kinases." J Mol Biol **413**(1): 222-235.

Biondi, EG, Reisinger, SJ, *et al.* (2006). "Regulation of the bacterial cell cycle by an integrated genetic circuit." Nature **444**(7121): 899-904.

Capra, EJ and Laub, MT (2012). "Evolution of two-component signal transduction systems." Annu Rev Microbiol **66**: 325-347.

Cock, PJ and Whitworth, DE (2007). "Evolution of prokaryotic two-component system signaling pathways: Gene fusions and fissions." Mol Biol Evol **24**(11): 2355-2357.

Dutta, R, Qin, L, *et al.* (1999). "Histidine kinases: Diversity of domain organization." Mol Microbiol **34**(4): 633-640.

El-Showk, S, Ruonala, R, *et al.* (2013). "Crossing paths: Cytokinin signalling and crosstalk." Development **140**(7): 1373-1383.

Galperin, MY (2006). "Structural classification of bacterial response regulators: Diversity of output domains and domain combinations." J Bacteriol **188**(12): 4169-4182.

Galperin, MY, Nikolskaya, AN, *et al.* (2001). "Novel domains of the prokaryotic two-component signal transduction systems." FEMS Microbiol Lett **203**(1): 11-21.

Gao, R, Mack, TR, *et al.* (2007). "Bacterial response regulators: Versatile regulatory strategies from common domains." Trends Biochem Sci **32**(5): 225-234.

Gao, R and Stock, AM (2010). "Molecular strategies for phosphorylation-mediated regulation of response regulator activity." Curr Opin Microbiol **13**(2): 160-167.

Goodman, AL, Merighi, M, *et al.* (2009). "Direct interaction between sensor kinase proteins mediates acute and chronic disease phenotypes in a bacterial pathogen." Genes Dev **23**(2): 249-259.

Hwang, I, Chen, HC, *et al.* (2002). "Two-component signal transduction pathways in arabidopsis." Plant Physiol **129**(2): 500-515.

Hwang, I, Sheen, J, *et al.* (2012). "Cytokinin signaling networks." Annu Rev Plant Biol **63**: 353-380.

Janiak-Spens, F, Sparling, DP, *et al.* (2000). "Novel role for an hpt domain in stabilizing the phosphorylated state of a response regulator domain." J Bacteriol **182**(23): 6673-6678.

Kim, D and Forst, S (2001). "Genomic analysis of the histidine kinase family in bacteria and archaea." Microbiology **147**(Pt 5): 1197-1212.

Koretke, KK, Lupas, AN, *et al.* (2000). "Evolution of two-component signal transduction." Mol Biol Evol **17**(12): 1956-1970.

Mahonen, AP, Bishopp, A, *et al.* (2006). "Cytokinin signaling and its inhibitor ahp6 regulate cell fate during vascular development." Science **311**(5757): 94-98.

Martin, W, Rujan, T, *et al.* (2002). "Evolutionary analysis of arabidopsis, cyanobacterial, and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus." Proc Natl Acad Sci U S A **99**(19): 12246-12251.

Moglich, A, Ayers, RA, *et al.* (2009). "Structure and signaling mechanism of per-arnt-sim domains." Structure **17**(10): 1282-1294.

Moreira, S, Bishopp, A, *et al.* (2013). "Ahp6 inhibits cytokinin signaling to regulate the orientation of pericycle cell division during lateral root initiation." PLoS One **8**(2): e56370.

Parkinson, JS (2010). "Signaling mechanisms of hamp domains in chemoreceptors and sensor kinases." Annu Rev Microbiol **64**: 101-122.

Posas, F, Wurgler-Murphy, SM, *et al.* (1996). "Yeast hog1 map kinase cascade is regulated by a multistep phosphorelay mechanism in the sln1-ypd1-ssk1 "two-component" osmosensor." Cell **86**(6): 865-875.

Price, MN, Dehal, PS, *et al.* (2008). "Horizontal gene transfer and the evolution of transcriptional regulation in escherichia coli." Genome Biol **9**(1): R4.

Qian, W, Han, ZJ, *et al.* (2008). "Two-component signal transduction systems of xanthomonas spp.: A lesson from genomics." Mol Plant Microbe Interact **21**(2): 151-161.

Rabin, RS and Stewart, V (1993). "Dual response regulators (narl and narp) interact with dual sensors (narx and narq) to control nitrate- and nitrite-regulated gene expression in escherichia coli k-12." J Bacteriol **175**(11): 3259-3268.

Ren, B, Liang, Y, *et al.* (2009). "Genome-wide comparative analysis of type-a arabidopsis response regulator genes by overexpression studies reveals their diverse roles and regulatory mechanisms in cytokinin signaling." Cell Res **19**(10): 1178-1190.

Schaller, GE, Shiu, SH, *et al.* (2011). "Two-component systems and their co-option for eukaryotic signal transduction." Curr Biol **21**(9): R320-330.

Shi, X and Rashotte, AM (2012). "Advances in upstream players of cytokinin phosphorelay: Receptors and histidine phosphotransfer proteins." Plant Cell Rep **31**(5): 789-799.

Szurmant, H and Hoch, JA (2010). "Interaction fidelity in two-component signaling." Curr Opin Microbiol **13**(2): 190-197.

Wegener-Feldbrugge, S and Sogaard-Andersen, L (2009). "The atypical hybrid histidine protein kinase rodk in myxococcus xanthus: Spatial proximity supersedes kinetic preference in phosphotransfer reactions." J Bacteriol **191**(6): 1765-1776.

Weigt, M, White, RA, *et al.* (2009). "Identification of direct residue contacts in protein-protein interaction by message passing." Proc Natl Acad Sci U S A **106**(1): 67-72.

2 Aim of this work

Besides its functions in ethylene signal transduction, cytokinin and H_2O_2 signalling *etc*., the two-component-signalling system seems to play very significant roles in crosstalk and fine-tuning between distinct signalling pathways. These additional functions of the TCS might be essential for the plant's fitness within the natural environment and also for their general survival. Based on these observations, this thesis intends to contribute to a better understanding of these special features of TCS and describe them. The specific aims of this thesis are:

- To investigate and determine the functional (physiological) role of ARR2 interactions with biotic stress-related members of MAPK cascade, obtaining new data by expanding the comprehensive ARRs/MAPKs module interaction map and determine of the roles of *ARR1* and *ARR2* in response to pathogen attack and different elements that cause biotic stress in plants (see Chapter 1).

- Characterisation of a novel flowering time phenotype in the *ARR1* and *ARR2* lacking mutants under non-inductive short day conditions discovered during this dissertation by resolving which floral pathway(s), their mechanism and nature of interaction, these two TCS elements are involved in (see Chapter 2).

3 Materials and Methods

3.1 Chemicals

All chemicals were ordered from Biorad (München), Fluka (Buchs, CH), Merck (Darmstadt), Carl Roth (Karlsruhe), Duchefa (Haarlem, NL) or Sigma (Steinheim). Organic solvents were delivered by Brenntag Chemiepartner GmbH NL (Plochingen) and Merck. BASTA pesticide was ordered from Bayer Crop Science. The ingredients used for culture media were ordered from Invitrogen (Carlsbad, USA), Merck, Sigma and Duchefa (Haarlem, NL). Restriction enzymes, ligase and DNA modification enzymes used for nucleic acids studies were ordered from Invitrogen, Stratagene (La Jolla, USA), New England Biolabs (Beverly, USA), Promega (Mannheim) and Amersham Pharmacia Biotech. Oligonucleotides were received from Eurofins MWG Operon (Ebersberg) and antibodies from the companies Sigma-Aldrich (Taufkirchen) and New England Biolabs (Beverly, USA). The synthetically generated fg22, elf18 peptides, PEN and Xac extracts were a kind gift from the Laboratory of Georg Felix.

3.1.1 Media

The different media used are presented in the following table. For media preparation deionized water was used and the media was sterilized by autoclaving for 20 minutes at 121°C. Bacto-agar 15g/L was used for solid media preparation, (BD) or 8g/L Select-Agar for MS plates (Sigma-Aldrich). Where it was needed, filter sterilized antibiotics were added to the sterilized medium at appropriate final concentrations as listed in Table 3.1

Table 3.1 List with recipes of common used media in the thesis

Medium	Ingredients per 1 liter	Species
LB	10 g Bacto-Tryptone, 5 g NaCl, 5 g Yeast extract (YE)	*Escherichia coli*
Kings's B	20 g glycerol, 40 g Proteose Pepton 3, after autoclaving addition of 0.1 % (v/v) $MgSO_4$ and KH_2PO_4	*Pseudomonas syringae*
½ MS	2.2 g MS (Duchefa), pH 5.7 (KOH)	*Arabidopsis thaliana*
YPD	20g Bactopeptone (BD #211677), 20g Glucose (monohydrate), 10g Yeast extract (BD #212750), 1000ml ddH_2O	*Saccharomyces cerevisiae*
CSM	20g Glucose (monohydrate), 6,6g Yeast nitrogen base (BD #291940), 0,64g CSM-(L-W, or L-W-A, Qbiogene #4520-012), 1000ml ddH_2O	*Saccharomyces cerevisiae*
Z buffer	10,68g Na_2HPO_4, 5,5g NaH_2PO_4, 0,75g KCl, 246mg $MgSO_4$, 1000ml ddH_2O, adjust PH to 7,0	*Saccharomyces cerevisiae*

3.1.2 Antibiotics

Media were supplemented when required with antibiotics at the final concentrations listed in the following table:

Table 3.2 List of used antibiotics and applied concentrations

Antibiotics	Concentration (µg/µl)	Solvent
Carbenicillin	100	Water
Kanamycin	50	Water
Rifampicin	50	Methanol
Spectinomycin	100	Water
Tetracyclin	50	Ethanol

3.2 Vectors and Primers

All the vectors used for this thesis are listed directly within each method text section. Primers were synthesized by Eurofins MWG Operon. Primers stocks were kept at a 100 µM concentration diluted in nuclease-free water and stored at -20°C.

Table 3.3 List of primers (primer sequences) used for genotyping and cloning

Primers used for genotyping		
Name of the mutant line	**Sequence (5´-> 3´)**	**Source**
flc-3	AAA ATA TCT GGC CCG ACG AAG	Johanne Lempe, PhD Thesis, University of Tübingen, 2007
	CGA CGA GAA GAG CGA CGG ATG	
arr2-4	GAACGGGAGGAGCTCGAG	Laboratory Harter, ZMBP, MV
	GACCTGGATATTATCGATGGAGTATCC	
arr1-4	GAAGAACAAACATGGATTCGATATAGTA	Laboratory Harter, ZMBP, MV
	CCGTCATAAACGAGTTGTTAAGATTG	
tDNA(SALK)	TGGTTCACGTAGTGGGCCATCG	Laboratory Harter, ZMBP, MV
co-9	CAACTCTATCTCCCCCGTAGC	Balasubramanian et al., PloS Gen. 2006
	GATGCTCAAGTTCACTCTGCC	
soc1-2	GGATCCATGGTGAGGGGCAAAACTC	Yoo et al., Plant Phys 2005
	CTGAAACATCTGATCAAAAGCTG	
	TTGGGTTCACGTAGTGGGCCATCG	
flm-3	GATGCGGTTTTTGGTGTTATG	Laboratory Harter, ZMBP, MV
	GCCTAGAATATGGCCTTTATCG	
Primers used for cloning		
Truncation size/position	**Sequence (5´-> 3´)**	**Source**
ARR2$^{1-300}$	ATGGTAAATCCGGGTCACGGAAG	Laboratory Harter, ZMBP, MV
	GATCAAATCCATTCAACGAAGA	
ARR2$^{300-664}$	TCTTCGTTGAATGGATTTGATC	Laboratory Harter, ZMBP, MV
	TCAGACCTGGATATTATCGATG	
ARR2$^{1-145}$	ATGGTAAATCCGGGTCACGGAAG	Laboratory Harter, ZMBP, MV
	CCACTCGTTACGCTTCTTCCG	
ARR2$^{145-664}$	CGGAAGAAGCGTAACGAGTGG	Laboratory Harter, ZMBP, MV
	TCAGACCTGGATATTATCGATG	
ARR2$^{51-81}$	CTCTACAGAGTAACTAAATGTA	Laboratory Harter, ZMBP, MV
	AACATCACTAATGACAATATC	
ARR2$^{215-260}$	AAGAAACCACGCGTGGTTTGGTC	Laboratory Harter, ZMBP, MV
	TACGTTTTCTCGCGTTAGCCCG	

Table 3.4 List of primers (primer sequences) used for RTq-PCR

Primers used for RTq-PCR				
Gene Name	**Probe**	**Sequence (5´-> 3´)**	**Size (bp)**	**Source**
SOC1	#69	CACAAACCCTTTATCCTCGAA TTGCCCCTCACCATATCTTC	103	Laboratory Harter, ZMBP, MV
CO	#77	AACAATGACCGATCCAGAGAA CCTCCTTGGCATCCTTATCA	77	Laboratory Harter, ZMBP, MV
FD	SYBR	GGCAGAAAATGCAAGACTCA TCTTTTTGGGTTGCTGAATTG	74	Laboratory Harter, ZMBP, MV
GA4	#127	TGCCTTCCAAATCTCAAACC ACCGGTGAGAAACTCAATGTC	67	Laboratory Harter, ZMBP, MV
GA5	#45	CATGGGTTTCAGCCATTTG CTCTAAAGTAGTCCCGTTTTACGC	121	Laboratory Harter, ZMBP, MV
LFY	#69	TTGATGCTCTCTCCCAAGAAG TTGACCTGCGTCCCAGTAA	113	Laboratory Harter, ZMBP, MV
SHY2	#9	TGATCCTTAGTCTCTTGCACGTA CAAAGATGGTGATTGGATGCT	77	Laboratory Harter, ZMBP, MV
ARR7	#68	TCATCTGAGAACATCTTACCTCGT TTCACCGGTTTCAACAAGAAT	77	Laboratory Harter, ZMBP, MV
WUS	#33	AACCAAGACCATCATCTCTATCATC TCAGTACCTGAGCTTGCATGA	86	Laboratory Harter, ZMBP, MV
AP1	#68	AAAACAGCATGCTTTCTAAACAGA GTGGCCTTGGTTCTGCTG	89	Laboratory Harter, ZMBP, MV
FDP	#112	AACTTGAGCTTGAAATTGCTCAC GAGTTGCTTCGGCTATTTTCA	87	Laboratory Harter, ZMBP, MV
TFL	#140	CCTGCACTGGATCGTTACAA TGGCAATTCATAGCTCACCA	79	Laboratory Harter, ZMBP, MV
TSF	#138	TGGAGGAGACGACTTCAGAAA GCTTGGACTCGGCACATC	67	Laboratory Harter, ZMBP, MV
FLM	#65	CGGACAGAGCAGTCTCAAGTT TGAAGAACCAAATGTCGATAATGT	108	Laboratory Harter, ZMBP, MV
SVP	#67	TGACTGCAAGTTATGCCTCTCT CCGGAAAACTGTTCGAGTTC	68	Laboratory Harter, ZMBP, MV
FT	#22	TCAAAACAAGCCAAGAGTTGAG CATCTGGATCCACCATAACCA	77	Laboratory Harter, ZMBP, MV
FLC	#65	GGAGAGGGCAGTCTCAAGGT GCTACTTGAACTTGTGGATAGCAA	103	Laboratory Harter, ZMBP, MV
ARR1	SYBR	GCGCACTTCTTAAGCAGGAA TGGAGTATGCGTCAAAGTCG	66	Laboratory Harter, ZMBP, MV
ARR2	SYBR	CGTTGATGATGATCCAACTTGT TCCGAAGCAGAGACAATGC	94	Laboratory Harter, ZMBP, MV
ARR4	SYBR	GTCATCGAGAGATTGCTTCGT ACGCCATCCACTATCTACCG	66	Laboratory Harter, ZMBP, MV
ARR5	SYBR	TCAGAGAACATCTTGCCTCGT ATTTCACAGGCTTCAATAAGAAATC	94	Laboratory Harter, ZMBP, MV
PR1	#135	TGATCCTCGTGGGAATTATGT TGCATGATCACATCATTACTTCAT	76	Laboratory Harter, ZMBP, MV
PDF1.2	#139	GTTCTCTTTGCTGCTTTCGAC GCAAACCCCTGACCATGT	87	Laboratory Harter, ZMBP, MV
EF-1- α	#5	TCATGGATCAAGCGGTGA CGCAACCAAACCTTCCATA	63	Laboratory Harter, ZMBP, MV

3.3 Bacterial strains

3.3.1 *Escherichia coli* strain DH5α

The *E. coli* strain DH5α [F-(Φ80*lac*ZΔM15) Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1] was used for cloning of the different constructs. The *E. coli* strain DB3.1 [F- gyrA462 endA1 glnV44 Δ(sr1-recA) mcrB mrr hsdS20(r_B^-, m_B^-) ara14 galK2 lacY1 proA2 rpsL20(Sm^r) xyl^5 Δleu mtl1] was used for the cloning and propagation of Gateway™ vectors carrying the *ccdb* gene.

3.3.2 *Agrobacterium* strain

The *Agrobacterium tumefaciens* strain GV3101 (T-DNA^- vir^+ rif^r) with helper plasmid pMP90 (gen^r) or pMP90RK (gen^r, kan^r) was used.

3.4 Organisms

3.4.1 Organisms used for pathogen assays

Two biotrophs *Pseudomonas syringae* pv. tomato Pto DC3000 (marked with Rif^r resistance) and the fungus *Peronospora parasitica* were used. Two necrothrophic fungi *Alternaria brasisicola* and *Botrytis cinerea* were used.

3.4.2 Plant lines

For all experiments Columbia-0 (Col-0) ecotype of *Arabidopsis thaliana* was used, and all transgenic lines were generated in this ecotype. For some experiments *Nicotiana benthamiana* was also used. The T-DNA insertion mutant lines used in this work include *arr1-4* (SALK 042196), *arr2-4 (SALK 016143), flm-3 (*SALK 141971), *co* (SAIL_24_H04), *soc1-2* (Lee *et al.* 2000; Yoo *et al.* 2005) and the deletion mutant *flc-3* (Michaels and Amasino 1999). The double mutant *arr1-4 arr2-4* was crossed and isolated by Dr. Katharina Caesar. Other mutants listed in the work were made by crossing; the lines obtained are: *flm-3 arr2-4*, *co arr2-4*, *soc1-2 arr2-4*, *flm-3 arr1-4 arr2-4, flc-3 arr1-4 arr2-4*. All of the crossed lines were genotyped by PCR for known insertion markers and characterised by checking mRNA expression. Single mutants *flc-3*, *flm*-3, *soc1-2* and *co* were kindly donated by Dr. Markus Schmid and Dr. Rebecca Schwab (Max-Planck Institute for Developmental Biology, Tübingen). *ARR2* and *ARR2D80N* loss-of-function genes were (both in pDONR207 Gateway vector; previously cloned in the laboratory of Prof. Dr. Klaus Harter) and pJL-Blue Gateway entry plasmid (kindly donated by Dr. Rebecca Schwab) were recombined into the respective pGREEN-IIS Gateway vectors pFK-101 (BAR_FD_rfA*) and pHW-059 (BAR_SUC2_rfA+) described by (Mathieu *et al.* 2007). These constructs were transformed into Col-0 wild-type, *arr1-4*, *arr2-4* or *arr1-4 arr2-4* mutants. Transgenic plants were selected with BASTA (Bayer) at a dilution of 1:1000 (§3.5.6.1).

3.5 Cultivation

3.5.1 Growth of *Escherichia coli*

E.coli strains were cultivated overnight at 37°C either on LB-plates or in liquid LB medium at 230 rpm. Antibiotics were added into the media according to the resistance cassettes the strains were harboring.

3.5.2 Growth of *Pseudomonas syringae*

P. syringae Pto pv. DC3000 strains were grown for 24 to 48 hours at 28°C either on King's B plates or in liquid King's B medium at 180 rpm. For the determination of bacterial growth in infection assays the *Pseudomonas* strain was re-isolated from plant material and plated on LB plates containing cycloheximide in addition to rifamycin.

3.5.3 Growth of *Agrobacterium tumefaciens*

A.tumefaciens strains were cultivated for 48 hours at 28°C on LB-plates or liquid LB medium at 230 rpm. Additional antibiotics were added into the media according to the plasmid-DNA the strains were carrying.

3.5.4 Growth of *Alternaria brassicicola*

The cultivation of *A. brassicicola* and the preparation of the spores for the infection assays were personally performed in the Laboratory of Dr. Birigit Kemmerling exactly as previously described (Kemmerling *et al.* 2007).

3.5.5 Growth of *Peronospora parasitica* and *Botrytis cinerea*

Assays with *Peronospora parasitica* and *Botrytis cinerea* were both performed by our collaboration partners. The *Peronospora parasitica* assay was performed by the research group of Professor Dr. Volker Lipka from University of Göttingen and the B*ortytis cinerea* assay was done in the laboratory of Professor Dr. Jean-Pierre Métraux at the University of Fribourg, Switzerland. *Peronospora parasitica* was grown as previously described by (Jacobs *et al.* 2003) and *Botrytis cinerea* as described by (Ferrari *et al.* 2003).

3.5.6 Growth of *Arabidopsis thaliana* and *Nicotiana benthamiana*

3.5.6.1 Growth conditions for flowering time analysis

For flowering time analysis, plants were first stratified by being kept at 4°C for 2 to 4 days and then transferred to 22°C; this was defined as day 0. *Arabidopsis thaliana* plants were grown on steam-sterilized GS90-soil (Gebr. Patzer GmbH) and for pathogen experiments soil was mixed with Vermiculite. Plants were grown in growth chambers on soil at 22°C under long days (LD) 16 hours light / 8 hours dark or short days (SD) 8 hours light / 16 hours dark (110 mEm^{-2} s^{-1}, 50-60% humidity). Plants used for pathogen experiments were grown only under SDs. Plants complemented under tissue specific promoters were used in T2 generation and therefore they were pre-selected with 1:1000 dilution of BASTA (dilution from the stock, 183 g/L glufosinate; 200 g/L ammonium salt, Bayer CropScience). The soil was directly soaked in the BASTA solution.

3.5.6.2 Growth of Nicotiana benthamiana

Nicotiana benthamiana plants were cultivated in a mixture of soil and sand containing 0.1 % (v/v) Confidor by the ZMBP Greenhouse (13 h light, 11 h darkness).

3.6 Standard molecular biology methods

General protocols were used for PCR, side directed mutagenesis, agarose gel electrophoresis, western blot, restriction digestion, ligation, transformation of bacteria and plasmid isolation (Sambrook and Russell 2001), Techniques done differently are explained directly in the text. Restriction enzymes were used according the manufacturer's protocols (Fermentas and NEB). For the generation of PCR fragments either the *Taq* DNA-Polymerase or the *Pfu* DNA polymerase (cloning purposes; Fermentas) were used. GeneRuler DNA Ladder Mix (Fermentas) was used as size marker for the agarose gel electrophoresis. DNA fragments were extracted out of agarose gels or purified out of PCR reactions by using the Qiagen Gel Extraction Kit and Qiagen PCR Purification Kit (Qiagen).

3.6.1 Yeast-two-hybrid

Yeast two-hybrid experiments were performed using the Matchmaker™System (Clontech). Plasmids were constructed by LR-reaction of corresponding Entry clones and destination vectors pGBKT7-DEST or pGADT7-DEST (Horak *et al.* 2008). Primers for clones produced during this thesis project are listed in the general primer list (§3.2), the rest of the clones were made by Achim Hahn and can be found in the plasmid repositories of Prof. Dr. Klaus Harter. Clones with *ARR18* were kindly provided by Manikandan Veerabagu and clones with *ARR4* are from the lab of Dr. Virtudes Mira-Rodado. Yeast strain PJ69-4A (James *et al.* 1996) was transformed using lithium acetate/SS-DNA/PEG method (James *et al.* 1996; Horak *et al.* 2008). After 3 days of growth on vector selective media (CSM, -L, -W), 6 independent clones were picked, resuspended in ddH_2O and 10µl were dropped on vector-selective media. Subsequently, 10µl of culture were dropped on vector- and interaction-selective media (CSM, -L, -W, -A) and incubated at 28°C. At day 3 the growth of the clones was monitored. In addition, yeasts from selective media were inoculated in selective media (CSM,-L,-W) harvested and analysed by western-blot using α-HA and α-myc antibodies to determine the correct expression of the fusion proteins (Horak *et al.* 2008).

3.6.2 Transient expression in tobacco leaves

Expression of proteins expressed transiently in tobacco leaves was performed as described previously (Marion *et al.* 2008). A single colony of *Agrobacterium tumefaciens* strain GV3101 pMP90 transformed with the desired constructs and was inoculated in 5ml of YEB-Medium (0.5% beef extract, 0.5% sucrose, 0.1% yeast extract, 0.05% $MgSO_4 \cdot 7H_2O$) containing Rif/Gent/ and vector-specific antibiotic at 28°C overnight. In the morning, 1 ml of the pre-culture was taken and re-inoculated into 5 ml of the same Medium. The same was done for *Agrobacterium* strain carrying the p19 RNAi-suppressor protein from tomato bushy stunt virus (Voinnet *et al.* 2003). Each culture was collected in a 15 ml Falcon Tube and centrifuged at 4000 rpm for 20 min. Bacteria pellets were then resuspended in AS-Medium (10 mM $MgCl_2$, 10 mM MES [pH 5.6], 150 µM acetosyringone) to an optical density at 600 nm of

about 0.7-0.8. The resuspended bacteria (two potential interaction partners and p19 strain) were mixed 600 ml each, a 1:1:1 ratio, in a 2ml Eppendorf tube and incubated for 0.5 to 1 hours at 4°C.

The bacterial solution was injected into the entire leaf area through the abaxial sides using a 1 ml syringe; two leaves per plant were inoculated. After inoculation, the plants were kept in a tray with a hood at 25°C. Two days after the bacterial inoculation, the β-estradiol responsive promoters in the *pABind* vectors (Zimmermann and Nentwig 1989) and the N-terminal *mRFP* vector pB7WGR2,0 (Plant Systems Biology, Gent) were induced by application of β-estradiol by brushing a 20 µM β-estradiol (in 0.1% Tween-20) solution onto the abaxial leaf surface. FRET measurements and localization studies were performed 24 to 48 hours after β-estradiol application. Localization studies were performed with confocal laser scanning microscopy using a Leica TCS SP2 confocal microscope (Leica Microsystems GmbH). Microscopy was carried out as previously described (Horak *et al.* 2008). Images were taken with HCX APO LW 20×/0.5 or the HCX PL APO 63×/1.2 W water-immersion objective.

3.6.3 Ethylene accumulation measurements

For ethylene measurements, leaf material of 4 week-old plants were cut into 1 mm thick strips and floated overnight in water. Afterward three leaf stripes (20 mg) were transferred in 6 ml glass vials containing 0.5 ml of an aqueous solution of the elicitor to be tested. The tubes were closed with a rubber septa and ethylene accumulating in the free air space was measured by gas chromatography (GC-14A Shimadzu) after 3h incubation at 170 rpm at room temperature. Following PAMPs and their working concentrations were used: PEN from *Penicilium schizogonium* 1 µL/500 µL, *Xanthomonas axonopodis citri* 3 µL/500 µL, flg22 (0,1 nM, 1 nM, 10 nM, 100 nM, 1 µM), elf 18(0,1 nM, 1 nM, 10 nM, 100 nM, 1 µM).

3.6.4 ROS (Reactive Oxygen Species) measurements

Leaf discs were excised from 6 week-old *Arabidopsis* plants and incubated in water overnight. The following day, the discs were transferred to a solution of 20 µM luminol L-012 (Wako Pure Chemical Industries) and 10 µg/mL (w/v) horseradish peroxidase in a 96-well-plate and treated with 100 nM flg22, 100 nM elf18, 1 µM chitin octamers or 1 mg/mL chitin (crab). The plates were analysed for a period of at least 30 min using a multiplate reader Centro LB 900 (Berthold Technologies). For each data point at least 9 replicates were measured.

3.6.5 Statistical analysis

Statistical analysis was performed using Microsoft Office Excel or JMP (SAS). The data represent the average of replicates with plus or minus standard error of the mean (SE). The significance of the differences was calculated using Student's t-test, ANOVA or Fisher's Least Significant Difference (LSD) test; which test was used is explicitly mentioned in the text, figures and tables.

3.6.6 ELM software for *in silico* predictions

ELM software (http://elm.eu.org) was used for MKK docking site identification in ARR2.

3.6.7 Cloning and site-directed mutagenesis

All the clones used in our experiments were constructed using Gateway™ technology (Invitrogen™ | Life Technologies). The Entry clones were obtained *via* BP-reaction in pDONR207. cDNA preparations derived from *Arabidopsis* leaves were used as template to clone *ARR2* (*AT4G16110*). For the generation of truncated ARR2 versions, $ARR2^{1-300,}$ $ARR2^{300-664,}$ $ARR2^{1-165}$, $ARR2^{145-664}$, $ARR2^{165-664}$ and $ARR2^{1-145}$, primers were made corresponding to the *ARR2* cDNA and the fragments were amplified by PCR using the *ARR2* Entry clone (see primer table §3.2). Site-directed mutagenesis of *ARR2* was carried out on the *ARR2* Entry clones using QuikChange® Site-Directed Mutagenesis Kit (Stratagene) and the *D80E*, *D80N ARR2* mutants were already present at the Laboratory of Klaus Harter at the time when the experiments were performed. The binary constructs for FRET-FLIM measurements (*pABind* vectors) under the control of the β-estradiol inducible promoter and for stable *Arabidopsis* transformations under the control of the *FD* and *SUC2* promoters were obtained *via* LR-reactions. LR-reactions were also done for yeast-two hybrid system destination vectors pGADT7 and pGBKT7.

3.6.8 Quantitative RT-PCR (RT-qPCR)

Total RNA from corresponding material (leaves, shoot apical meristem or whole plant) was isolated using the "Universal RNA Purification Kit" (Roboklon, Germany) including a DNA digestion step to ensure the absence of genomic DNA contamination/presence. The cDNA was synthetized using oligo-dT or random hexamer primers with H-Minus Reverse Transcriptase (Fermentas). qPCR primer were tested for doubling time by diluting 1:2 and ensuring that there was exactly a loss of 1 CT; primer efficiency was thereafter assumed to be 100%. At least two PCR reactions (in triplicate repeats each) using two independent cDNA synthesis were performed for every treatment. The amplification of cDNA was performed with PerfeCta qPCR SuperMix or PerfeCta SYBR Green Super Mix (Quanta Biosciences, distributed by VWR) using, when necessary, the corresponding Probe (Universal ProbeLibrary Single Probes, Roche) accordingly to manufacturer. The PCR reactions were ran in the Bio-Rad CFX384 Real Time PCR system (Bio-Rad). The used primers are listed in the §3.2. Expression levels of each gene were normalized according to the expression of the following housekeeping genes: *EF-1-α* (*AT5G60390*). The CFX Manager software (Version 1.1; Bio-Rad) has been used for the quantification of relative expression levels except for §4.2.3.4 for which the CT values were exported and ΔΔCT was manually processed in Excel to ensure proper normalisation.

3.6.9 ONPG assay

Before the Day One, 3 to 4 transformed yeast colonies were pooled and inoculated and incubated overnight at 28°C while shaking in 0.5ml of CSM-L/W liquid medium at 180 rpm.

Day 1: the tubes were taken from the shaker and 3 ml of YPD liquid medium was added in each one and put back to the shaker for an additional 3 hours. After that, the optical density at OD_{600} was measured. The tubes were then centrifuged for one minute at 11000 rpm and

the pellet was re-suspended in 1 ml of Z Buffer (the same procedure was repeated three times). The tubes were frozen in liquid nitrogen and stored at -80°C.

Day 2: Frozen tubes were thawed in a 37°C water bath and then returned to liquid nitrogen; this cycle freezing/thawing was repeated at least 4 times. Blank control (with all following solutions) was set and OD_{600} was measured. Immediately after adding of 500 µl of Z buffer-ME (38.6mM ß-Mercaptoethanol) and 160 µl of Z buffer/ONPG (13,3mM ß-Mercaptoethanol) the timer was started and the tubes were incubated at 30°C until yellow colouring appeared. The reaction was stopped by the addition of 400 µl 1M Na_2CO_3. The tubes were centrifuged afterwards and the supernatant was transferred to another tube. The optical density was measured at OD_{420}. Galactosidase units were calculated as follows:

$$\text{Galactosidase units} = {}^{1000 \times OD_{420}}/_{t \times V \times OD_{600}}$$

with t being elapsed time in min, V is the volume of the yeast culture used for the assay, OD_{420} and OD_{600} the optical density of the yeast cultures used for the assay.

3.6.10 MPK assay using anti-phospho antibodies

Anti-phospho antibodies p44/42 (Cell Signaling Technology, NEB #9101) were used as they are known to specifically detect phosphorylated MPK3, MPK4 and MPK6 in *Arabidopsis thaliana*. 5 week-old plants grown under short day conditions were used. Total protein amount was extracted from 50-100 mg of leaf material. For protein extraction 50mM Tris/HCl [pH 7.5], 5mM EDTA pH 8 and 2mM DTT was used. Protease inhibitor cocktail tablets (Roche) were used. Protein concentration was determined with Bradford Reagent (Biorad-System) where 10µl of protein extract was added to 990µl Bradford-Solution (Bradford Reagent diluted 1: 5 in H_2O), exposed for 5 min at RT; and then the optical density (OD) was measured at 595 nm. To estimate protein concentration the following formula was used:

$$\text{Protein concentration } \left[\frac{\text{mg}}{\text{ml}}\right] = {}^{OD_{595}}/_{(0{,}0283 \times vol.protein\ extract\ used)}$$

3.6.11 Stomata measurement assays

Leaves from 5 week-old *Arabidopsis thaliana* plants were floated for 2 h under continuous illumination (120–150 µE m^{-2} s^{-1}) in MES/KCl buffer (5mM KCl/10mM MES/50µM $CaCl_2$, pH 6,15) as previously described by (Mira-Rodado *et al.* 2012). Once the stomata were fully open, leaves were treated with either 1µM t-zeatin or 10µM ABA for further 2 h. The leaves were subsequently homogenized individually in a Waring blender for 30 sec and the epidermal fragments collected on a 100 µm nylon mesh (SpectraMesh, BDH-Merck, UK) and placed on a microscope slide and covered with a coverslip. Stomata apertures from epidermal fragments were then measured using a calibrated light microscope attached to an imaging system (Leica QWin software, Leica, UK). Lens 20X-0.75 magnification was used plus. Leaves were collected from plants also treated with *Pseudomonas syringae* DC3000 3 days after infection and directly homogenized without being exposed to additional light or being put in water. To

calculate stomata aperture, relative pixel distance values were measured in ImageJ software. For statistical analyses ANOVA was used.

3.6.12 Day length shifting experiments

This method was used for the synchronized flowering experiments. Plants were first grown at 8h light/16h dark (short days) at 23°C for 30 days and then shifted to 16h light/8h dark conditions (long days), (Maizel and Weigel 2004; Wahl *et al.* 2013). Apical centres (meristems) were harvested after removal of all visible leaves at day 0 and 5 (counting from the shifting day).

3.6.13 Generation of stable *Arabidopsis thaliana* transgenic lines

Stable transgenic plants were generated by *Agrobacterium tumefaciens*-mediated transformation using the floral-dip method (Clough and Bent 1998). For all transformations *Agrobacteria* GV3101::pMP90 or GV3101::pMP90RK strain were used. The RK strain possesses the RK2 replicase and *trf* gene required for the replication of RK plasmids. The *Agrobacterium* RK strain was co-transformed with pSoup vector which provides replication functions in trans for pGREEN-IIS destination vectors into which the different promoters used in this study (*FD*, *SUC2*) which had been cloned in front of a modified Gateway recombination cassette. Transgenic plants were selected with BASTA (Bayer) at a dilution of 1:1000.

3.6.14 Tissue fixation, embedding and sectioning of *Arabidopsis thaliana* apical meristem

3.6.14.1 Embedding

Day 1: Material was directly harvested into fixative FAA (Formaldehyde: Acetic Acid) in a little glass beaker. Fixative: FAA (Formaldehyde: Acetic Acid):

50%	Ethanol
5%	Glacial Acetic Acid
3.7%	Formaldehyde
41.3%	Water

Vacuum was applied and released (soft up and down with the vacuum) for 1 to 4 hours until the material became translucent and sank to the bottom. The FAA was afterwards changed and left in a cold-room overnight. FAA was replaced with 70% Ethanol; this step was repeated once to be sure all FAA is gone, after that material can be stored in falcon tubes for a long time.

Day 2: These steps were done at 4°C in a cold-room. 70% Ethanol was replaced with 85% Ethanol for 60 min. After one hour previous solution was replaced with 96% ethanol with Eosin and it was left overnight until a light pink colour emerged and tissue could be visualized.

Day3: These steps were done at room temperature. The previous solution was replaced by 96% ethanol with Eosin for one hour, and then two times replaced by the same solution for one hour each time. After that the tissue was treated with 25% Histoclear with75% Ethanol for 30 minutes and after each thirty minutes the percentage of Histoclear was increased by

25% where the percentage was decreased at the same amount until the percentage of Histoclear reached 100%. The tissue was further treated with the 100% Histoclear twice for 1 hour each time. After that paraplast chips were added 25% (v/v) of the solution and so left overnight at room temperature.

Day4: The tubes were placed at 42°C until the chips completely melted and one more ¼ volume of paraplast chips was constantly added until they completely melted. The tubes were afterwards moved to 60°C for several hours and parallel in another tube wax was melted overnight at 60°C to prepare for the next day.

Day5: Two wax changes approximately ¼ volume separated by several hours, were performed. The same procedure is for days 6 and 7.

Day8: The tissue was placed in sectioning moulds and stored at 4°C. The tissue was then ready for sectioning.

3.6.14.2 Sectioning

The sections made were 8µm thick. Slides were ProbeOne Plus from Fisher Biotechnology; they were pre-cleaned and charged. Moulds were trimmed in order to get as close to the tissue as possible. The ribbons were placed in 40°C water bath to remove compressions, and then they were fished out with a slide. They slides were kept on a heating plate at 42°C overnight. Thereafter, slides can be stored in a box at 4°C for a long time. Sections were scanned for clear visibility of two big air vacuoles; always the same plane was used for comparative measurements and statistics. Images were captured using a Leica DM-IRB microscope with a 20x lens.

3.6.15 Pathogen Assays with *Pseudomonas syringae* DC3000

Bacteria were inoculated from an LB plate into 50 ml LB medium with the corresponding antibiotics (rifampicin) and incubated over night at 28°C and shaken at 180 rpm in a shaker. The cells were harvested in the next morning by centrifugation in 50-ml falcon tubes at 3500 rpm at 4°C for 10 min. The pellet was resuspended in 10 mM $MgCl_2$ and the OD_{600} was set to OD_{600}=0.2 which refers to approximate concentration of 10^8 cfu/ml. The cells were afterwards diluted up to density $1x10^4$ cfu/ml and used likewise for the assays. Bacteria were inoculated on two leaves per 5-week-old plant by infiltration with a needless syringe in the middle of a leaf apoplast half (two leaves per plant) ensuring that an area was covered that was always bigger than the cork borer used for extracting leaf discs. The infected leaves were marked for easy identification.

The leaves were harvested at the time points 0 h and 1, 2 and 4 days past infection. For each time point, five plants and two leaves from each plant per line were used. One disc was made from each leaf with the cork borer. The leaf discs were surface was sterilised by washing them in 70% (v/v) ethanol first for about 1 minute and then 1 minute in water. Afterwards the leaf discs were placed into Eppendorf tubes containing 100µl 10mM $MgCl_2$. Immediately after placing the leaf in the Eppendorf tube, it was homogenized with the sterile pistil and

additional 100µl 10mM $MgCl_2$ was added on the pistil in order to wash out the remains of the leaf discs. The homogenised material was plated on LB-plates with rifampicin and 50µg/ml cycloheximide (it prevents growth of eukaryotes like fungi). Extracts were serially diluted 5 times at 10-fold per dilution and dilutions anticipated to yield evaluable data were scored by plating out 10 µl (see Table 3.3). At least two technical replicates per sample were evaluated.

For those experiments involving a cytokinin treatment, trans-zeatin in a concentration of 1µM was used. Cytokinin was given directly to the $1x10^4$ cfu/ml bacterial dilution before infiltration. Three days before the experiment was performed plants were also treated with cytokinin by mixing it with water to the working concentration and applied by watering.

Table 3.5 Predicted evaluable dilutions for PSt DC3000 in 10µl volume

Days/Dilutions	Undiluted	$1:10^1$	1.10^2	$1:10^3$	$1:10^4$	$1:10^5$
0h	*	*				
1st day	*	*	*			
2nd day		*	*	*	*	
4th day			*	*	*	*

3.6.16 Infection with *Alternaria brassicicola*

Alternaria brassicicola spores used for infection assays were obtained as previously published (Thomma *et al.* 1999). Leaves of 5 week-old *Arabidopsis* plants were drop-inoculated with two 5 µl droplets of spore solution ($5*10^5$ Spores/ml). Two leaves per plant and a minimum of 20 plants per line were infected. Plants of different lines were randomly distributed in the tray and incubated at 100% relative humidity. Fungal infection was scored using the following table (Table 3.4). The Disease Index was defined as previously published (see below and (Kemmerling *et al.* 2007)). For experiments with cytokinin, *trans*-zeatin was used at concentration of 1µM. Shortly before drop-inoculation, cytokinin was given to the $5*10^5$ Spores/ml bacterial dilution. Three days before the experiment was performed plants were also treated with cytokinin by mixing it with water to the working concentration and applied by watering.

3.6.16.1 Disease indexes assigned with their description

Scoring system was adopted from the laboratory of Dr. Birgit Kemmerling at the department of Biochemistry at ZMBP.

A

RANK	Description
1	No symptoms
2	Light brown spots at infection site
3	Dark spots brown spots at infection site
4	Spreading necrosis
5	Leaf maceration
6	Sporulation

B

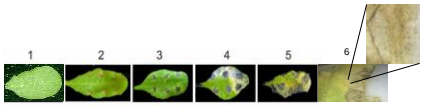


Figure 3.1 Scoring scale for determination of disease index for plants treated with *Alternaria brassicicola* (A) Description of different disease indexes **(B)** Photograph of treated *Arabidopsis* leaves with spores where is the phenotype related with appropriate disease indexes (image provided by courtesy of Thierry Halter, Dr. Birgit Kemmerling's group)

3.6.17 Infection with *Peronospora parasitica*

Treatment with the virulent *Peronospora parasitica* isolate was done on 4 to 6 week-old soil-grown plants. Spore suspensions of *Peronospora parasitica* were prepared as described (Shah *et al.* 2001). Plants were sprayed with a suspension of conidiospores diluted in water ($1x10^6$spores ml^{-1}). Inoculated plants were kept under humid conditions at 18°C for 1 week and then scored for fungal sporulation as previously published (Delaney *et al.* 1995).

3.6.18 Infection with *Botrytis cinerea*

Botrytis cinerea strain BMM was used (Zimmerli *et al.* 2000). The strain was grown on Difco potato dextrose agar (Becton Dickinson). Spores were harvested in water and then filtered and diluted in quarter-strength Difco potato dextrose broth (PDB) for inoculation as previously described (La Camera *et al.* 2011). For disease assays, 6 µL of spore solution (5×10^4 spores mL^{-1}) were deposited on three leaves (detached leaf assay) of 5 weeks old plants. Lesion diameters were measured after 3 days. Fungal growth was measured as previously described (Gachon and Saindrenan 2004). Plants were drop-inoculated and leafs discs were harvested at the indicated time points (0h, 12h, 24h, 36h, 48h post infection). The inoculated plants were kept under high humidity in a tray closed with a water-sprayed transparent lid.

3.6.19 FRET-FLIM and microscopy

Two days after the infection of Nb abaxial leaf surface with Agrobacteria carrying the desired binary plamids *pABind* vectors (Zimmermann and Nentwig 1989) were induced by application of β-estradiol by brushing a 20 µM β-estradiol (in 0.1% Tween-20) solution onto the abaxial leaf surface. FRET measurements were performed 24 to 48 hours after β-estradiol application as described in (Berendzen et al. 2012). The FRET-FLIM measurements were performed with a custom-built CSSM (confocal stage scanning microscope), based on a Zeiss Axiovert 135 TV, and equipped with a pulsed supercontinuum laser–source (SuperK™, NKT Photonics) as excitation light source operating at 471 nm and a repetition rate of 40 MHz. A microscope objective with high numerical aperture (Plan-Neofluar, 100×/1.30 oil, Zeiss) was used to focus the excitation light as well as to collect the fluorescence emission. The setup was equipped with a 500 nm dichroic mirror (FF500-Di01-25×36, Semrock) to block back-scattered excitation light and with a 527 nm bandpass filter (Semrock BrightLine BL527/20) to detect GFP-fluorescence. An avalanche photo diode (PDM series, MicroPhotonDevices (MPD), Italy) served as a spectrally integrating detector to record fluorescence intensity. Lifetime decays were recorded using a time-correlated single photon counting board for data acquisition (PicoHarp 300, Picoquant, Software: SymPhoTime, Picoquant) and the MPD as a detector (Wanke et al. 2011).

3.7 References for Materials and Methods

Berendzen, KW, Bohmer, M, *et al.* (2012). "Screening for in planta protein-protein interactions combining bimolecular fluorescence complementation with flow cytometry." Plant Methods **8**(1): 25.

Clough, SJ and Bent, AF (1998). "Floral dip: A simplified method for agrobacterium-mediated transformation of arabidopsis thaliana." Plant J **16**(6): 735-743.

Delaney, TP, Friedrich, L, *et al.* (1995). "Arabidopsis signal transduction mutant defective in chemically and biologically induced disease resistance." Proc Natl Acad Sci U S A **92**(14): 6602-6606.

Ferrari, S, Plotnikova, JM, *et al.* (2003). "Arabidopsis local resistance to botrytis cinerea involves salicylic acid and camalexin and requires eds4 and pad2, but not sid2, eds5 or pad4." Plant J **35**(2): 193-205.

Gachon, C and Saindrenan, P (2004). "Real-time pcr monitoring of fungal development in arabidopsis thaliana infected by alternaria brassicicola and botrytis cinerea." Plant Physiol Biochem **42**(5): 367-371.

Horak, J, Grefen, C, *et al.* (2008). "The arabidopsis thaliana response regulator arr22 is a putative ahp phospho-histidine phosphatase expressed in the chalaza of developing seeds." BMC Plant Biol **8**: 77.

Jacobs, AK, Lipka, V, *et al.* (2003). "An arabidopsis callose synthase, gsl5, is required for wound and papillary callose formation." Plant Cell **15**(11): 2503-2513.

James, P, Halladay, J, *et al.* (1996). "Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast." Genetics **144**(4): 1425-1436.

Kemmerling, B, Schwedt, A, *et al.* (2007). "The bri1-associated kinase 1, bak1, has a brassinolide-independent role in plant cell-death control." Curr Biol **17**(13): 1116-1122.

La Camera, S, L'Haridon, F, *et al.* (2011). "The glutaredoxin atgrxs13 is required to facilitate botrytis cinerea infection of arabidopsis thaliana plants." Plant J **68**(3): 507-519.

Lee, H, Suh, SS, *et al.* (2000). "The agamous-like 20 mads domain protein integrates floral inductive pathways in arabidopsis." Genes Dev **14**(18): 2366-2376.

Maizel, A and Weigel, D (2004). "Temporally and spatially controlled induction of gene expression in arabidopsis thaliana." Plant J **38**(1): 164-171.

Marion, J, Bach, L, *et al.* (2008). "Systematic analysis of protein subcellular localization and interaction using high-throughput transient transformation of arabidopsis seedlings." Plant J **56**(1): 169-179.

Mathieu, J, Warthmann, N, *et al.* (2007). "Export of ft protein from phloem companion cells is sufficient for floral induction in arabidopsis." Curr Biol **17**(12): 1055-1060.

Michaels, SD and Amasino, RM (1999). "Flowering locus c encodes a novel mads domain protein that acts as a repressor of flowering." Plant Cell **11**(5): 949-956.

Mira-Rodado, V, Veerabagu, M, *et al.* (2012). "Identification of two-component system elements downstream of ahk5 in the stomatal closure response of arabidopsis thaliana." Plant Signal Behav **7**(11): 1467-1476.

Sambrook, J and Russell, DW (2001). Molecular cloning: A laboratory manual, third edition (3 volume set), Cold Spring Harbor Laboratory Press.

Shah, J, Kachroo, P, *et al.* (2001). "A recessive mutation in the arabidopsis ssi2 gene confers sa- and npr1-independent expression of pr genes and resistance against bacterial and oomycete pathogens." Plant J **25**(5): 563-574.

Thomma, BP, Nelissen, I, *et al.* (1999). "Deficiency in phytoalexin production causes enhanced susceptibility of arabidopsis thaliana to the fungus alternaria brassicicola." Plant J **19**(2): 163-171.

Voinnet, O, Rivas, S, *et al.* (2003). "An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus." Plant J **33**(5): 949-956.

Wahl, V, Ponnu, J, *et al.* (2013). "Regulation of flowering by trehalose-6-phosphate signaling in arabidopsis thaliana." Science **339**(6120): 704-707.

Wanke, D, Hohenstatt, ML, *et al.* (2011). "Alanine zipper-like coiled-coil domains are necessary for homotypic dimerization of plant gaga-factors in the nucleus and nucleolus." PLoS One **6**(2): e16070.

Yoo, SK, Chung, KS, *et al.* (2005). "Constans activates suppressor of overexpression of constans 1 through flowering locus t to promote flowering in arabidopsis." Plant Physiol **139**(2): 770-778.

Zimmerli, L, Jakab, G, *et al.* (2000). "Potentiation of pathogen-specific defense mechanisms in arabidopsis by beta -aminobutyric acid." Proc Natl Acad Sci U S A **97**(23): 12920-12925.

Zimmermann, M and Nentwig, GH (1989). "[survival rate of desmodontal cells in relation to their extraoral dehydration]." Schweiz Monatsschr Zahnmed **99**(9): 1007-1010.

4 Chapter 1

Interaction of Type-B TCS components with MAPK kinase cascade members and a putative crosstalk function in pathogen response

4.1 Introduction

4.1.1 Two-component system (TCS) in *Arabidopsis thaliana*

Like in all plants, the TCS in *Arabidopsis thaliana* is also a multi-step phosphorelay system (Hwang *et al.* 2002; Heyl and Schmulling 2003; El-Showk *et al.* 2013). TCS mediates physiological response to hormones (cytokinin and ethylene, cross-talk with auxin), nutrients, light and osmotic stress. Its components have important roles in the regulation of several developmental processes such as control of stem cell number in SAM, female gametophyte development, differentiation of root vascular tissue, just to name a few (Hwang *et al.* 2012; El-Showk *et al.* 2013). This involves members of three protein families: *Arabidopsis* histidine kinases (AHKs), *Arabidopsis* histidine phosphotransferase proteins (AHPs) and *Arabidopsis* response regulators (ARRs). *Arabidopsis* possess 11 AHKs and they are hybrid kinases, with an exception of *ETHYLENE RESPONSE SENSOR 1 (ERS1)* kinase (Guo and Ecker 2004). Based on their functional and structural characteristics the AHKs can be grouped into three subfamilies (Grefen and Harter 2004). Five *AHKs* have roles as ethylene receptors (Grefen and Harter 2004): *ERS1, ERS2, ETHYLENE RESISTANT 1 (ETR1), ETR2* and *ETHYLENE INSENSITIVE 4 (EIN4*); *ERS1* and *ETR1* belong to the class I subfamily of AHKs (Grefen and Harter 2004) and are localised to the endoplasmic reticulum (Guo and Ecker 2004). *ETR2*, *ERS2* and *EIN4* belong to the class II of ethylene receptors (Grefen and Harter 2004; Guo and Ecker 2004) and have degenerated transmitter domains, *i.e.* they do not have histidine kinase activity as they lack at least one crucial amino acid to be functional (Guo and Ecker 2004). Kinase *AHK1* is playing role in stress signalling as membrane-bound osmosensor (Urao *et al.* 1999) and *CYTOKININ INSENSITIVE I (CKI1)* is enrolled in female gametophyte development (Pischke *et al.* 2002; Hejatko *et al.* 2003). *AHK5,* or also known as *CKI2,* is missing a transmembrane domain (Urao *et al.* 1999; Pischke *et al.* 2002) and therefore some authors classify it as a separate group. The *AHK5/CKI2* is playing a role in stomata closure signalling (Desikan *et al.* 2008) and it is predicted to be a cytoplasmic hybrid histidine kinase (Grefen and Harter 2004; Hass *et al.* 2004).

Members of the final subfamily respond to cytokinin: *AHK2, AHK3* and *AHK4/WOODEN LEG (WOL)/ CYTOKININ RESPONSE 1 (CRE1)* (Hwang *et al.* 2002; Grefen and Harter 2004; El-

Showk *et al*. 2013). Briefly, these AHKs contain a ligand-binding CYCLASE HISTIDINE KINASE ASSOCIATED SENSORY EXTRACELLULAR (CHASE) domain at their N-terminus which is flanked by two transmembrane domains and followed on the cytoplasmic side by the C-terminus with a histidine kinase domain and output domain (Suzuki *et al*. 2001; Ueguchi *et al*. 2001; Hwang *et al*. 2002). This sensor domain is like other bacterial histidine kinases PAS domains and their mechanism of function (Hothorn *et al*. 2011). The cytokinin receptors are localised not only on plasma membrane but also at the endoplasmic reticulum membrane highlighting that compartmentalization is important for cytokinin signal transduction (Caesar *et al*. 2011). These receptors show different sensitivity to different types of cytokinin hormones and also are expressed and functional in different tissues (Stolz *et al*. 2011; Lomin *et al*. 2012). AHK2 and AHK4/CRE1 have roughly the same high affinity to isopentenyladenine (iP) and *t*-zeatin but a lower affinity to dihydrozeatin whereas AHK3 has high affinity to dihydrozeatin compared to isopentenyladenine (Lomin *et al*. 2012). P_{ARR5}*::GUS* experiments in *ahk2*, *ahk3* and *cre1* mutant backgrounds demonstrated that loss of *cre1* affected promoter responses in the root while loss of *ahk2*, *ahk3* crippled shoot responses (Stolz *et al*. 2011). Interestingly, *AHK4* is known to be prevalent in the roots and *AHK3* in the shoots (Lomin *et al*. 2012). If correctly interpreted, the receptors are expressed and receptive to the corresponding cytokinins that are transported from the opposing tissue, *i.e*. *t*-zeatin from root to shoot and iPs from shoot to root (Lomin *et al*. 2012).

There are 6 AHPs in *Arabidopsis* that mediate phosphorelay and shuttle through the nucleus between AHKs and ARRs. *AHP1* to *AHP5* are positive regulators in cytokinin signalling (Hutchison *et al*. 2006) and are also known as "canonical" AHPs because they contain the conserved histidine HPt residue thus they can carry the TCS-phosphate group on further to ARRs (Suzuki *et al*. 1998). Despite their partial redundancy, different subsets of AHPs have been shown to trigger cytokinin responses in specific tissues and organs. For example the *ahp2/ahp3/ahp5* triple loss-of-function mutant showed increased seed size similar to that observed in the *ahk2/ahk3/ahk4* triple mutant (Hutchison *et al*. 2006; Riefler *et al*. 2006). Different combinations of *ahp1-5* mutants do not show the same responses to exogenous cytokinin and its effects on chlorophyll content, lateral root formation or hypocotyl elongation (Hutchison *et al*. 2006). Generally speaking however, the loss of *AHPs* seems to be additive, as expected due to redundancy. *AHP6* is called a "pseudo AHP" because it lacks the conserved histidine residue required for TCS-dependent phosphorylation: nevertheless, it is an active cytokinin signalling inhibitor (Suzuki *et al*. 1998; Grefen and Harter 2004; Mahonen *et al*. 2006) (Moreira *et al*. 2013). Recently it has been reported that *AHP2*, *AHP3*, and *AHP5*, also work redundantly to control responses to drought stress in a negative and redundant manner as loss of these three *AHPs* resulted in a strong drought-tolerant phenotype that was associated with the stimulation of protective mechanisms (Nishiyama *et al*. 2013).

There are 23 functional ARRs in *Arabidiopsis* and based on their protein properties and phylogeny they are divided into 3 subgroups: the type-A, type-B and pseudo-response regulators. The Type-A subfamily of *ARRs* are single-domain response regulators that

contains the members: *ARR3, ARR4, ARR5, ARR6, ARR7, ARR8, ARR9, ARR15, ARR16* and *ARR17*. Members of this subfamily only have a functional receiver domain (with functional Asp residue) and a short C-terminal tail (Grefen and Harter 2004). Members of the type-A subfamily generally act as negative regulators of cytokinin signalling based on genetic and transgenic studies (Lee *et al.* 2008; Hwang *et al.* 2012; El-Showk *et al.* 2013). So far only ARR4 has been described with a function outside of the TCS. ARR4 positively interacts with phytochrome B and stabilizes the active PhyB-Pfr form under extended red light conditions and thus functions as modulator of photomorphogenesis (Sweere *et al.* 2001; Mira-Rodado *et al.* 2007).

The type-B response regulators have additional effector domains on their extended C-termini and are therefore able to activate the transcription in a TCS-dependent manner (Sakai *et al.* 2001). Their expression is not regulated by any stimulus tested so far (Grefen and Harter 2004). This subfamily consists of 11 members: *ARR1, ARR2, ARR10, ARR11, ARR12, ARR13, ARR14, ARR18, ARR19, ARR20* and *ARR21*. The output domain of the B-Type members usually is composed of the GARP (named after Golden2 found in maize) DNA-binding domain, at least one NLS and C-terminal transactivation domain (Lohrmann and Harter 2002). B-Type response regulators work as transcription factors binding to target promoters that contain 5´-W/GAT/W-3´ motif (W represents either A or T) (Lohrmann and Harter 2002). Last group of response regulators are C-Type *ARRs* (Horak *et al.* 2008). Both type-A and type-B ARRs are now know to be regulated by the 26S proteasome (Ren *et al.* 2009; Kim *et al.* 2013; Kurepa *et al.* 2013).

Two genes (*ARR22* and *ARR24*) encode single-response domain response regulators and are structurally very similar to type-A response regulators but their expression is not regulated by cytokinin. It has been recently suggested that they function as phosphatases for phosphates on histidine moiety of AHPs, but their expression domain is restricted to reproductive organs (Kiba *et al.* 2003; Gattolin *et al.* 2006; Horak *et al.* 2008).

Beside these three groups of the *ARRs* there is one more group, composed out of 9 members, called *pseudo-response regulators* (*pseudo-RRs*). These are response regulator proteins that have a mutation in the conserved response regulator aspartate (Asp) residue (Hwang *et al.* 2002). It has already been proven that pseudo-RRs regulate flowering time in *Arabidopsis thaliana* and that *TOC1/APRR1*, an *Arabidopsis* pseudo response regulator, is part of the plant circadian clock (Farre and Liu 2013). Even though pseudo-RRs have lost their ability to accept the phosphorelay phosphate, they are constitutively active because the highly conserved Asp is mutated to a glutamate naturally mimicking a phosphorylated Asp (Grefen and Harter 2004). The pseudo-response regulators can be split into two subfamilies (Matsushika *et al.* 2000): The APRR2 family which more similar to a classical response regulator structure with an N-terminal receiver domain followed by a DNA binding domain (also Myb-like GARP) and C-terminal effector extension; the spacing between the RR domain and the GARP domain is however larger than that of the type-B ARRs (Makino *et al.* 2000; Matsushika *et al.* 2000). The APRR1 family has an "inverted" response regulator structure:

there is an N-terminal RR domain, a DNA-binding CCT domain at the C-terminal end, and, presumptuously, the effector domain in-between these two domains (Makino *et al.* 2000). PRR9, PRR7, and PRR5 function as transcriptional repressors of *CCA1* and *LHY* (Nakamichi *et al.* 2010) recently shown to bind to DNA via their C-terminal CCT domains (Gendron *et al.* 2012). The pseudo-response regulators are also targeted by the 26S proteasome (Baudry *et al.* 2010).

4.1.2 Crosstalk of TCS-related pathways in plants

Although most type-B *ARRs* have been described to only be involved in cytokinin signalling, an exception is *ARR2*. Recently it was discovered that cytokinin-activated ARR2 promotes plant immunity in *Arabidopsis* via salicylic acid signalling whereby ARR2 directly interacts with salicylic acid response factor TGA3 and increases pathogen resistance (Choi *et al.* 2010). *ARR2* is also known to be involved in ethylene signalling by. working downstream of *ETR1* (Hass *et al.* 2004). Furthermore, *ARR2* is involved in ethylene and H_2O_2-mediated stomatal closure (Desikan *et al.* 2006). The crosstalk of TCSs with other plants signalling pathways became more evident after a comparative microarray analysis was done on 30-day-old *arr2* mutant and control Landsberg erecta (Ler) plants where the results showed altered expression of about 600 genes mostly related to biotic and abiotic stresses, ethylene and auxin signalling (Hass *et al.* 2004). The expression of dominant-active, non-phosphorylatable, $ARR2^{D80E}$ (aspartate-to-glutamate mutation) caused even more dramatic changes in more than 16000 genes, and most of the disturbed genes are involved in hormone homoeostasis and its signal transduction, biotic and abiotic stress, photomorphogenesis, and others (Hass *et al.* 2004).

4.1.3 Mitogen-activated protein kinase cascade

Eukaryotic mitogen-activated protein kinase (MAPK) cascades transduce environmental and developmental signals into adaptive and programmed responses. In plants, MAPK cascades have evolved to regulate innate immunity, hormonal response, stress and developmental processes (Colcombet and Hirt 2008; Rodriguez *et al.* 2010). MAPK signalling cascade(s) are evolutionary conserved within eukaryotes (Schwartz and Madhani 2004) and have roles in abiotic stress, response to pathogens and pathogen-derived elicitors, plant hormones (ethylene, auxin), cell cycle and developmental processes (Tena *et al.* 2011; Rasmussen *et al.* 2012; Pathak *et al.* 2013; Smekalova *et al.* 2013). MAPK cascades transduce and amplify their signal by three different types of kinase members: *MAP kinase kinase kinase (MKKK), MAP kinase kinase (MKK)* and *MAP kinase (MPK)* (Rodriguez *et al.* 2010). In general, they function as a three tiered kinase cascade with each upstream member required to active the lower level eventually influencing genes expression (Rodriguez *et al.* 2010). Initially, a ligand activates plasma membrane receptors and these stimulated receptors activate MKKKs that are serine or threonine kinases that in turn phosphorylate MKKs at a conserved S/T-X_{3-5}-S/T motif (X can be any amino acid) (Chang and Karin 2001; Rodriguez *et al.* 2010). The MKKs afterwards phosphorylate MPKs on threonine and tyrosine residues at a conserved T-X-Y repeat (Chang and Karin 2001; Rodriguez *et al.* 2010). Activity of MAPKs can be regulated/deactivated by serine/threonine or tyrosine phosphatases (Luan 2003). Different cascades can share kinase components but still maintain their signalling specificity by various

mechanisms like protein-protein interactions, scaffolding, cross-inhibition and feedback control (Whitmarsh and Davis 1998; Bardwell *et al.* 2001; Takekawa *et al.* 2005). MAPK cascades are present in all eukaryotes and is evolutionarily highly conserved which confirms its essential function in these organisms (Kim *et al.* 2003).

4.1.4 Mitogen-activated protein kinase cascade and its signalling in *Arabidopsis thaliana*

Around 60 *MKKKs*, 10 *MKKs* and 20 *MPKs* are found in the genome of *Arabidopsis thaliana* (Group 2002). Besides having the largest number of members, *MKKKs* also have the greatest variety in domain composition and primary structures among all the MAPK cascade members (Group 2002). Their nomenclature is still not totally defined as some authors have different opinions about this topic. Based on amino acid sequence of the kinase catalytic domains the *Arabidopsis MKKKs* are grouped into 2 main classes which are further divided into different subclasses (Group 2002; Rodriguez *et al.* 2010): *MEKKs* and the Raf-like kinases. The *MEKK* MKKKs family comprises members who kinase domains share significant similarity to previously described *MKKKs*, for example MEKK1 in mammals or to yeast STE1 (Group 2002). Members of this family are generally active in response to abiotic stress, especially drought, touch and high salinity (Mizoguchi *et al.* 1996). The *Raf-like kinase* class is named after RAF1 mammalian kinase. Interestingly, most members of this class have a PAS domain which is the most frequent sensor domain present among TCS histidine kinases and other pathways (Zhulin *et al.* 1997; Zwerger and Hirt 2001). The most studied members of this *MKKK* class are *CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1)* and *ENHANCED DISEASE RESISTANCE 1 (EDR1)* which are both involved in ethylene-mediated signalling and defence responses (Kieber *et al.* 1993; Huang *et al.* 2003). Among the *Raf-like* class there is also the ZIK sister clade for which no phosphorylation activity has been shown on MKKs (Figure 4.1) (Rodriguez *et al.* 2010).

The small number of putative *MKKs* in *Arabidopsis* suggests that crosstalk between various signal-transduction pathways might be concentrated at this level of the MAPK signalling cascade. It is known that one MKK can activate various different MPKs (Popescu *et al.* 2009). Considering their small number, the MKKs are very well described in *Arabidopsis* and rice and both are classified into 4 groups (A-D) (Group 2002; Hamel *et al.* 2006; Rodriguez *et al.* 2010). Members of the group A are *MKK1, MKK2* and *MKK6*. Kinases MKK1 and MKK2 are involved in abiotic (cold, salinity) and biotic (especially innate immune response) stresses (Teige *et al.* 2004; Meszaros *et al.* 2006; Rodriguez *et al.* 2010), both acting upstream of MPK4 (Ichimura *et al.* 1998). Group B includes only one member: *MKK3* which is distinguishing from the other kinases by the presence of a nuclear transport factor 2 (NTF2) domain (Kiegerl *et al.* 2000; Group 2002). Group C includes *MKK4* and *MKK5* (Group 2002) and they play very important roles in biotic stresses by providing resistance to bacterial and fungal pathogens controlling converging signals initiated by diverse pathogens (Asai *et al.* 2002; Group 2002; Rodriguez *et al.* 2010). In addition, both MKK4 and MKK5 are in the pathway downstream of YODA (MAPK kinase kinase) regulating cell fate specification in stomata development (Wang *et al.* 2007) and the ethylene synthesis pathway (Babula *et al.* 2006). Members of group D are *MKK7, MKK8, MKK9* and *MPK10* whose physiological roles are not yet precisely described. It is known that the MKK9–MPK3/MPK6 cascade promotes *ETHYLENE-INSENSITIVE 3 (EIN3)*-

mediated transcription in response to ethylene signalling (Yoo *et al.* 2008; Hahn and Harter 2009) and that MKK7 repressed expression causes deficiency or enhancement in auxin transport (Dai *et al.* 2006).

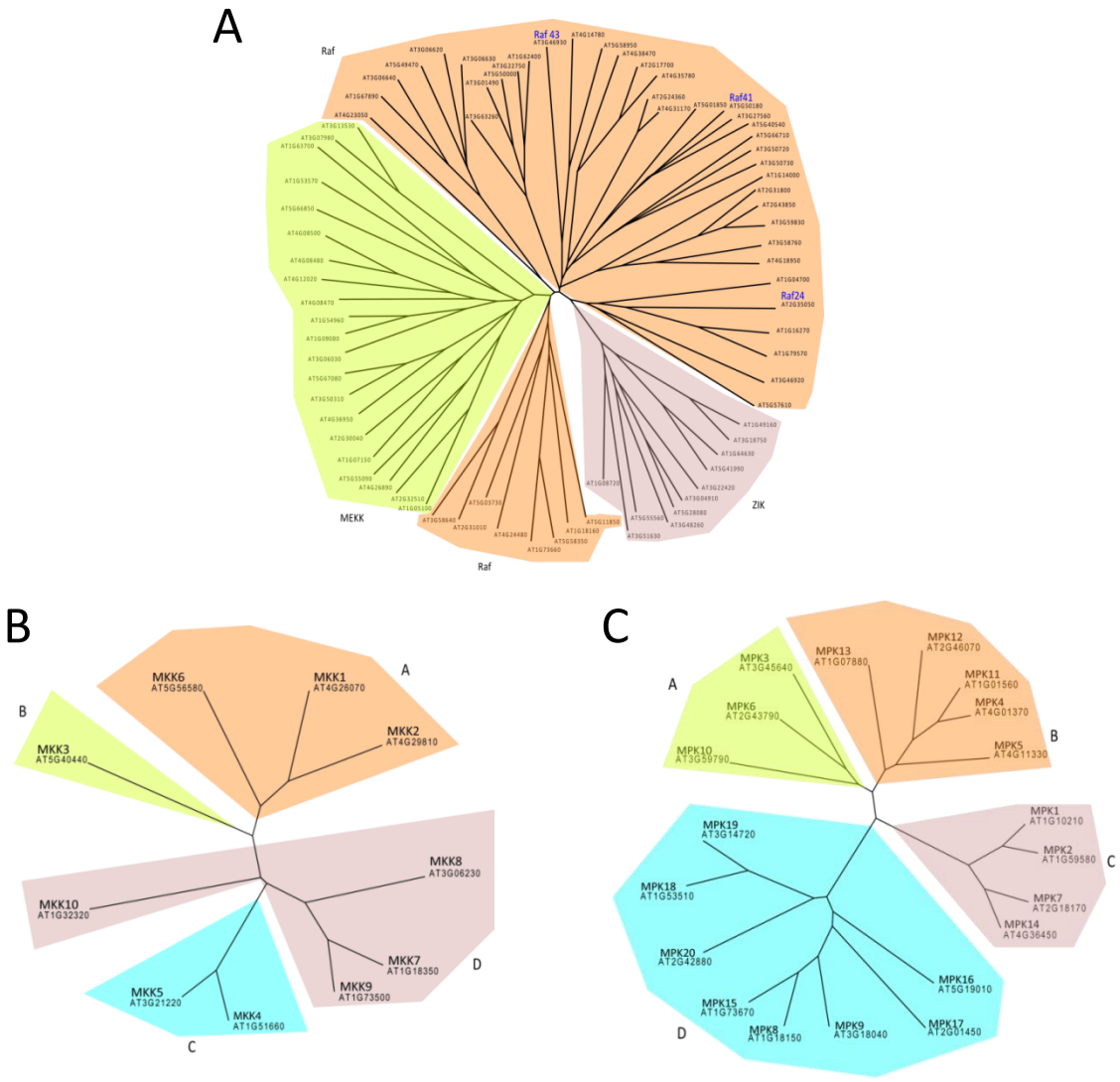


Figure 4.1 Relatedness of *Arabidopsis MKKKs* based on their protein sequence
The unrooted tree was made by aligning complete protein sequences with Clustal W (default settings) available on TAIR (http://www.arabidopsis.org/) of the ***(A)*** *AtMKKKs,* ***(B)*** *AtMKKs and* ***(C)*** *AtMPKs. S*ubfamilies are marked according to the classifications proposed in (Group 2002). The trees were draw with TreeView X.

The last members of the MAPK signalling cascade are MPKs. They are also grouped into 4 groups (A-D) based on their sequence homology (Group 2002; Rodriguez *et al.* 2010). In plants, MPKs contain either the TDY (present also in all mammals) or TEY phosphorylation motif which is phosphorylated by MKKs and causing MPK activation (Group 2002; Rodriguez *et al.* 2010). Members of groups A, B and C families contain TEY whereas only group D has the TDY phosphorylation motif. The most studied group of MPKs is Group A. Generally, members of this group are involved in environmental and hormonal responses (Group 2002). *MPK3* and *MPK6* are members of this group and they have been the most studied group

among all MAP kinases. MPK3/MPK6 play roles in ethylene signalling downstream of MKK9 (Hahn and Harter 2009) whereas MPK6 is additionally required for interaction with ETHYLENE RESPONSE FACTOR 104 (ERF104) in ethylene responses regulated by flg22 (Bethke *et al.* 2009). Findings for the group B are mostly based on physiological studies of *Arabidopsis* MPK4 involved in pathogen defence and abiotic stresses (Widmann *et al.* 1999; Yuasa *et al.* 2001; Qiu *et al.* 2008). MPK4 is known to play important role in plant immunity, it functions as a negative regulator of pathogen defence and also interferes with stress signalling pathways at several distinct steps in pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) as well as in effector-triggered immunity (ETI) (Berriri *et al.* 2012; Colcombet *et al.* 2012). Furthermore, it is also known that the *Pseudomonas syringae* HopAI1 effector irreversibly inactivates MPK4 to prevent immune responses (Rasmussen *et al.* 2012). Data on C group are very limited and based on *MPK7* whose expression is regulated by the circadian rhythm (Group 2002). Group D also has a C-terminal MKKs docking domain (Rodriguez *et al.* 2010). It was found that some members of this group are induced by fungi and wounding in rice and alfalfa (He *et al.* 1999; Schoenbeck *et al.* 1999). One described member is *MPK17*. Generally not much is known about *MPK17* but it is thought to be important, like whole group D, in plant sugar signal transduction (Sheen *et al.* 1999).

4.1.5 Plant pathogens and mechanisms of plant defence

4.1.5.1 Immunity

Plants are often attacked by a huge variety of microbial pathogens and herbivore insects. In order to response to these threats they have developed numerous defence mechanisms and many of them are directly induced by pathogen attack (Glazebrook 2005). Generally, defence often begins with gene-for-gene pathogen recognition where the production of pathogen virulence effectors leads to their recognition by plants that carry correspondent resistance *(R)* genes (Glazebrook 2005). This kind of resistance is accompanied by fast reactive oxygen species (ROS) production also known as oxidative burst which is needed for a further process known as hypersensitive cell death (HR). Reactive oxygen species (ROS) were initially recognized as toxic by-products of aerobic metabolism, removed by means of antioxidants and antioxidative enzymes (Kawano 2003) and was not suspected to provide meaningful information for the plant.

On one side, R gene-driven resistance activates salicylic acid (SA)-dependent signalling pathways which in turn lead to the activation of pathogenesis-related proteins (PR) that also contribute to resistance. This rapid activation of defence results in the limitation of pathogen growth. Innate immunity is generally initiated with recognition of conserved pathogen/microbial-associated molecular patterns (PAMPs/MAMPs) (Nishimura and Dangl 2010). Pathogen-associated molecular patterns, or PAMPs, are molecules associated with groups of pathogens that are recognized by cells of the innate immune system. These molecules can be referred to as small molecular motifs conserved within a class of microbes (Boller and He 2009) and they are typically essential components of whole classes of pathogens, such as bacterial flagellin or fungal chitin (Dodds and Rathjen 2010). PAMPs are

perceived by pattern recognition receptors (PRRs), leading to activation of a series of immune responses, including the expression of defence genes, Reactive Oxygen Species (ROS) production, nitric oxide, ethylene, jasmonic acid (JA), and salicylic acid (SA) and activation of MAP kinase cascades (Jones and Dangl 2006; Tena *et al.* 2011; Yamaguchi *et al.* 2013). Activation of PRRs leads to PAMP-triggered immunity (PTI); PTI is often the first inducible response of a plant to PAMPs (Jones and Dangl 2006; Nishimura and Dangl 2010). The largest group within the *R genes* is binding site-leucine rich repeats (NBS-LRR) (Belkhadir *et al.* 2004), now mostly called NLR-dependent effector-triggered immunity (ETI) (Dangl *et al.* 2013). ETI is a second class of perception called effector-triggered immunity. This way of perception involves recognition by molecules called effectors which present intracellular receptors of pathogen virulence presenting, in contrast to PTI, co-evolutionary dynamics between the plant and pathogen. Generally, PTI and ETI give rise to similar responses. PTI is generally effective against non-adapted pathogens in a phenomenon called non-host resistance, whereas ETI, which is stronger and faster than PTI, is mostly active against adapted pathogens (Dodds and Rathjen 2010). Plants also respond to endogenous molecules which are released by pathogen invasion, cell wall or cuticular fragments, called danger-associated molecular patterns (DAMPs) (Dodds and Rathjen 2010).

Beside defence response controlled by SA pathways, defence responses can also be controlled by ethylene and/or jasmonic acid (JA) and this responses are mostly overlapping with response to wounding (Glazebrook 2005). Nevertheless, SA, JA and ethylene interacting between each other, SA and JA are reported to mutually inhibit the expression of many target genes whereas JA and ethylene are sometimes both acquired for expression of some genes but there are also some cases of negative interaction between their signalling.

4.1.5.2 Biotrophic and necrotrophic pathogens

According to their lifestyle pathogens are divided into biotrophs and necrotrophs. Biotrophs are pathogens that live and feed on living tissue; necrotrophs are defined as organisms that live and feed on dead tissue (Mcdowell and Dangl 2000; Thaler *et al.* 2004). Besides their different life style more important is the fact that Necrotrophic and biotrophic pathogens have evolved differently leading to distinct defence strategies in plants and thus activation of different genes required for pathogenicity (Idnurm and Howlett 2001; Oliver and Ipcho 2004). SA is linked to resistance to biotrophic pathogens and is important to trigger the HR, a programmed cell death (PCD) to locally counteract pathogen attack and progression. ET and JA play a role in the control of PCD spreading (Glazebrook 2005; Colcombet and Hirt 2008) and regulate resistance against necrotrophic pathogens (Colcombet and Hirt 2008). However, some pathogens cannot be assigned as biotrophs or necrotrophs There are also hemibiotrophic pathogens: hemibiotrophic pathogens incorporate aspects of both biotrophic and necrotrophic infection strategies. Often this involves an initial biotrophic infection phase during which the pathogen spreads in host tissue, followed by a necrotrophic phase during which host cell death is induced (Dodds and Rathjen 2010). However, the interactions between SA-, JA-, and ethylene-dependent pathways do not appear to be simple.

Complications could arise from different roles of these pathways in different stages of plant-pathogen interactions (Katagiri *et al*. 2002).

In order SA to accumulate two genes encoding enzymes similar to triacyl-glycerol lipases are required *PHYTOALEXIN DEFICIENT 4 PAD4* and *ENHANCED DISEASE SUSCEPTIBILITY 1 EDS1* (Zhou *et al*. 1998; Falk *et al*. 1999), raised SA levels in turn are activating various defense effector genes including *PR-1* (Glazebrook 2005). The *PR-1* effector gene for plant defense is activated by NPR1 and TGA-type transcription factors. *NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1)* has function in regulation of systemic acquired resistance (SAR) in *Arabidopsis* (Rochon *et al*. 2006) and NPR1, as recently reported, plays the role of an SA receptor *in vivo*, in the signalling cascade leading to *PR-1* activation (Wu *et al*. 2012). Systemic acquired resistance (SAR) means a mechanism of induced defence and implies long lasting protection against a broad spectrum of microorganisms. Salicylic acid is absolutely required by SAR as signalling molecule (Durrant and Dong 2004). Based on their physical interaction *TGA* transcription factors are considered as regulators of pathogenesis-related (*PR*) genes because of their physical interaction with the known positive regulator NPR1 (Kesarwani *et al*. 2007). Using reverse genetics approach it has been characterized 10 *TGA* factors in *Arabidopsis* so far and they all belong to the bZIP transcription factor family (Jakoby *et al*. 2002). When the level of SA are low, NPR1 exists as oligomeric form in cytoplasm, the monomers are held together by disulfide linkages which are getting reduced after the level of NPR1 increases and NPR1 is now present in monomeric form which as such can enter the nucleus and interact with the nuclear TGAs and induce expression of *PR-1* (Zhang *et al*. 2003).

As response to pathogen attack the levels of JA can also increase and as a consequence to that defence effector genes are increasing as well, especially *VSP1* (Otani *et al*. 1998) and *PDF1.2*. The *VEGETATIVE STORAGE PROTEIN 1 (VSP1)* gene of *Arabidopsis thaliana* encodes a storage protein that accumulates in vegetative organs and it is jasmonate-responsive element (Guerineau *et al*. 2003). The *PLANT DEFENSIN PDF1.2* gene in *Arabidopsis* encodes a plant defensin and it is commonly used as a marker for characterization of the jasmonate-dependent defence responses (Brown *et al*. 2003). Expression of the *PDF1.2* requires both JA and ethylene (Glazebrook 2005). For known signalling activities of JA in *Arabidopsis CORONATINE INSENSITIVE 1 (COI1)* is required. *COI1* gene encodes protein from F-box group and it is believed to act in proteolysis (Xie *et al*. 1998) but it can also bind histone deacetylases (Devoto *et al*. 2002). Beside the essential COI1, JA also requires members of MAPK cascade for its activity, the MPK4. MPK4 knock-out mutant failed to express the *PD1.2*. gene (Petersen *et al*. 2000). For ethylene connection to pathogen defence *EIN2* is involved but that is not the only ethylene-related gene involved in pathogen defence (Lorenzo *et al*. 2003).

Pseudomonas syringae is a gram-negative, rod-shaped bacterium with polar flagella and it infects a wide variety of plants (Katagiri *et al*. 2002). *Pseudomonas* mostly acts as biotrophic pathogen (Zhao *et al*. 2003; Tsai *et al*. 2011). *Peronospora parasitica* is an oomyceteous

fungus from the *Peronosporaceae* family. In contrast to *Pst* DC3000, *Peronospora* is an obligate biotroph that is controlled by the salicylic acid-dependent SAR (systemic acquired resistance) pathway (Nawrath and Metraux 1999; Jambunathan *et al.* 2001). Defence responses under SA control are critical for resistance to both *Pst* DC3000 and *Peronospora parasitica (Glazebrook 2001; Van Wees et al. 2003)* (Nawrath and Metraux 1999; Jambunathan *et al.* 2001). *Alternaria brassicicola* belongs to phylum *Ascomycota* (family *Pleosporaceae*) and it known to be a very common plant pathogen. Black spot disease caused by *Alternaria brassicicola* is an important fungal disease affecting cruciferous crops (*Brassicaceae*). The interaction between *Arabidopsis thaliana* and *A. brassicicola* is a representative model system and objective estimation of disease progression is indispensable for accurate functional analyses (Su'udi *et al.* 2013). Resistance against *Alternaria* requires the *Arabidopsis* phytoalexin, camalexin and jasmonic acid (JA)-dependent signalling, respectively (Van Wees *et al.* 2003). *Botrytis cinerea* is necrotrophic fungus belonging to the phylum *Ascomycota* (family *Sclerotianiaceae*) and it is a natural pathogen of *Arabidopsis thaliana* (P *et al.* 2007). *Botrytis* belongs in top 10 fungal pathogens in molecular plant pathology because it has an impact because of its broad host range causing severe damage: *Botrytis* causes bunch rot in viticulture or grey mould in horticulture (Dean *et al.* 2012). The fungus is considered as a typical necrotroph, which co-opts programmed cell death pathways in the host to achieve infection (Amselem *et al.* 2011; Dean *et al.* 2012).

4.1.6 TCS type-B response regulator and MAPK cascade members are involved in pathogen signalling

MAPK cascade members found to be involved in pathogen signalling are *MPK3, MPK4* and *MPK6* (Colcombet and Hirt 2008). These kinases in *Arabidopsis* are activated by PAMPs, mostly by flg22 (Droillard *et al.* 2004). Very soon other MAPK cascade members, working upstream of MPK3/4/6 and downstream of the FLS2-BAK1, were found (Colcombet and Hirt 2008). These are MKKK1 and two MKKs, MKK4 and MKK5 (Colcombet and Hirt 2008). Nevertheless, in *mekk1* knock-out plants, flg22 was still able to activate MPK3 and MPK6 suggesting redundant effects at the MKKK level (Ichimura *et al.* 2006; Suarez-Rodriguez *et al.* 2007). Interestingly, biosynthesis of ethylene is also triggered by flg22 via MPK6 which in turn activates *ACS6 (1-amino-cyclopropane-1-carboxylic acid) synthase 6* a crucial element in ethylene synthesis (Liu and Zhang 2004; Joo *et al.* 2008). *Pst* DC3000 induces *MKK3* to positively regulate expression of *PR1*. On the other hand *mkk3-1* knock-out plants showed high susceptibility to *Pst* DC3000 and this effect was the opposite when the *mpk3-1* mutant was complemented by *MKK3* wild-type form or constitutive active *MKK3-EE* form (S235E and S411E, MKKK phosphorylation residues) (Doczi *et al.* 2007), that is, the downstream MPK targets are needed for a functional readout of MKK activity. Downstream elements for MKK3 thus appear to be Group C MPKs based on yeast-two-hybrid interaction studies; physiological roles were only confirmed with MPK7 (Zhang *et al.* 2007). Recently was found that ARR2 also contributes resistance against *Pst* DC3000. ARR2 cannot directly do this, but interacts with salicylic acid response factor TGA3 after it is picks up a TCS phosphate at D80, thereafter interacting with the TGA3/NPR1 dimer making ARR2/TGA3/NPR1 trimer complex

that can bind to the *PR1* promoter, activate it, and increase pathogen resistance. *arr2-4* mutant plants in that study showed susceptibility to *Pseudomonas* (compared to the wild-type Col-0 plants) and also retarded induction of *PR1* in response to SA (Choi *et al.* 2010). ARR2 was shown to pull down *PR1*, *PR2*, and *ARR6* promoter DNA via ChIP assays. The association to DNA at *PR1* however was mediated by TGA3 and not ARR2 (Choi *et al.* 2010), is suggests that a type of scaffolding role for ARR2 was discovered. Possibly other such roles for ARR2 and or type-B RRs independent of direct DNA binding could be discovered.

4.1.7 Cell-specificity effect of the MAPK cascade

Despite the fact that only couple members of the MAPK cascade control huge range of physiological responses, these signalling cascades are quite specific because they show cell specificity (Colcombet and Hirt 2008). For example, activity of *MPK4* is increased in response to osmotic shock in *Arabidopsis* but only in suspension cells not in plantlets (Droillard *et al.* 2004). Subcellular-dependent function can be very well observed on ethylene-related roles of *MPK6*. The cascade MKK4-MPK6-ACS6 is involved in ethylene production whereas MKK9-MPK6-EIN3 cascade plays role in ethylene signalling. The MKK9–MPK3/MPK6 cascade promotes *ETHYLENE-INSENSITIVE 3 (EIN3)*-mediated transcription in ethylene signalling (Yoo *et al.* 2008; Hahn and Harter 2009). Ethylene as a ligand binds and inactivates the negative regulator CTR1 (MKKK family member) to activate the positive MKK9–MPK3/6 cascade (Yoo *et al.* 2008). This signalling pathway breaks a linear model and common MAPK signalling, and opens many questions, for example, both the real connection between CTR1 and MKK9 or how CTR1 is actually inhibiting the autophosphorylation of MKK9 through direct protein-protein interaction or in some other way (Hahn and Harter 2009). Supporting cell-specificity idea, scaffolding proteins have extremely important functions because they help MPK kinases to build physiologically functional cascades. Such an example is present in yeast where the C-terminal of the SSK1 RR binds to the N-terminus of the MKKK SSK2 inducing a conformational change which induces its autophosphorylation and activation of the kinase cascade (Posas and Saito 1998). Therefore it is very important to identify and describe these proteins in their crosstalk points and this work contributes to this issue.

4.2 Results

4.2.1 Background

The TCS and MAPK signal transduction pathways are two differently evolved signalling pathways both crucial for mediating control of physiological responses and development in plants (Grefen and Harter 2004; Hass *et al.* 2004; Mira-Rodado *et al.* 2007; Pathak *et al.* 2013; Smekalova *et al.* 2013). It has been known that *ARR2* also functions in ethylene signalling as an *arr2 Ler-0* knock-out shows an hyposensitive phenotype (Hass *et al.* 2004). Transcriptomic data of *arr2 Ler-0* knock-out and *ARR2* overexpression lines revealed drastic mis-regulation of various defence-related genes some of which are well known to be controlled/activated by MAPK cascade signalling (Hass *et al.* 2004). Preliminary data suggested that ARR2 co-localises and interacts with several MAP kinase kinases in Y2H and *in vivo* BIFC (Achim Hahn, personal correspondence). These findings indicated that there might be a functional connection of ARR2-dependent TCS signalling and MAPK cascades which were further explored in this thesis.

4.2.2 Interaction of ARR2 with MAPK cascade members

4.2.2.1 Response regulators Type A or B do not interact with MKKKs

MKKKs were tested for protein-protein interaction against ARR2 using the yeast-two-hybrid system as an *in vivo* heterologous approach. The large number of MKKK proteins made it impossible to test all of them, but nevertheless, three representative members were chosen for this experiment: *Raf24, Raf43* and *Raf41* due to their distinct sequence and functions (Jouannic *et al.* 1999; Group 2002). Besides ARR2, two other members of type-B family were chosen, ARR1 and ARR18, and a well-known type-B family member, ARR4. ARR1 has very high sequence homology with ARR2 and it is proposed to be redundant to ARR2 in many physiological functions (Sakai *et al.* 2000). ARR18 on the other hand is different from its group members ARR1 and ARR2 in sequence, expression pattern and presumably function (Mason *et al.* 2004; Veerabagu *et al.* 2012) even though these three type-B response regulators are all members of the subgroup I. ARR4 was taken as a typical member of type-A group of response regulators to distinguish B-type and A-type characteristics, if there are any.

After cloning the ORFs into the yeast-two-hybrid expression vectors, yeast strain PJ69-4A (James *et al.* 1996) was transformed and colonies carrying the auxotrophic markers were selected on CSM –W-L media. MKKKs were cloned with the GAL4-BD fusion and ARRs were cloned as GAL4-AD fusions. None of tested MKKK showed transactivation in yeast (only shown for Raf41) indicating that all three MKKKs could be evaluated in yeast. Nevertheless, when the colonies were plated on interaction-selective media (-W, L, A), none of these MKKKs interacted with any of tested ARRs (Figure 4.2). This experiment was repeated three times using all interaction elements and each time the same result was obtained. Based on this experiment, if there is intercommunication between this two signaling pathways, then it is downstream of MKKKs or requires other MKKK elements.

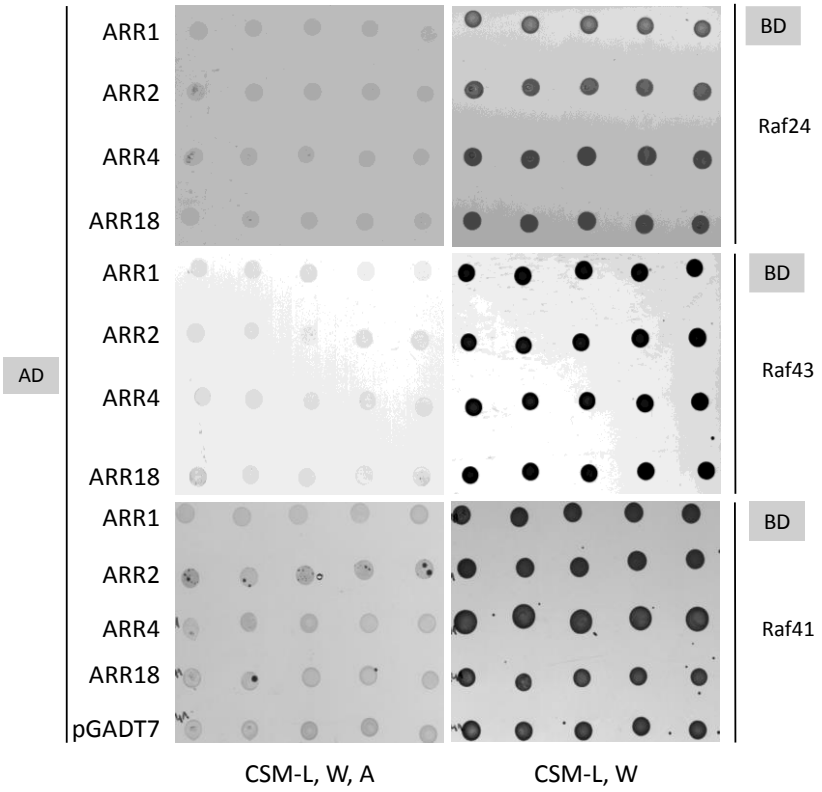


Figure 4.2 *Arabidopsis* response regulators do not interact with selected MKKKs in yeast-two-hybrid assays.

Proteins were expressed either as GAL4-AD-fusions (ARR1, ARR2, ARR4, ARR18) or as GAL4-BD-fusions (Raf24, Raf43, Raf41). Yeast cells were incubated for 4 days at 28°C on either vector selective (CSM-L, W) or interaction selective (CSM-L, W, A) media. The free AD domain was expressed along with BD-fusions for transactivation controls; only Raf41 is shown. For all interactions PJ96-4A was used. This experiment was repeated at least three times with similar results.

4.2.2.2 B-Type response regulators interact with MKK members

MKKs represent a point of signal convergence within MAPK signalling cascade (Mordret 1993). After cloning the ORFs into the yeast-two-hybrid expression vectors, yeast strain PJ69-4A was transformed and colonies carrying the auxotrophic markers were selected on CSM-W, L medium. MKK were cloned with a GAL4-BD fusion and ARRs were cloned as GAL4-AD fusions. As before ARR1, 2, 4 and 18 were tested against MKKs: MKK1, MKK2, MKK4, MKK5. MKK3 is transitive in yeast and cannot be analyzed as a full-length protein and therefore was not tested (not shown). None of other tested MKK constructs showed transactivation in yeast (Figure 4.3) indicating that all three MKKs could be evaluated in yeast. Interaction was observed for ARR1, 2 and 18 with MKK1, MKK2, MKK4, MKK5, whereas no interaction was observed for ARR4 with any MKK tested (Figure 4.3).

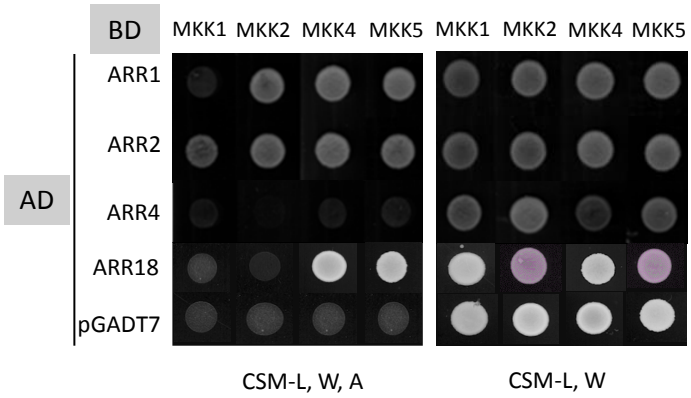


Figure 4.3 Type-B *Arabidopsis* response regulators interacted with MKKs in yeast-two-hybrid assays.

Proteins were expressed either as GAL4-AD-fusions (ARR1, ARR2, ARR4, ARR18) or as GAL4-BD-fusions (MKK1, MKK2, MKK4 and MKK5). Yeast cells were incubated for 4 days at 28°C on either vector selective (CSM-L, W) or interaction selective (CSM-L, W, A) media. The free AD domain was expressed along with BD-fusions for transactivation controls. For all interactions PJ96-4A was used. This experiment was repeated at least three times with similar results; representative colonies are shown for simplification.

4.2.2.3 ARR2 response regulator interacts also with MPKs

As the last members of MAPK signalling cascades, MPKs were also tested against ARR2. For this experiment MPK4 and MPK17 were chosen as they both have been described in the literature and have distinct evolutionally histories (Group 2002).

After cloning the ORFs into the yeast-two-hybrid expression vectors, yeast strain PJ69-4A was transformed and colonies carrying the auxotrophic markers were selected on CSM –W-L media. MPKs were cloned with a GAL4-BD fusion and ARRs were cloned as GAL4-AD fusions. ARR1, 2, 4 and 18 were tested against MPK4 and MPK17 (Figure 4.4). Neither MPK showed transactivation in yeast indicating that each MPKs could be evaluated in yeast (data for MPK17 shown).

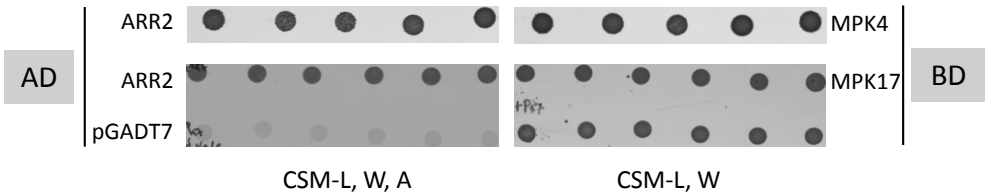


Figure 4.4 ARR2 interacted with MPKs in yeast-two-hybrid assays.

Proteins were expressed either as GAL4-AD-fusions (ARR2) or as GAL4-BD-fusions (MPK4 and MPK17). Yeast cells were incubated for 4 days at 28°C on either vector selective (CSM-L, W) or interaction selective (CSM-L, W, A) media. The free AD domain was expressed along with BD-fusions for transactivation controls; only MPK17 is shown. For all interactions PJ96-4A was used. This experiment was repeated at least three times with similar results.

Both MPK4 and MPK17 showed interaction with ARR2. It is known that MKKs and MPKs interactions can be observed in yeast. Therefore, the MKK1/2/4/5 were tested against the two MPKs, MPK4 and MPK17. Interestingly, only MPK17 interacted with MKK5. MPK4 interacted with all four MKKs but a stronger interaction was observed with MKK4 and MKK5, especially with a preference for with MKK5 (Figure 4.5).

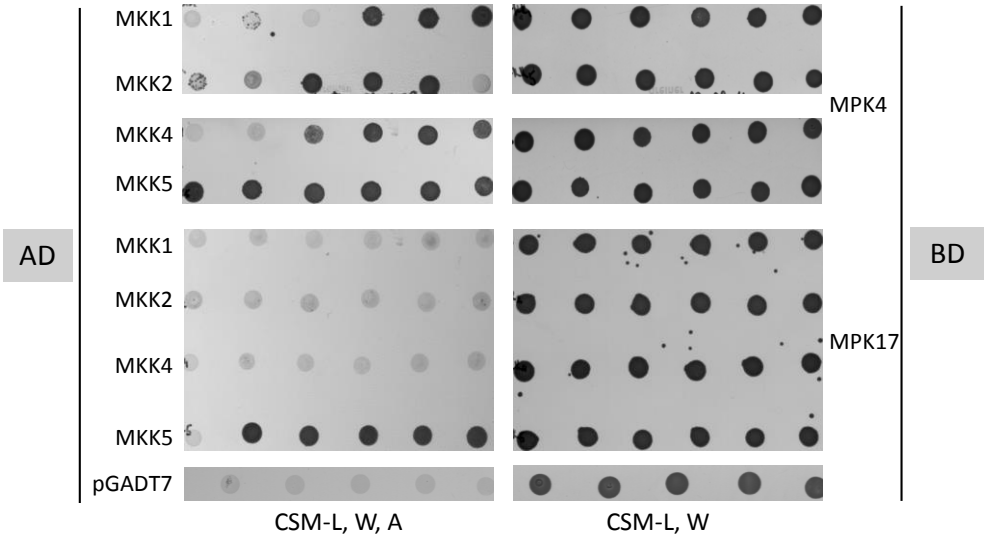


Figure 4.5 MPK4 and MPK17 interact with biotic stress-related MKKs in yeast-two-hybrid assays.

Proteins were expressed either as GAL4-AD-fusions (MKK1, MKK2, MKK4 and MKK5) or as GAL4-BD-fusions (MPK4 and MPK17). Yeast cells were incubated for 4 days at 28°C on either vector selective (CSM-L, W) or interaction selective (CSM-L, W, A) media. The free AD domain was expressed along with BD-fusions for transactivation controls; only MPK4 is shown. For all interactions PJ96-4A was used. This experiment was repeated at least three times with similar results.

4.2.2.4 MKK4 and MKK5 interacted with truncated versions of ARR2 containing only receiver or output domains

In order to get a closer look at which regions of ARR2 are essential for ARR2-MKK interactions, *ARR2* was truncated (Figure 4.6, C). Protein truncation $ARR2^{1-300}$ contains receiver domain, part of the DNA-binding GARP domain and two out of three nuclear localisation signals (NLSs) present in ARR2. $ARR2^{300-664}$ carries the other half of the DNA-binding GARP domain and the third NLS motif. $ARR2^{1-145}$ contains a receiver domain only. $ARR2^{145-664}$ contains the output domain with all three NLSs. $ARR2^{1-165}$ contains the receiver domain and one of three NLSs while the part. $ARR2^{165-664}$ contains other two NLSs and the output domain. This information is summarized in Figure 4.6C as a graphic representation of the ARR2 protein domains, the location of MKK docking sites and the truncation positions.

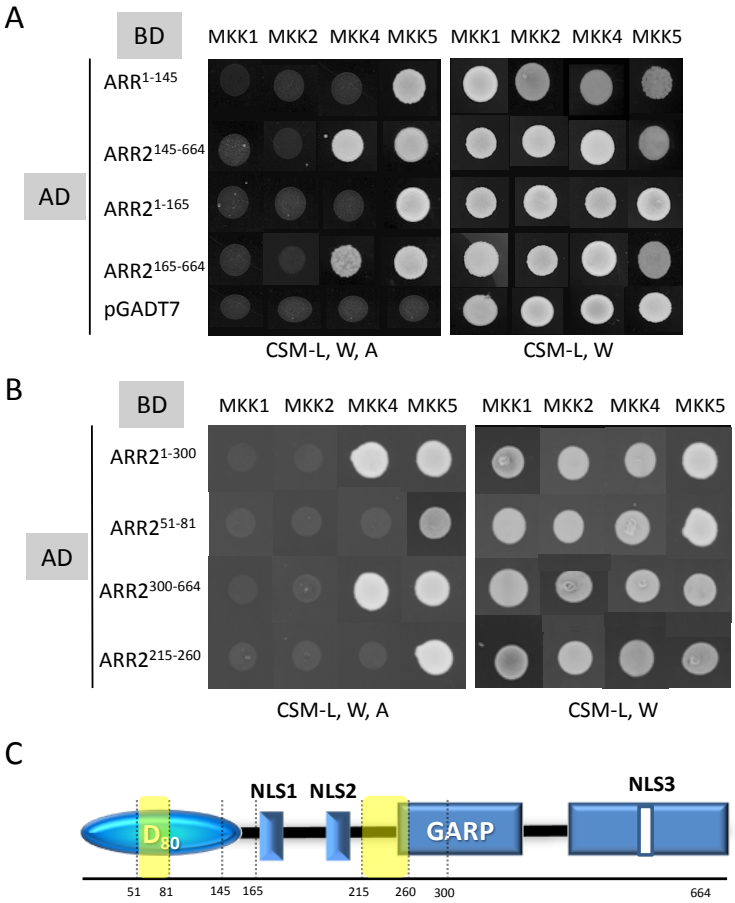


Figure 4.6 MKK docking motifs are present on both receiver and output domains in ARR2. **(A, B)** Proteins were expressed either as GAL4-AD-fusions ($ARR2^{1-145}$, $ARR2^{145-664}$, $ARR2^{1-165}$, $ARR2^{165-664}$, $ARR2^{1-300}$, $ARR2^{300-664}$, $ARR2^{51-81}$ and $ARR2^{215-260}$) or as GAL4-BD-fusions (MKK1, MKK2, MKK3, MKK4 and MKK5). **(C)** Cartoon presentation of the location of the truncated parts of ARR2 with MKK binding sites indicated in yellow. Yeast cells were incubated for 4 days at 28°C on either vector selective (CSM-L, W) or interaction selective (CSM-L, W, A) media. The free AD domain was expressed along with BD-fusions for transactivation controls shown in A. For all interactions PJ96-4A was used. This experiment was repeated at least three times with similar results; representative colonies are shown.

The various ARR2 truncations were tested against MKK1, MKK2, MKK4 and MKK5 for interaction using yeast-two-hybrid system (Figure 4.6, A,B). The receiver domain-containing truncations $ARR2^{1-145}$, $ARR2^{1-165}$ and $ARR2^{1-300}$ extended variant showed positive interaction only with MKK5; for all other tested kinase members no interaction was observed. On the other hand, the truncation containing the output domain $ARR2^{145-664}$, $ARR2^{165-664}$ and the $ARR2^{300-664}$ truncation showed very strong interaction with MKK4 and MKK5, but not with MKK1 or MKK2. These results suggested that there are a couple of different MKK docking sites within ARR2 that are widely dispersed along the protein. It is therefore possible that more than one, or maybe all, binding sites are needed. In summary, the type-B receiver domain and output domain can interact with MKKs.

4.2.2.5 MKK docking motifs are present on ARR2 in both receiver and output domains

MKK4 and MKK5 play an important role in the plant´s response to biotic stress, such as pathogen attack (Asai *et al.* 2002) and stomata development (Wang *et al.* 2007). The interaction of ARR2 with MKK4 and MKK suggested it would be useful to find out more about the number and position of MKK docking sites in ARR2 by applying an *in silco* approach. For this purpose ELM software (http://elm.eu.org) was used. ELM is a computational biology resource for investigating candidate functional sites in eukaryotic proteins reported to have high significance value (Dinkel *et al.* 2012). The docking interaction in the MAP kinase cascades is achieved through specific conserved regions on MKKs (docking groove) and MAPK-interacting molecules (the MKK docking motif). After evaluation of the raw output from ELM (Supplemental figure 1), docking motifs for MAPK cascade interacting molecules (e.g. MKKs, substrates, phosphatases) were predicted. There are at least two different MKK docking types: the classical docking motif and short peptide containing the sequence FxF (Sharrocks *et al.* 2000; Bardwell *et al.* 2001; Galanis *et al.* 2001). The classical docking motif is characterized by a cluster of at least two positively charged amino acids followed by a spacer of 2-to-6 residues from a hydrophobic-X-hydrophobic sequence, where the hydrophobic residues are long-chain aliphatics (usually Leu or Ile).

In the spacer and in the sequence immediately C-terminal to the hydrophobic-X-hydrophobic element, there is a high propensity for the presence of Pro, Asn, and/or Gly, which are residues that are both turn-forming and helix-breaking (Bardwell *et al.* 2001). The classic motif approximates (R/K)X_{2-6}ΨXΨ where Ψ is a hydrophobic residue and X any residue. Four such docking motifs were found in ARR2 (Supplemental figure 1, figure 4.6). Interestingly, two of them were found on the receiver domain of the ARR2 at 56-66 (KCNRAESALSL) and 68-78 (RKNKNGFDIVI) amino acid positions. Two other potential docking domains were located in the output domain of ARR2 at amino acid positions 215-225 (KKPRVVWSVEL) and 244-252 (KKILEMMNV) (Figure 4.6, C). In order to check if these motifs are really docking sites for MKKs two truncation peptides of ARR2 to were made: $ARR2^{51-81}$ and $ARR2^{215-260}$. Only two fragments were made because of the fact that between the potential receiver domain docking sites only a very small physical distance lies between them and such small peptides would most likely be degraded in yeast. Surprisingly, both peptides $ARR2^{51-81}$ and $ARR2^{215-260}$ interacted only with MKK5 and that very strongly; No interactions were obtained with the other MKKs (Figure 4.6, B). This suggests that for all of the other MKKs other docking sites must be present which do not fit into the typical pattern for MKK docking domains, or, an alternative type of interaction domain exists. These experiments confirmed the previous observations in yeast, where truncated versions of ARR2 containing only receiver domain sequence were used, that receiver domain may also play role in ARR2-MKKs interactions and not just the output domain only. Thus it can be concluded that the inability of the ARR4 type-A RR to interact with MKKs is not due to an absence of output domain but rather due to difference in sequence of receiver domains between type-A and the types-B ARRs.

4.2.2.6 *ARR2 does not need to be phosphorylated by TCS elements in order to interact with MKKs in Y2H*

The ARR2 can obtain a phosphoryl group from the AHP TCS elements on its aspartate at the 80th position (Grefen and Harter 2004). Mutation of D80 to E (glutamate, Glu) increases the transactivation activity of ARR2 (Hwang and Sheen 2001) and leads to severe developmental defects if expressed in plants (Hass *et al.* 2004). Mutation of D80 to N (asparagine, Asn) however, prevents TCS dependent phosphorylation (Kim *et al.* 2006) and impairs basal reporter-gene activation capacity. To check if the phosphorylation state of ARR2 at the D80 position influences MPK(K)-ARR2 protein interaction loss-of-function ARR2D80N mutant and gain of function ARRD80E protein variants were tested in the yeast-two-hybrid system.

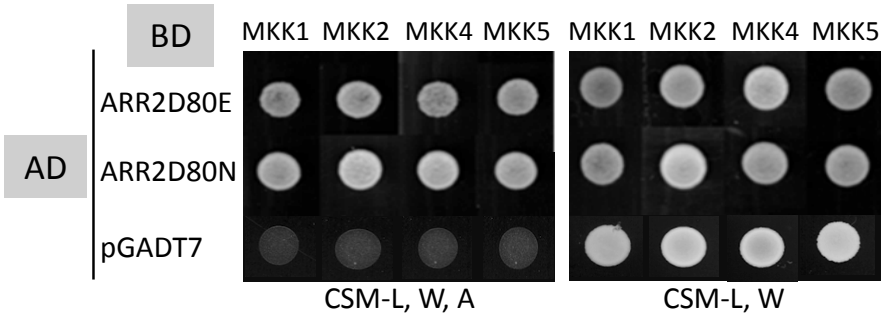


Figure 4.7 ARR2 interacts with the MKKs independent of TCS-mediated phosphorylation. Proteins were expressed either as GAL4-AD-fusions (ARR2D80E and ARR2D80N) or as GAL4-BD-fusions (MKK1, MKK2, MKK4 and MKK5). Yeast cells were incubated for 4 days at 28°C on either vector selective (CSM-L, W) or interaction selective (CSM-L, W, A) media. The free AD domain was expressed along with BD-fusions for transactivation controls. For all interactions PJ96-4A was used. This experiment was repeated at least three times with similar results.

MKK1-5 were used for this experiment. Both mutant versions of ARR2 showed exactly the same pattern like the wild-type protein (Figure 4.7). This suggests that ARR2 does not need to be phosphorylated by TCS elements for successful interaction with MKKs and that ARR2 might serve as scaffold protein in signal transduction between MAPK cascade elements and the TCS or yet other unknown, undescribed proteins.

4.2.2.7 *ARR2 shows very strong interaction with biotic-stress-related MKKs in yeast*

MKK1 and MKK2 are known to be involved in response to abiotic stress (*e.g.* osmotic stress, temperature change, high salinity) (Teige *et al.* 2004) whereas MKK4 and MKK5 are known for their role in biotic stress (*e.g.* pathogen interactions) (Asai *et al.* 2002). In the previous yeast experiments some interactions appeared stronger as judged by both the growth intensity of the colonies and the number of colonies that displayed positive interactions. In particular, MKK4 and MKK5 repeatedly displayed stronger interactions with ARR2, its variants and other B-types. Therefore, the interaction strength of the MKK interactions with ARR2 was quantified in yeast using serial dilutions and quantification of reporter-gene strength by the O-NPG assay.

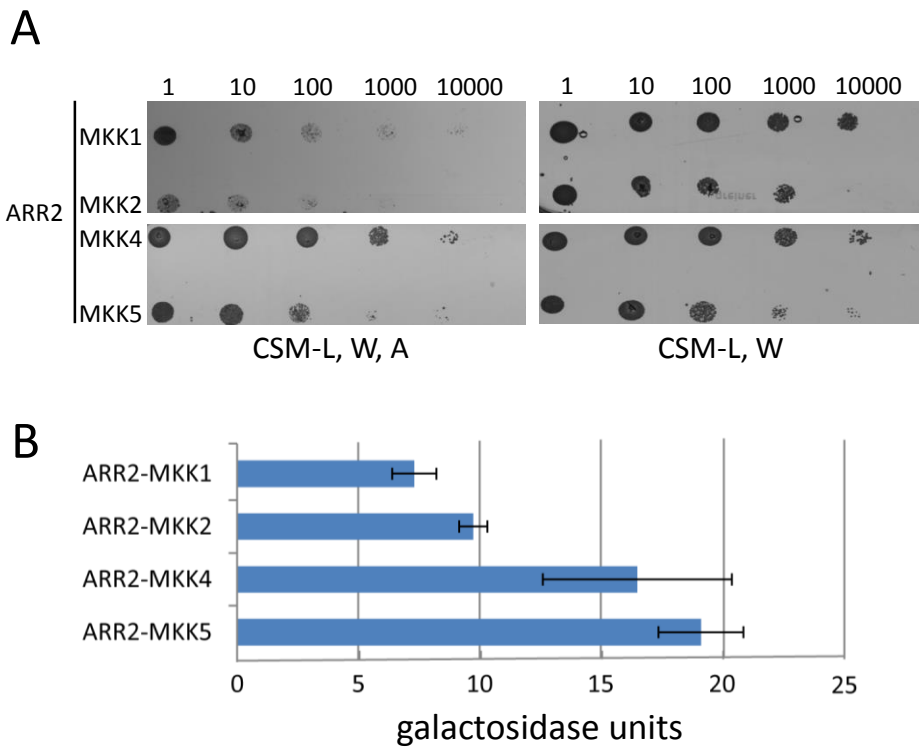


Figure 4.8 ARR2 shows strong interaction with biotic-stress-related MKKs in yeast.
(A) Dilution series of fusion proteins co-expressed from the yeast-two-hybrid expression vectors: GAL4AD-ARR2 and MKK1, MKK2, MKK4 and MKK5 fused to N-terminal GAL4BD. **(B)** The β-galactosidase assay of the same constructs used for dilution series. The β-galactosidase activity was measured in the extracts of three independent yeast clones. Diluted yeast colonies were incubated for 2 days at 28°C on interaction selective (CSM-L, W, A) media. The experiment was repeated at least two times.

Serial dilutions (1, $1:10^1$, $1:10^2$, $1:10^3$ and $1:10^4$) of the corresponding yeast culture was grown on selective medium and transformed PJ64-4A yeast cells with corresponding vectors were used for the O-NPG assay (β-galactosidase assay). Five colonies containing ARR2 and MKK1/2/4/5 were tested. As suspected, ARR2 interacted much stronger with biotic-related MKKs (MKK4 and MKK5) than the abiotic-related ones (MKK1 and MKK2) by the growth assay (Figure 4.8).

While the serial dilution method is informative, the more quantitative and less error-prone method is measuring β-galactosidase reporter-gene activity *via* the O-NPG assay. In this case, the O-NPG assay was extended to include ARR1 and ARR18. As before, ARR2 interacted stronger with MKK4/5 (~17 and ~20 O-NPG units for MKK4 and MKK5 respectively) than MKK1/2 (~5 and ~7 O-NPG units for MKK1 and MKK2 respectively). The same tendency was observed for ARR1 and ARR18 (Supplemental figure 2). It can be therefore concluded that. in general, MKK4 and MKK5 have a stronger affinity for B-type subclass I response regulators.

4.2.2.8 ARR2 shows very strong interaction with biotic-stress-related MKKs in planta

In order to see if this strong and specific interaction between ARR2 and MKK4 and MKK5 obtained in yeast is also the case in plant cells, FRET-FLIM (Förster Resonance Energy Transfer-Fluorescence Life Time Imaging) microscopy analysis was performed *in planta*. For this experiment the fluorescence lifetime of a Donor (i.e. GFP) is measured. A reduction in the lifetime is observed when an Acceptor (i.e. mCherry or mRFP) is within a distance of 10 Å or less; this distance is what is expected of protein-protein interactions (Caesar *et al.* 2011). Since MKK3 is also known to be involved in biotic stress in a JA dependent manner (Doczi *et al.* 2007) and was previously not characterized in yeast due to its strong transactivation effect, MKK3 was also tested against ARR2 *in planta* using the FRET-FLIM approach.

ARR2 was cloned in-frame with *GFP* to make an *ARR2::GFP* fusion. The *MKKs* were therefore cloned in-frame with *mCherry*. A positive readout control was created by fusing *ARR2* to a tandem *GFP::mCherry* coding frame via a short linker and subsequently called *ARR2::FRET*. After cloning, the constructs were transformed into *Agrobacteria tumefaciens* and transiently expressed in tobacco (*Nicothiana bethamiana*) epidermal cells as a heterologous plant system. The negative control was *ARR2::GFP* transformed alone.

All three tested MKKs interacted with ARR2 (Figure 4.9). Both MKK4 and MKK5 interacted with ARR2 stronger than MKK3, with MKK5 having the strongest interaction with ARR2 (Figure 4.9, B). These *in planta* data confirmed the previous data obtained in the yeast-two hybrid system and lead to more stable and concrete evidence that these interactions, especially ones with MKK4 and MKK5, might be have physiologically functional roles in *Arabidopsis* to biotic stresses.

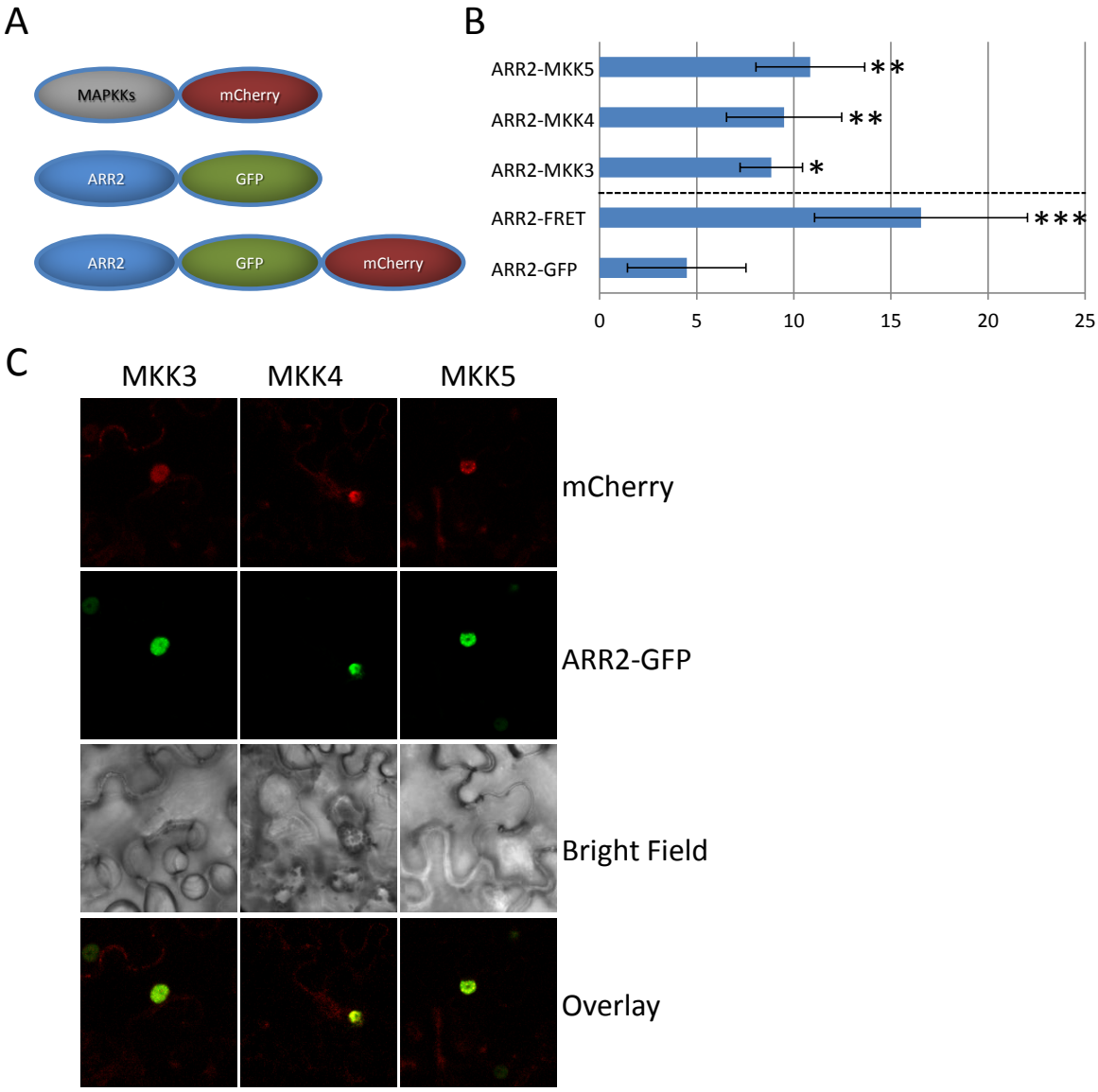


Figure 4.9 ARR2 interacts very strongly with biotic-stress-related MKKs *in planta*.
(A) Cartoon representation of ARR2 and MKKs proteins fused for fluorescent proteins for FRET-FLIM measurements. **(B)** FRET-FLIM results of ARR2-GFP interaction with MKK3, MMK4 and MKK5 fused to mCherry. **(C)** CLSM photos of tobacco leaves showing localization of expressed ARR2-GFP fusion protein with indicated MKKs fused to mCherry fluorescent protein. The fusion proteins were transiently expressed in tobacco cells and the results are given as function of Chi^2 test (measurements and evaluation courtesy of Dr. Kirstin Elgass). ARR2-GFP and ARR2-GFP-mCherry were used as negative and positive controls respectively. This experiment was repeated two times with similar results.

4.2.3 Roles of *ARR2* in pathogen-related phenomenon

As previously shown, FRET-FLIM *in planta* measurements and experiments using the yeast-two-hybrid system connect TCS type-B RRs to MKKs *via* physical interaction. ARR2 especially interacted strongly with MKK3, MKK4 and MKK5. Additionally, ARR1 and ARR2 interacted with MPK4 and MPK17 as well. MKKs play an important role in the plant´s response to biotic stress, such as pathogen attack (Asai *et al.* 2002). And interestingly, this is also the case for all three *in planta* tested MKKs: MKK4 and MKK5 (Nakagami *et al.* 2005; Popescu *et al.* 2009) as well as MKK3 (Doczi *et al.* 2007) clearly implicating the involvement of *ARR1* and *ARR2* in pathogen defence.

4.2.3.1 Reactive Oxygen Species (ROS) and ethylene measurements in ARR1 and ARR2 mutants after treatment with Pathogen-Associated Molecular Patterns (PAMPs)

As a first look into pathogen-related responses in ARR2, wild-type and mutants *arr1-4, arr2-4* and *arr1-4 arr2-4* lines were treated with PAMPs. As output, ROS and ethylene production were measured.

4.2.3.1.1 *The arr1-4 arr2-4* double mutant showed differences in ROS production after treatment with flg22 and elf18

For ROS experiments, leaves from 5 to 6 week-old plants were used. Leaves were cut in discs from the same leaf surface and incubated in water overnight.

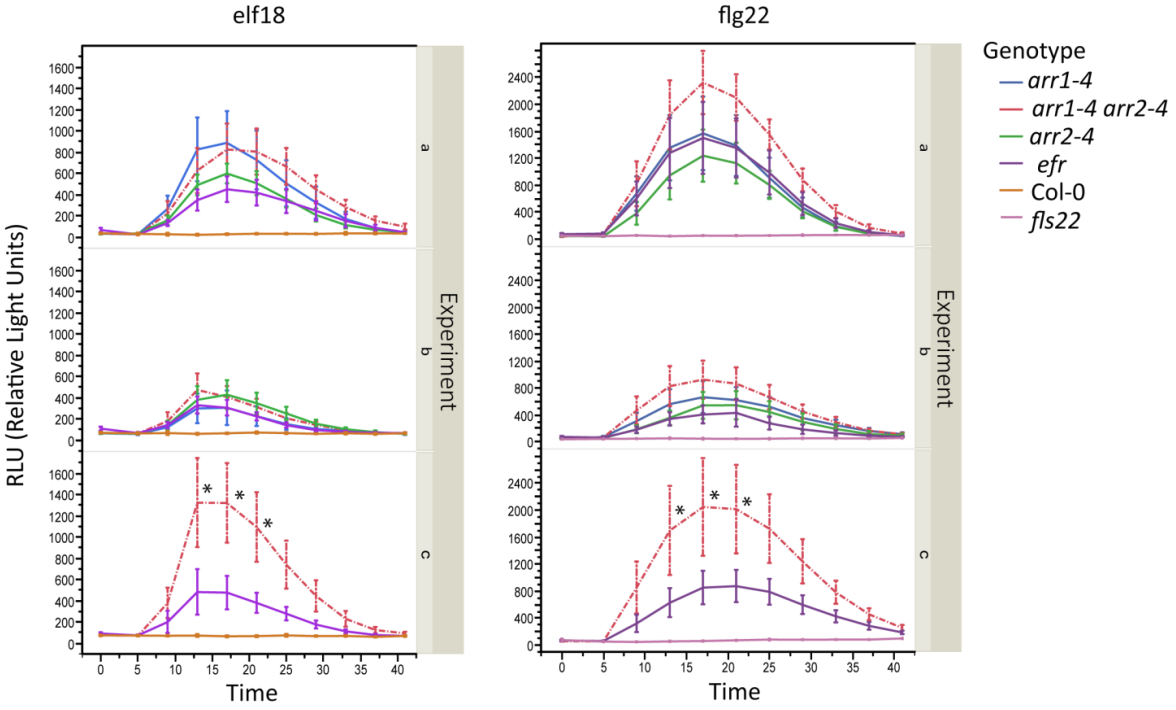


Figure 4.10 *arr1-4 arr2-4* double mutant shows differences in ROS production after treatment with PAMPs.

Arabidopsis leaves from different mutants were treated with elf18 (left) and flg22 (right). Mutants *efr* and *fls22* were used as negative response controls. Letters a, b and c stand for biological experimental repetitions whereas in the c repetition only the *arr1-4 arr2-4* double mutant was used along with control plants. Fisher's Least Significant Difference (LSD) test was used to compute significance levels at the 0.05 α level separately for the time points 13, 17 and 21 minutes.

The following day, the discs were transferred to a reaction solution and the ROS production was triggered by treatment with 100nM of flg22 or 100nM elf18. In addition to the type-B mutant lines, *fls2* (Dunning *et al.* 2007; Krol *et al.* 2010) and *efr* mutants lacking leucine rich repeat-receptor-like kinases *FLS2 (Flagellin-Sensitive 2)* and *EFR (Elongation Factor-Tu receptor*), respectively (Krol *et al.* 2010). FLS2 recognizes bacterial flagellum (active epitope flg22) and EFR recognizes elongation factor Tu (active epitopes elf 18, elf13 and elf26).

Both times the same tendency was observed: *arr1-4 arr2-4* double mutant significantly produced more ROS than the wild-type ($p \leq 0,05$ at time-points 13, 17, 21 min post treatment) in response to both applied PAMPs. Although there were differences between the single mutants, these observations were not consistent between experiments; the double mutant consistently however showed more ROS than the wild-type (Figure4.10, experiment a verses b). Therefore, a third experiment was conducted using more replicates to confirm the previous two experiments. It could be concluded that the *arr1-4 arr2-4* double mutant significantly produces more ROS in response to applied PAMPs, flg22 ($p \leq 0,05$ at time points 13, 17, 21 min post treatment) and elf18 ($p \leq 0,05$ at time-points 13, 17, 21 min post treatment) but it is unclear what the effect of the single mutants have on ROS production (Figure 4.10).

4.2.3.1.2 *The arr1-4 arr2-4* double mutant did not show any difference in ethylene production after treatment with different PAMPs

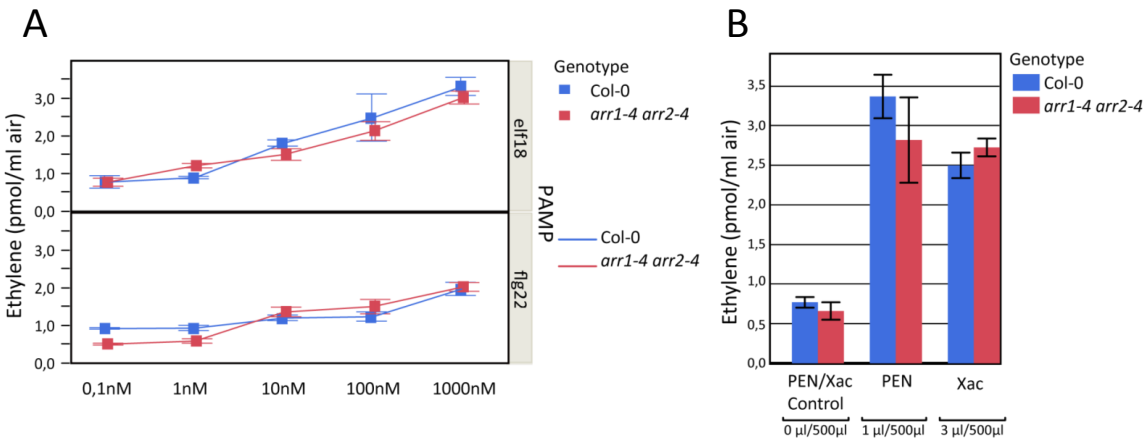


Figure 4.11 *arr1-4 arr2-4* double mutant shows no difference in ethylene production after treatment with PAMPs

Arabidopsis leaves from *arr1-4 arr2-4* double mutant and Col-0 wild-type were treated with **(A)** elf18 and flg22 using different concentrations: 0nM, 1nM, 1nM, 10nM, 100nM and 1000nM **(B)** Fungal extracts used were 1µl of PEN and 3µl of Xac in a final volume of 500µl. Fisher's Least Significant Difference (LSD) test was used to compute significance levels at the 0,05 α level. This experiment was performed as two biological replicates each containing three technical replicates.

Ethylene production was measured as a response to different PAMPs flg22 and elf18 or crude extracts PEN from *Penicilium schizogonium* and Xac from *Xanthomonas axonopodis citri*. A dose response curve was obtained for Flg22 and Elf18 (0.1nM, 1nM, 10nM, 100nM, 1µM) but only single concentrations 1µL/500µL PEN and Xac 3µL/500µL for the crude extracts was

conducted. Based on results from obtained the previous ROS measurements and due to lack of growth space, only Col-0 wild-type and *arr1-4 arr2-4* double mutant lines were used. The double mutant did not show any statistically significant difference in ethylene production to any of the tested PAMPs compared to the wild-type (Figure 4.11, A, B). Based on this experiment, it can be concluded that either there is no significant difference in ethylene production. Alternatively, perhaps the analytical system was not sensitive enough to detect subtler changes in ethylene production compared to that of the ROS system.

4.2.3.1.3 *The arr1-4 arr2-4* double mutant did not show any difference in activation pattern of MPK3, MPK4 and MPK6 after treatment with flg22

The MPK kinases MPK3, MPK4 and MPK6 are known to be activated by phosphorylation in a flg22 dependent manner (Droillard *et al.* 2004). Activation of these MPKs can be analysed by using the phospho-p44/p42 MPK antibody (Brock *et al.* 2010; Montillet *et al.* 2013). As previously observed, flg22 was able to elicit a higher ROS response in the *arr1-4 arr2-4* mutant background (§4.2.3.1.1). Thus by conclusion, it is possible that these MPK kinases are differentially activated in the *arr1-4 arr2-4* mutant background compared to the wild-type. For this experiment 4 week-old plants were used and their leaves were infected by dropping of 1µM solution of flg22 or mock onto a leaf surface. Samples were collected at three time points: immediately after infection, 15 minutes and 30 minutes after treatment (Figure 4.12).

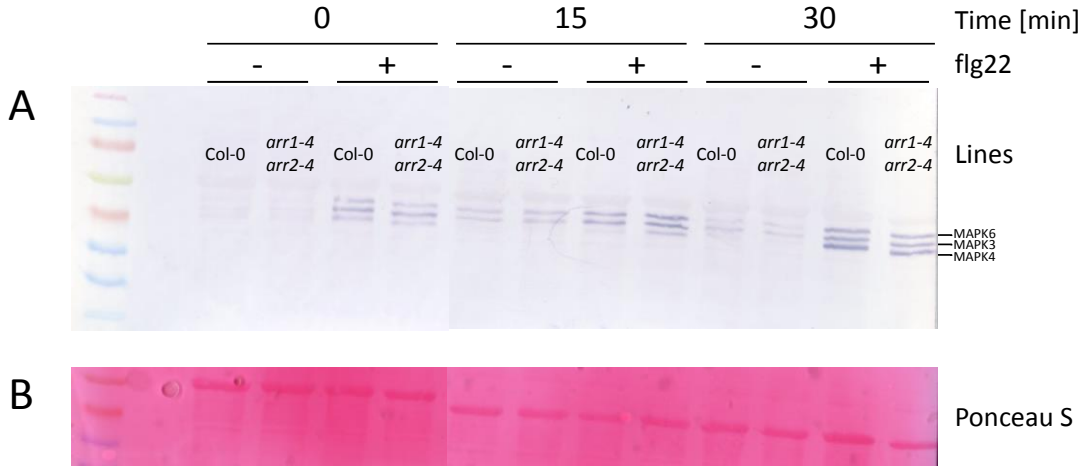


Figure 4.12 No differences in MPK3/4/6 phosphorylation pattern induced by flg22 elicitor are observed between *arr1-4 arr2-4* double mutant and Col-0 wild-type.
(A) Western blot of protein extracts from *Arabidopsis* leaves taken from *arr1-4 arr2-4* double mutant and Col-0 treated with flg22. The phospho-p44/p42 MPK Antibody was used to detect phosphorylated MPK3/4/6. **(B)** Membrane stained with Ponceau S shows equal loading quantity for all samples. PAMP flg22 was used in 1µM concentration and the samples were collected at 0 min, 15min, and 30 min after treatment. At least five different plants per line were used and two leaves per plant were treated with flg22 or MOCK and the material pooled. This experiment was repeated three times with similar results.

Before flg22 treatment only weak signals were obtained for MPK6 and MPK3 showing that these two kinases are phosphorylated at some basal level. After 15 minutes of flg22 treatment, the signals for MPK3/6 became much stronger and a third band representing MPK4 appeared (Figure 4.12). Half an hour after infection the phosphorylation pattern stayed

the same as the 15 minute time point, indicating that the peak flg22-depdenent MPK activation level was already reached at the 15th minute or before. Mock treated leaves at 15 and 30 minutes after treatment maintained the same basal MPK3/6 pattering as the zero time point. In conclusion, no dramatic differences were observed between the wild-type and the double mutant in activation pattern or intensity with any of these three kinases.

4.2.3.2 Pathogen assays using biotrophs and necrotrophs

A broad spectrum of different pathogens was used in this work to cover both necrotrophic and biotrophic types of infection strategies. These pathogens were challenged on Col-0, *arr1-4, arr2-4* and *arr1-4 arr2-4* plant lines. It was very important to obtain various measurements of a given interaction with high spatial and temporal resolution. For necrotrophs, *Alternaria brasisicola* and *Botrytis cinerea* were tested. For biotrophs, the obligate biotrophic fungus *Peronospora parasitica* was tested as well as the biotrophic/hemibiotrophic bacterium *Pseudomonas syringae pv. tomato* DC3000 *(Pto* DC3000*).*

4.2.3.2.1 Pathogen assays using biotrophs

Pathogen assays with biotrophs were always performed using one bacterial and one fungal pathogen: the bacterium *Pto* DC3000 and fungus *Peronospora parasitica*.

4.2.3.2.1.1 There is no difference in response of ARR1 and ARR2 mutants to biotrophic Pst DC3000

This pathogenic assay was done using five-week old plants and treating them with *Pto* DC3000 bacterial strain. Bacterial cells were infiltrated into two leaves per plant. Bacterial cells at an approximate concentration of 10^4 cfu/ml were infiltrated with a needless syringe into the middle of the leaf apoplastic space (two leaves per plant) to an area was always bigger than the cork borer used for cutting the leaf discs with which the experiment was further performed. Afterwards the leaves were harvested, at the time point 0h and 1st, 2nd and 4th days past infection, material was plated on LB-plates with antibiotics and number of colonies per plate/mutant formed was counted.

Based on number of grown colonies per plate the difference in resistance between the mutants was determined. Although this experiment was repeated six times none of them consistently showed any pattern that could be attributed to the loss of *ARR1* or *ARR2* or both. That is, any differences that could be observed in the second and fourth day were never reproduced in any other experiment (all repetitions are not shown but compare 4.13A with the 4.13B mock for an example.

Knowing that ARR2 has been implemented in *Pto* DC3000 pathogen resistance and that this response required both cytokinin and SA (Choi *et al.* 2010), a cytokinin treatment was added to the experimental design. *t*-zeatin was used at a working concentration of 1µM. Before infiltration cytokinin was given to the 1x10^4 cfu/ml bacterial dilution. The plants were also treated with cytokinin 3 days before the experiment by adding it to the water used for watering. No clear trend could be observed that could be ascribed to a combined effect of exogenous cytokinin and loss of *ARR1* or *ARR2* could be found. In fact, in this experiment, the cytokinin treatment resulted in reduced bacterial growth in all plant lines which was highly

statistically significant by day 4 (Supplement figure 3). This effect of cytokinin on *Pto* DC3000 growth has been observed before (Choi *et al.* 2010; Choi *et al.* 2011).

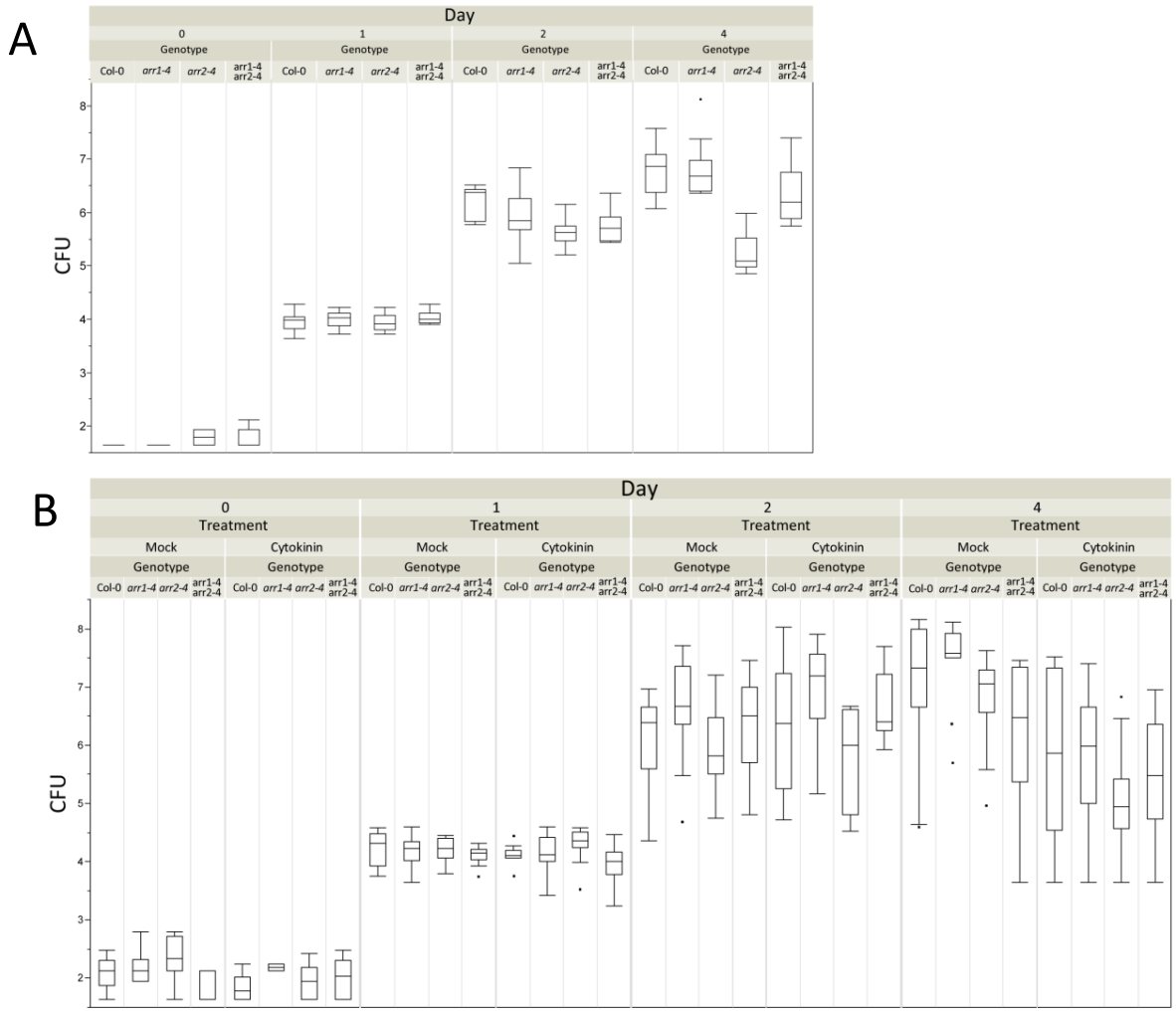


Figure 4.13 *ARR1* and *ARR2* mutant plants do not show reproducible differences in response to biotrophic *Pseudomonas syringae* pv. tomato DC3000.

Leaves of *Arabidopsis ARR1* and *ARR2* single and double mutants and corresponding wild-type (Col-0) were treated with *Pseudomonas*. The evaluation of infection was done at day 0, 1, and 4. **(A)** Plant lines treated only with *Pseudomonas syringae* pv. tomato DC3000. **(B)** Plant lines treated either with *Pseudomonas syringae pv. tomato* DC3000 only (Mock) and also with 1µM t-zeatin (cytokinin). Fisher's Least Significant Difference (LSD) test was used to compute significance levels at the 0.05 α level (Supplemental figure 3). These experiments were repeated at least three times each with similar results. CFU stands for Colony Forming Units. Data are represented as box-and-whisker plots: the central horizontal line is the median (2nd quartile) and the boxed region extends from the beginning of the 1st quartile to the end of the 3rd quartile. Lines extending outside of the boxed region are 1.5 x (the respective interquartile range). Data points outside this region are shown as dots and are called outliers.

4.2.3.2.1.2 There is no difference in response of ARR1 and ARR2 mutants to Peronospora parasitica

Next *arr1-4 arr2-4* plants were challenged with *Peronospora parasitica*. This experiment was performed in collaboration with the research group of Prof. Dr. Volker Lipka from University of Göttingen. Four to six-week old plants were inoculated by spraying the leaves with a *Peronospora parasitica* conidial suspension and the infected plants were observed after seven days.

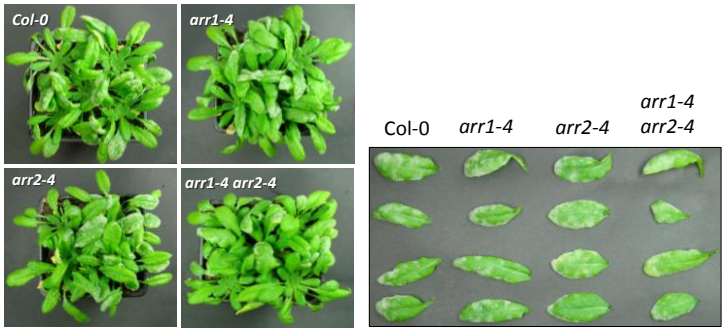


Figure 4.14 *ARR1* and *ARR2* mutant plants do not show differences in response to biotroph *Peronospora parasitica*.
Leaves of *Arabidopsis* wild-type (Col-0), *ARR1* and *ARR2* single and double mutants were sprayed with *Peronospora*. After seven days there were no visible differences among the mutant plants and the wild-type. This experiment was repeated once with similar results. Data were produced by the research group of Prof. Dr. Volker Lipka.

If there is any difference in resistance to *Peronospora*, a clear phenotypic difference would have been visible. However, since there was no visual difference obtained between the wild-type and the mutant plants there was not any need for counting of sporangiophores on the leaves (pers. communication V. Lipka). It was concluded that the mutant plants do not show any difference in resistance to *P. parasitica* compared to the wild-type (Figure 4.14).

4.2.3.2.1.3 Lack of differential response to biotrophs Pst DC 3000 and Peronospora parasitica is not due to perturbation of stomata aperture regulation

It was proposed that a larger stomata aperture would make it easier for bacteria to enter into the plant, infect cells and form a stabile microfilm in vascular bundles allowing it to further multiply and invade the plant. It is also known that cytokinin can cause stomata to open (Tanaka *et al.* 2006) and abscisic acid (ABA) causes them to close (Desikan *et al.* 2006). Initially, *Pst* DC3000 also causes stomata to close (Zeng *et al.* 2010; Desclos-Theveniau *et al.* 2012), however after 3 hrs, the bacteria produce diffusible phytotoxin COR that reopens closed stomata to increase the number of sites for bacterial invasion (Melotto *et al.* 2008). Therefore an experiment was designed to test this hypothesis.

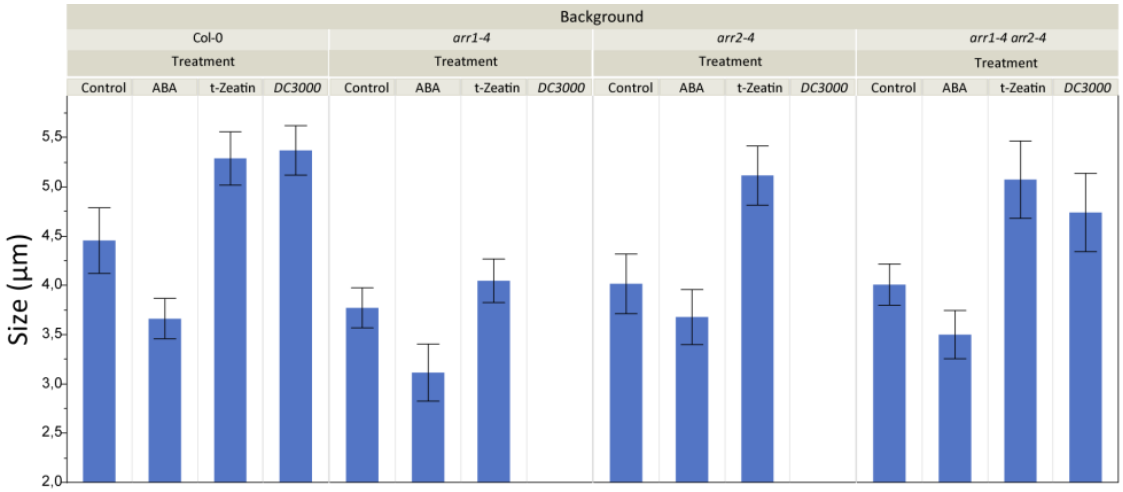


Figure 4.15 Regulation of stomata aperture in *ARR1* and *ARR2* lacking mutants in response to hormonal treatment with ABA and *t*-zeatin and *Pst DC 3000 Pseudomonas* strain

For treatments with hormones at least five plants per line were used and from those 2 leaves per plant were treated with the hormones. Experiments with *Pst DC 3000* were done only for *arr1-4 arr2-4* double mutant and Col-0 whereby five plants per line were used and the stomata aperture size was measured 3 days after infection. This experiment was repeated once with similar results. The mean and SE is shown. Leaves were floated on water or water containing 10µM ABA or 1µM *t*-zeatin (see §3.6.11 for details). For statistical results see Supplemental Figure 4.

Leaves were treated with water (Control), ABA, *t*-zeatin for 2 hours or three days after *Pst* DC3000 infection. The results are displayed as bar graphs (Figure 4.15) and the significance tests results are given in Supplemental Figure 4. Importantly for this work, it is known that ABA-dependent stomata closure is not dependent on ARR2, as the *arr2-4* responds like wild-type (Desikan *et al.* 2006). This response was replicated; that is, the stomata apertures were significantly closed in response to ABA treatment. On the contrary, treatment with *t*-zeatin resulted in a significant increase in stomata aperture for all plant lines. Plants sprayed with *Pst* DC3000 also had stomata apertures wider than the non-treated controls (data only for Col-0 and *arr1-4 arr2-4* were obtained). The data showed that there was no difference in stomata aperture response in the mutant backgrounds compared to the wild-type. Furthermore, all plant lines responded the same way after treatment with different hormones and after application of *Pst* DC3000 by spraying (Figure 4.15).

In conclusion, based on the preceding pathogen experiments and this stomata aperture measurement, there is no difference in the resistance of *arr1-4, arr2-4* or *arr1-4 arr2-4* plants to *Pseudomonas syringae Pst DC3000* under my experimental conditions.

4.2.3.2.2 Pathogen assays using necrotrophs

Two necrotrophic fungi *Alternaria brassicola* and *Botrytis cinerea* were challenged with Col-0 wild-type and the *arr1/2 mutants*. Resistance against *Alternaria* requires the *Arabidopsis* phytoalexin, camalexin and jasmonic acid (JA)-dependent signalling pathways, respectively (Van Wees *et al.* 2003). *Botrytis cinerea* is a natural necrotrophic pathogen of *Arabidopsis thaliana* (P *et al.* 2007) and is considered as a typical necrotroph that co-opts programmed cell death pathways in the host to achieve infection (Amselem *et al.* 2011; Dean *et al.* 2012).

4.2.3.2.2.1 arr2-4 single and arr1-4 arr2-4 double mutants are more susceptible to Alternaria brassicicola

Alternaria brassicicola was applied as a sporal suspension dropped (5µl droplets of spore solution of $5*10^5$ Spores/ml) onto *Arabidopsis* leaves (two leaves per plant) and scored on the 7^{th} and 10^{th} day after infection using a disease progression index as in (Kemmerling *et al.* 2007) and summarized in Methods (§3.6.16). Statistically significant differences were obtained between *arr2-4* single, and *arr1-4 arr2-4* double mutants compared to the WT (Col-0) were computed using the mean. Single *arr2-4* ($p \leq 0.001$) and the double *arr1-4 arr2-4* mutants ($p \leq 0.001$) showed statistically significant susceptibility compared to the wild-type where the difference in *arr1-4* single mutant ($p \leq 0.01$) was not statistically significant from wild-type (Figure 4.16, A).

The same experiment was therefore repeated in presence of 1 µM exogenous cytokinin, *t*-zeatin. Similar to the previous experiment with *Pst* DC3000, before the assay was performed, cytokinin was added to the fungal dilution. The plants were also treated with cytokinin by mixing it with water used for watering 3 days before the experiment was performed. Cytokinin induced some kind of resistance in all of the plants (Figure 4.16, B) and this resistance was statistically significant (see Supplementary Figures 5 and 6, Figure 4.16B). At the day 7 after treatment the disease index of cytokinin treated plants decreased from 270.8 by mock treated plants to 257.5 ($p \leq 0.0029$, LSD (Fisher's Least Squared Difference Test). The same tendency was observed 10^{th} day after treatment where the disease index of treated plants decreased from 306.0 to 294.4 ($p \leq 0.0066$ LSD (Fishers Least Squared Difference Test).

The *arr2-4* and the double mutant are more sensitive irrespective of the cytokinin treatment to *Alternaria* which was statistically confirmed (see Supplemental Figure 5 and 6). In contrast, wild-type plants and the *arr1-4* single mutant did not show differences in their disease indices. This means that *arr2-4* is responsible for susceptibility phenotype observed in the double mutant (Figure 4.16, B).

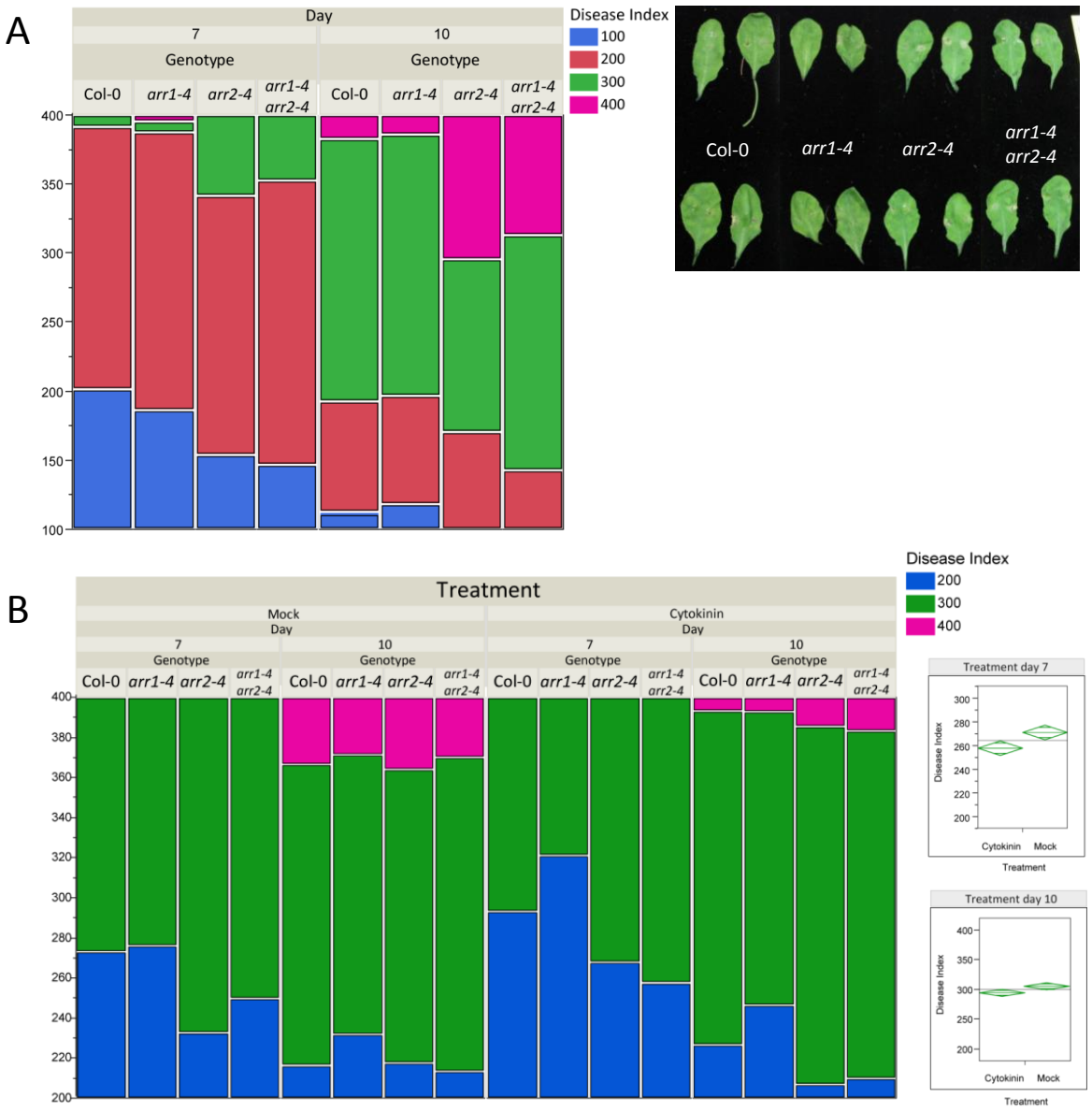


Figure 4.16 *Arabidopsis thaliana arr2-4* single and *arr1-4 arr2-4* double mutants are susceptible to necrotroph *Alternaria brassicicola*

Leaves of *arr1-4, arr2-4, arr1-4 arr2-4* and Col-0 were treated with *Alternaria* spores. The infection was evaluated at day 7 and 10. The data are shown as mosaic plots which are a graphical representation of an automatically computed two-way frequency table (also called a Contingency Table) (A) Plant lines only treated with *Alternaria brassicicola* spores. Image insert to the right shows a representative leaf composition at Day 7 (B) Plant lines treated either with *Alternaria brassicicola* spores only (Mock) and also with 1µM t-zeatin. To the right, the pooled mean and ANOVA 95% confidence interval for all samples either treated or not treated with cytokinin is shown for Day 7 (ANOVA $p \leq 0.0029$) and Day 10 (ANOVA $p \leq 0.0066$); the cytokinin treated samples showed less disease symptoms. LSD (Fisher's Least Squared Difference Test) was also used to compute significance levels at the $\alpha=0,05$ level based on the mean (Supplemental Figures 5 and 6). For this experiment at least 20 plants per line were used and two leaves per plant were infected with *Alternaria* spores. This experiment was repeated at least three times with similar results.

4.2.3.2.2.2 arr1-4 arr2-4 double mutant is resistant to Botrytis cinerea

Botrytis cinerea is especially interesting as it is known to be a natural pathogen of *Arabidopsis thaliana*. *Botrytis cinerea was* applied to the single mutants, double mutant and wild-type by dropping a fungal suspension on the leaves. Susceptibility to *B. cinerea* was determined using a detached leaf assay and a visually identified by a lesion disease index. Lesion size has been used as an indicator of susceptibility to *B. cinerea* (Ferrari *et al.* 2003; Denby *et al.* 2004) and this has been shown to correlate with whole plant susceptibility and pathogen growth within the plant (Govrin and Levine 2000; Denby *et al.* 2004).

This experiment was performed in collaboration with Floriane L'Haridon from research group of Prof. Dr. Jean-Pierre Métraux from University of Fribourg, Switzerland. Hyphae and conidiophores were visually identified throughout the lesions and the infection sites were categorized according to their lesion sizes which are divided into three groups: Group 1 are lesions less than or equal to 2mm, group 2 lesions between 2 mm and 6 mm and the group 3 are lesions bigger than 6 mm. To reflect different severities of infection (Figure 4.17, A) the outgrowing lesion size was also determined (Figure 4.17, B) as well as the average lesion size (Figure 4.17, C). The lesions were measured three days after application of the pathogen.

As the data are categorical, they are presented in Figure 4.17 A and B as mosaic plots; alternative plots and statistics can be found in Supplemental Figure 7. The categorized lesion size is shown in Figure 4.17A and average lesion size is shown as a bar graph of the mean (Figure 4.17, C). As can be seen in Figure 4.17 A and B the severity of in infection was reduced in all mutant lines compared to Col-0. Col-0 had the majority of its lesions in Category 3 and its overall outgrowth index at 75% or more (87.5% of all lesions). In contrast, each *ARR* mutant had more lesions in Category 1 and 2, less in 3 (Figure 4.17, A). In fact looking only at Category 3 results, Col-0 was infected better with over 58.3% its lesions bigger than 6 mm. In contrast, the double mutant had only 31.3% of its lesions bigger than 6 mm. The single mutants showed in infection levels in-between wild-type and the double mutant: *arr2-4* (40.6% ≥6 mm) and *arr1-4* single mutant (43.8% ≥6 mm). This difference in infection efficiency is also evident from inspection of the outgrowth index, whereby only 58.3% of *arr1-4*, 54.2% of *arr2-4* and 50.0% of *arr1-4 arr2-4* leaves had an outgrowth index at 75% or more.

The average lesion size (Figure 4.17, C) again showed a clear picture of mutant resistance. Average lesion size for the wild-type was about 5.77 mm whereas the single mutants this value decreased to 4.39 mm for *arr1-4* and 3.99 mm for *arr2-4*. In the double mutant the average lesion size was 3.52 mm only. Leaves treated with inoculation media alone did not develop any lesions or lesion-like symptoms (data not shown).

All of these parameters very clearly show that *arr1-4 arr2-4* double mutant is more resistant to *Botrytis* compared to Col-0 wild-type. Interestingly the same tendency was observed in the single mutants as well, but only the difference by the double mutant was statistically significant when ascertained for average lesion size. These results clearly show the redundancy (additive effect) of the *ARR1* and *ARR2* in respect to defense against *Botrytis*.

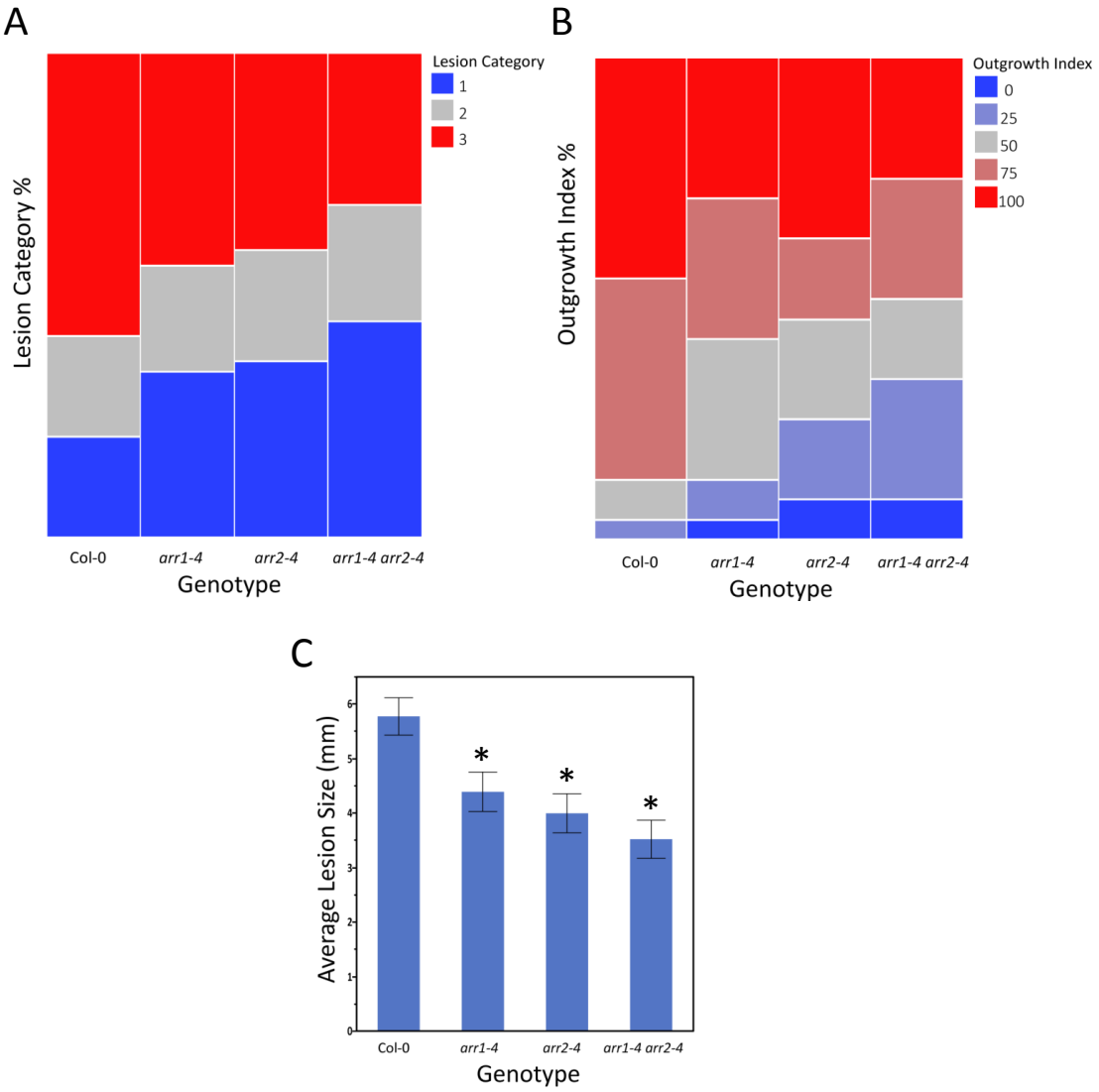


Figure 4.17 *Arabidopsis thaliana arr1-4 arr2-4* double mutants is susceptible to necrotroph *Botrytis cinerea*

Leaves of *Arabidopsis ARR1* and *ARR2* single and double mutants and corresponding wild-type (Col-0) were treated with *Botrytis cinerea*. The evaluation of infection was done 48hrs after the treatment. Data for (A,B) are shown as mosaic plots which are a graphical representation of an automatically computed two-way frequency table (also called a Contingency Table). Data in C is the mean and SE. **(A)** Susceptibility of plant lines to *Botrytis cinerea* are visually presented in a mosaic plot by lesion disease index (Category 1: ≤ 2mm, Category 2: 2-6mm, Category 3: >6mm). **(B)** Susceptibility of plant lines to *Botrytis cinerea* are visually presented in a mosaic plot by outgrowth index in % **(C)** Average lesion size of the tested plant lines in mm. LSD (Fishers Least Squared Difference Test) was used to compute significance levels at the α ≤ 0.05 level using the mean; asterisk shows a significance difference compared to Col-0 only. For this experiment at least 20 plants per line were used. This experiment was repeated two times with similar results.

4.2.3.3 Response of common marker genes PR-1 and PDF1.2 with respect to Botrytis cinerea

Knowing that the double mutant was more resistant to *Botrytis* compared to the wild-type, leaf material of treated and not treated plants was harvested and the transcript levels of *PR-1* and *PDF 1.2* were quantified by RT-qPCR. *PR-1* and *PDF1.2* are induced in response to a variety of pathogens (Penninckx *et al.* 1996; Schenk *et al.* 2000). The *PR-1* gene has been used to elucidate transcriptional control mechanisms regulating SAR (Pape *et al.* 2010) and expression of *PR-1* is salicylic-acid responsive (Metraux *et al.* 1990; Zhang *et al.* 2013).

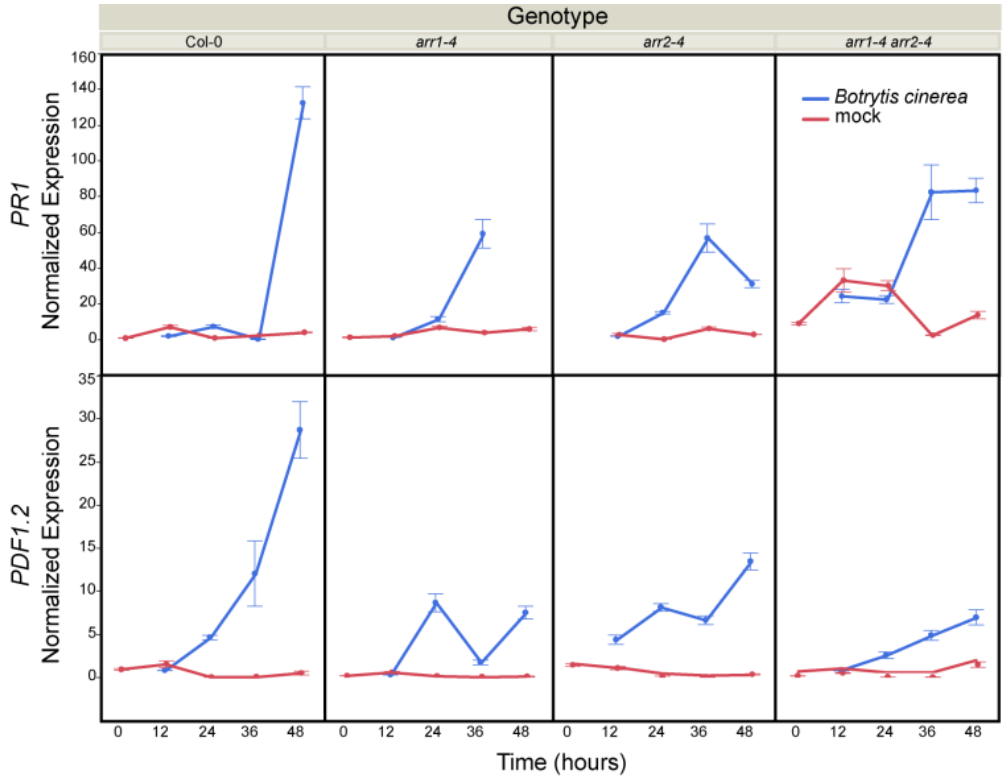


Figure 4.18 Response of common marker genes *PR-1* and *PDF1.2* after treatment with *Botrytis cinerea*

Relative expression levels of *PR-1* and *PDF1.2* genes in *arr1-4, arr2-4, arr1-4 arr2-4* and Col-0. Gene expression was measured after 0, 12, 24, 36 and 48 hrs after treatment with *Botrytis*. For this experiment all of the treated material for each plant line from both biological experiments (§4.2.3.3) was pooled together. This mRNA extraction and RT-qPCR was repeated two times with similar results. For each gene, time point 0 for Col-0 was used as reference and *EF-1-α* was used as the internal control to calculate ΔΔCT values.

PDF1.2 belongs to the plant *defensin (PDF)* family encodes an ethylene- and jasmonate-responsive plant defensin protein (Penninckx *et al.* 1998; Ferrari *et al.* 2003). *PDF1.2* mRNA levels are not responsive to salicylic acid treatments (Manners *et al.* 1998) although jasmonate and salicylic acid can act synergistically to enhance the expression of this gene (Mur *et al.* 2006; Koornneef and Pieterse 2008).

For this experiment all of the treated material for each plant line from both biological experiments (§4.2.3.3) was pooled together. Infected leaves from both treated and non-treated plants were harvested after 0 hrs, 12 hrs, 24 hrs, 36 hrs and 48 hrs post inoculation

and the mRNA expression levels for *PR-1* and *PDF1.2* were quantified. In non-treated plants *PR-1* was, as expected, very low expressed in all harvested time points. The first significant up-regulation of the *PR-1* was after 24 h where the mRNA level was higher in all mutants compared to the wild-type. This trend in rising expression continued until 36 hrs after inoculation and the levels of *PR-1* were still higher in the mutants. However, there was no difference in expression among the mutants themselves (Figure 4.18). Two days after inoculation (48 hrs) the expression of *PR-1* in all mutants maintained the same level as seen at 36 hrs while expression in the wild-type was increased. Thus, although the basal levels of *PR-1* was higher in the mutant plants, the pattern of induction with respect to *Botrytis* was not altered in any way.

Expression level of *PDF1.2* was very low in non-treated plants independently of the time point and the first increase of expression among the inoculated plants was visible 24 hrs after inoculation. The same tendency in all mutants and the wild-type plants was observed as an up-regulation at 24 hrs and 36 hrs post inoculation. Up until the 36 hrs time point, no difference was observed between either single mutants or the wild-type. However a difference was obtained by the double mutant with the level of *PDF1.2* down-regulated. After 48 hrs, the expression level of *PDF1.2* in the wild-type strongly went up compared to any of the mutants.

Based on these observations it must be concluded that even though regulation of *PR-1* and *PDF1.2* is slightly perturbed in *arr1/2* plants, it does not appear reflect the resistance of *arr1-4 arr2-4* to *Botrytis*. Thus even though *PR-1* and *PDF 1.2* are commonly used marker genes for these kind of studies, in this case, the genes themselves do not correlate directly to the resistance phenotype and are more a reflection of the current molecular status in the plants after infection.

4.3 Discussion

4.3.1 ARR2 interacts with MKK members

For the first time a connection has been shown between MAPK signalling cascades and the two-component signalling system in plants. These findings supported earlier presumptions and previous data (Hass *et al.* 2004) that *ARR2* might play role in response in biotic stresses, especially in response to pathogens. This crosstalk was observed strictly downstream of MKKKs, at the level of MKKs and MPKs. This is because none of the tested response regulators (A- and B-type) interacted with any of tested MKKKs. In comparison, only type-B RRs interacted with MKKs and MPKs.

Nevertheless, although only type-B interacted with MKKs, they did not interact with all of them. All MAPK kinase kinase members, except MKK3, were tested against: ARR1, ARR2, ARR10, ARR14, ARR18 and ARR4 (type-A) using yeast-two-hybrid method (studies with MKK6-10, and with ARR10 and ARR14 were done in our laboratory in collaboration with Niklas Wallmeroth (Wallmeroth, Diplom, Tübingen). Besides MKK3, MKK9 showed also transactivation in yeast, but its growth on control medium still allowed for evaluation of protein-protein interactions. ARR10 interacted only with MKK4, MKK5 and MKK9 while ARR14 interacted only with MKK5. ARR1, ARR2, and ARR18 interacted with more MKKs whereas the A-type ARR ARR4 did not interact with any. This information has been summarized below (Table 4.1).

Table 4.1 Summary table of protein-protein interactions between MKKs and RRs

+ means positive scored interaction, - stands for negative scored interaction, (+) stands for slightly transactive protein but a scorable interaction could still be observed, and blank means interaction was not tested. All interactions, except the one with MKK3 (grey labelled), were observed based on yeast two-hybrid system. Fields in blue represent my own results and the rest of data were produced in our laboratory by Niklas Wallmeroth during his Diploma thesis.

	ARR1	ARR2	ARR10	ARR14	ARR18	ARR4
MKK1	-	+	-	-	-	-
MKK2	+	+	-	-	-	-
MKK3		+				
MKK4	+	+	+	-	+	-
MKK5	+	+	+	+	+	-
MKK6	-	-	-	-	-	-
MKK7	-	+	-	-	+	-
MKK8	-	-	-	-	-	-
MKK9	(+)	(+)	(+)	-	(+)	-
MKK10	-	-	-	-	-	-

Despite very high sequence homology between ARR1 and ARR2, they did not interact with the same MKK members suggesting that the interactions could be rather specific. Interestingly, ARR2 interacted with the largest number of MKKs. On the converse side, MKK4, MKK5 and MKK9 interacted with the largest number of type-B ARRs.

MKK7 and MKK9 both are class D MKKs and also play important roles in biotic stress responses. Only ARR2 and ARR18 interacted with MKK7. MKK7 is very well known as a negative regulator of polar auxin transport, defence response to bacterium and salicylic acid mediated signalling pathways (Dai *et al.* 2006). ARR1 is known to control polar auxin transport by directly controlling *SHY2/IAA3*, an Aux/IAA transcriptional repressor, expression, which in turn controls expression of *PIN1*, *PIN3* and *PIN7* in the vascular tissue (Taniguchi *et al.* 2007). As ARR1 did not interact with MKK7 in the yeast-two-hybrid, it is unclear if this rules out a role for MAPK cascades *in planta*. The high homology between *ARR2* and *ARR1*, plus the fact that *ARR12*, the closest homolog to *ARR10*, also has been shown to have a role in regulating *SHY2* (Moubayidin *et al.* 2010) leaves this possibility still open. ARR1, ARR2, ARR10, and ARR12 all complement the root cytokinin response phenotype of the *arr1 arr12* mutant and therefore can functionally replace each other in root elongation assays (Hill *et al.* 2013). Taken together, it is worth speculating that ARR2 also has a role in polar auxin transport by controlling *SHY2* expression and integrating signal information from MKK7. This hypothesis of course must be tested experimentally.

MKK9 is known to be enrolled in phosphorylation between MPK3 and MPK6 and it is independently involved in ethylene and calmalexin biosynthesis, response to salt stress and wounding (Xu *et al.* 2008). In addition, both MKK7 and MKK9 are thought to be involved in the innate immunity cell death response (Popescu *et al.* 2009). ARR1, ARR2, ARR10 and ARR18 all interacted with MKK9. That ARR2 has been described to also function downstream of ethylene signalling (Popescu *et al.* 2009) this implies that these B-types could mediate allosteric functions. Expression of constitutive active version $MKK9^{EE}$ lead to enhanced cell death in *N. benthamiana* transfection assays (Popescu *et al.* 2009). Whether or not type-B ARRs could be part of this process is not yet known.

As mentioned, ARR2 interacted with the most MKKs and of those most of them are hormonal/stress-related MKKs (MKK3, MKK4, MKK5, MKK7 and MKK9). Moreover, ARR2 strongly interacted with MKK4 and MKK5 compared to the abiotic-stress related MKKs MKK1 and MKK2. This difference of interaction strength was conferred by O-NPG assay and by making serial dilution of transformed yeast cells. Therefore, MKK4, MKK5, MKK3 were tested *in planta* by quantifying protein-protein interaction strength using FRET-FLIM. The FRET-FLIM results revealed strong interaction between ARR2 and MKK4, MKK5, MKK3 *in planta*. Thus, there is little doubt that these two protein classes do interact in living plant cells.

Two MPKs were tested against ARR2 and MKKs, the biotic stress-related MPKs MPK4 and MPK17 (Group 2002). Both MPK4 and MPK17 interacted with ARR2, ARR18 and ARR1. MPK4 is especially known to play very important roles in pathogen response (Rasmussen *et al.* 2012). It has been known that *mpk4* knockout mutant plants show constitutive activation of SA-mediated defences (Petersen *et al.* 2000). Furthermore, MPK4 activity inhibits basal defences to virulent *Pst* DC3000 and pathogen-induced SA accumulation, MPK4 also plays role in ETI as compromising it and its activity negatively regulate ROS production induced by PAMPs (Berriri et al. 2012). The interaction of MPK4 with both biotic stress-related MKKs

MKK4 and MKK5 could be confirmed in this thesis. MPK17, whose physiological function(s) are not described yet, only interacted with MKK5. Recall however that MKK5 interacted with all of the type-B ARRs. MKK9 also interacted with the majority of type-B ARRs, albeit over its yeast-two-hybrid transactivation capacity. However, only MKKs 1 to 5 were tested against MPK17 in this thesis. Nevertheless, it is reported that MPK17 interacts with MKK9 and MKK10 in yeast-two-hybrid assays (Lee *et al.* 2008). Thus, although the interaction of MPK17 with MKK9 was not confirmed in this thesis, it suggests that a *bona fide* interaction network has been documented. Furthermore, expounding on the knowledge of roles for MKK5 and MKK9, the type-B ARRs are somehow involved in physiological responses related to biotic stress and/or hormonal regulation. With regards to MKK9 which is known to be involved in ethylene signalling downstream of CTR1 (Yoo *et al.* 2008) as a MKK9–MPK3/6 module. Haß et al. 2004 from our laboratory showed that *ARR2* also responds to ethylene in an ETR1-TCS dependent manner. In addition, MKK4/5 have also been placed in a MKK4/5/9-MPK6 ethylene biosynthesis model (Hahn and Harter 2009; Zhao and Guo 2011). Interestingly, the most type-B ARR interactive MKKs MKK4/5/7/9 were shown to activate signalling cascades controlling cell death pathways in plants (Popescu *et al.* 2009). How these two signalling pathways converge on ethylene signalling is still a mystery, yet it suggests that part of the signal integration can occur at a protein-protein interaction level.

The FRET-FLIM analysis revealed that MKK3 and ARR2 interact in living plant cells. MKK3 is an activator of group C MPKs (MPK1, MPK2, MPK7, and MPK14) of which MKK3 also activates MPK7 in response to plant pathogen *Pst* DC3000 and ROS (Doczi *et al.* 2007). MKK3 is interesting as it is shown to play a physiological role in response to biotic stresses as a MKK3-MPK6 cascade important for JA signal transduction in *Arabidopisis* (Takahashi et al. 2007) and the same cascade plays crucial role in resistance to *Salmonella* infections in *Arabidopsis* (Schikora *et al.* 2008). Additionally, ARR2 showed very strong interaction with MKK4 and MKK5 compared to MKK1 and MKK2 and this interaction was also confirmed *in planta*. The MKK1 and MKK2 are mostly involved in abiotic stresses (Asai *et al.* 2002). For example MKK2 is specially activated by cold and salt stress and by stress-induced MKK1 (Teige *et al.* 2004). They have also been described with a role in biotic stress as a part of MKK1/2-MPK4 cascade functioning as negative regulator of SA-dependent systemic required resistance (Petersen *et al.* 2000) and positive regulator of ET- and JA-mediated defences (Brodersen *et al.* 2006; Qiu *et al.* 2008). Here, it is also possible the MKK1/2-MPK4 module is allosterically influenced by ARR2 and ARR1.

The presumptuously stronger interaction of MKK4 and MKK5 over the other MKKs with B-types was confirmed in yeast-serial dilution and quantitative O-NPG assays. MKK4/5 are known to be active in response to different biotic stresses but especially active and described in pathogen defence (Asai *et al.* 2002) where MKK4 is strongly induced when plants treated with *Pseudomonas syringae* or together with MKK9 against *Phytophthora infestans* (Menges *et al.* 2008). Even newly characterised homologue of *MKK5* in cotton *(Gossypium hirsutum) GhMKK5* affects pathogen resistance to the bacterial pathogen *Ralstonia solanacearum* by

elevating the expression of pathogen resistance *(PR)* genes (*PR1a, PR2, PR4, PR5,* and *NPR1*) (Zhang *et al.* 2012). Interestingly, the interaction capability of ARR2 was not due to the presence of the ARR2 type-B output domain, since a truncated version of ARR2 containing only its receiver domain was able to interact with MKK4 and MKK5. Furthermore, using an *in silico* approach, it was shown that MKK docking domains are dispersed throughout ARR2 and not restricted to its output domain. In total, four different MKK binding domains were predicted to be present in ARR2. All of these regions seem to be functional when tested by yeast-two-hybrid system based on the larger protein truncations. When tested as peptides, the receiver domain-based and output domain-based MKK docking motifs showed interaction only with MKK4 and MKK5. This observation was taken to confirm previous observations that these two MKKs interact much stronger with ARR2 than the others. Furthermore, the interaction of ARR2 with MKKs does not require phosphorylation by TCS elements on its D80 position in order to interact with any of tested MPK(K) cascade elements. This suggests that ARR2 might interact with MAPK cascade members independent of cytokinin or ethylene, *i.e.* TCS signalling. In fact, this would put *ARR2* in novel signalling pathways which may or may not require its transcription factor roles. This potential function of *ARR2* might make signalling pathways and protein-protein interaction more specific. One way to unravel TCS dependent signalling and the physiological role of MAPK cascades would be possible with constitutive expression of non-phosphorylatable $ARR2^{D80N}$ (Hwang and Sheen 2001; Choi *et al.* 2010). This was not performed at this time, but similar experiments with the constitutive TCS active form $ARR2^{D80E}$ were attempted. Readout however was not possible as mutant lines complemented with $ARR2^{D80E}$ driven under a constitutively active promoter were lethal in embryonic stage (Hass *et al.* 2004). In this work, attempts to bypass constitutive expression of $ARR2^{D80E}$ using a β-estradiol inducible promoter only for a few days after treatment still showed huge developmental defects, early necrosis and most likely embryonic lethality (see Supplemental Figure 10).

Taken together, the protein-protein interaction data suggest involvement of ARR2 in a biotic stress direction concerning its association with biotic stress-related MKKs and MPKs, and in particular pathways mediated by MKK4/5/7/9. This crosstalk of ARR2 with MKKs and MPKs could be a conformation of the early presumptions that TCS and B-type response regulators *per se* play an important role in fine-tuning of different signals and in mediating crosstalk between different signalling pathways in aim of improving the fitness of plants for better adaptation within their natural ecosystems. Therefore, a connection to biotic stress and *ARR1* and/or *ARR2* was pursued in this thesis to provide a physiological basis for which a functional output could be eventually be assigned to. Provided that this is successful, cross-talk relationships between ARR1/2 and MKKs could be pursued.

4.3.2 *arr2-4* can be shown to be involved in pathogen responses to necrotrophs

Innate immunity is generally initiated with recognition of conserved pathogen-associated molecular patterns (PAMPs). As a first look into pathogen-related responses in *ARR2*, wild-type and *arr1-4*, *arr2-4* and *arr1-4 arr2-4* mutant lines were treated with PAMPs and as output, ROS and ethylene production was measured.

Indeed, ROS measurement triggered by flg22 and elf18 showed that *arr1-4 arr2-4* double mutant significantly produced more ROS than the wild-type or single mutants in response to both applied PAMPs. Although there were differences between the single mutants, these observations were not consistent between experiments but the double mutant however consistently showed more ROS than the wild-type. This was first hint that *ARR1* and *ARR2* are enrolled in response to pathogen defence. Since the double mutant showed the strongest effect in ROS production only the *arr1-4 arr2-4* double mutant and Col-0 wild-type were taken and tested against PAMPs with fungal origin (PEN, Xac) for ethylene production.

Double mutant here did not show any statistically significant difference in ethylene production to any of tested PAMPs compared to the wild-type. This approach only showed that there is no significant difference in ethylene production between the mutant and the wild-type but it doesn't necessarily mean that the signalling pathways controlled by ethylene are not affected in the double mutant since differences in ROS production were observed. It could be that the differences in ethylene production between the double mutant and the wild-type are below the detection range of the machine used for ethylene measurements. Similarly, it could be that the ROS measurements were much more sensitive than those of ethylene.

Changes in ROS have been correlated with MKK and MPK activity (Pitzschke and Hirt 2009). Unfortunately, the lack of *ARR1* and *ARR2* did not cause any dramatic differences between the wild-type and the double mutant in MPK phosphorylation status in response to flg22 of pathogen-response related MPK4, MPK3 and MPK6. This does not exclude that actually ARR1 and ARR2 may serve here as MKK scaffold proteins which redirect and specify pathogen-related signalling pathways, but it does suggest the function of ARR1 and ARR2 does not regulate MKK kinase activity.

The data up to this point suggested that ARR2 (and other B-types) interact with MKKs and MPKs. The interaction with ARR2 in yeast-two-hybrid and *in planta* was preferentially with MKK4 and MKK5, which are known to be involved in biotic stress. Furthermore, changes in ROS production with respect to flg22 were also observed although this could not be corroborated with ethylene production or MPK phosphorylation status. Recent publications about *ARR2* and cytokinin have indicated a growing connection to pathogen stress (Choi *et al.* 2010). Therefore, pathogen assays were performed on *ARR1* and *ARR2* single and double mutants. For these assays necrotrophic and biotrophic pathogens were taken since each has evolved differently leading to distinct defence strategies in plants and thus activation of different genes required for pathogenicity (Idnurm and Howlett 2001; Oliver and Ipcho 2004).

No any statistically significant differences between wild-type (Col-0) and the mutant lines were obtained using *Pst* DC3000 *when* applying it by leaf infiltration at 3 x 10^6 CFU. The same mutant lines were also twice challenged with *Pst* DC3000 in presence of 1µM exogenous cytokinin (*t-zeatin*). No biologically significant differences were found either. These results are in contrast to data who published a weak *arr2-4* phenotype for resistance to *Pst* DC3000 at one log difference (Choi *et al.* 2010). The majority of their assays worked with overexpressor lines to show the effects of cytokinin and *ARR2* variants however. Considering that our data are of very high quality as they were reproduced at least 5 times, we conclude that *arr1-4*, *arr2-4* and *arr1- arr2-4* do not confer any significant resistance to *Pst* DC3000 infection. *Peronospora* is known to activate defence pathways via salicylic acid (SA) accumulation, which is a general characteristic of biotrophic pathogens (Glazebrook 2005), and independently of SA via *RPPs* (*RECOGNITION OF PERONOSPORA PARASITICA*) genes, especially via *RPP7*. *RPPs* represent R (resistance) genes, genes responsible for gene-dependent defence activation in *Arabidopsis* (Mcdowell *et al.* 2000). Here seems that *ARR1* and *ARR2* are not involved in these signalling pathways or that the other B-types work in a redundant fashion no evident from the yeast-two-hybrid data.

It could be postulated that the defence response could depend on stomata developmental/response defects. This is because *ARR2* has been described to be involved in mediating stomatal closure in an H_2O_2-depdenent manner (Desikan *et al.* 2006; Mira-Rodado *et al.* 2012) and that *MKK4* and *MKK5* are known to be involved in stomatal patterning (Lampard *et al.* 2009). Such same effects should be seen by spraying the bacteria onto plants. Stomata aperture was measured in mutant backgrounds and the wild-type treated with hormones (ABA and cytokinin) and after *Pst* DC3000 application. Although the wild-type and mutants closed in response to ABA as previously described (Desikan *et al.* 2006), wild-type and mutants also responded equally to cytokinin application or *Pst* DC3000 challenge. In fact, stomata in all the plant lines treated with *Pst* DC3000 were more open than non-treated control consistent with the release of diffusible phytotoxin COR by the bacteria which reopen closed stomata to increase the number of bacterial invasion sites (Melotto *et al.* 2008). Stomata aperture measurements were done 3 days after pathogen application which was more than enough to cause reopening of stomata. Here was shown that *ARR1* and *ARR2* are not any how involved in mechanism for recognition of phytotoxin COR and in this case they are not part of apparatus for regulation of stomata aperture. Therefore under our conditions and experimental approach we could not confirm the previously published observation that cytokinin modulates SA signalling by augmenting resistance against *Pst* DC3000, a process in which the SA/cytokinin-dependent interaction TGA3 and ARR2 is important (Choi *et al.* 2010). This discrepancy, despite the performed experiment where stomata aperture was measured and also no difference obtained, may still be due to differences in infection conditions.

Interestingly, cytokinin caused the stomata to open in the wild-type and the *arr1/2* mutant lines. This means, that although there is a lack of two cytokinin responsive (Hill *et al.* 2013), type-B they are not crucial for stomatal aperture responses. This indicates that this response

is more than likely redundantly controlled by other B-types. On the other hand, the strong effects of previously reported for *arr2-4* on H_2O_2-dependent closure point towards explaining MKK4/5/9 cross-talk by the fact that ARR2 interacts with more MKKs than does all the other type-B ARRs.

Generally speaking, JA-dependent signalling pathways play a crucial role in defence against necrotrophic pathogens (Glazebrook 2005). Therefore assays done with nectrotrophic pathogens were performed in order to see if there could be a defect in regulation of jasmonic acid (JA)-dependent signalling pathways. The *arr2-4* single mutant and *arr1-4 arr2-4* double mutant but not *arr1-4* single mutant, showed enhanced susceptibility to the necrotrophic fungus *Alternaria brassicicola* compared to the wild-type. This experiment strongly suggested that, despite their high homology, *ARR2* but not *ARR1,* is the only one of the two involved in signalling pathways responsible for defence against *Alternaria*. It is known that *COI1* (CORONATINE INSENSITIVE 1), *COI1*-dependent genes and camalexin biosynthesis-dependent genes are primarily responsible for its defence against *Alternaria brassicicola* (Van Wees *et al.* 2003). In almost all *Arabidosis* mutants that show susceptibility to *Alternaria*, a perfect correlation has been observed between camalexin deficiency after *A. brassicicola* infection and susceptibility (Van Wees *et al.* 2003). However, it is known that even though infection by *A. brassicicola* and infection by *P. syringae* are quite different stimuli, genes under *COI1* control are common for plant resistance against both pathogens but this is not the case with camalexin biosynthesis-dependent genes (Van Wees *et al.* 2003; Jones and Dangl 2006). Based on the data obtained with *arr2-4* mutants, *ARR2* is more likely involved in camalexin biosynthesis pathways where it might positively regulate its synthesis. Furthermore, ARR2 interacted with MKK3 further strengenthing a role of ARR2 in the MKK3-MPK6 JA signal transduction. How these two are intertwined is yet unclear at this point.

The effects of the necrotrophic fungus *Botrytis cinerea* was challenged on *arr1/2*. This pathogen is important for two reasons: *Botrytis cinerea* is known to activate distinct signalling pathways from *Alternaria* and more importantly, *Arabidopsis thaliana* is natural host of *Botrytis cinerea*. Based on measured disease index parameters, the *arr1-4 arr2-4* double mutant clearly showed resistance to *Botrytis* compared to the wild-type and an additive effect compared to the single mutant parents. That is, the double mutant twice as less lesions (~30%) than the wild-type (~60%) lesions greater for equal to 6 mm (≥6 mm). Both single mutants showed the same resistance tendency and were statistically different from the wild-type. It was also observed that *arr2-4* single mutant had a slight, non-significant tendency to be more resistant than the *arr1-4* single mutant: it had 40% of lesions ≥6 mm whereas the *arr1-4* had only 45%. Again this favours the role of *ARR2* compared to *ARR1* with respect to resistance against necrotrophic pathogens.

The common marker genes *PR-1* and *PDF1.2* were tested from the pooled material from the *Botrytis cinerea* challenged *Arabidopsis* plants. Despite the fact that *PR-1* is rather SA marker and not suitable for infection with necrotrophs, expression of *PR-1* was induced 36 hrs post infection, 12 hrs earlier than the wild-type. This expression was maintained at the same level

in the mutants one day later whereas *PR-1* expression in the wild-type was strictly upregulated in the wild-type at 48 hrs. This would imply that the *arr1/2* caused early induction of *PR-1* in response to *Botrytis* infection. Based on the current models, the expression level of *PDF1.2* should be increased in the mutant plants compared to the wild-type knowing the role of *PDF1.2* as JA marker and is upregulated in response to necrotroph infection (Seo *et al.* 2001; Qi *et al.* 2012; Lu *et al.* 2013). Although *PDF1.2* was induced in response to *Botrytis* infection as expected in all samples, it was not as highly induced in the mutants compared to the wild-type. Thus, the expression of this gene cannot be correlated with the mutant resistance phenotype. These results present a conundrum as to the mechanism of resistance to *Botrytis*. On one hand, we expected that *PDF1.2* expression to be increased in the mutant plants, however dampened expression kinetics were observed. On the other hand, *PR-1* was upregulated at an earlier time in the mutant plants but did not reach the same maximum as the wild-type at 48 hrs. Therefore, it is unclear if these two markers really can be correlated with the resistance phenotype of *arr1/2*. It is known that despite the wide use of *PR-1* and *PDF1.2* as markers for the induction SA- and JA-dependent pathogen pathways, the levels of both *PR-1* and *PDF1.2* expression do not clearly correlate with the level of susceptibility to *Botrytis cinerea* (Ferrari *et al.* 2003). So the observed differences in expression of these genes in the *arr1/2* mutants are more a reflection of the current molecular status in the plants after infection, that is, it definitely showed that the current status was of plants under pathogen attack.

The dramatic difference in resistance between *Botrytis cinerea* and *Alternaria brassisicola* could be explained by the very wide, naturally variable resistance to necrotrophic plant pathogens which appears to be quantitative and polygenic (Micic *et al.* 2004; Rowe and Kliebenstein 2008). This tendency is exactly visible in the *arr1-4 arr2-4* double mutant when the same mutants tested against two different necrotrophic pathogens showed completely opposite phenotypes. Furthermore, all of the parameters very clearly showed the redundancy (additive effect) of the *ARR1* and *ARR2* in respect to defence against *Botrytis* but not *Alternaria*, of which *arr2-4* played the major role. In addition, the nature and extent of isolate-specific interaction between plants and necrotrophic pathogens is relatively unknown, and no qualitative naturally variable resistance genes effective against necrotrophic pathogens have been yet described (Jones and Dangl 2006; Rowe and Kliebenstein 2008). Provocatively, infection by the fungal pathogen *Phytophthora infestans* led to the rapid transcriptional induction of *MKKK19, MKK9 and MKK4*, while *Botrytis cinerea* infection led to the rapid transcriptional induction of completely different genes *MKKK18, 19* and *20, Raf43, ZIK2, 8* (Menges *et al.* 2008). Although no direct genetic or transgenic connection has been shown between ARR1/2 and MKKs, a role for ARR1/2 is more than likely evaluable in necrotrophic but not biotrophic pathogen resistance as neither *Pst* DC3000 nor *Peronospora parasitica* differentially infected *arr1/2* compared to their wild-type control. There are also reports that have shown signalling responses to bacterial and fungal pathogen attack are distinct, *i.e.* different genes are induced, especially with regard to MAPK cascades. It is still open if differences in resistance of *arr1/2* mutants when tested against distinct pathogens is

correlated with MKK/type-B ARR crosstalk. If valid, the role of TCS-dependent phosphorylation, at least for protein-protein interaction is not required (so far judged by yeast-two-hybrid assays). In addition, *arr1/2* mutants did not change the phosphorylation level of MPK3/4/6 in response to flg22. Therefore, regulation of MKK activity also does not appear to be mediated by type-B ARRs, although it was not tested if modulating the TCS-dependent phosphorylation state of ARR2 could influence this. It is therefore proposed that the proposed protein-protein crosstalk mediates protein target networks dynamics, *i.e.* their interaction affects binding/signalling kinetics of the two systems without actually controlling the physical TCS or MAPK cascade phosphorylation mechanism. As both the TCS and MAPK cascades target gene expression, the interaction of the two networks would modify their own information flow leading to changes in output gene expression similar to what has been observed for a TCS and MAPK cascade in yeast (Xu and West 1999).

4.4 References for Chapter 1

Amselem, J, Cuomo, CA, *et al.* (2011). "Genomic analysis of the necrotrophic fungal pathogens sclerotinia sclerotiorum and botrytis cinerea." PLoS Genet **7**(8): e1002230.

Asai, T, Tena, G, *et al.* (2002). "Map kinase signalling cascade in arabidopsis innate immunity." Nature **415**(6875): 977-983.

Babula, D, Misztal, LH, *et al.* (2006). "Genes involved in biosynthesis and signalisation of ethylene in brassica oleracea and arabidopsis thaliana: Identification and genome comparative mapping of specific gene homologues." Theor Appl Genet **112**(3): 410-420.

Bardwell, AJ, Flatauer, LJ, *et al.* (2001). "A conserved docking site in meks mediates high-affinity binding to map kinases and cooperates with a scaffold protein to enhance signal transmission." J Biol Chem **276**(13): 10374-10386.

Baudry, A, Ito, S, *et al.* (2010). "F-box proteins fkf1 and lkp2 act in concert with zeitlupe to control arabidopsis clock progression." Plant Cell **22**(3): 606-622.

Belkhadir, Y, Subramaniam, R, *et al.* (2004). Curr. Opin. Plant Biol. **7**: 391.

Berriri, S, Garcia, AV, *et al.* (2012). "Constitutively active mitogen-activated protein kinase versions reveal functions of arabidopsis mpk4 in pathogen defense signaling." Plant Cell **24**(10): 4281-4293.

Bethke, G, Unthan, T, *et al.* (2009). "Flg22 regulates the release of an ethylene response factor substrate from map kinase 6 in arabidopsis thaliana via ethylene signaling." Proc Natl Acad Sci U S A **106**(19): 8067-8072.

Boller, T and He, SY (2009). "Innate immunity in plants: An arms race between pattern recognition receptors in plants and effectors in microbial pathogens." Science **324**(5928): 742-744.

Brock, AK, Willmann, R, *et al.* (2010). "The arabidopsis mitogen-activated protein kinase phosphatase pp2c5 affects seed germination, stomatal aperture, and abscisic acid-inducible gene expression." Plant Physiol **153**(3): 1098-1111.

Brodersen, P, Petersen, M, *et al.* (2006). "Arabidopsis map kinase 4 regulates salicylic acid- and jasmonic acid/ethylene-dependent responses via eds1 and pad4." Plant J **47**(4): 532-546.

Brown, RL, Kazan, K, *et al.* (2003). "A role for the gcc-box in jasmonate-mediated activation of the pdf1.2 gene of arabidopsis." Plant Physiol **132**(2): 1020-1032.

Caesar, K, Elgass, K, *et al.* (2011). "A fast brassinolide-regulated response pathway in the plasma membrane of arabidopsis thaliana." Plant J **66**(3): 528-540.

Caesar, K, Thamm, AM, *et al.* (2011). "Evidence for the localization of the arabidopsis cytokinin receptors ahk3 and ahk4 in the endoplasmic reticulum." J Exp Bot **62**(15): 5571-5580.

Chang, L and Karin, M (2001). "Mammalian map kinase signalling cascades." Nature **410**(6824): 37-40.

Choi, J, Choi, D, *et al.* (2011). "Cytokinins and plant immunity: Old foes or new friends?" Trends Plant Sci **16**(7): 388-394.

Choi, J, Huh, SU, *et al.* (2010). "The cytokinin-activated transcription factor arr2 promotes plant immunity via tga3/npr1-dependent salicylic acid signaling in arabidopsis." Dev Cell **19**(2): 284-295.

Colcombet, J, Berriri, S, *et al.* (2012). "Constitutively active mpk4 helps to clarify its role in plant immunity." Plant Signal Behav **8**(2).

Colcombet, J and Hirt, H (2008). "Arabidopsis mapks: A complex signalling network involved in multiple biological processes." Biochem J **413**(2): 217-226.

Dai, Y, Wang, H, *et al.* (2006). "Increased expression of map kinase kinase7 causes deficiency in polar auxin transport and leads to plant architectural abnormality in arabidopsis." Plant Cell **18**(2): 308-320.

Dangl, JL, Horvath, DM, *et al.* (2013). "Pivoting the plant immune system from dissection to deployment." Science **341**(6147): 746-751.

Dean, R, Van Kan, JA, *et al.* (2012). "The top 10 fungal pathogens in molecular plant pathology." Mol Plant Pathol **13**(4): 414-430.

Denby, KJ, Kumar, P, *et al.* (2004). "Identification of botrytis cinerea susceptibility loci in arabidopsis thaliana." Plant J **38**(3): 473-486.

Desclos-Theveniau, M, Arnaud, D*, et al.* (2012). "The arabidopsis lectin receptor kinase lecrk-v.5 represses stomatal immunity induced by pseudomonas syringae pv. Tomato dc3000." PLoS Pathog **8**(2): e1002513.

Desikan, R, Horak, J*, et al.* (2008). "The histidine kinase ahk5 integrates endogenous and environmental signals in arabidopsis guard cells." PLoS One **3**(6): e2491.

Desikan, R, Last, K*, et al.* (2006). "Ethylene-induced stomatal closure in arabidopsis occurs via atrbohf-mediated hydrogen peroxide synthesis." Plant J **47**(6): 907-916.

Devoto, A, Nieto-Rostro, M*, et al.* (2002). Plant J. **32**: 457.

Dinkel, H, Michael, S*, et al.* (2012). "Elm--the database of eukaryotic linear motifs." Nucleic Acids Res **40**(Database issue): D242-251.

Doczi, R, Brader, G*, et al.* (2007). "The arabidopsis mitogen-activated protein kinase kinase mkk3 is upstream of group c mitogen-activated protein kinases and participates in pathogen signaling." Plant Cell **19**(10): 3266-3279.

Dodds, PN and Rathjen, JP (2010). "Plant immunity: Towards an integrated view of plant-pathogen interactions." Nat Rev Genet **11**(8): 539-548.

Droillard, MJ, Boudsocq, M*, et al.* (2004). "Involvement of mpk4 in osmotic stress response pathways in cell suspensions and plantlets of arabidopsis thaliana: Activation by hypoosmolarity and negative role in hyperosmolarity tolerance." FEBS Lett **574**(1-3): 42-48.

Dunning, FM, Sun, W*, et al.* (2007). "Identification and mutational analysis of arabidopsis fls2 leucine-rich repeat domain residues that contribute to flagellin perception." Plant Cell **19**(10): 3297-3313.

Durrant, WE and Dong, X (2004). "Systemic acquired resistance." Annu Rev Phytopathol **42**: 185-209.

El-Showk, S, Ruonala, R*, et al.* (2013). "Crossing paths: Cytokinin signalling and crosstalk." Development **140**(7): 1373-1383.

Falk, A, Feys, BJ*, et al.* (1999). Proc. Natl. Acad. Sci. USA **96**: 3292.

Farre, EM and Liu, T (2013). "The prr family of transcriptional regulators reflects the complexity and evolution of plant circadian clocks." Curr Opin Plant Biol **16**(5): 621-629.

Ferrari, S, Plotnikova, JM*, et al.* (2003). "Arabidopsis local resistance to botrytis cinerea involves salicylic acid and camalexin and requires eds4 and pad2, but not sid2, eds5 or pad4." Plant J **35**(2): 193-205.

Galanis, A, Yang, SH*, et al.* (2001). "Selective targeting of mapks to the ets domain transcription factor sap-1." J Biol Chem **276**(2): 965-973.

Gattolin, S, Alandete-Saez, M*, et al.* (2006). "Spatial and temporal expression of the response regulators arr22 and arr24 in arabidopsis thaliana." J Exp Bot **57**(15): 4225-4233.

Gendron, JM, Pruneda-Paz, JL*, et al.* (2012). "Arabidopsis circadian clock protein, toc1, is a DNA-binding transcription factor." Proc Natl Acad Sci U S A **109**(8): 3167-3172.

Glazebrook, J (2001). "Genes controlling expression of defense responses in arabidopsis--2001 status." Curr Opin Plant Biol **4**(4): 301-308.

Glazebrook, J (2005). "Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens." Annu Rev Phytopathol **43**: 205-227.

Glazebrook, J (2005). "Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens." Annu Rev Phytopathol **43**(1): 205-227.

Govrin, EM and Levine, A (2000). "The hypersensitive response facilitates plant infection by the necrotrophic pathogen botrytis cinerea." Curr Biol **10**(13): 751-757.

Grefen, C and Harter, K (2004). "Plant two-component systems: Principles, functions, complexity and cross talk." Planta **219**(5): 733-742.

Group, M (2002). "Mitogen-activated protein kinase cascades in plants: A new nomenclature." Trends Plant Sci **7**(7): 301-308.

Guerineau, F, Benjdia, M*, et al.* (2003). "A jasmonate-responsive element within the a. Thaliana vsp1 promoter." J Exp Bot **54**(385): 1153-1162.

Guo, H and Ecker, JR (2004). "The ethylene signaling pathway: New insights." Curr Opin Plant Biol **7**(1): 40-49.

Hahn, A and Harter, K (2009). "Mitogen-activated protein kinase cascades and ethylene: Signaling, biosynthesis, or both?" Plant Physiol **149**(3): 1207-1210.
Hamel, LP, Nicole, MC*, et al.* (2006). "Ancient signals: Comparative genomics of plant mapk and mapkk gene families." Trends Plant Sci **11**(4): 192-198.
Hass, C, Lohrmann, J*, et al.* (2004). "The response regulator 2 mediates ethylene signalling and hormone signal integration in arabidopsis." EMBO J **23**(16): 3290-3302.
He, C, Fong, SH*, et al.* (1999). "Bwmk1, a novel map kinase induced by fungal infection and mechanical wounding in rice." Mol Plant Microbe Interact **12**(12): 1064-1073.
Hejatko, J, Pernisova, M*, et al.* (2003). "The putative sensor histidine kinase cki1 is involved in female gametophyte development in arabidopsis." Mol Genet Genomics **269**(4): 443-453.
Heyl, A and Schmulling, T (2003). "Cytokinin signal perception and transduction." Curr Opin Plant Biol **6**(5): 480-488.
Hill, K, Mathews, DE*, et al.* (2013). "Functional characterization of type-b response regulators in the arabidopsis cytokinin response." Plant Physiol **162**(1): 212-224.
Horak, J, Grefen, C*, et al.* (2008). "The arabidopsis thaliana response regulator arr22 is a putative ahp phospho-histidine phosphatase expressed in the chalaza of developing seeds." BMC Plant Biol **8**: 77.
Hothorn, M, Dabi, T*, et al.* (2011). "Structural basis for cytokinin recognition by arabidopsis thaliana histidine kinase 4." Nat Chem Biol **7**(11): 766-768.
Huang, Y, Li, H*, et al.* (2003). "Biochemical and functional analysis of ctr1, a protein kinase that negatively regulates ethylene signaling in arabidopsis." Plant J **33**(2): 221-233.
Hutchison, CE, Li, J*, et al.* (2006). "The arabidopsis histidine phosphotransfer proteins are redundant positive regulators of cytokinin signaling." Plant Cell **18**(11): 3073-3087.
Hwang, I, Chen, HC*, et al.* (2002). "Two-component signal transduction pathways in arabidopsis." Plant Physiol **129**(2): 500-515.
Hwang, I and Sheen, J (2001). "Two-component circuitry in arabidopsis cytokinin signal transduction." Nature **413**(6854): 383-389.
Hwang, I, Sheen, J*, et al.* (2012). "Cytokinin signaling networks." Annu Rev Plant Biol **63**: 353-380.
Ichimura, K, Casais, C*, et al.* (2006). "Mekk1 is required for mpk4 activation and regulates tissue-specific and temperature-dependent cell death in arabidopsis." J Biol Chem **281**(48): 36969-36976.
Ichimura, K, Mizoguchi, T*, et al.* (1998). "Isolation of atmekk1 (a map kinase kinase kinase)-interacting proteins and analysis of a map kinase cascade in arabidopsis." Biochem Biophys Res Commun **253**(2): 532-543.
Idnurm, A and Howlett, BJ (2001). "Pathogenicity genes of phytopathogenic fungi." Mol Plant Pathol **2**(4): 241-255.
Jakoby, M, Weisshaar, B*, et al.* (2002). "Bzip transcription factors in arabidopsis." Trends Plant Sci **7**(3): 106-111.
Jambunathan, N, Siani, JM*, et al.* (2001). "A humidity-sensitive arabidopsis copine mutant exhibits precocious cell death and increased disease resistance." Plant Cell **13**(10): 2225-2240.
James, P, Halladay, J*, et al.* (1996). "Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast." Genetics **144**(4): 1425-1436.
Jones, JD and Dangl, JL (2006). "The plant immune system." Nature **444**(7117): 323-329.
Joo, S, Liu, Y*, et al.* (2008). "Mapk phosphorylation-induced stabilization of acs6 protein is mediated by the non-catalytic c-terminal domain, which also contains the cis-determinant for rapid degradation by the 26s proteasome pathway." Plant J **54**(1): 129-140.
Jouannic, S, Hamal, A*, et al.* (1999). "Characterisation of novel plant genes encoding mekk/ste11 and raf-related protein kinases." Gene **229**(1-2): 171-181.
Katagiri, F, Thilmony, R*, et al.* (2002). "The arabidopsis thaliana-pseudomonas syringae interaction." Arabidopsis Book **1**: e0039.
Kawano, T (2003). "Roles of the reactive oxygen species-generating peroxidase reactions in plant defense and growth induction." Plant Cell Rep **21**(9): 829-837.
Kemmerling, B, Schwedt, A*, et al.* (2007). "The bri1-associated kinase 1, bak1, has a brassinolide-independent role in plant cell-death control." Curr Biol **17**(13): 1116-1122.

Kesarwani, M, Yoo, J, *et al.* (2007). "Genetic interactions of tga transcription factors in the regulation of pathogenesis-related genes and disease resistance in arabidopsis." Plant Physiol **144**(1): 336-346.

Kiba, T, Yamada, H, *et al.* (2003). "The type-a response regulator, arr15, acts as a negative regulator in the cytokinin-mediated signal transduction in arabidopsis thaliana." Plant Cell Physiol **44**(8): 868-874.

Kieber, JJ, Rothenberg, M, *et al.* (1993). "Ctr1, a negative regulator of the ethylene response pathway in arabidopsis, encodes a member of the raf family of protein kinases." Cell **72**(3): 427-441.

Kiegerl, S, Cardinale, F, *et al.* (2000). "Simkk, a mitogen-activated protein kinase (mapk) kinase, is a specific activator of the salt stress-induced mapk, simk." Plant Cell **12**(11): 2247-2258.

Kim, CY, Liu, Y, *et al.* (2003). "Activation of a stress-responsive mitogen-activated protein kinase cascade induces the biosynthesis of ethylene in plants." Plant Cell **15**(11): 2707-2718.

Kim, HJ, Chiang, YH, *et al.* (2013). "Scf(kmd) controls cytokinin signaling by regulating the degradation of type-b response regulators." Proc Natl Acad Sci U S A **110**(24): 10028-10033.

Kim, HJ, Ryu, H, *et al.* (2006). "Cytokinin-mediated control of leaf longevity by ahk3 through phosphorylation of arr2 in arabidopsis." Proc Natl Acad Sci U S A **103**(3): 814-819.

Koornneef, A and Pieterse, CM (2008). "Cross talk in defense signaling." Plant Physiol **146**(3): 839-844.

Krol, E, Mentzel, T, *et al.* (2010). "Perception of the arabidopsis danger signal peptide 1 involves the pattern recognition receptor atpepr1 and its close homologue atpepr2." J Biol Chem **285**(18): 13471-13479.

Kurepa, J, Li, Y, *et al.* (2013). "Proteasome-dependent proteolysis has a critical role in fine-tuning the feedback inhibition of cytokinin signaling." Plant Signal Behav **8**(3): e23474.

Lampard, GR, Lukowitz, W, *et al.* (2009). "Novel and expanded roles for mapk signaling in arabidopsis stomatal cell fate revealed by cell type-specific manipulations." Plant Cell **21**(11): 3506-3517.

Lee, DJ, Kim, S, *et al.* (2008). "Phosphorylation of arabidopsis response regulator 7 (arr7) at the putative phospho-accepting site is required for arr7 to act as a negative regulator of cytokinin signaling." Planta **227**(3): 577-587.

Lee, JS, Huh, KW, *et al.* (2008). "Comprehensive analysis of protein-protein interactions between arabidopsis mapks and mapk kinases helps define potential mapk signalling modules." Plant Signal Behav **3**(12): 1037-1041.

Liu, Y and Zhang, S (2004). "Phosphorylation of 1-aminocyclopropane-1-carboxylic acid synthase by mpk6, a stress-responsive mitogen-activated protein kinase, induces ethylene biosynthesis in arabidopsis." Plant Cell **16**(12): 3386-3399.

Lohrmann, J and Harter, K (2002). "Plant two-component signaling systems and the role of response regulators." Plant Physiol **128**(2): 363-369.

Lomin, SN, Krivosheev, DM, *et al.* (2012). "Receptor properties and features of cytokinin signaling." Acta Naturae **4**(3): 31-45.

Lorenzo, O, Piqueras, R, *et al.* (2003). Plant Cell **15**: 165.

Lu, X, Jiang, W, *et al.* (2013). "Aaerf1 positively regulates the resistance to botrytis cinerea in artemisia annua." PLoS One **8**(2): e57657.

Luan, S (2003). "Protein phosphatases in plants." Annu Rev Plant Biol **54**: 63-92.

Mahonen, AP, Bishopp, A, *et al.* (2006). "Cytokinin signaling and its inhibitor ahp6 regulate cell fate during vascular development." Science **311**(5757): 94-98.

Makino, S, Kiba, T, *et al.* (2000). "Genes encoding pseudo-response regulators: Insight into his-to-asp phosphorelay and circadian rhythm in arabidopsis thaliana." Plant Cell Physiol **41**(6): 791-803.

Manners, JM, Penninckx, IA, *et al.* (1998). "The promoter of the plant defensin gene pdf1.2 from arabidopsis is systemically activated by fungal pathogens and responds to methyl jasmonate but not to salicylic acid." Plant Mol Biol **38**(6): 1071-1080.

Mason, MG, Li, J, *et al.* (2004). "Type-b response regulators display overlapping expression patterns in arabidopsis." Plant Physiol **135**(2): 927-937.

Matsushika, A, Makino, S, *et al.* (2000). "Circadian waves of expression of the aprr1/toc1 family of pseudo-response regulators in arabidopsis thaliana: Insight into the plant circadian clock." Plant Cell Physiol **41**(9): 1002-1012.

McDowell, JM, Cuzick, A*, et al.* (2000). "Downy mildew (peronospora parasitica) resistance genes in arabidopsis vary in functional requirements for ndr1, eds1, npr1 and salicylic acid accumulation." Plant J **22**(6): 523-529.

McDowell, JM and Dangl, JL (2000). "Signal transduction in the plant immune response." Trends Biochem Sci **25**(2): 79-82.

Melotto, M, Underwood, W*, et al.* (2008). "Role of stomata in plant innate immunity and foliar bacterial diseases." Annu Rev Phytopathol **46**: 101-122.

Menges, M, Doczi, R*, et al.* (2008). "Comprehensive gene expression atlas for the arabidopsis map kinase signalling pathways." New Phytol **179**(3): 643-662.

Meszaros, T, Helfer, A*, et al.* (2006). "The arabidopsis map kinase kinase mkk1 participates in defence responses to the bacterial elicitor flagellin." Plant J **48**(4): 485-498.

Metraux, JP, Signer, H*, et al.* (1990). "Increase in salicylic acid at the onset of systemic acquired resistance in cucumber." Science **250**(4983): 1004-1006.

Micic, Z, Hahn, V*, et al.* (2004). "Qtl mapping of sclerotinia midstalk-rot resistance in sunflower." Theor Appl Genet **109**(7): 1474-1484.

Mira-Rodado, V, Sweere, U*, et al.* (2007). "Functional cross-talk between two-component and phytochrome b signal transduction in arabidopsis." J Exp Bot **58**(10): 2595-2607.

Mira-Rodado, V, Veerabagu, M*, et al.* (2012). "Identification of two-component system elements downstream of ahk5 in the stomatal closure response of arabidopsis thaliana." Plant Signal Behav **7**(11): 1467-1476.

Mizoguchi, T, Irie, K*, et al.* (1996). "A gene encoding a mitogen-activated protein kinase kinase kinase is induced simultaneously with genes for a mitogen-activated protein kinase and an s6 ribosomal protein kinase by touch, cold, and water stress in arabidopsis thaliana." Proc Natl Acad Sci U S A **93**(2): 765-769.

Montillet, JL, Leonhardt, N*, et al.* (2013). "An abscisic acid-independent oxylipin pathway controls stomatal closure and immune defense in arabidopsis." PLoS Biol **11**(3): e1001513.

Mordret, G (1993). "Map kinase kinase: A node connecting multiple pathways." Biol Cell **79**(3): 193-207.

Moreira, S, Bishopp, A*, et al.* (2013). "Ahp6 inhibits cytokinin signaling to regulate the orientation of pericycle cell division during lateral root initiation." PLoS One **8**(2): e56370.

Moubayidin, L, Perilli, S*, et al.* (2010). "The rate of cell differentiation controls the arabidopsis root meristem growth phase." Curr Biol **20**(12): 1138-1143.

Mur, LA, Kenton, P*, et al.* (2006). "The outcomes of concentration-specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death." Plant Physiol **140**(1): 249-262.

Nakagami, H, Pitzschke, A*, et al.* (2005). "Emerging map kinase pathways in plant stress signalling." Trends Plant Sci **10**(7): 339-346.

Nakamichi, N, Kiba, T*, et al.* (2010). "Pseudo-response regulators 9, 7, and 5 are transcriptional repressors in the arabidopsis circadian clock." Plant Cell **22**(3): 594-605.

Nawrath, C and Metraux, JP (1999). "Salicylic acid induction-deficient mutants of arabidopsis express pr-2 and pr-5 and accumulate high levels of camalexin after pathogen inoculation." Plant Cell **11**(8): 1393-1404.

Nishimura, MT and Dangl, JL (2010). "Arabidopsis and the plant immune system." Plant J **61**(6): 1053-1066.

Nishiyama, R, Watanabe, Y*, et al.* (2013). "Arabidopsis ahp2, ahp3, and ahp5 histidine phosphotransfer proteins function as redundant negative regulators of drought stress response." Proc Natl Acad Sci U S A **110**(12): 4840-4845.

Oliver, RP and Ipcho, SV (2004). "Arabidopsis pathology breathes new life into the necrotrophs-vs.-biotrophs classification of fungal pathogens." Mol Plant Pathol **5**(4): 347-352.

Otani, H, Kohnobe, A*, et al.* (1998). Physiol. Mol. Plant Pathol. **52**: 285.

P, VANB, Woltering, EJ*, et al.* (2007). "Histochemical and genetic analysis of host and non-host interactions of arabidopsis with three botrytis species: An important role for cell death control." Mol Plant Pathol **8**(1): 41-54.

Pape, S, Thurow, C, *et al.* (2010). "The arabidopsis pr-1 promoter contains multiple integration sites for the coactivator npr1 and the repressor sni1." Plant Physiol **154**(4): 1805-1818.

Pathak, RK, Taj, G, *et al.* (2013). "Modeling of the mapk machinery activation in response to various abiotic and biotic stresses in plants by a system biology approach." Bioinformation **9**(9): 443-449.

Penninckx, IA, Eggermont, K, *et al.* (1996). "Pathogen-induced systemic activation of a plant defensin gene in arabidopsis follows a salicylic acid-independent pathway." Plant Cell **8**(12): 2309-2323.

Penninckx, IA, Thomma, BP, *et al.* (1998). "Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in arabidopsis." Plant Cell **10**(12): 2103-2113.

Petersen, M, Brodersen, P, *et al.* (2000). Cell **103**: 1111.

Petersen, M, Brodersen, P, *et al.* (2000). "Arabidopsis map kinase 4 negatively regulates systemic acquired resistance." Cell **103**(7): 1111-1120.

Pischke, MS, Jones, LG, *et al.* (2002). "An arabidopsis histidine kinase is essential for megagametogenesis." Proc Natl Acad Sci U S A **99**(24): 15800-15805.

Pitzschke, A and Hirt, H (2009). "Disentangling the complexity of mitogen-activated protein kinases and reactive oxygen species signaling." Plant Physiol **149**(2): 606-615.

Popescu, SC, Popescu, GV, *et al.* (2009). "Mapk target networks in arabidopsis thaliana revealed using functional protein microarrays." Genes Dev **23**(1): 80-92.

Posas, F and Saito, H (1998). "Activation of the yeast ssk2 map kinase kinase kinase by the ssk1 two-component response regulator." EMBO J **17**(5): 1385-1394.

Qi, L, Yan, J, *et al.* (2012). "Arabidopsis thaliana plants differentially modulate auxin biosynthesis and transport during defense responses to the necrotrophic pathogen alternaria brassicicola." New Phytol **195**(4): 872-882.

Qiu, JL, Fiil, BK, *et al.* (2008). "Arabidopsis map kinase 4 regulates gene expression through transcription factor release in the nucleus." EMBO J **27**(16): 2214-2221.

Qiu, JL, Zhou, L, *et al.* (2008). "Arabidopsis mitogen-activated protein kinase kinases mkk1 and mkk2 have overlapping functions in defense signaling mediated by mekk1, mpk4, and mks1." Plant Physiol **148**(1): 212-222.

Rasmussen, MW, Roux, M, *et al.* (2012). "Map kinase cascades in arabidopsis innate immunity." Front Plant Sci **3**: 169.

Ren, B, Liang, Y, *et al.* (2009). "Genome-wide comparative analysis of type-a arabidopsis response regulator genes by overexpression studies reveals their diverse roles and regulatory mechanisms in cytokinin signaling." Cell Res **19**(10): 1178-1190.

Riefler, M, Novak, O, *et al.* (2006). "Arabidopsis cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism." Plant Cell **18**(1): 40-54.

Rochon, A, Boyle, P, *et al.* (2006). "The coactivator function of arabidopsis npr1 requires the core of its btb/poz domain and the oxidation of c-terminal cysteines." Plant Cell **18**(12): 3670-3685.

Rodriguez, MC, Petersen, M, *et al.* (2010). "Mitogen-activated protein kinase signaling in plants." Annu Rev Plant Biol **61**: 621-649.

Rowe, HC and Kliebenstein, DJ (2008). "Complex genetics control natural variation in arabidopsis thaliana resistance to botrytis cinerea." Genetics **180**(4): 2237-2250.

Sakai, H, Aoyama, T, *et al.* (2000). "Arabidopsis arr1 and arr2 response regulators operate as transcriptional activators." Plant J **24**(6): 703-711.

Sakai, H, Honma, T, *et al.* (2001). "Arr1, a transcription factor for genes immediately responsive to cytokinins." Science **294**(5546): 1519-1521.

Schenk, PM, Kazan, K, *et al.* (2000). "Coordinated plant defense responses in arabidopsis revealed by microarray analysis." Proc Natl Acad Sci U S A **97**(21): 11655-11660.

Schikora, A, Carreri, A, *et al.* (2008). "The dark side of the salad: Salmonella typhimurium overcomes the innate immune response of arabidopsis thaliana and shows an endopathogenic lifestyle." PLoS One **3**(5): e2279.

Schoenbeck, MA, Samac, DA, *et al.* (1999). "The alfalfa (medicago sativa) tdy1 gene encodes a mitogen-activated protein kinase homolog." Mol Plant Microbe Interact **12**(10): 882-893.

Schwartz, MA and Madhani, HD (2004). "Principles of map kinase signaling specificity in saccharomyces cerevisiae." Annu Rev Genet **38**: 725-748.

Seo, HS, Song, JT, *et al.* (2001). "Jasmonic acid carboxyl methyltransferase: A key enzyme for jasmonate-regulated plant responses." Proc Natl Acad Sci U S A **98**(8): 4788-4793.

Sharrocks, AD, Yang, S-H, *et al.* (2000). "Docking domains and substrate-specificity determination for map kinases." Trends Biochem Sci **25**(9): 448-453.

Sheen, J, Zhou, L, *et al.* (1999). "Sugars as signaling molecules." Curr Opin Plant Biol **2**(5): 410-418.

Smekalova, V, Doskocilova, A, *et al.* (2013). "Crosstalk between secondary messengers, hormones and mapk modules during abiotic stress signalling in plants." Biotechnol Adv.

Stolz, A, Riefler, M, *et al.* (2011). "The specificity of cytokinin signalling in arabidopsis thaliana is mediated by differing ligand affinities and expression profiles of the receptors." Plant J **67**(1): 157-168.

Su'udi, M, Park, JM, *et al.* (2013). "Quantification of alternaria brassicicola infection in the arabidopsis thaliana and brassica rapa subsp. Pekinensis." Microbiology.

Suarez-Rodriguez, MC, Adams-Phillips, L, *et al.* (2007). "Mekk1 is required for flg22-induced mpk4 activation in arabidopsis plants." Plant Physiol **143**(2): 661-669.

Suzuki, T, Imamura, A, *et al.* (1998). "Histidine-containing phosphotransfer (hpt) signal transducers implicated in his-to-asp phosphorelay in arabidopsis." Plant Cell Physiol **39**(12): 1258-1268.

Suzuki, T, Sakurai, K, *et al.* (2001). "Two types of putative nuclear factors that physically interact with histidine-containing phosphotransfer (hpt) domains, signaling mediators in his-to-asp phosphorelay, in arabidopsis thaliana." Plant Cell Physiol **42**(1): 37-45.

Sweere, U, Eichenberg, K, *et al.* (2001). "Interaction of the response regulator arr4 with phytochrome b in modulating red light signaling." Science **294**(5544): 1108-1111.

Takahashi, F, Yoshida, R, *et al.* (2007). "The mitogen-activated protein kinase cascade mkk3-mpk6 is an important part of the jasmonate signal transduction pathway in arabidopsis." Plant Cell **19**(3): 805-818.

Takekawa, M, Tatebayashi, K, *et al.* (2005). "Conserved docking site is essential for activation of mammalian map kinase kinases by specific map kinase kinase kinases." Mol Cell **18**(3): 295-306.

Tanaka, Y, Sano, T, *et al.* (2006). "Cytokinin and auxin inhibit abscisic acid-induced stomatal closure by enhancing ethylene production in arabidopsis." J Exp Bot **57**(10): 2259-2266.

Taniguchi, M, Sasaki, N, *et al.* (2007). "Arr1 directly activates cytokinin response genes that encode proteins with diverse regulatory functions." Plant Cell Physiol **48**(2): 263-277.

Teige, M, Scheikl, E, *et al.* (2004). "The mkk2 pathway mediates cold and salt stress signaling in arabidopsis." Mol Cell **15**(1): 141-152.

Tena, G, Boudsocq, M, *et al.* (2011). "Protein kinase signaling networks in plant innate immunity." Curr Opin Plant Biol **14**(5): 519-529.

Thaler, JS, Owen, B, *et al.* (2004). "The role of the jasmonate response in plant susceptibility to diverse pathogens with a range of lifestyles." Plant Physiol **135**(1): 530-538.

Tsai, CH, Singh, P, *et al.* (2011). "Priming for enhanced defence responses by specific inhibition of the arabidopsis response to coronatine." Plant J **65**(3): 469-479.

Ueguchi, C, Koizumi, H, *et al.* (2001). "Novel family of sensor histidine kinase genes in arabidopsis thaliana." Plant Cell Physiol **42**(2): 231-235.

Urao, T, Yakubov, B, *et al.* (1999). "A transmembrane hybrid-type histidine kinase in arabidopsis functions as an osmosensor." Plant Cell **11**(9): 1743-1754.

van Wees, SC, Chang, HS, *et al.* (2003). "Characterization of the early response of arabidopsis to alternaria brassicicola infection using expression profiling." Plant Physiol **132**(2): 606-617.

Veerabagu, M, Elgass, K, *et al.* (2012). "The arabidopsis b-type response regulator 18 homomerizes and positively regulates cytokinin responses." Plant J **72**(5): 721-731.

Wang, H, Ngwenyama, N, *et al.* (2007). "Stomatal development and patterning are regulated by environmentally responsive mitogen-activated protein kinases in arabidopsis." Plant Cell **19**(1): 63-73.

Whitmarsh, AJ and Davis, RJ (1998). "Structural organization of map-kinase signaling modules by scaffold proteins in yeast and mammals." Trends Biochem Sci **23**(12): 481-485.

Widmann, C, Gibson, S*, et al.* (1999). "Mitogen-activated protein kinase: Conservation of a three-kinase module from yeast to human." Physiol Rev **79**(1): 143-180.

Wu, Y, Zhang, D*, et al.* (2012). "The arabidopsis npr1 protein is a receptor for the plant defense hormone salicylic acid." Cell Rep **1**(6): 639-647.

Xie, DX, Feys, BF*, et al.* (1998). Science **280**: 1091.

Xu, J, Li, Y*, et al.* (2008). "Activation of mapk kinase 9 induces ethylene and camalexin biosynthesis and enhances sensitivity to salt stress in arabidopsis." J Biol Chem **283**(40): 26996-27006.

Xu, Q and West, AH (1999). "Conservation of structure and function among histidine-containing phosphotransfer (hpt) domains as revealed by the crystal structure of ypd1." J Mol Biol **292**(5): 1039-1050.

Yamaguchi, K, Yamada, K*, et al.* (2013). "Receptor-like cytoplasmic kinases are pivotal components in pattern recognition receptor-mediated signaling in plant immunity." Plant Signal Behav **8**(10).

Yoo, SD, Cho, YH*, et al.* (2008). "Dual control of nuclear ein3 by bifurcate mapk cascades in c2h4 signalling." Nature **451**(7180): 789-795.

Yuasa, T, Ichimura, K*, et al.* (2001). "Oxidative stress activates atmpk6, an arabidopsis homologue of map kinase." Plant Cell Physiol **42**(9): 1012-1016.

Zeng, W, Melotto, M*, et al.* (2010). "Plant stomata: A checkpoint of host immunity and pathogen virulence." Curr Opin Biotechnol **21**(5): 599-603.

Zhang, J, Shao, F*, et al.* (2007). "A pseudomonas syringae effector inactivates mapks to suppress pamp-induced immunity in plants." Cell Host Microbe **1**(3): 175-185.

Zhang, L, Li, Y*, et al.* (2012). "Cotton ghmkk5 affects disease resistance, induces hr-like cell death, and reduces the tolerance to salt and drought stress in transgenic nicotiana benthamiana." J Exp Bot **63**(10): 3935-3951.

Zhang, PJ, Li, WD*, et al.* (2013). "Feeding by whiteflies suppresses downstream jasmonic acid signaling by eliciting salicylic acid signaling." J Chem Ecol **39**(5): 612-619.

Zhang, Y, Tessaro, MJ*, et al.* (2003). Plant Cell **15**: 2647.

Zhao, Q and Guo, HW (2011). "Paradigms and paradox in the ethylene signaling pathway and interaction network." Mol Plant **4**(4): 626-634.

Zhao, Y, Thilmony, R*, et al.* (2003). "Virulence systems of pseudomonas syringae pv. Tomato promote bacterial speck disease in tomato by targeting the jasmonate signaling pathway." Plant J **36**(4): 485-499.

Zhou, N, Tootle, TL*, et al.* (1998). Plant Cell **10**: 1021.

Zhulin, IB, Taylor, BL*, et al.* (1997). "Pas domain s-boxes in archaea, bacteria and sensors for oxygen and redox." Trends Biochem Sci **22**(9): 331-333.

Zwerger, K and Hirt, H (2001). "Recent advances in plant map kinase signalling." Biol Chem **382**(8): 1123-1131.

5 Chapter 2

ARR1 and *ARR2* are involved in flowering time regulation of *Arabidopsis thaliana* under short days

5.1 Introduction

Flowering is a crucial step in plant development and it is characterised as a transition from vegetative to reproductive phase (Koornneef *et al.* 1998). Due to its importance of ensuring reproductive success, flowering is controlled by complex networks of genetic regulatory pathways (Coupland 1995; Srikanth and Schmid 2011; Poethig 2013). In the end, successful reproduction not only includes seed formation but also dispersion, germination and growth and complex regulatory mechanisms need to integrate both environmental and endogenous signals to ensure the next generation can also reproduce (Bernier *et al.* 1993; Koornneef *et al.* 1998; Srikanth and Schmid 2011). However, it is clear today that all these signalling pathways that control flowering are not strictly separated but that they work together, *i.e.* crosstalk in the regulation of flowering (Genoud and Metraux 1999; Franklin 2009). Surprisingly the number of common targets regulated by huge number of signalling pathways is quite small and they present central floral pathway integrator or "integrator genes" (Li *et al.* 2008; Srikanth and Schmid 2011). Even in self-fertile plants, flowering time is still strictly controlled by environmental and endogenous factors (Srikanth and Schmid 2011). Exogenous factors that strongly influence the flowering are day length, light quality and temperature (Coupland 1995; Reeves and Coupland 2000; Chen *et al.* 2004). On the other side, common endogenous factors that are regulating flowering are hormones (hormonal status of the plant), sugars and plant age (Blazquez *et al.* 1998; Wahl *et al.* 2013). Five genetic pathways have been identified that play role in control of flowering: the vernalization (originally jarovization) pathway, the photoperiod pathway (related to the day length and quality of perceived light), the gibberellin (GA) pathway, the autonomous pathway (endogenously controlled independent of photoperiod or gibberellin pathways), and plant age (also an endogenous pathway) (Srikanth and Schmid 2011).

5.1.1 Photoperiod-dependent flowering control

Photoperiod pathways present a cascade of events in charge for measurement day length and consequently initiating flowering (Valverde *et al.* 2004; Sawa *et al.* 2007). The organ for photoperiod perception is located exclusively in the leaves. Light perception in leaves is enabled by the presence of three main classes of photoreceptors specialized for different wavelengths: phototropins (blue light), phytochromes (red/far red light) and cryptochromes (blue light) (Franklin *et al.* 2005; Lariguet and Dunand 2005). Once a particular type of light-signal is perceived (varies between species) the flower-triggered substance called "florigen" is

produced and afterwards transmitted to the shoot apex to induce flowering (Colasanti and Sundaresan 2000). Even before the era of molecular biology had begun, it was very well known that plants measure and respond to the day length (Srikanth and Schmid 2011). This is due to the presence of the internal oscillators, genes that are regulated by the circadian clock (Harmer *et al.* 2000). The circadian clock is explained as a timekeeping mechanism in photoperiodism (Mizoguchi *et al.* 2005).

5.1.2 Photoperiod-dependent flowering control in *Arabidopsis thaliana*

In *Arabidopsis thaliana*, a circadian clock controlled flowering pathway includes the following gene members *GIGANTEA (GI), CONSTANS (CO)* and *FLOWERING LOCUS T (FT)* that promote flowering specifically under long days (Fowler *et al.* 1999; Mizoguchi *et al.* 2005). Briefly, the circadian clock in *Arabidopsis thaliana* is composed and works as follows: The central oscillator generates a self-sustaining rhythm driven by two interacting feedback loops that are active at different times of day. The first one so called "morning loop" consists of *CCA1 (CIRCADIAN AND CLOCK ASSOCIATED 1)* and *LHY (LATE ELONGATED HYPOCOTYL)*, which encode closely related MYB transcription factors that regulate circadian rhythms in *Arabidopsis*, as well as *PRR 7* and *9 (Pseudo-Response Regulators)*. The second "evening loop" consists of *GI (GIGANTEA)* and *ELF4 (EARLY FLOWERING 4)*, both are involved in regulation of flowering time genes (Fowler *et al.* 1999; Mizoguchi *et al.* 2005; Kolmos and Davis 2007).

Classification of plants due to their photoperiodic responses is usually based on flowering. The two main photoperiodic response categories are short-day and long day-plants (Garner 1933). Short-day plants (SDPs) flower in short days (qualitative SDPs) or their flowering is accelerated by short days (quantitative SDPs). Long-day plants (LDPs) flower only in long days (qualitative LDPs) or their flowering is accelerated by long days (quantitative LDPs). The essential distinction between long-day and short-day plants is that flowering in LDP is promoted only when the day length exceeds a certain duration. This time course is called critical day length. To flower SDPs require a day length less than a critical day length (Yano *et al.* 2001; Kojima *et al.* 2002). Plants that flower under any photoperiodic condition are referred to as day-neutral plants (DNPs) (Wu *et al.* 2004; Lifschitz *et al.* 2006); examples are most of the desert annuals that flower quickly whenever the water is available. *Arabidopsis thaliana* is a facultative LDP, *i.e.* quantitative LDP means it flowers also under SDs but its flowering is accelerated by inductive long days (Hicks *et al.* 1996). Important to photoperiodic flowering is the so-called phenomenon "coincidence model" proposed in the mid-1930s by the German botanist Professor Erwin Bünning which brings together endogenous and exogenous signals. The circadian oscillator controls the timing of light-sensitive and light-insensitive phases only when the light signal is coincident with the appropriate phase of the circadian rhythm thereby allowing flowering induction under both non-inductive and inductive days (Srikanth and Schmid 2011). The first mutant discovered in *Arabidopsis thaliana* incapable of a photoperiodic flowering response was *co* mutant: a mutant lacking in *CO (CONSTANS)* which encodes a zinc finger transcription factor family protein which is controlling expression of other floral regulators (Putterill *et al.* 1995; Tran *et al.* 2007). The *co*

mutant has a very strong late flowering phenotype under inductive LD conditions only whereas at non-inductive SD, *co* flowers identical like the wild-type plant suggesting the CO to be a flower activator (Putterill *et al.* 1995). It was later discovered that the expression *CO* is regulated by the circadian clock with pick expression of *CO* around dusk (Suarez-Lopez *et al.* 2001). Expression pattern of *CO* represents an example of "coincidence model". The CO proteins levels are also controlled however and CO only accumulates enough to promote flowering under LD when the light phase overlaps/coincides with the maximal pick of the *CO* mRNA (Valverde *et al.* 2004; Bohlenius *et al.* 2006). Research has shown that CO is posttranscriptional regulated and that during the dark phase the protein is tagged for degradation by ubiquitin and afterwards degraded in 26s proteasome and part of this is controlled by different photoreceptors (Valverde *et al.* 2004). It has been shown that PhyB signalling in the morning negatively regulates *CO* whereas PhyA and cryptochromes positively regulate the accumulation of CO protein in the evening (Lin 2000; Endo *et al.* 2013). This entire regulation of *CO* happens only in leaves (An *et al.* 2004; Ayre and Turgeon 2004), *i.e.* it does not transfer signal from the leaves to the shoots. Further downstream CO activates expression of other flowering-related genes which are transferring the signal information to the apical meristem and initiating flowering. One of them is *FT (FLOWERING LOCUS T)* gene (Kardailsky *et al.* 1999; Hisamatsu and King 2008). *FT* belongs to a small group of proteins that show structural similarities to mammalian phosphatidylethanolamine-binding protein (Ahn *et al.* 2006; Kim *et al.* 2013). Five more members belong to this family: *TSF (TWIN SISTER OF FT), MFT (MOTHER OF FT AND TFL1), BFT (BROTHER OF FT AND TFL1), ATC (ARABIDOPSIS THALIANA CENTRORADIALIS HOMOLOGUES)* and their major role is the regulation of photoperiodic flowering (Wigge 2011; Pin and Nilsson 2012; Kim *et al.* 2013). In the shoot apical meristem of *Arabidopsis thaliana* it seems that the FT protein, but not *FT* mRNA (Notaguchi *et al.* 2008), is needed to induce flowering, although there are some indications that *FT* mRNA could also be contributing flower induction but only together with its protein form (Li *et al.* 2009). In the shoot apex FT makes a complex with FD (Abe *et al.* 2005; Wigge *et al.* 2005). *FD* belongs to the bZIP transcription factor family and it is preferentially expressed in the shoot apex and required there for FT to promote flowering (Abe *et al.* 2005). This complex afterwards activates downstream targets such as *SOC1 (SUPPRESSOR OF OVEREXPRESSION OF CO 1)* (Searle *et al.* 2006), APETELA 1 (*AP1)* (Wigge *et al.* 2005) and indirectly *LFY (LEAFY)* (Abe *et al.* 2005; Smith *et al.* 2011). *SOC1* is floral activator required for *CO* to promote flowering, it acts downstream of *FT* (Yoo *et al.* 2005), *AP1* and *LFY* are meristem identity genes which generally promote the transition to flowering by further induction of floral homeotic genes (Yu *et al.* 2004).

5.1.3 Photoperiod-dependent flowering control in rice and other plants

The coincidence model is also present within neutral and short day plants like in tomato (*Solanum lycopersicum*) or rice (*Oryza sativa*), respectively. In tomato, the orthologue of *FT* is *SFT (SINGLE-FLOWER TRUSS)* and it is a floral activator just like in *Arabidopsis* (Lifschitz *et al.* 2006). Besides its commercial value and, in contrast to *Arabidopsis*, *Oryza sativa* belongs to the group of short day plants (Yano *et al.* 2001; Kojima *et al.* 2002). The rice genes *Hd1*

(Heading-date 1) and *Hd3a (Heading-date 3a)* encode homologs to *Arabidopsis CO* and *FT* respectively. Like *CO* in *Arabidopsis, Hd1* has an identical expression pattern in rice (Kojima *et al.* 2002) with only difference between rice and *Arabidopsis* is that under short day conditions *Hd1* acts as repressor of *Hd3a* expression. That is the reason why is the flowering in rice, in contrast to *Arabidopsis*, actually inhibited by coincidence of light and the *Hd1* expression; when the light overlaps the maximum expression pick of *Hd1* mRNA it blocks afterwards expression of *Hd3a* and inhibits the flowering. These three examples showed that *CO-FT* regulatory module is conserved among plants pointing its importance and differences in flowering time are most likely mediated through different strategies for regulating this module (Srikanth and Schmid 2011). Summed together, photoperiodism is a very complex mechanism and it is highly controlled and fine-tuned by different regulatory elements which impend on key, evolutionarily conserved genetic networks (Hayama and Coupland 2004; Franklin *et al.* 2005; Imaizumi and Kay 2006).

5.1.4 Connection between two-component system and flowering regulation in rice and *Arabidopsis*

Interestingly, in rice, FT-like gene expression can be controlled independently of *Hd1* by the so-called *Ehd1 (Early heading Date 1)* gene encoding a B-type response regulator, part of two-component signal (TCS) transduction systems (Doi *et al.* 2004) suggesting an important role of TCS in regulating flowering. *Ehd1* functions as a floral inducer and it induces *FT-like* and MADS box genes (*e.g.* the *AP1* ortholog in *Arabidopsis*) (Doi *et al.* 2004).

TCS systems typically transduce a receptor-mediated signal into a phosphorelay from a histidine-kinase onto a conserved aspartate residue of a response regulator (RR) protein. In *Arabidopsis*, there are response regulator proteins that have a mutation in this conserved aspartate (Asp) residue: these proteins are called pseudo response regulators (pseudo-RRs) (Doi *et al.* 2004). It has been already proven that pseudo-RRs regulate flowering time in *Arabidopsis thaliana* and that *TOC1/APRR1*, an *Arabidopsis* pseudo response regulator, is part of the plant circadian clock. *TOC1/APRR1* belongs to a small gene family in *Arabidopsis* (Matsushika *et al.* 2000) and it influences flowering time via the phase setting of *CO* expression (Yanovsky and Kay 2002). Even though pseudo-RRs have lost their ability to accept the phosphorelay phosphate as they are constitutively active because the highly conserved Asp is mutated to a glutamate naturally mimicking a phosphorylated Asp (Doi *et al.* 2004).

In *Arabidopsis thaliana*, ARR4 (an A-type response regulator) plays a very important role in stabilization of active PhyB-Pfr under extended red light conditions and thus functions as modulator of photomorphogenesis (Sweere *et al.* 2001; Mira-Rodado *et al.* 2007). PhyB, as above mentioned, is actively involved in expression control of *CO* in *Arabidopsis thaliana* and also *Hd3a* in *Oryza sativa*.

On the other hand it has been proven in various ways that B-Type response regulators are directly, positively regulating expression of the A-Type response regulators in *Arabidopsis*

(Grefen and Harter 2004; Brenner *et al.* 2012; Veerabagu *et al.* 2012). In addition to this, it was recently shown that floral regulator *SVP (SHORT VEGETATIVE PHASE)* is directly regulating A-type response regulators as well as *CRFs (CYTOKININ RESPONSE FACTORS)* (Gregis *et al.* 2013) was well as directly binding to *STIP (STYMPY)* newly reported component of cytokinin signalling pathway mediationg cytokinin signalling during shoot meristem establishment in *Arabidospsis thaliana* (Skylar *et al.* 2010). Furthermore, it was also recently reported that cytokinin is influencing flowering time in *Arabidopsis*, especially under non inductive short days (Bernier 2011; D'aloia *et al.* 2011). B-type response regulators are being discovered to be involved in fine-tuning and crosstalk of a multitude of signalling pathways in higher plants (Veerabagu *et al.* 2012; Zwack and Rashotte 2013). For example, *ARR2,* a member of B-type response regulators, is part of TCS signalling important for signal transmission of cytokinin and ethylene (detail about TCS signalling and members can be found in the introduction of the first chapter) (Rashotte *et al.* 2003; Grefen and Harter 2004; Hass *et al.* 2004; Mason *et al.* 2005; Zwack and Rashotte 2013). Despite the great sequence similarity of the B-types within the same subgroup they still show very high specificity among each other (Hill *et al.* 2013). The *arr2* loss-of-function mutant plant in Landsberg displayed retarded growth and development including early flowering (Hass *et al.* 2004). *ARR2* was also reported to function in ethylene signalling (Hass *et al.* 2004) and in response to pathogen attack (Choi *et al.* 2010) further demonstrating that B-types can be involved in multiple signalling pathways.

It was discovered that, when applied in hydroponic culture from the roots, in SDs, cytokinin bypassies *FT* and activates transcription of *TSF* in leaves to induce flowering (D'aloia *et al.* 2011). Furthermore, TSF interacts with FD and regulates expression of *SOC1* which also seems to be controlled by cytokinin (D'aloia *et al.* 2011). On the other hand *TSF* and *SOC1* also work independently of *FD* suggesting that *FD* and its paralogue *FDP (FD PARALOG)* (Abe *et al.* 2005) might share this function of *SOC1* controle (Bernier 2011; D'aloia *et al.* 2011). Cytokinin itself cannot induce flowering (Bernier 2011) but the fact that when cytokinin was added to roots of hydroponic-grown *Arabidopsis* plant was sufficient to induce flowering in SDs and that the cytokinin contents of leaves, phloem sap, and SAM (SHOOT APICAL MERISTEM) increased in response to LDs induction in *Arabidopsis* (Bernier 2011; D'aloia *et al.* 2011). In *Sinapis cytokinin* level is also increased in response to LDs induction (Corbesier et al., 2003) and it was reported co-dependent transportation of *SaFT* and cytokinin to the meristem suggests that cytokinin may play a part to regulatory effects attributed to "florigen" (Bernier 2011). However, biological functions of *Arabidopsis* B-type response regulators with respect to flowering have not yet been identified.

5.1.5 Temperature as floral regulator

Another factor, beside photoperiod, that is important in the regulation of flowering is temperature (Blazquez *et al.* 2003). Flowering can be controlled by temperature in two manners: by the process called vernalisation and also by ambient temperatures under which plants exposed to during their vegetative phase (Alonso-Blanco and Koornneef 2000; Koornneef *et al.* 2004).

5.1.5.1 Vernalisation

Vernalization is a process by which flowering is promoted when plants sense exposure to the prolonged cold temperatures (≤7°C). This is an adaptive trait that helps prevent flowering before winter is over thus permitting flowering in the favourable conditions of spring (Kim *et al.* 2009). In winter annual biennial and perennial plants, vernalization is an obligate process, whereas for summer annuals vernalization is facultative. For vernalization to work the temperature has to be a in range between 1 and 7°C for a time of 1 to 3 months depending on the plant species (Srikanth and Schmid 2011). In *Arabidopsis,* vernalization involves the recruitment of chromatin-modifying complexes to a clade of flowering repressors that are silenced epigenetically via histone modifications (Kim *et al.* 2009). This effect is known as "memory of winter" and it does not pass to the progeny but is reset during meiosis (Amasino 2004; Heo and Sung 2011). In *Arabidopsis* and cereals, vernalization results in the suppression of genes that repress flowering (Kim *et al.* 2009). Interestingly, in *Arabidopsis*, the most commonly used laboratory strains do not need to be vernalized, but some do flower very late if they are not vernalized first (Reeves *et al.* 2007; Srikanth and Schmid 2011). There are two dominant genes for vernalization in *Arabidopsis thaliana*: *FRI (FRIGIDA)* and *FLC (FLOWERING LOCUS C)* (Lee and Amasino 1995; Song *et al.* 2012) wehreby FRI up-regulates the expression of *FLC* (Geraldo *et al.* 2009). *FRI* encodes a nuclear protein found only in plants (Johanson *et al.* 2000; Bari and Jones 2009) and *FLC* encodes a MADS-box DNA binding protein that functions as a repressor of flowering, *i.e.* flowering-time related genes (Deng *et al.* 2011). FLC represses expression of *FT*, *FD*, and *SOC1* floral activators by direct interaction with their promoters (Hepworth *et al.* 2002; Helliwell *et al.* 2006). It has been proven that FLC also directly interacts with another MAD-box member, *SVP* which is also floral repressor that functions within the thermosensory pathway (Li *et al.* 2008). This interaction makes physiological and biological sense because it partially supresses *FLC*-mediated delay in flowering (Li *et al.* 2008; Bari and Jones 2009; Yoshida *et al.* 2009). Briefly, *FLC* is silenced in response to vernalization, after *FLC* been silenced VIN3 (VERNALIZATION INSENITIVE 3) initially repressing *FLC* during the cold exposure by remodelling the chromation, *i.e.* by methylating lysine residues of histone H3 (Sung and Amasino 2004; Bond *et al.* 2009) than at least two crucial genes *VRN1* and *VRN2 (VERNALIZATION 1 and 2)* maintain the epigenetic state of *FLC* (Bastow *et al.* 2004). Vernalization as a phenomenon is probably result of convergent evolution as outside of *Brassicaceae* no clear orthologs of *FLC* has been identified, making this a complicated research field (Srikanth and Schmid 2011).

5.1.5.1.1 Ambient temperature

Another factor that controls flowering is ambient temperature during its vegetative stage. Higher temperatures (27°C) accelerate flowering under non-inductive SDs for different *Arabidopsis* accessions serving as a substitute to inductive LDs (Balasubramanian *et al.* 2006). Ambient temperature effects on flowering are very diverse among different species, and even between different accessions of *Arabidopsis thaliana*. Many flowering time mutants also show temperature dependence, *i.e.* when exposed to higher temperatures they flowered earlier (e.g. photoreceptor mutants *phyB*, *cry2* and most *Arabidopsis fri/flc* natural accessions)

(Blazquez *et al.* 2003). Nevertheless, this is not the case when *Nd-1* or *svp* were tested. *Nd-1* accession mutant showed masked effect when grown at 27°C (Balasubramanian *et al.* 2006) as this mutant was shown to have a deletion of the floral repressor *FLM (FLOWERING LOCUS/MADS AFFECTING FLOWERING1))* as a major cause for early flowering of *Nd-1* in short days at 23 °C (Werner *et al.* 2005; Balasubramanian *et al.* 2006).

FLM is like FLC a MADS-box protein and shares strong sequence similarity with *FLC* but despite this their physiological roles seem to be different (Scortecci *et al.* 2003). Microarray data showed that genes associated with alternative splice site selection are affected by thermal induction and it has been also proven that *FLM* has temperature-dependent alternative splicing and this splicing is an important regulator of flowering. (Werner *et al.* 2005; Srikanth and Schmid 2011) In the Waissilewskija ecotype *FLM* gene has four splice variants (α, β, γ and δ) whereas in Columbia (Col-0) accession *FLM* has two splice variants (β and δ) and they are both translated (Lee *et al.* 2013; Pose *et al.* 2013). FLM- β is the prevalent splice variant at lower temperatures (16°C) whereas FLM- δ dominates at higher temperatures (27°C) (Lee *et al.* 2013; Pose *et al.* 2013). Both splice variants are interact and make heterodimers with SVP independent of the transcriptional level which does not change with variation in temperature. Interestingly, they can also interact among each other making FLM-β x FLM-δ heterodimers but only FLM-β x FLM-β homodimers are possible (Lee *et al.* 2013; Pose *et al.* 2013). Proposed model of activity says that dependent of which splice variant β or δ is making heterodimers with SVP determinates later the activity of SVP-FLM heterocomplex (Lee *et al.* 2013). At low ambient temperatures formation of SVP-SVP and SVP-FLM-β complexes is favoured and they both repressing flowering. As the temperature is rising the FLM-β splice form is down-regulated and therefore SVP-FLM- δ complex formation is increased and it now accelerates flowering. Based on this model the role of temperature-dependent mRNA splicing in adaptation to climate change is observed and explained (Pose *et al.* 2013). Other known genes which are involved in regulating flowering in response to ambient temperatures are *HSP70 (HEAT SHOCK PROTEIN 70)* found to be highly correlated with an increase in temperature (Balasubramanian *et al.* 2006; Kumar and Wigge 2010), and also *ARP6 (ACTIN RELATED PROTEIN 6)* which is known to be a nuclear protein that represses flowering as maintaining the expression of *FLC* (Choi *et al.* 2005; Deal *et al.* 2005).

5.1.6 The autonomous pathway

Beside the endogenous hormonal factors that are influencing flowering it is important to mention that autonomous pathways are defined as those that delay flowering irrespective of day length (Mouradov *et al.* 2002). Genes involved in autonomous pathway can be grouped into two broad functional categories of genes that act by repressing *FLC*; these are: general remodelling and maintenance factors of *FLC*, and proteins that effect RNA processing (He *et al.* 2003; Srikanth and Schmid 2011).

5.1.7 Gibberellic acid pathway as a regulator of flowering

One of most potent endogenous control of flowering is hormonal control regulated by the gibberellic acid (GA) pathway (Blazquez *et al.* 1998). The effect of gibberellic acid on plant

growth and development has been known since the beginning of the last century when the rice plants infected with fungus *Gibberella fujikuroi* were growing so fast that they tipped over (Tudzynski 1999; Hsuan *et al.* 2011). Other developmental roles of GA, like its influence on flowering time, became familiar much later. Numerous GAs were discovered in plants but they are not all of them are biologically active; the active ones are GA1, GA3, GA4 and GA7 named according the order of their discovery (Hedden and Phillips 2000). After huge mutagenesis screenings for mutants that affect GA biosynthesis were detected. These mutants showed, beside various developmental defects, delay of flowering by *Arabidopsis* mostly under the non-inductive SDs (Wilson *et al.* 1992). The *ga1-3* mutant, lacking the gene for synthesis of ent-kaurene needed for GA1 formation (Sun *et al.* 1992) showed under inductive LDs almost normal flowering pattern whereas it was not able to flower at all at SDs, even when previously treated with exogenous GAs. Nevertheless, this flowering effect of GA is not strictly limited to SDs as demonstrated by the triple *gid1* mutant (lacking in all three *GID-GIBBERELLIC INSENSITIVE DWARF* receptors for GA). The *gid1* mutant flowers extremely late or not even at all under inductive LDs (Griffiths *et al.* 2006; Willige *et al.* 2007). Interestingly, GA works in parallel (*i.e.* independently) of *CO* as the *ga1-3 co* double mutant showed an additive late-flowering phenotype. of *co* under LD but on the other hand the levels of *FT* mRNA expression were increased after addition of exogenous GAs suggesting that GA are on some other way independently controlling expression of *FT* (Hisamatsu and King 2008). It was later shown that *FT* expression is controlled by GA through interaction of the GID1 receptor with members of the DELLA protein family, totally independent of *CO* (Sun 2010). DELLA proteins belong to the GRAS family of transcriptional regulators and work as repressors of plant growth and development (Hirsch and Oldroyd 2009).

5.1.8 Other factors affecting flowering

Other important endogenous factors that are promoting flowering are sugars (Bernier *et al.* 1993). It is known that sucrose can promote flowering in some plant species but this effect might be rather species-specific (Srikanth and Schmid 2011). Trehalose is another sugar that promotes flowering (Schluepmann *et al.* 2003). Furthermore, T6P (trehalose-6-phosphate) was proven to be a proxy for carbohydrate status in plants and that it is absolutely essential for expression of *FT* and *TSF* in the phloem companion cells (Wahl *et al.* 2013). The age of the plant was recently reported to play a role in the regulation in flowering time independent of photoperiod, vernalization or GA pathways, and instead works via the *miR156* microRNA level which decreases with increasing age of the plant (Wang *et al.* 2009). In this work an early flowering phenotype of *ARR1* and *ARR2* lacking mutants (*arr1-4, arr2-4* single and *arr1-4 arr2-4* double mutants) under SD was further investigated. This is the first time that B-type response regulators in *Arabidopsis* (LD facultative plant) have been shown to regulate flowering. TCS and probably cytokinin were suggested to play roles as regulators of flowering. Further *ARR1* and *ARR2* are acting independent of *FLC* floral repressor but therefore *FLM* floral repressor seems to be epistatic to both *ARR1* and *ARR2* response regulators.

5.2 Results

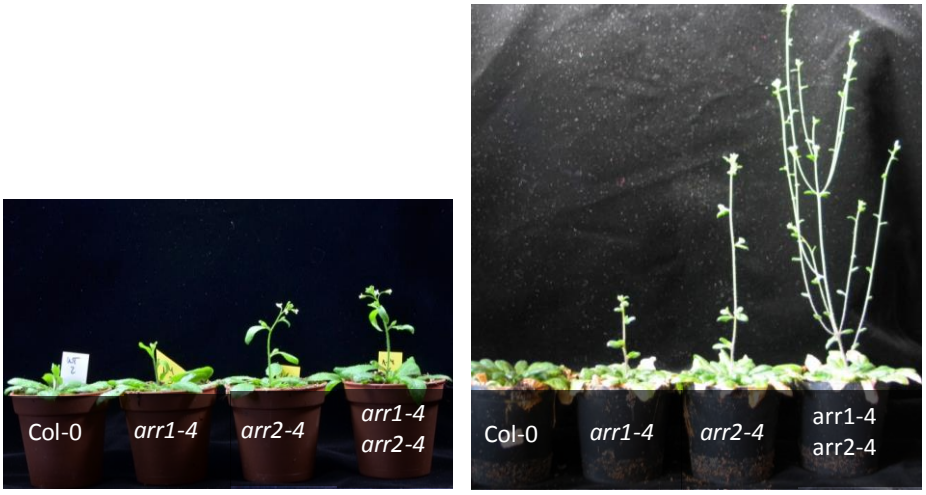
5.2.1 *ARR2* mutants show early flowering phenotype under short day (SD) conditions

Previous work on *ARR2* had been conducted with the *arr2-1 to arr2-4* mutant allele in the Ler-0 ecotype (Hass *et al.* 2004). Work on this mutant showed connections between the two-component signalling pathway, genes related to defence and abiotic stress signalling and adaptation and ethylene signalling (Hass *et al.* 2004). In order to investigate further roles of *ARR2* with its closest homolog *ARR1*, a mutant of *ARR2* was obtained in the Col-0 background. This mutant, and a mutant of *ARR1*, *arr1-4,* also in the Col-0 background, were shown to be full knock-outs. Interestingly, *arr1-4*, *arr2-4* single mutants and the *arr1-4 arr2-4* double mutant displayed precocious flowering under non-inductive short day (SD) conditions, with the double mutant having an additive effect (Figure 5.1).

Despite the high sequence similarity and reported functional redundancy of *ARR1* and *ARR2* in regulating expression of certain common targets in response to cytokinin signal (Mason *et al.* 2005), the loss of *ARR1* and *ARR2* effects flowering time to a different degree.

The single *arr1-4* and arr2-4 mutants flower one and two weeks earlier than wild-type control plants, respectively (Figure 5.1, B). In contrast, the *arr1-4 arr2-4* double mutant flowers three weeks before the Col-0 control (Figure 5.1, B). The early flowering phenotype can be also observed as a reduction of rosette leaf number. The *arr1-4* mutant induces flowering by more than 4 leaves, *arr2-4* mutant by more than 7 leaves, while *arr1-4 arr2-4* produces 15 leaves less than Col-0 (Figure 5.1, B). A trend towards early flowering was also observed when the *arr1/2* mutants were grown under inductive long day (LD) conditions. However, only the *arr1-4 arr2-4* double mutant flowered significantly earlier than wild-type ($p = 0{,}0052$; $\alpha = 0{,}05$) and produced 3 leaves less than the wild-type (Figure 5.1, C). In conclusion, the loss of *ARR1*, *ARR2*, or both genes simultaneously, resulted in early flowering under SD conditions. As ARR1 and ARR2 are known B-Type transcription factors, some of known flowering time-related genes (*CO, SOC1, AP1, FT, FLC, FLM, TSF, TFL, LFY* and *FD)* were profiled for alterations in expression patterns in the various mutants under short day and long day conditions.

A



B

	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Short Day							
Col-0 (wild type)	112,8	51,5	±11,8	±4,6	102-129	43-60	20
arr1-4	108,9	48,0	±10,3	±5,6	95-119	39-56	20
arr2-4	103,8	46,2 ‡	±10,6	±6,7	85-116	36-54	20
arr1-4 arr2-4	91,7 ‡	33,7 ‡	±12,4	±7,4	79-110	20-45	20

C

	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Long Day							
Col-0 (wild type)	31,2	13,4	±3,7	±1,4	26-36	12-16	20
arr1-4	29,0	12,9	±4,6	±1,6	24-36	10-15	20
arr2-4	27,7 ‡	12,5	±4,8	±1,2	21-35	10-15	20
arr1-4 arr2-4	26,9 ‡	11,0 ‡	±2,9	±1,1	23-31	9-13	20

Figure 5.1 Novel early flowering phenotype of *arr1/2* mutants.
Photographs illustrating the phenotypes of wild-type (Col-0) and *arr1-4, arr2-4* single mutants and *arr1-4 arr2-4* double mutant grown under LDs (left panel) and SDs (right panel). Differences in Days Until Flowering (DUF) and number of Rosette Leaf Number (RLN). ‡Significance differences (α=0,05) with the wild-type were determined by LSD (Fischer's Least Significant Differences) after the data passed one-way ANOVA (α=0,05). Each day length experiment was repeated twice with similar results. Flowering time data of the mutants grown under SDs **(B)** and LDs **(C)**. DUF, days until flowering; RLN, rosette leaf number; n, number of individuals.

5.2.2 Lack of the activity of *ARR1/ARR2* genes causes down-regulation of specific floral repressors

In order to identify which flowering time pathways are disturbed in the *arr1-4*, *arr2-4* and *arr1-4 arr2-4* mutant lines, key flowering loci were tested for changes in expression with respect to Col-0. Gene expression data quantified by RT-qPCR was firstly obtained from leaves of 70-days-old SD-grown plants shortly before the *arr1-4 arr2-4* double mutant, which displays the strongest acceleration in flowering, entered the reproductive phase. In agreement with the early flowering, expression of the floral repressors *FLC* and *FLM* were 2-fold and 4-fold reduced in leaves, respectively (Figure 5.2, A). In contrast, expression of the known floral repressor *SVP* was not affected. Similarly, expression if the floral activators *CO*, *SOC1* and *FT* were also not affected with only *FD* showing a slight up-regulation (Figure 5.2,

B). Based on these observations the *arr1-4 arr2-4* double mutant causes a depression of floral repressors in leaf and the key LD inductive-pathway genes are not perturbed. Genes that are predominantly expressed at the meristem are also known to have very important roles in flowering initiation, especially in early flowering under short days (Koornneef *et al.* 1998). Therefore, some key meristem-localized genes were examined with the aim of profiling the state of the SAM from 70-day-old SD-grown plants.

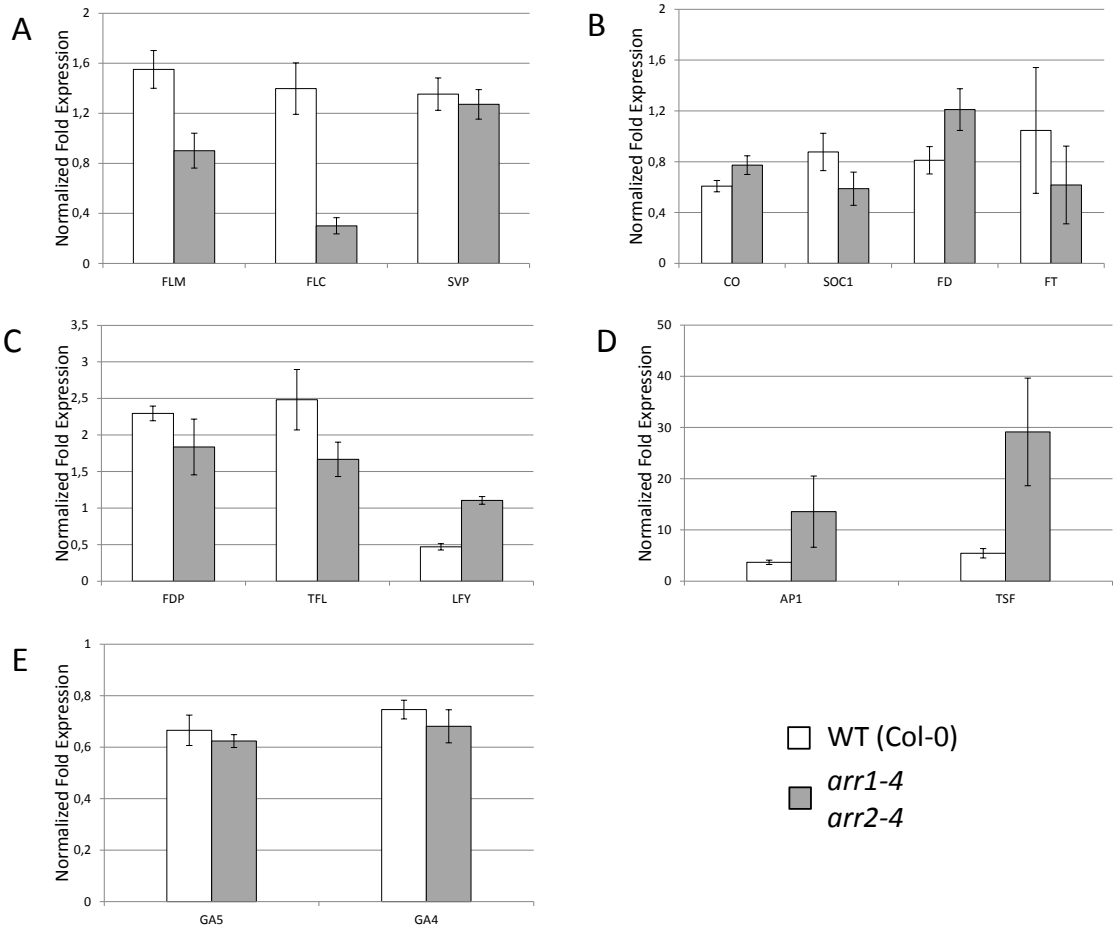


Figure 5.2 The transcript levels of the flowering time pathway-specific marker genes in the wild-type and a*rr1-4 arr2-4* double mutant at DUF 70.

(A) Expression of floral repressor genes *FLM*, *FLC* and *SVP* in the wild-type and double mutant plants. The expression of *FLM* and *FLC* in double mutant plants is down-regulated compared to the wild-type which is not the case with *SVP*. **(B)** Expression of floral activators CO, *SOC1, FD* and *FT*. *FT* levels are very low under the short days and it was taken as a control (*i.e.* levels are qPCR noise). **(C** and **D)** The transcript levels of the flowering time pathway-specific marker genes *FDP, TFL, LFY, AP1* and *TSF*. **(E)** Expression of *GA4* and *GA5*, marker genes for gibberellic acid pathway. RNA was isolated from SD plants short before flowering of the double mutants, tissue from leaves and/or shoot apical meristem was taken. For each line 15 plants were taken and pooled together, RT-qPCR was done using three biological replicates were used with three technical repetitions each and normalized by *ELF-1-α*. Error bars indicate SD of the mean. Leaf material was used for **(A, B)** and for **(C-E)** material was taken from SAM.

In the SAM, the expression of the floral repressor *TERMINAL FLOWER 1* (*TFL1*) was reduced 1.7-fold, whereas expression of the floral activator *FD PARALOG* (*FDP*) was not significantly changed (Figure 5.2, C). In contrast, the meristem identity genes *LEAFY* (*LFY*) was 2-fold increased (Figure 5.2, B) and *APETALA* (*AP1*) and *TWIN SISTER OF FT* (*TSF*) were up-regulated

in the double mutant, 3-fold and 6-fold respectively. The meristem identity genes like *AP1* and *LFY* play an important role in early flower initiation (Ferrandiz *et al.* 2000) and their higher expression confirms the early flowering state of the *arr1-4 arr2-4* double mutant compared to Col-0.

Flower initiation in *Arabidopsis thaliana* under non-inductive short day conditions (Eriksson *et al.* 2006) is dependent on the biosynthesis of the plant hormone gibberellin (GA) and cross-talk between cytokinin and GA has been described (Greenboim-Wainberg *et al.* 2005). This dependency can be explained, at least partly, by GA regulation of the flower meristem identity gene *LFY* and the flowering time gene *SOC1* (Eriksson *et al.* 2006). Gibberellic acid flowering pathway is mostly typical for non-inductive conditions, based on the observations of *ga1* mutant (Wilson *et al.* 1992). GA4 and GA5 *(*gibberellin 3 ß-hydroxylase 1 and 2) are involved in later steps of the gibberellic acid biosynthetic pathway (Hedden and Phillips 2000) and their mRNA levels were shown to somewhat parallel active GA levels *in vivo* (Achard *et al.* 2008). Therefore, the mRNA levels of *GA4* and *GA5* were examined in the SAM at DUF 70. Both *GA4* and *GA5* were unaffected in the double mutant in the SAM (Figure 5.2, E), presumptively indicating that *ARR1* and *ARR2* are not involved in the gibberellic acid flowering pathway.

In conclusion, the expression data obtained by RT-qPCR suggests that *ARR1* and *ARR2* are mostly involved in short-day, non-inductive flowering pathways. This is supported by the fact that the expression of floral repressors *FLC* and *FLM* was lower in the double mutant, whereas known floral activators were not dramatically altered in leaf. The SAM at DUF70 in the double mutant was clearly committed to flowering compared to the wild-type as evident by the high expression levels of *AP1*, *LFY*, and *TSF*. Double mutant was clearly, based on *AP1* expression, in different developmental stage than the wild-type. In order to investigate the previous observations experiments with different approach were further done.

5.2.3 Change in expression of floral repressors is strictly due to early flowering and not developmental effects

To ensure that the data reported above reflect true expression differences rather than differences in developmental stage, *arr1-4 arr2-4* plants were grown for 30 days under non-inductive SD conditions and then shifted to the inductive photoperiodic long days. It has previously been shown that plants grown for 30 days under SDs are large and developed enough to rapidly enter the reproductive phase after a shift to LDs (Schmid *et al.* 2003). For *Arabidopsis*, plants are irreversibly committed to flowering after 3 days in LDs as indicated by the expression of the floral homeotic gene *AP1* (Schmid *et al.* 2003). Therefore flowering was induced in *arr1-4 arr2-4* and wild-type plants at 30 days after germination by shifting them LD and plant material was harvested 0 (control) and 5 days after the shift.

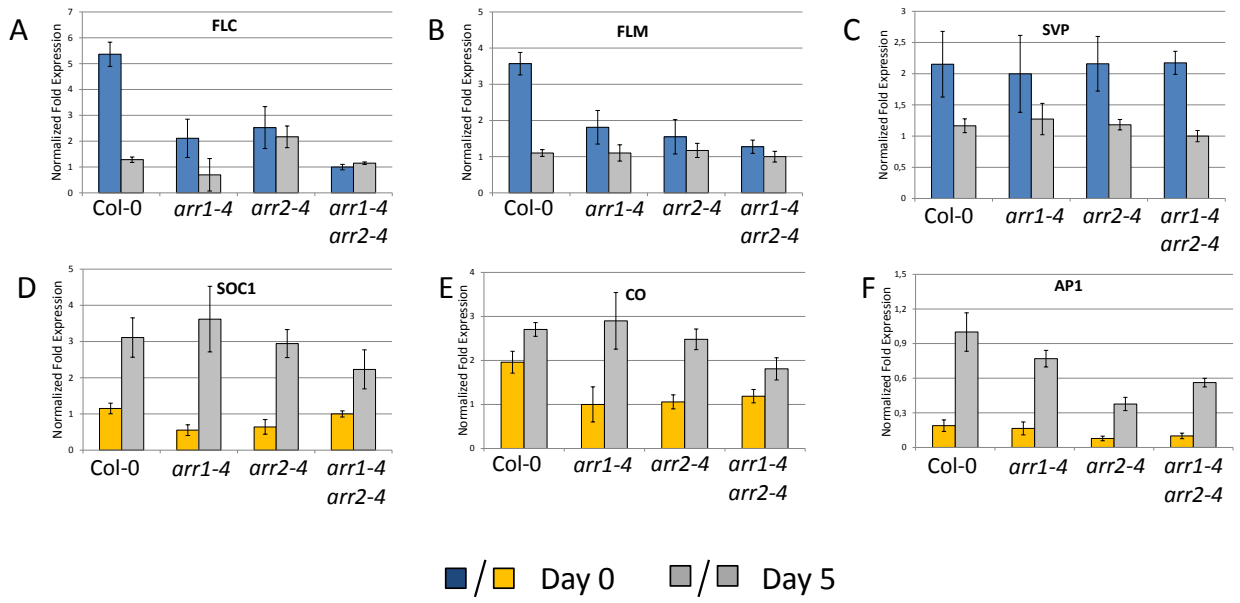


Figure 5.3 Expression of floral regulators in SAM before and after photoperiodic induction.

Transcription levels of genes known floral repressors (**A-C**), activators/integrators (**D,E**) or meristem identity genes (**F**) in wild-type Col-0, *arr1-4, arr2-4* and *arr1-4 arr2-4*. RNA was isolated from the shoot apical meristems (SAM) of 30-day-old plants grown in SD at DUF 30 (Day 0) and after being shifted to LD (Day 5). For each line at least 15 plants were taken and pooled together, RT-qPCR was performed using two biological replicates and three technical replicates each and normalized to *ELF-1-α*. Error bars indicate SD of the mean.

After shifting, the expression of the floral repressors *FLM*, *FLC* and *SVP* was reduced in Col-0 while the expression of floral activator *CO* and floral pathway integrator *SOC1* increased (Figure 5.3) indicating that SAM to the shift in photperiod. *AP1* expression (Figure 5.3, F) is suggests that plants were still in vegetative state before the shift and first after the shift under the inductive long days flowering was induced (the experiment as such was working).

Thus a vegetative phase was replaced by a reproductive phase. The expression level of the floral repressor *FLC* in *arr1-4*, *arr2-4* and *arr1-4 arr2-4* is decreased (about 2-fold) before the shift relative to Col-0 and stays reduced after the shift (Figure 5.3, A). *FLM* also follows this pattern, in that it is already reduced in expression before the shift and maintains this level after the shift (Figure 5.3, B). In contrast, the expression of floral repressor *SVP* decreased after the shift from SDs to LDs independently of the loss of *ARR1* or *ARR2* (Figure 5.3, C). Similarly to *SVP*, the expression of *SOC1* is unchanged in the mutant backgrounds and *SOC1* reached induction levels like that in Col-0 (Figure 5.3, D). *CO* also follows a similar pattern as *SOC1*, in that after the flowering shift induction, the expression of *CO* is increased in response to the shift to LD (Figure 5.3, E).

Taken together, these results suggest that the reduction of *FLC* and *FLM* expression might be causal for the early flowering observed in *arr1-4* and *arr2-4* single mutants and the *arr1-4 arr2-4* double mutant.

5.2.4 Changes in expression of floral repressors are not due to an increase in size of the apical meristem

ARR1 and *ARR2* are known to have roles in cytokinin signalling (Grefen and Harter 2004; Zwack and Rashotte 2013) and it is known that cytokinin has a positive role in regulating SAM size and activity (Tucker and Laux 2007; Werner and Schmulling 2009; Gupta and Rashotte 2012). Cytokinin is also required in the SAM for maintenance of cell division and prevention of cell differentiation (Jasinski *et al.* 2005; Gupta and Rashotte 2012). Therefore, the size of shoot apical meristem SAM was measured in mutant plants and compared to its wild-type in order to see if the early flowering phenotype could be ascribed to a difference size or in morphology. For this experiment SAM of 30-day-old plants grown under SDs were examined (Figure 5.4).

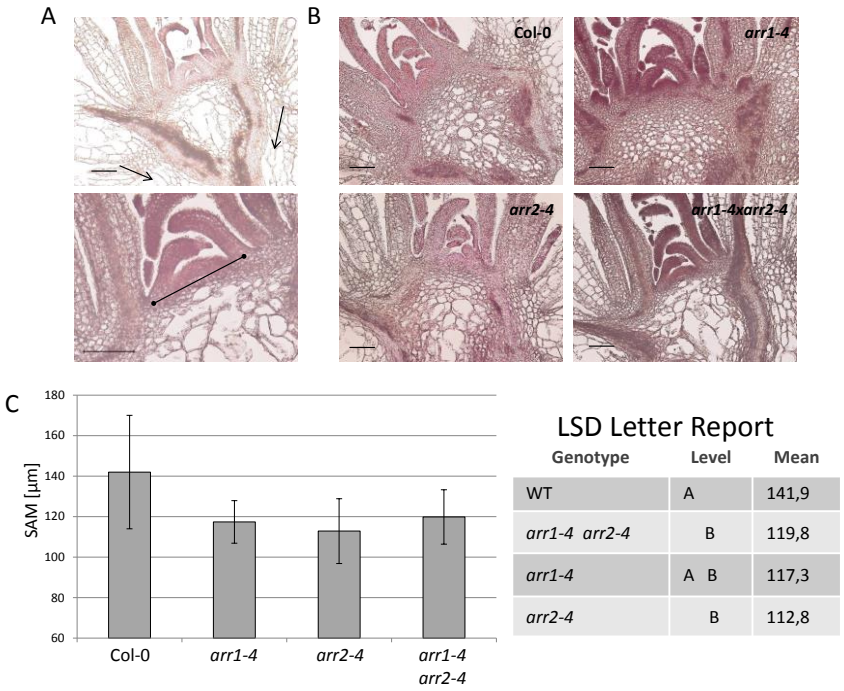


Figure 5.4 Size of shoot apical meristem of Col-0 *arr1-4* and *arr2-4* single mutants and *arr1-4 arr2-4* double mutant.

(A) Examples of the tissue taken and how measurements were made. Sections were scanned for clear visibility of the two large air vacuoles (arrows, upper image). Once found then the SAM width was measured (solid line, lower image). **(B)** Representative sections of shoot apical meristems of Col-0, *arr1-4, arr2-4* and *arr1-4 arr2-4* respectively. **(C)** Size of shoot apical meristems in single mutants and the double mutants from 30 day-old plants grown under SDs. At least 10 plants per line were used for statistical evaluation. Significance classes were computed using the Fisher's Least Significant Difference (LSD, α=0,05) test after passing one-way ANOVA (α=0.05 , p ≥0.0339). Classes *not connected* by a letter are significantly different. All of the mutants have means that are smaller than Col-0, but only *arr2-4* and *arr1-4 arr2-4* are statistically smaller than the wild-type. Error bars indicate SD of the mean. Scale bars, 100µm

SAMs of Col-0 and *arr1-4* single mutant did not show a statistical significant difference in their size (LSD, α=0.05) even though the mean of the *arr1-4* similar to the *arr2-4* and *arr1-4 arr2-4* and are not statistically different (Figure 4, C). On the other hand, the SAMs of *arr2-4* single mutant and the *arr1-4 arr2-4* double mutant are statistically significantly smaller than in the wild-type (LSD, α=0.05). The SAM of the *arr1-4 arr2-4* double mutant is not significantly different in size from *arr2-4* or *arr1-4* single mutants however (Figure 5.4, C). This

suggests that the difference in SAM size is only due to lack of *ARR2* and not *ARR1*, however one should keep in mind that the *arr1-4* SAM was never observed to be in the same range as that of Col-0 (Figure 5.4, C) and was not statistically different from *arr2-4* or the double mutant. It is possible that with a larger sample size the *arr1-4* would have also been statistically smaller. For now, it is evident that the SAMs of *arr1-4*, *arr2-4* and *arr1-4 arr2-4* are not larger than Col-0. Thus, despite of even smaller size of SAM in the mutant plants they manage to flower earlier than the wild-type. Differences in anatomy or morphology of SAMs in mutant plants and the wild-type were not observed (Figure 5.4, A, B).

5.2.5 ARR2 expressed either in the SAM or from phloem companion cells recues the early flowering phenotype of *arr2-4* and *arr1-4 arr2-4*

The RT-qPCR results from the previous experiments indicated that there were changes in floral regulator expression in both leaf and the SAM. Investigation of the SAM indicated the early flowering phenotype could not be attributed to an increase in meristem size. Therefore as the action of *ARR2* with respect to flowering time was unclear, the double mutant was complemented with two different tissue specific promoters, *pSUC2* and *pFD*. *pSUC2* is a vasculature-specific promoter expressed only in phloem (companion cells) of all green tissues of *Arabidopsis* such as rosette leaves, stems, and sepals. *SUC2* encodes a plasma-membrane sucrose-H+ symporter (Truernit and Sauer 1995; Wippel and Sauer 2012). *pFD* is a meristem-specific promoter expressed in the shoot apex (Abe *et al.* 2005). FD is bZIP protein required for positive regulation of flowering (Abe *et al.* 2005; Wigge *et al.* 2005). Furthermore, it is know that ARR2 can be activated by phosphorylation of an aspartate (D80) in a Two-Component System (TCS) dependent manner (Grefen and Harter 2004). Mutation of D80 to E (Glutamate) mimics the phosphorylated state, increases the transactivation capacity of ARR2 (Hwang and Sheen 2001) and leads to severe developmental defects (Hass *et al.* 2004). Mutation of D80 to N (Asparagine), however, prevents TCS dependent phosphorylation (Kim *et al.* 2006) and impairs basal gene activation capacity, yet D80N still appears to respond to cytokinin according to reporter gene assays (Hwang and Sheen 2001; Kim *et al.* 2006). Therefore, the *arr2-4* and *arr1-4 arr2-4* mutants were complemented using wild-type *ARR2* and $ARR2^{D80N}$ cDNA versions under the control of the *pSUC2* and *pFD* promoters (Tables 5.1 to5. 4). Due to the stronger flowering time effect observed by the loss of *ARR2*, *arr1-4* plants were included as controls only and not complemented with the *ARR2* at this time, thus this complementation assay focuses on the effect of the *ARR2* gene.

All plants used for this experiment were in examined in the T2 generation and therefore preselected with BASTA. For each construct two or three independent lines were chosen and a minimum of 15 plants were used for the experiment. To rule out any secondary effects from the BASTA application and ensure that the vector backbones did not disturb the early flowering phenotype, all the mutant lines as well as the wild-type plants were transformed with empty vectors. This was done by using the "pJL blue" gateway compatible vector which only has a MCS (multiple cloning site (MCS) sequence between the gateway cassette. The results will be presented in the same order as they are given in Tables 5.1 to 5.4.

Table 5.1 shows the complementation results for *ARR2* and *ARR2D80N* driven under the *pSUC2* promoter in LDs. Two to three transgenic lines were chosen for each construct and genotype pair. The table presents the data for each line analysed; statistical analyses were performed by pooling the results from independent lines for each transgene. With regards to the two parameters DUF and rosette leaf number, DUF is more predictive and has a higher resolution as the number of rosette leaves was nearly invariant under our LD condition (total range 2 leaves). Therefore, only the results with respect to DUF will be presented for LDs. The control vector construct, here called "pJL-blue", did not complement the flowering time phenotype. The wild-type flowered the latest (mean: 29 DUF), the double mutant the earliest (mean: 24 DUF) and the two single mutants in-between (means: 26 DUF for *arr1-4* and 25 DUF for *arr2-4*). Thus even though the early flowering phenotype is comparably weak in LDs, the flowering time trend previously observed was not disturbed by the vector backbone nor the BASTA treatment. *ARR2* driven by *pSUC2* was able to complement the flowering defect to near wild-type levels in the *arr2-4* single mutant (mean: DUF 28 same significance class as Col-0). Most remarkably, *ARR2 D80N* loss-off-function version driven under the *pSUC2* was also able to partially complement (mean: 27 DUF) the early flowering phenotype of the single mutant under LDs. Neither construct was able to complement the double mutant however.

The complementation results for *ARR2* and *ARR2 D80N* driven under the *pFD* promoter in LDs are given in Table 5.2. Like the *pSUC2* experiment in LDs, the DUF parameter is more informative as the rosette number again is nearly invariant and has a small range (2 leaves). The control pJL-blue constructs lead to the same tendency with the wild-type flowering the latest (mean: 29 DUF), the double mutant earliest (mean: 25 DUF) and the single mutants in-between as previously observed. This again demonstrated that neither the BASTA application nor vector backbone disturbed the flowering phenotype of the respective genotypes. Expression of *ARR2* from the *pFD* promoter was able to complement the single and double mutant (means: 28.5 and 29 DUF, respectively). A partial complementation was also observed with *ARR2 D80N* in the *arr2-4* mutant but not the *arr1-4 arr2-4* double mutant. Table 5.3 summarises the results for complementation of the various mutant lines for *ARR2* and *ARR2 D80N* driven under the *pSUC2* promoter in SDs. The control pJL-blue constructs once again did not disturb the flowering time with the wild-type flowering the latest (mean: 101 DUF with 53 leaves), the double mutant earliest (mean: 81 DUF with 41 leaves) and the single mutants in-between as previously observed. All the complemented lines used for these experiments showed within the same complementation same tendency (supplemental figures 8 and 9). The rosette leaf range is larger this time (range of 13 leaves) and overall reflects the DUF parameter but not only for the double mutant. The best complementation was observed in the double mutant (mean: 90 DUF with 48 leaves) with *ARR2 D80N* also partially, but significantly, complementing the mutant (mean: 86 DUF with 45 leaves). In contrast to the *pSUC2* under LD conditions, neither *ARR2* nor *ARR2 D80N* was able to even partially complement *arr2-4* based on the significance classes. Nevertheless, this complementation of *ARR2* did have a mean DUF of 89, nearly identical to that of the partially complemented double mutant.

Table 5.1 Flowering time of transgenic lines driven under tissue specific *SUC2* promoter (*pSUC2*) under long day inductive conditions.

DUF, days until flowering; RLN, rosette leaf number; n, number of individuals; #, identifier for individual transgenic line. All transgenic lines used for this experiment were in T2 generation and preselected with BASTA. Significance levels were computed after passing ANOVA (α=0.05) by using Fischer's Least Significance Difference (LSD) with alpha levels α=0.01 for DUF and α=0.05 or RLN. Classes *not connected* by a letter are significantly different.

	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Long Day							
pSUC2:pJL-blue #6	29,4	10,3	±1,6	±1,1	27-32	9-12	15
pSUC2:pJL-blue #5	29,0	11,7	±1,6	±1,4	27-32	10-13	15
pSUC2:pJL-blue #2	28,6	11,0	±2,5	±2,2	26-33	7-13	15
arr1-4 pSUC2:pJL-blue #1	26,6	11,0	±1,0	±1,0	25-28	10-12	15
arr1-4 pSUC2:pJL-blue #5	25,6	10,2	±1,3	±0,9	24-28	9-11	15
arr1-4 pSUC2:pJL-blue #4	26,3	10,7	±2,8	±1,4	24-32	9-14	15
arr2-4 pSUC2:pJL-blue #5	25,2	9,0	±1,9	±1,2	25-29	7-10	15
arr2-4 pSUC2:pJL-blue #3	25,2	10,1	±1,7	±0,9	23-26	9-12	15
arr2-4 pSUC2:pJL-blue #4	26,3	10,1	±2,5	±1,1	23-31	8-12	15
arr1-4 arr2-4 pSUC2:pJL-blue #5	25,9	9,4	±0,8	±1,3	25-27	7-11	15
arr1-4 arr2-4 pSUC2:pJL-blue #2	25,3	9,6	±1,3	±1,3	24-28	7-12	15
arr1-4 arr2-4 pSUC2:pJL-blue #3	24,9	9,5	±1,7	±0,8	23-27	8-10	15
arr2-4 pSUC2:ARR2 #1	28,1	10,9	±1,8	±1,0	25-30	10-12	15
arr2-4 pSUC2:ARR2 #2	28,1	10,7	±1,8	±1,0	25-31	9-12	10
arr2-4 pSUC2:ARR2D80N #5	27,4	11,3	±3,2	±2,3	24-33	8-14	20
arr2-4 pSUC2:ARR2D80N #6	26,3	10,4	±1,9	±1,8	24-29	8-13	10
arr1-4 arr2-4 pSUC2:ARR2 #3	25,7	10,7	±1,5	±0,5	24-29	9-14	15
arr1-4 arr2-4 pSUC2:ARR2 #1	25,0	10,3	±1,4	±1,1	23-28	9-12	15
arr1-4 arr2-4 pSUC2:ARR2D80N #6	23,8	10,8	±2,0	±1,0	22-29	9-12	15
arr1-4 arr2-4 pSUC2:ARR2D80N #2	23,7	10,9	±1,3	±0,9	21-25	10-12	15
arr1-4 arr2-4 pSUC2:ARR2D80N #X	24,4	10,6	±1,4	±1,4	23-27	9-14	15

Genotype	LSD DUF	Mean
WT (Col-0) pSUC2::Jlblue	A	29,0
arr2-4 (Col-0) pSUC2::ARR2	A	28,1
arr2-4 (Col-0) pSUC2::ARR2D80N	B	26,8
arr1-4 (Col-0) pSUC2::Jlblue	B C	26,1
arr2-4 (Col-0) pSUC2::Jlblue	C	25,7
arr1-4Xarr2-4 (Col-0) pSUC2::Jlblue	C	25,4
arr1-4Xarr2-4 (Col-0) pSUC2::ARR2	C	25,3
arr1-4Xarr2-4 (Col-0) pSUC2::ARR2D80N	D	24,0

Genotype	LSD Rosette	Mean
WT (Col-0) pSUC2::Jlblue	A	11,0
arr2-4 (Col-0) pSUC2::ARR2D80N	A	10,8
arr1-4Xarr2-4 (Col-0) pSUC2::ARR2D80N	A	10,8
arr2-4 (Col-0) pSUC2::ARR2	A	10,8
arr1-4 (Col-0) pSUC2::Jlblue	A B	10,5
arr1-4Xarr2-4 (Col-0) pSUC2::ARR2	A B	10,3
arr2-4 (Col-0) pSUC2::Jlblue	B C	9,9
arr1-4Xarr2-4 (Col-0) pSUC2::Jlblue	C	9,5

Table 5.2 Flowering time of transgenic lines driven under tissue specific *FD* promoter under long day inductive conditions.

DUF, days until flowering; RLN, rosette leaf number; n, number of individuals; #, identifier for individual transgenic line. All transgenic lines used for this experiment were in T2 generation and preselected with BASTA. Significance levels were computed after passing ANOVA (α=0.05) by using Fischer's Least Significance Difference (LSD) with alpha levels α=0.01 for DUF and α=0.05 or RLN. Classes *not connected* by a letter are significantly different.

	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Long Day							
pFD:pJL-blue #1	29,3	11,5	±1,7	±1,7	27-32	8-13	15
pFD:pJL-blue #3	29,5	11,0	±1,7	±1,7	27-32	8-13	15
pFD:pJL-blue #5	29,3	11,4	±1,7	±1,4	27-32	9-13	15
arr1-4 pFD:pJL-blue #1	26,8	10,0	±1,6	±1,9	24-29	7-12	15
arr1-4 pFD:pJL-blue #2	26,5	10,5	±1,5	±2,1	24-28	7-14	15
arr1-4 pFD:pJL-blue #3	27,2	10,2	±1,2	±1,4	25-28	7-12	15
arr2-4 pFD:pJL-blue #1	25,1	9,6	±1,0	±2,2	23-26	7-11	15
arr2-4 pFD:pJL-blue #5	24,8	10,1	±1,2	±1,2	23-26	8-11	15
arr2-4 pFD:pJL-blue #4	25,0	10,3	±1,2	±1,5	23-26	8-13	15
arr1-4 arr2-4 pFD:pJL-blue #1	24,6	9,4	±1,3	±1,5	23-27	7-12	15
arr1-4 arr2-4 pFD:pJL-blue #3	25,1	9,6	±1,1	±1,7	24-27	7-13	15
arr1-4 arr2-4 pFD:pJL-blue #6	24,8	9,6	±1,4	±1,5	23-27	7-11	15
arr2-4 pFD:ARR2 #8	28,0	10,5	±1,6	±1,1	27-30	9-13	15
arr2-4 pFD:ARR2 #5	28,0	11,1	±1,2	±1,6	27-30	8-13	15
arr2-4 pFD:ARR2 #7	29,8	11,8	±2,3	±1,2	27-30	10-13	15
arr2-4 pFD:ARR2D80N # 1	26,2	11,4	±2,0	±1,4	24-30	9-14	10
arr2-4 pFD:ARR2D80N #2	26,6	11,4	±2,0	±1,4	24-30	9-13	15
arr1-4 arr2-4 pFD:ARR2 #4	29,4	12,1	±2,6	±2,0	26-33	9-15	15
arr1-4 arr2-4 pFD:ARR2 #2	28,1	11,6	±1,6	±1,3	26-31	10-13	15
arr1-4 arr2-4 pFD:ARR2 #6	30,0	12,0	±2,0	±1,7	28-32	10-13	15
arr1-4 arr2-4 pFD:ARR2D80N #1	25,4	10,9	±1,6	±1,7	24-29	7-12	15
arr1-4 arr2-4 pFD:ARR2D80N #2	25,6	10,7	±1,5	±1,5	24-29	8-12	15

Genotype	LSD DUF	Mean
WT (Col-0) pFD::Jlblue	A	29,3
arr1-4Xarr2-4 (Col-0) pFD::ARR2	A	28,9
arr2-4 (Col-0) pFD::ARR2	A	28,4
arr1-4 (Col-0) pFD::Jlblue	B	26,8
arr2-4 (Col-0) pFD::ARR2D80N	B C	26,4
arr1-4Xarr2-4 (Col-0) pFD::ARR2D80N	C D	25,5
arr2-4 (Col-0) pFD::Jlblue	D	25,0
arr1-4Xarr2-4 (Col-0) pFD::Jlblue	D	24,8

Genotype	LSD Rosette	Mean
arr1-4Xarr2-4 (Col-0) pFD::ARR2	A	11,9
arr2-4 (Col-0) pFD::ARR2D80N	A B	11,4
WT (Col-0) pFD::Jlblue	A B	11,3
arr2-4 (Col-0) pFD::ARR2	A B	11,0
arr1-4Xarr2-4 (Col-0) pFD::ARR2D80N	B C	10,8
arr1-4 (Col-0) pFD::Jlblue	C D	10,2
arr2-4 (Col-0) pFD::Jlblue	C D	10,0
arr1-4Xarr2-4 (Col-0) pFD::Jlblue	D	9,6

Finally, the complementation results for *ARR2* and *ARR2 D80N* driven under the *pFD* promoter under SDs are in Table 5.4. As observed in the other three experiments, the control pJL-blue constructs did not disturb the flowering time: wild-type flowered the latest (mean: 108 DUF with 54 leaves), *arr1-4* (mean: 88 DUF with 50 leaves), *arr2-4* (mean: 84 DUF with 48 leaves) and the double mutant the earliest (mean: 81 DUF with 41 leaves). In contrast to the previous experiments *ARR2* driven by the *pFD* promoter was rescued both the *arr2-4* (mean: 97 DUF with 50 leaves) and the *arr1-4 arr2-4* (mean: 95 DUF with 50 leaves) mutants almost completely. Most remarkably, the $ARR2^{D80N}$ was also able to, partially, and significantly, complement both the single *arr2-4* (mean: 89 DUF with 47 leaves) and the double mutant (mean: 86.5 DUF with 45.5 leaves).

Taken together, even though full complementation was only observed under LDs, the complementation of the early flowering of *arr2-4* under SDs was also partial, yet statistically significant, suppressed. This suggests that lack of *ARR2* is indeed causal for the observed early flowering phenotype. *ARR2* driven under *pFD* showed the strongest effect and can almost completely complement the flowering phenotype of both the single and the double mutant in SDs where the *arr2-4* and *arr1-4 arr2-4* mutation effects strongest. The rescue is slightly weaker when *ARR2* is driven under *pSUC2* but still significant in the double mutant. In LD conditions, *ARR2* expressed from either promoter was able to complement the *arr2-4* mutation, but only *ARR2* expressed under the *pFD* promoter was able to partially rescue the *arr1-4 arr2-4* mutant. Mutant plants were also slightly complemented with loss-of-function *ARR2 D80N* gene in the single and double mutants in SDs when driven under the *pFD* promoter or by *pSUC2* in *arr1-4 arr2-4*. This effect of *ARR2 D80N* was also observed in LDs but only in the *arr2-4* mutant background. The *ARR2D80N*-cDNA mutant version cannot be phosphorylated by the TCS anymore, and is less responsive to cytokinin than the wild-type *ARR2*-cDNA (Hwang and Sheen 2001; Choi *et al.* 2010; Veerabagu *et al.* 2012). The reason for this is most likely due to an incomplete inactivation of the *ARR2* by the D80N mutation. Considering this fact, it can be still concluded that ARR2 needs to be phosphorylated by the TCS (Two-Component System) in order to best rescue the early flowering phenotype as only partial complementation was observed for the *ARR2D80N* loss-of-function transgene. This strongly suggests that ARR2 may be a point of integration for the TCS and other flowering time pathways under SDs.

Table 5.3 Flowering time of transgenic lines driven under tissue specific *SUC2* promoter under short day non-inductive conditions.

DUF, days until flowering; RLN, rosette leaf number; n, number of individuals; #, identifier for individual transgenic line. All transgenic lines used for this experiment were in T2 generation and preselected with BASTA. Significance levels were computed after passing ANOVA (α=0.05) by using Fischer's Least Significance Difference (LSD) with alpha levels α=0.01 for DUF and α=0.05 or RLN. Classes *not connected* by a letter are significantly different.

	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Short Day							
pSUC2:pJL-blue #6	101,3	54,3	±5,3	±4,4	90-109	47-60	15
pSUC2:pJL-blue #5	96,8	52,0	±7,9	±4,3	90-108	48-57	15
pSUC2:pJL-blue #2	103,5	53,2	±6,9	±2,9	94-115	50-58	15
arr1-4 pSUC2:pJL-blue #1	86,6	49,4	±6,3	±2,3	84-97	46-52	15
arr1-4 pSUC2:pJL-blue #5	92,7	49,6	±4,0	±2,0	89-97	45-52	15
arr1-4 pSUC2:pJL-blue #4	90,7	48,3	±4,0	±1,5	86-93	47-50	15
arr2-4 pSUC2:pJL-blue #5	85,4	47,9	±3,2	±3,1	82-90	44-53	15
arr2-4 pSUC2:pJL-blue #3	87,1	47,6	±5,6	±2,6	81-96	45-52	15
arr2-4 pSUC2:pJL-blue #4	87,2	46,8	±4,3	±3,5	82-93	43-51	15
arr1-4 arr2-4 pSUC2:pJL-blue #5	78,7	39,8	±4,3	±3,5	74-85	35-45	15
arr1-4 arr2-4 pSUC2:pJL-blue #2	82,9	41,5	±3,9	±3,7	78-90	35-47	15
arr1-4 arr2-4 pSUC2:pJL-blue #3	81,6	42,3	±5,4	±3,7	75-89	38-47	15
arr2-4 pSUC2:ARR2 #1	91,5	48,0	±4,8	±3,5	87-98	43-52	15
arr2-4 pSUC2:ARR2 #2	88,4	47,6	±4,3	±4,4	79-93	38-54	10
arr2-4 pSUC2:ARR2D80N #5	85,7	46,4	±3,8	±3,0	79-89	39-49	20
arr2-4 pSUC2:ARR2D80N #6	84,0	45,6	±5,4	±3,6	77-90	39-49	10
arr1-4 arr2-4 pSUC2:ARR2 #3	89,8	46,9	±4,0	±2,7	84-93	43-51	15
arr1-4 arr2-4 pSUC2:ARR2 #1	90,3	47,9	±6,1	±2,2	80-100	45-52	15
arr1-4 arr2-4 pSUC2:ARR2D80N #6	87,3	45,3	±4,9	±2,5	78-93	42-51	15
arr1-4 arr2-4 pSUC2:ARR2D80N #2	87,7	45,0	±8,	±3,0	75-100	39-49	15
arr1-4 arr2-4 pSUC2:ARR2D80N #X	83,0	44,3	±8,8	±1,8	75-100	42-47	15

Genotype	LSD DUF	Mean
WT (Col-0) pSUC2::Jlblue	A	100,8
arr1-4Xarr2-4 (Col-0) pSUC2::ARR2	B	90,0
arr2-4 (Col-0) pSUC2::ARR2	B C	89,1
arr1-4 (Col-0) pSUC2::Jlblue	B C	88,9
arr2-4 (Col-0) pSUC2::Jlblue	B C	86,6
arr1-4Xarr2-4 (Col-0) pSUC2::ARR2D80N	C	86,0
arr2-4 (Col-0) pSUC2::ARR2D80N	C D	85,0
arr1-4Xarr2-4 (Col-0) pSUC2::Jlblue	D	81,1

Genotype	LSD Rosette	Mean
WT (Col-0) pSUC2::Jlblue	A	53,1
arr1-4 (Col-0) pSUC2::Jlblue	B	49,1
arr2-4 (Col-0) pSUC2::ARR2	B C	47,8
arr2-4 (Col-0) pSUC2::Jlblue	B C	47,4
arr1-4Xarr2-4 (Col-0) pSUC2::ARR2	B C	47,4
arr2-4 (Col-0) pSUC2::ARR2D80N	C D	46,0
arr1-4Xarr2-4 (Col-0) pSUC2::ARR2D80N	D	44,9
arr1-4Xarr2-4 (Col-0) pSUC2::Jlblue	E	41,3

Table 5.4 Flowering time of transgenic lines driven under tissue specific *FD* promoter under short day non-inductive conditions.

DUF, days until flowering; RLN, rosette leaf number; n, number of individuals; #, identifier for individual transgenic line. All transgenic lines used for this experiment were in T2 generation and preselected with BASTA. Significance levels were computed after passing ANOVA (α=0.05) by using Fischer's Least Significance Difference (LSD) with alpha levels α=0.01 for DUF and α=0.05 or RLN. Classes *not connected* by a letter are significantly different.

	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Short Day							
pFD:pJL-blue #1	108,1	54,6	±4,5	±3,3	102-115	49-58	15
pFD:pJL-blue #3	106,7	54,1	±6,3	±4,3	98-115	47-59	15
pFD:pJL-blue #5	107,7	52,5	±6,4	±4,5	99-118	45-56	15
arr1-4 pFD:pJL-blue #1	86,9	49,4	±4,1	±5,7	78-93	40-57	15
arr1-4 pFD:pJL-blue #2	86,9	50,5	±6,4	±4,3	79-97	41-53	15
arr1-4 pFD:pJL-blue #3	89,0	50,8	±5,1	±4,0	79-97	47-55	15
arr2-4 pFD:pJL-blue #1	82,7	48,7	±5,2	±4,8	74-89	39-53	15
arr2-4 pFD:pJL-blue #5	83,6	48,3	±4,6	±3,2	75-89	47-53	15
arr2-4 pFD:pJL-blue #4	83,5	46,7	±4,3	±4,3	74-89	39-54	15
arr1-4 arr2-4 pFD:pJL-blue #1	80,2	39,3	±4,3	±3,9	73-86	35-47	15
arr1-4 arr2-4 pFD:pJL-blue #3	81,4	41,8	±3,4	±2,9	75-87	39-46	15
arr1-4 arr2-4 pFD:pJL-blue #6	81,1	41,3	±4,7	±2,8	74-89	37-45	15
arr2-4 pFD:ARR2 #8	96,4	49,6	±4,0	±3,7	95-102	42-53	15
arr2-4 pFD:ARR2 #5	97,7	50,5	±3,9	±2,8	93-105	45-53	15
arr2-4 pFD:ARR2 #7	95,8	48,0	±5,6	±1,9	90-100	46-50	15
arr2-4 pFD:ARR2D80N # 1	88,4	47,3	±3,9	±2,3	83-94	41-50	18
arr2-4 pFD:ARR2D80N #2	89,8	46,2	±3,5	±3,1	82-94	41-53	10
arr1-4 arr2-4 pFD:ARR2 #4	95,3	49,8	±3,7	±3,0	90-100	47-54	15
arr1-4 arr2-4 pFD:ARR2 #2	93,4	48,3	±5,9	±4,0	88-106	40-54	15
arr1-4 arr2-4 pFD:ARR2 #6	96,8	51,0	±2,8	±3,1	94-101	46-55	15
arr1-4 arr2-4 pFD:ARR2D80N #1	85,9	45,3	±4,2	±2,7	79-91	39-48	15
arr1-4 arr2-4 pFD:ARR2D80N #2	87,1	45,7	±3,8	±2,7	79-91	39-49	15

Genotype	LSD DUF	Mean
WT (Col-0) pFD::Jlblue	A	107,6
arr2-4 (Col-0) pFD::ARR2	B	96,7
arr1-4Xarr2-4 (Col-0) pFD::ARR2	B	95,4
arr2-4 (Col-0) pFD::ARR2D80N	C	89,0
arr1-4 (Col-0) pFD::Jlblue	C	87,6
arr1-4Xarr2-4 (Col-0) pFD::ARR2D80N	C D	86,5
arr2-4 (Col-0) pFD::Jlblue	D E	83,3
arr1-4Xarr2-4 (Col-0) pFD::Jlblue	E	80,9

Genotype	LSD Rosette	Mean
WT (Col-0) pFD::Jlblue	A	53,5
arr1-4 (Col-0) pFD::Jlblue	B	50,3
arr1-4Xarr2-4 (Col-0) pFD::ARR2	B C	49,7
arr2-4 (Col-0) pFD::ARR2	B C	49,6
arr2-4 (Col-0) pFD::Jlblue	C D	47,9
arr2-4 (Col-0) pFD::ARR2D80N	D E	46,8
arr1-4Xarr2-4 (Col-0) pFD::ARR2D80N	E	45,5
arr1-4Xarr2-4 (Col-0) pFD::Jlblue	F	40,8

5.2.6 *ARR1* and *ARR2* work mostly independent of *FLC*

FLOWERING LOCUS C (FLC) is a transcription factor that functions as a repressor of the floral transition. It is the main target of the vernalization pathway in *Arabidopsis thaliana* and is epigenetically silenced by the prolonged exposure of plants to cold. FLC was shown to bind to and directly block the transcriptional activation of *SOC1* and *FT* (Helliwell *et al.* 2006; Srikanth and Schmid 2011).

Based on RT-qPCR data obtained from *ARR1* and *ARR2* knockouts, the *arr1-4, arr2-4* and *arr1-4 arr2-4* double mutants showed reduced levels of *FLC* compared to wild-type. In order to find out more about the nature of this interaction the *arr1-4 arr2-4* double mutant was crossed with a deletion mutant for *FLC, flc-3* (Michaels and Amasino 1999). The triple mutant was identified by PCR (see Methods) and confirmed at the expression level (supplemental figure 11).

The *flc-3 arr1-4 arr2-4* (mean: 28 DUF) triple mutant under inductive LD conditions behaved like the *flc-3* single mutant (mean: 28 DUF), flowering about 6 days earlier (mean: 34 DUF) and producing 2 leaves less than the Col-0. The *arr1-4 arr2-4* double mutant displayed an intermediate phenotype (mean: 32 DUF) (Figure 5.5, A (left panel), B). Under LDs, *FLC* appears to be epistatic to *ARR1* and *ARR2*. Under non-photoperiodic SD conditions the *flc-3 arr1-4 arr2-4* triple mutant (mean: 78 DUF) very clearly shows an additive effect. It flowers earlier than both parents, 8 days earlier than the *flc-3* (mean: 87 DUF) about 2 weeks before *arr1-4 arr2-4* (mean: 92 DUF) and more than 3 weeks before the Col-0 (mean: 111 DUF). The same flowering time was observed when this experiment was repeated under SD conditions but under a different light quality(. These results confirm the consistency of an additive early flowering phenotype of the *flc-3 arr1-4 arr2-4* triple mutant in SDs (Figure 5.5, A (right panel), D).

The *flc-3 arr1-4 arr2-4* triple mutant repeatedly showed a clear tendency towards early flowering when compared to the *flc-3* single mutant. However, the differences were not statistically significant. Therefore, the same experiment was repeated again under LDs but at 16°C temperature, which results in a mild delay in flowering. Interestingly, under these conditions, *flc-3 arr1-4 arr2-4* flowered earlier than *flc-3*. The triple mutant *flc-3 arr1-4 arr2-4* flowered over a week earlier that the wild-type (mean: 34 DUF) which was almost five days before *arr1-4 arr2-4* double mutant (mean: 39 DUF) and two days earlier than *flc-3* single mutant (mean: 36 DUF) (Figure 5.5, C), suggesting an additive effect on of *FLC* and *ARR1* and *ARR2* under inductive LD conditions.

Based on these experiments, the *flc-3 arr1-4 arr2-4* triple mutant shows very consistent additive effect in early flowering under SDs and LDs (statistically significant only at 16°C). Based on this result *ARR2* seems to work mainly independent of *FLC*. If there is any intercommunication between these two signalling pathways then it is rather small.

A

B

	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Long Day							
Col-0 (wild type) A	33,9	13,9	±1,5	±1,2	32-37	11-15	15
arr1-4 arr2-4 B	32,3	13,6	±1,4	±1,0	31-35	12-15	15
flc-3 C	28,2	11,4	±0,4	±0,7	28-29	10-12	15
flc-3 arr1-4 arr2-4 C	27,6	11,8	±0,5	±0,4	27-28	11-12	15

C

	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Long Day (16°C)							
Col-0 (wild type) A	42,05	16,74	±2,3	±1,9	38-45	14-22	19
arr1-4 arr2-4 B	39,12	15,82	±2,2	±1,4	35-42	13-18	17
flc-3 C	36,75	14,31	±2,1	±1,9	33-40	12-18	16
flc-3 arr1-4 arr2-4 D	34,75	12,95	±1,6	±1,5	32-37	10-16	20
Long Day (23°C)							
Col-0 (wild type) A	29,00	11,65	±2,7	±0,8	22-32	10-13	20
arr1-4 arr2-4 B	27,21	11,00	±2,6	±0,8	23-31	9-12	19
flc-3 C	22,43	9,67	±2,1	±0,9	20-28	8-11	21
flc-3 arr1-4 arr2-4 C	22,06	9,47	±2,3	±0,8	19-26	8-11	17

D

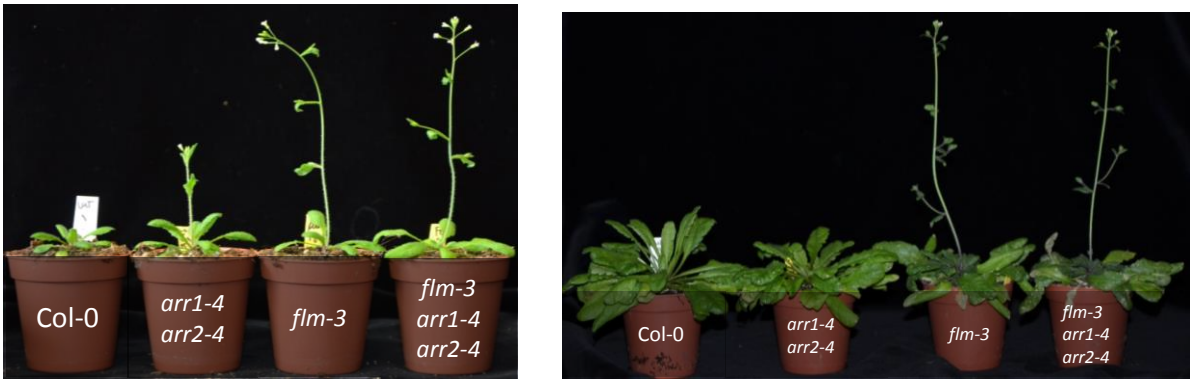
	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Short Day (Chamber 1)							
Col-0 (wild type) A	111,2	34,7	±6,4	±4,6	100-118	28-43	15
arr1-4 arr2-4 B	92,1	27,7	±4,7	±2,6	84-102	24-32	15
flc-3 C	86,6	27,3	±4,7	±4,4	78-93	21-36	15
flc-3 arr1-4 arr2-4 D	77,9	24,0	±4,6	±2,4	72-85	21-28	15
Short Day (Chamber2)							
Col-0 (wild type) A	113,0	47,6	±4,2	±4,2	107-120	43-51	15
arr1-4 arr2-4 B	91,4	37,9	±6,3	±1,7	81-99	40-45	15
flc-3 C	83,8	35,7	±3,0	±2,2	78-87	37-41	15
flc-3 arr1-4 arr2-4 D	76,8	27,8	±4,2	±1,8	71-83	27-32	15

Figure 5.5 Flowering phenotype of *arr1-4 arr2-4 flc-3* triple mutant.
(A) Photographs showing early flowering phenotype of *arr1-4 arr2-4* double mutant, *flc-3* single mutant and *arr1-4 arr2-4 flc-3* triple mutant under LDs (left panel) and SDs (right panel) compared to wild-type. The arrow indicates the position of floral bud of Col-0. **(B)** Flowering times of mutant plants grown under long day conditions. **(B)** Flowering times of mutant plants grown at 16°C and 23° C under LD conditions. **(C)** Flowering times of mutant plants under SD conditions. Additive effect on flowering time by the triple mutant is very visible and consistent even under different light conditions (*i.e.* Chamber 1 and Chamber 2). DUF, days until flowering; RLN, rosette leaf number; n, number of individuals. Significance tests with LSD (α=0.05) were performed on DUF after passing one-way ANOVA (α=0.05). LSD classes are given as letters in superscript. Classes *not connected* by a letter are significantly different.

5.2.7 *ARR1* and *ARR2* work in the same pathway upstream of *FLM*

FLOWERING LOCUS M (FLM) is a MADS-domain gene that acts as an inhibitor of flowering in *Arabidopsis*. Although the sequence of *FLM* is similar to that of *FLC*, *FLM* and *FLC* interact with different flowering pathways (Scortecci *et al.* 2003). The *FLM* levels were also strongly down-regulated in both *ARR1* and *ARR2* knockouts and in the double mutant. Therefore, to get more insight into the potential interaction with FLM in respect to flowering time, the *arr1-4 arr2-4* double mutant was crossed with *flm-3* single knock-out mutant for *FLM* (Balasubramanian *et al.* 2006). The triple mutant was identified by PCR (see Methods) and confirmed at the expression level (supplemental figure 12).

A



B

	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Long Day							
Col-0 (wild type) A	30,1	10,9	±2,2	±1,7	27-34	8-15	20
arr1-4 arr2-4 B	26,9	9,8	±1,4	±1,4	25-30	8-12	20
flm-3 C	23,8	7,9	±0,9	±0,9	23-25	7-9	20
flm-3 arr1-4 arr2-4 C	24,3	8,4	±1,3	±0,9	23-27	7-10	20

C

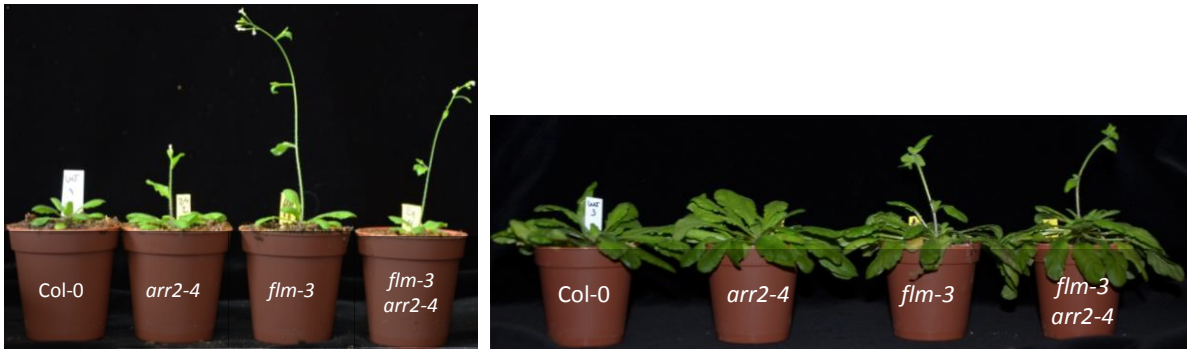
	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Short Day							
Col-0 (wild type) A	110,0	50,3	±6,4	±4,8	100-121	40-56	20
arr1-4 arr2-4 B	87,6	40,1	±5	±2,8	83-95	36-45	20
flm-3 C	77,4	28,8	±5,1	±4,3	69-85	27-34	20
flm-3 arr1-4 arr2-4 C	77,3	28,8	±7,3	±5,8	68-95	20-39	20

Figure 5.6 Flowering phenotype of *flm-3 arr1-4 arr2-4* triple mutant.
(A) Photographs showing early flowering phenotype of *arr1-4 arr2-4* double mutant, *flm-3* single mutant and *flm-3 arr1-4 arr2-4* triple mutant under LDs (left panel) and SDs (right panel) compared to wild-type. Epistatic effect of *FLM* on *ARR1* and *ARR2* on early flowering is very good visible in both long-and short days. **(B)** Flowering time of mutant plants grown under long day conditions. **(C)** Flowering time of mutant plants grown under short day conditions. DUF, days until flowering; RLN, rosette leaf number; n, number of individuals. Significance tests with LSD (α=0.05) were performed on DUF after passing one-way ANOVA (α=0.05). LSD classes are given as letters in superscript. Classes *not connected* by a letter are significantly different.

The flowering time was first determined for the *flm-3 arr1-4 arr2-4* triple mutant under LD conditions. The triple mutant showed very early flowering (compared to the Col-0) and was indistinguishable from the *flm-3* single mutant. The triple mutant *flm-3 arr1-4 arr2-4* flowered in average 6 days earlier (mean: 24 DUF) which was the time same as *flm-3* (mean:

24 DUF), and both developed 3 rosette leaves less than the Col-0 (mean: 30 DUF). The double mutant *arrr1-4 arr2-4* (mean: 27 DUF) flowered somewhat later than the *flm-3* mutants and produced one leaf less than Col-0 (Figure 5.6, A left panel ,B). Clearly *FLM* is epistatic to *ARR1* and *ARR2*.

A



B

	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Long Day							
Col-0 (wild type) A	30,1	10,9	±2,2	±1,7	27-34	8-15	20
arr2-4 A	28,8	10,9	±1,9	±1,6	27-34	8-15	20
flm-3 B	23,8	7,9	±0,9	±0,9	23-25	7-9	20
flm-3 arr2-4 B	24,7	8,2	±1,3	±1,0	22-27	6-9	7

C

	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Short Day							
Col-0 (wild type) A	110,0	50,3	±6,4	±4,8	100-121	40-56	20
arr2-4 B	95,8	45,3	±4,5	±2,7	87-105	40-48	20
flm-3 C	77,4	28,8	±5,1	±4,3	69-85	27-34	20
flm-3 arr2-4 C	87,4	28,3	±5,4	±3,1	81-99	22-31	9

Figure 5.7 Flowering phenotype of *flm-3 arr2-4* double mutant.
(A) Photographs showing early flowering phenotype of *arr2-4* and *flm-3* single mutants and *flm-3 arr2-4* double mutant under LDs (left panel) and SDs (right panel) compared to wild-type. **(B)** Flowering time of mutant plants grown under long day conditions. **(C)** Flowering time of mutant plants grown under SD conditions. DUF, days until flowering; RLN, rosette leaf number; n, number of individuals. Significance tests with LSD (α=0.05) were performed on DUF after passing one-way ANOVA (α=0.05). LSD classes are given as letters in superscript. Classes *not connected* by a letter are significantly different.

The *flm-3 arr1-4 arr2-4* triple mutant and the *flm-3 single* mutant grown under non-inductive SD condition showed epistasis to *flm-3* single mutant as well (Figure 5.6, A(right panel), C). The *flm-3* and *flm-3 arr1-4 arr2-4* triple mutant mutants flowered approximately at the same time, i.e. more than 30 days earlier (mean: 77 DUF) than the wild-type (mean: 110 DUF) and over 10 days earlier than the *arr1-4 arr2-4* double mutant (mean: 87 DUF). Both *flm-3* containing mutants developed 20 rosette leaves less (mean: RLN 29) than Col-0 (mean: RLN 50). In contrast the *arr1-4 arr2-4* double mutant made 10 leaves less than Col-0 (mean: RLN 40). Based on these results *FLM* is epistatic to *ARR1* and *ARR2* in LDs and SDs, and therefore *ARR1* and *ARR2* appear to be genetically involved within the same flowering pathway as *FLM*. *ARR1* and *ARR2* are hypostatic to *FLM*, i.e. they both probably act upstream of *FLM*). Epistasis of *ARR2* to *FLM* was one more time confirmed on *flm-3 arr2-4* double mutant. Under long

day conditions *flm-3 arr2-4* (mean: 87 DUF) double mutant flowers at the same time like the *flm* (mean: 77 DUF) *parent* which is almost a week before the wild-type and 5 days before the *arr2-4* single mutant. Single mutant *flm-3 and* the *flm-3 arr2-4* double mutant also have the same number of rosette leaves (Figure 5.7, A (left panel) and B).

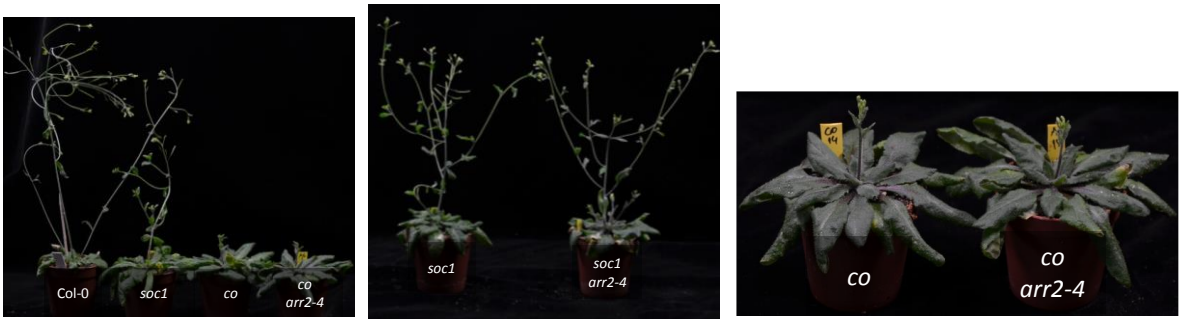
5.2.8 Initial experiments with crossings between *arr2-4* with *soc1-2* and *co* reveal unanticipated interactions

During this work it was possible to generate and analyse double mutants with *co* and *soc1-2* lines for placing *ARR2* within other known flowering time pathways. Recall that *arr2-4* had the stronger flowering time phenotype compared to *arr1-4*. Floral activators *SOC1* and *CO*, especially SOC1, are floral integrators converting most of the flowering pathways. CONSTANS (CO) promotes flowering of *Arabidopsis* in response to day length. Under LD conditios CO activates transcription of *FLOWERING LOCUS T* (*FT*) in the vascular tissue of leaves and initiates flowering (Corbesier *et al.* 2007). *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) is required for CO to promote flowering and integrates the long-day and vernalization/autonomous pathways (Samach *et al.* 2000). On the other hand *SOC1* also integrates signalling from the GA-dependent pathway, a major flowering pathway under non-inductive short days (Moon *et al.* 2003). The *SOC1* and *FT* genes are also regulated by a different flowering-time pathway that acts independently of CO and which is active also at the non-inductive SD conditions (Samach *et al.* 2000). As previously described, beside the similar role of *CO* and *SOC1* as floral activators, they can also act independently of each other. Therefore, both *arr2-4 soc1* and *arr2-4 co* double mutants were tested for flowering time under LD and SD conditions.

Under inductive LD conditions both double mutants were indistinguishable from their *soc1* and *co* single mutant parents. The *arr2-4 soc1* double mutant flowered 5 days later than the Col-0 and more than a week after its *arr2-4* parent developing 7 rosette leaves more than Col-0 and *arr2-4*. Late flowering phenotype is especially remarkable by *arr2-4 co* (mean: 45 DUF) double mutant where flowering was delayed for more than two weeks compared to Col-0 (mean: 30 DUF) and more than 17 days than by *arr2-4* (mean: 28 DUF), *arr2-4 co* double mutant developed 13 rosette leaves more than Col-0 and *arr2-4* (Figure 5.8, A and B).

Under non-inducible SD conditions CO is unable to activate the *FT* flowering-pathway. When grown under SD conditions *co* single mutant and *arr2-4 co* double mutant show very similar flowering phenotype. They flower around 10 days (*arr2-4 co* double mutant) and 15 days (*arr2-4* single mutant) (mean: 100 DUF) before the Col-0 (mean: 110 DUF) but make even 15 rosette leaves less than the wild-type). On the other hand, *co* and *arr2-4 co* double mutant flower only a couple of days later than the *arr2-4* (mean: 96 DUF although they both made 10 leaves less than *arr2-4* single parent (Figure 5.8, C).

A



B

	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Long Day							
Col-0 (wild type) C	30,1	10,9	±2,2	±1,7	27-34	8-15	20
arr2-4 C	28,8	10,9	±1,9	±1,6	27-34	8-15	20
co A	45,8	23,3	±2,5	±3,0	40-49	17-26	20
soc1 B	34,7	18,2	±2,1	±1,8	30-37	17-22	20
arr2-4 co A	45,7	23,8	±3,0	±2,3	37-48	20-28	20
arr2-4 soc1 B	33,5	17,6	±3,6	±2,5	27-57	12-22	6

C

	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Short Day							
Col-0 (wild type) B	110,0	50,3	±6,4	±4,8	100-121	40-56	20
arr2-4 E	95,8	45,3	±4,5	±2,7	87-105	40-48	20
co C D	102,6	35,1	±6,9	±4,1	93-112	30-43	20
soc1 A	116,4	45,1	±8,4	±4,1	105-125	42-55	20
arr2-4 co D E	99,9	35,3	±7,2	±6,0	90-111	25-46	20
arr2-4 soc1 B C	107,3	40,8	±5,6	±6,1	101-117	33-47	6

Figure 5.8 Flowering phenotype of *soc1 arr2-4* and co arr2-4 double mutants.
(A) Photographs showing late flowering phenotype of *soc1 arr2-4* (middle panel) and *co arr2-4* (left and right panel) double mutants under LDs compared to wild-type. **(B)** *ARR2* is hypostatic to *CO* and *SOC1*. **(C)** Flowering phenotype of *soc1 arr2-4 and co arr2-4* under SD conditions . DUF, days until flowering; RLN, rosette leaf number; n, number of individuals. Significance tests with LSD (α=0.05) were performed on DUF after passing one-way ANOVA (α=0.05). LSD classes are given as letters in superscript. Classes *not* connected by a letter are significantly different.

Double mutant *arr2-4 soc1* shows intermediate flowering phenotype compared to its parents. The *arr2-4 soc1* (mean: 107 DUF) double mutant flowers around 9 days earlier than *soc1* (mean: 116 DUF) single mutant and makes 5 rosette leaves less, the same double mutant flowers around 12 days later than *arr2-4* single mutant and makes around 5 rosette leaves more than the *arr2-4* single mutant (Figure 5.8, C).

Taken together, under inductive long days *ARR2* seems to be hypostatic or even works independently of *CO* and *SOC1*. Under non-inductive short days CO-related-flowering pathways are not very active and *arr2-4 co* double mutant shows very similar phenotype like *arr2-4* parent showing again indications that *ARR2* might be hypostatic or independent to *CO* under SDs as well. Another *arr2-4 soc1* double mutant shows similar phenotype like the wild-type which is in between of both single parents suggesting that under SD conditions *ARR2* works partially independent and partially dependent of SOC1-flowering pathways.

5.3 Discussion

During this work, a novel early flowering phenotype was observed and confirmed for single mutants *arr1-4* and *arr2-4* and the double mutant *arr1-4 arr2-4* with respect to flowering time. The early flowering phenotype is especially visible under non-inductive SD conditions for all three mutant lines. The *arr1-4 arr2-4* double mutant flowers even earlier than either single mutant or the wild-type and this tendency was statistically significant between wild-type and the both single mutants. In contrast, under LD conditions, only the *arr2-4* as single mutant showed slightly statistically significant differences as early flowering on the other hand was also observed for the *arr1-4* single mutant but this tendency could not be statistically confirmed in LD. Nevertheless, these differences under inductive long days are from a physiological/functional point of view very subtle and were only revealed when plants were exposed to cool temperatures (16°C). In this case, the double mutant clearly flowered earlier than wild-type. Thus, although *arr2-4*, and presumably *arr1-4*, also affect flowering in LDs, the loss of these two genes clearly points much stronger roles of *ARR2* as a flowering repressor under SD conditions.

Flowering of *arr1-4* mutant in SDs is approximately one week earlier, *arr2-4* flowers two weeks earlier, the *arr1-4 arr2-4* double mutant flowers three weeks before the Col-0. This effect on days until flowering (DUF) was also reflected in rosette leaf number. It has been already proven that generally B-types from the subgroup I (Kim *et al.* 2012) can complement the *arr10 arr12* cytokinin response-deficient mutant (Hill *et al.* 2013). This could be also the case with early flowering phenotype. Yet interestingly, despite redundancy and very similar sequence homology of *ARR1* and *ARR2* genes, we managed to prove that under the loss of *ARR2* effects flowering more strongly than *ARR1* suggesting specificity and affinity (dominance) of *ARR2* over *ARR1* with respect to flowering time despite their high sequence homology. Interestingly, overexpression of ARR2 was shown to have higher transactivation potential and response with and without the addition of cytokinin on the *ARR6* promoter compared to ARR1 (Hwang and Sheen 2001). In addition, even though *ARR2* contributes to primary root-elongation responses to exogenous cytokinin, ARR1 is the predominate cytokinin information carrier in this tissue (Mason *et al.* 2005). Thus, even though these two homologues can have different roles in different tissues, *ARR1* and *ARR2* showed an additive effect on flowering time, which is visible in the *arr1-4 arr2-4* double mutant under both inductive- and non-inductive conditions. One can therefore conclude that both genes are involved in the same flowering time pathway and their role is especially crucial in the signalling pathways which are dominantly active under non-inductive SD conditions.

To check which genes are miss-regulated in the double mutant RT-qPCR analysis of familiar and well described floral regulators was performed. One scenario that would explain the early flowering phenotype is that *ARR1* and *ARR2* genes/pathway (cytokinin) positively regulates the expression of certain floral repressors (such as *FLM*, *FLC*, *SVP*). Alternatively, early flowering could be induced by activation of positive regulators of flowering (*TSF, FD, CO)* or floral integrators (*SOC1*). The chosen markers were applied in order to profile the state of

SAM at this chosen time point. For the first hints, leaf material and material from shoot apical meristem (SAM) from the wild-type and the double mutant was taken depending on where the tested gene is best expressed. The material was taken from plants grown under SDs and it harvested shortly before the double mutant entered in the reproductive phase (based on previous results).

Gene expression of floral activators *CO*, *SOC1*, *FT* and *FDP* did not show any statistically significant difference in the double mutant compared to the wild-type. *FT* is known to be expressed in LDs conditions and acts as a long-range signal in *Arabidopsis* (Valverde *et al*. 2004; Jaeger and Wigge 2007; Sawa and Kay 2011) and here this marker gene was used as a "control" to make sure that the plants were strictly grown under SD conditions and this is confirmed by its very low expression. Floral activators *FD* and *TSF* showed slight up-regulation, although only *TSF* was statistically significantly different compared to the wild-type. Interestingly, it was shown that under non inductive SDs exogenous cytokinin promotes early flowering via activation of the *TSF* (D'aloia *et al*. 2011). The exact mechanism of *TSF* activation is not known and it could be that *ARR1* and *ARR2* play role in its regulation knowing that both *ARR1* and *ARR2* are part of TCS and react to cytokinin treatment (Brenner *et al*. 2012). It might be that non phosphorylated formof *ARR1* and *ARR2* by TCS are somehow disturbing transcription of *TSF* and control flowering on that way. Unfortunately we have failed to produce the *tsf-1 arr1-4 arr2-4* triple knock out mutant and make a closer look how these three genes are interacting on genetic level. In general this data clearly indicated that the expression profile of floral activators is not significantly changed in the double mutant and that these genes are mainly not responsible for the early flowering phenotype in the double mutant.

On the other hand, tested floral repressors showed quite a different expression pattern in the double mutant plants. The floral repressors *FLC, FLM, SVP* and *TFL1* cover most of the crucial signalling pathways responsible for the flowering regulation in *Arabidopsis* (Coupland 1995; Koornneef *et al*. 1998; Song *et al*. 2013). Interestingly, three of tested floral repressors showed significant down-regulation of their expression in double mutant: *FLC* and *FLM* were quite reduced whereas *TFL1* showed only slight reduction. These data show the importance of *FLC* and *FLM* among the rest floral repressors with respect to early flowering in *arr1-4 arr2-4*. On the other hand, the *SVP* floral repressor seems not to be miss-regulated by the lack of *ARR1* or *ARR2*. This observation is of paramount importance and will be address later.

GA promotes flowering transition most strongly under environmental conditions such as SDs, especially when other regulatory pathways that promote flowering in *Arabidopsis thaliana* are not active (Moon *et al*. 2003; Porri *et al*. 2012). In an attempt to address this, showed that *GA4* and *GA5* reflected the state of active GA signalling in healthy plants (Achard *et al*. 2008). Therefore the expression of *GA4* and *GA5* were also monitored. Based on these two genes, we conclude that the GA flowering pathway is not disturbed in the double mutant as both *GA4* and *GA5* marker genes for this pathway are unaffected in the SAM in the double mutant.

This presumptively indicates that *ARR1* and *ARR2* are not involved in the GA flowering pathway but in other flowering signalling pathways active under non-inductive conditions.

Since meristem identity genes and some other genes predominately expressed in meristem play very important role in the initiation of the early flowering they were applied in order to profile the state of SAM at the chosen time point. For this purpose especially *AP1* but also and *LFY* expression was tested. Expression of *LFY* and *AP1* was 2-fold and 3-fold increased with respect to the wild-type. These markers confirmed the early flowering state of the *arr1-4 arr2-4* double mutant compared to Col-0 at the time point where this process could not be visually obtained. Although the low expression of FT suggested that the plants were monitored before they were flowering, it could still have been possible that the shift to inflorescence meristem had already occurred. Therefore, to eliminate the possibility of unseen developmental effects, the flowering transition induced with 30-day old plants grown under non-inductive conditions and then shifted to inductive LDs. The material collected from SAM before and after the shift guaranteed that differences in expression profiles of floral regulators among the mutant plants were due to the miss-regulation of the flowering time only. Once more, it was demonstrated that the early flowering phenotype in both single parents and the wild-type comes from the miss-regulation, i.e. down-regulation of specific floral repressors, primarily *FLC* and *FLM*, as that their level was low even under non-inductive conditions and it remained unchanged after the shifting. Expression pattern of *SVP* floral regulator remained unaffected in any of mutant plants before the shift and responded as expected after the shift (was down-regulated) confirming the previous results and suggesting that *ARR1* and *ARR2* are specifically regulating only certain floral repressors under non-inductive SD conditions. Consistently, all of the tested floral activators showed their regular expression profile like wild-type,: they were up-regulated after the shift from vegetative into reproductive state and showing that neither *ARR1* nor *ARR2* are not playing any role in transcriptional regulators of these floral activators at the not expression level. In conclusion, the early flowering phenotype was most probably a consequence of down-regulation of floral repressors but not of miss-regulation of floral activators.

Considering roles of *ARR1* and *ARR2* in cytokinin signalling and knowing the fact that cytokinin has a positive-correlative role in regulating SAM size (Werner and Schmulling 2009; Gupta and Rashotte 2012) and that SAM size has been correlated with flowering time (Jeong and Clark 2005), the SAM size of *arr1-4*, *arr2-4*, and *arr1-4 arr2-4* warranted examination. Such information could be used to deduce if changes in expression of floral repressors were due to an increase in size of the apical meristem in the mutant plants. Therefore, plants were grown under the same conditions used in the shifting experiment. Both the *arr2-4* single and *arr1-4 arr2-4* double mutants had surprisingly statistically significant smaller SAMs than the wild-type and although the measurement population also showed a smaller size for *arr1-4*, its SAM did not pass the significance threshold (α=0.05). Thus, the smaller SAM fits more with a loss of cytokinin signal, which would be expected knowing that both ARR1 and ARR2 are active in cytokinin singling and despite the smaller SAM size in the mutant plants they

manage to flower earlier than the wild-type. Differences in anatomy or morphology of SAMs in mutant plants and the wild-type were not observed either. In conclusion, the changes in expression of floral repressors were not due to an increase in size of the apical meristem.

Although the *arr2-4* and *arr1-4* mutants have been previously described (Mason *et al*. 2005) and presumed free of any other background mutations, we wanted to be sure that the early-flowering phenotype was due to the lack of *ARR1* and *ARR2*. In order to check this presumption and to determine the origin/location of acting of the *ARR2* with respect to early flowering phenotype the *arr2-4* and *arr1-4 arr2-4* mutants which had the strongest phenotype were complemented with *ARR2*-cDNA and an *ARR2D80N*-cDNA mutant. The *ARR2D80N*-cDNA mutant version cannot be phosphorylated by the TCS anymore, and is less responsive to cytokinin than the wild-type *ARR2*-cDNA (Hwang and Sheen 2001; Choi *et al*. 2010; Veerabagu *et al*. 2012). Thus although it is not completely inert, *ARR2D80N* can be used to see if the wild-type protein, and therefore TCS phosphorylation-dependency, is necessary to rescue the early flowering phenotype.

The cDNA variants were driven under tissue-specific promoters *pFD* and *pSUC2*. For this purpose following vectors with promoter expressed in specific tissues were used: *pSUC2* vasculature-specific promoter expressed in phloem (companion cells) only (Truernit and Sauer 1995; Wippel and Sauer 2012)and *pFD*-meristem-specific promoter expressed in shoot apical meristem only (Mathieu *et al*. 2007). Based on their expression pattern one can determine the most likely location where ARR2 protein activity is needed to complement the flowering time phenotype. Only T2 plants were able to be analysed, therefore a pre-selection was needed to remove any non-transgenic plants using BASTA; secondary effects were controlled by using an empty-vector backbone transformed into all of the mutant lines as well as the wild-type.

ARR2 driven under *pFD* shows the strongest effect and can almost completely complement the flowering phenotype of both the single and the double mutant in SDs where the *arr2-4* and *arr1-4 arr2-4* mutation effects were most strongly observed. This effect in SDs is slightly weaker (partial) when *ARR2* is driven under *pSUC2* but still significant in the double mutant. In LDs conditions, *ARR2* expressed from either promoter was able to complement the *arr2-4* mutation, but only *ARR2* expressed under the *pFD* promoter was able to partially rescue the *arr1-4 arr2-4* mutant. Considered that both tissue-specific promoters are equally strong and having in mind that *pSUC2* expression is on whole plant level one can conclude that *ARR2* is most potently working in the SAM with respect to flowering time.

Some remarks are warranted: first, both promoters can lead to complementation (even if partial) meaning that function of ARR2 is deliverable (or needed) at the meristem but also throughout the plant. Taken together; the observed early flowering phenotype is truly a consequence of the lack of *ARR2* as it can be rescued when it is expressed directly in the SAM by the *pFD* promoter or when it is expressed in phloem companion cells by *pSUC2*. Although not directly shown in the work if the lack of *ARR2* and *ARR1* means that cytokinin signalling

per-se is the culprit for the early-flowering phenotype, the idea is still passed around, albeit lightly, that cytokinin(s) in some form or fashion also could works in a "florigen" type function (Bernier 2011). These results are based on work in *Sinapis alba* (*Sa*) where connections to a simultaneous, but not necessarily, co-dependent transportation of *SaFT* and cytokinin to the meristem. Bernier proposed that some aspect of cytokinin, possibly on the organizing centre, contributes to flowering, but does not in itself cause flowering in *Sinapis* (Bernier 2011). This idea is interesting in lieu of the results here where a stronger effect is seen when *ARR2* is expressed in the meristem, yet *ARR2* still expressed in the phloem (distally) can also lead to complementation. Conflicting with these ideas is the observation that *Arabidopsis* can be induced to flower in SD with an 8 hour hydroponic treatment with BAP (D'aloia *et al.* 2011). This effect requires *TSF* and not *FT* (D'aloia *et al.* 2011). Recall that expression of *TSF* was altered in *arr1-4 arr2-4*.

FLC is known to interact with directly interacts with another MAD-box member SVP, *SVP* which is also floral repressor that functions within the thermosensory pathway (Li *et al.* 2008). SVP and FLC also target *CYTOKININ RESPONSE 1 (CRE1)* and other genes in cytokinin signalling *(Gregis et al. 2013)*. SVP has been further connected to cytokinin via *SVP* regulation of STIMPY (*STIP)*, but this occurs independently and downstream of *FT* and *TSF* (Gregis *et al.* 2013). *STIP* works downstream of cytokinin in establishing the SAM (Liu *et al.* 2009). Interestingly, *FLC* has been shown to be active in the SAM and in the vascular tissue directly regulating *SOC1* and *FT* (reviewed in (Andres and Coupland 2012). *TSF* was shown not to be bound by SVP (Gregis *et al.* 2013). In this work, *arr1-4* and *arr2-4* were also crossed to *tsf-1* (Yamaguchi *et al.* 2005), but triple mutants were not identified, therefore it was not possible to determine the genetic interaction of *arr1-4* or *arr2-4* with *tsf-1*.

Knowing that the loss of *ARR1* and *ARR2* impair cytokinin signalling, it is tempting to speculate that this would explain the early flowering phenotype. In this regard, *SVP* has recently been uncovered to be involved in regulation of some cytokinin genes (Gregis *et al.* 2013). Considering that the expression data and mutant analysis (discussed below) suggest that *ARR1* and *ARR2* are not in the *SVP-FLC* pathway, it is still an open question if the early flowering phenotype can only be attributed to a loss of cytokinin single transmission. Second, other possible reasons for partial complementation could be that the plants used for this experiment were preselected in the T2 generation with most of them in the hemizygous state and thus not enough ARR2 was produced. Alternatively, the gene was not driven under its native promoter and perhaps ARR2 is also needed in other tissues. For example, it is known that *ARR1* is active in controlling auxin flux by regulation of the auxin-response repressor protein SHY2 (SHORT HYPOCOTYL2) (Taniguchi *et al.* 2007; Chapman and Estelle 2009) and the functional characterisation of *ARR1* and *ARR2* have mostly been done with regards to cytokinin responses in the root (Mason *et al.* 2005; Argyros *et al.* 2008; Hill *et al.* 2013).

As said before, the connection to cytokinin, is compelling but not conclusive. In rice, it is known that a cytokinin-dependent TCS pathway exists that requires a B-type (*EHD1*) for aviation of *FT* homologues *HD3A* and *RICE FLOWERING LOCUS T1 (RFT1)* under SDs (Andres

and Coupland 2012). Provided that more evidence is mounted that connects more TCS components to this flowering time phenotype, then (although highly speculative at this point) it could be proposed that a TCS network also works in *Arabidopsis* to modulate flowering which may be analogous to that of rice (as it is not likely it is the same considering no orthologues can be found) or more indicative to the observations made in *Sinapis*. Nevertheless, this regulation mechanism in rice only supports our observation that TCS, or at least its B-Type response regulators *ARR1* and *ARR2*, might also play role in flowering regulation of *Arabidopsis thaliana* under short day conditions as mutant plants lacking in *ARR1* and *ARR2* genes showed the early flowering phenotype under non-inductive short days in *Arabidopsis*. In support to this claim is a fact that *ARR4* (A-type response regulator) in *Arabidopsis thaliana* plays very important role in stabilization of active PhyB-Pfr under extended red light conditions and thus functions as modulator of photomorphogenesis (Sweere *et al.* 2001; Mira-Rodado *et al.* 2007) and PhyB, as above mentioned, actively involved in expression control of *CO* in *Arabidopsis thaliana* and *Hd3a* in *Oryza sativa (Ishikawa et al. 2011)*.

That said, is there any evidence that TCS signalling is required for the early-flowering time phenotype? Examination of the mutant plants showed that they were also slightly complemented with loss-of-function *ARR2D80N* in the single and double mutants under SDs when driven under the *pFD* promoter or by *pSUC2* (albeit only in the *arr1-4 arr2-4*). This slight effect of *ARR2D80N* was also observed in LDs but only in the *arr2-4* mutant background. As mentioned earlier, the reason for this is most likely due to an incomplete inactivation of the *ARR2* by the D80N mutation which was proven by other groups (Hwang and Sheen 2001). ARR2 has a strong basal transactivation capacity unlike the other B-types without the presence of exogenous cytokinin; yet, it still can strongly activate promoter-reporters in a cytokinin-dependent manner (Hwang and Sheen 2001; Veerabagu *et al.* 2012). The ARR2-D80N mutation impairs, sometimes strongly, this basal transactivation capacity without destroying the cytokinin-dependent response, which is highly enhanced compared to mock controls (Hwang and Sheen 2001). Considering this fact, it can be still concluded that ARR2 needs to be phosphorylated by the TCS in order to best rescue the early flowering phenotype as only weak partial complementation were observed for the *ARR2D80N* loss-of-function gene. Type-B response regulators mediate most, if not all, of the immediate-early changes of gene expression induced by cytokinin. This is also the case with *ARR1* and *ARR2* member of the largest sub-class of response regulators expressed in almost all tissues which consists of seven members (*ARR1, ARR2, ARR10, ARR12, ARR11, ARR14,* and *ARR18*) (Brenner *et al.* 2012). This additionally supports the claim that the early flowering phenotype in the *ARR1* and *ARR2* lacking plants is originating due to the impaired signalling of cytokinin. Furthermore, the expression pattern of the *ARR1* and *ARR2* in almost all tissues is showing the importance of cytokinin in every tissue of the plant, and also explains the observation that the early flowering phenotype was rescued also when *ARR2* expressed from phloem. On the other hand mutant complementation with *ARR2D80E* gain-of-function gene under constitutive active promoter is not possible while the plants showed embryo lethality (Hass *et*

al. 2004). Based on this fact the complementation with *pSUC2* and *pFD* tissue specific promoters could not possible while due to their high potency. Therefore *arr1-4 arr2-4* was complemented with ARR2D80E cloned into estradiol inducible *pABind::GFP* vector. As expected the effect of the gain-of-function *ARR2*D80E was very strong and lethal even for plants in T2 generation (supplemental figure 10) and was clearly visible 3 days after the hormonal treatment. Plants were getting various necrotic spots and dying very fast, whereas some of them managed to flower showing early flowering phenotype caused by stress. The formed siliques showed great variation in size and the seed yield was generally more than double lower compared with the wild-type containing certain number of aborted and sterile seed as well. This strongly suggests that *ARR2* may be a "crosstalk point" between the TCS and some flowering time pathways under the short day conditions. Expression analysis, via RT-qPCR, revealed which genes were miss-regulated in the single and double mutants compared to the wild-type and after conducting the flowering time shift, it could be confirmed that the repressors *FLC* and *FLM* are down-regulated in *arr1-4 arr2-4*. In order to determine which floral pathways *ARR1* and *ARR2* are involved in, *arr1-4 arr2-4* or *arr2-4* mutants were crossed to mutants in key floral regulatory pathways: *flc-3, flm-3, tsf-1, co, and soc1*. Triple mutants were obtained and evaluated with *flc-3* and *flm-3*, whereas *arr2-4* double mutants with *co* and *soc1* were also obtained and evaluated. Crosses with *tsf* unfortunately were not evaluable. For these experiments *arr2-4* single and *arr1-4 arr2-4* double mutants were crossed against *flc-3, flm-3* single mutants lacking in these floral repressors as they showed the highest miss-regulation pattern in *ARR1* and *ARR2* lacking mutants. On the other hand, despite the fact that all tested floral activators did not show any expression miss-regulation in *arr1-4 arr2-4*, *co* and *soc1* single mutants were crossed in order to see how and if these genes are interacting on genetic level.

When grown under inductive LD conditions at standard 22/23°C temperature the *flc-3 arr1-4 arr2-4* triple mutant behaved like its *flc-3* parent suggesting that FLC and the ARR1/2 are involved in the same pathway that controls flowering, *i.e. FLC* is epistatic to both *ARR1* and *ARR2*. Nevertheless, early flowering phenotype is under LDs is by all the mutants quite weak and the temporal resolution of flowering was very low. In addition to this *flc-3 arr1-4 arr2-4* triple mutant after each repeat under the LDs showed clear tendency to flower even earlier than the *flc-3* single parent but none of the time this tendency could be statistically proven. Therefore, the same experiment was repeated again under LDs but at 16°C temperature. Lower temperature should generally slow down the flowering time and the discrete differences in flowering time between the different mutant lines should be observed in higher resolution. This was indeed the case at 16°C, plants generally flowered later than at 23°C. Interestingly, this time *flc-3 arr1-4 arr2-4* triple mutant flowered even earlier that *flc-3* parent suggesting the additive effect of *FLC* under *ARR1* and *ARR2* under inductive LD conditions. Surprisingly, *flc-3* deletion mutant was in all this conditions showing the same ratio of flowering compared to the wild-type suggesting that FLC is probably active at the same level on both temperatures with respect to flowering time regulation, *i.e*. it is active at 16°C as a repressor. It have been published that that there are some natural accessions that are

unresponsive to thermal induction despite having non-functional *fri/flc* alleles (Srikanth and Schmid 2011). Recently was also reported that *flc-3* single mutant restores its temperature sensitivity fist below 16°C and not at higher temperatures (Lee *et al.* 2013). Under non-inductive SD conditions the *flc-3 arr1-4 arr2-4* triple mutant very clearly shows consistent additive effect in early flowering. The same pattern was observed also when this experiment was repeated under SD conditions in other growth chamber with different light intensity.

In conclusion, *FLC* appears to work partially independent of *ARR1* and *ARR2* which lack of expression has the same outcome with respect to early flowering phenotype making the triple mutant to flower even earlier then its parents in additive manner. This additive effect is especially good visible under SD conditions due to the enhanced activity of ARR1 and ARR2 under non-inductive short days with respect to early flowering. Under LD conditions at normal ambient temperature of 23°C additive effect could not be statistically proven, but there was each time the tendency present, due to very low involvement of ARR1 and ARR2 with respect to flowering time and therefore weak flowering time phenotype with the mutants. When plants grown under inductive LD conditions but at 16°C temperature additive effect could be observed and statistically proven. On the other hand, in *flm-3 arr2-4* double and *flm-3 arr1-4 arr2-4* triple mutants obtained early flowering phenotype was very clear. When grown on both inductive LD and non-inductive SD conditions both *flm-3 arr2-4* double and *flm-3 arr1-4 arr2-4* triple mutants showed identical phenotype like *flm-3* single parent supporting previous expression data obtained by RT-qPCR. Single *flm-3* mutant has very strong early flowering effect on both LD and SD conditions so it was very easy to proof and describe the obtained phenotype. Based on obtained results both ARR1 and ARR2 seem to work in the same pathway like FLM with respect to early flowering. Genetically observed *FLM* is epistatic to *ARR1* and *ARR2*.

Despite the fact that all tested floral activators and signal integrators did not show any miss-regulation of their expression in tested *ARR1* and *ARR2* knock outs, *CO* and *SOC1* lacking mutants were crossed with *arr2-4* single mutant in order to examine their genetic interaction/hierarchy and see if there is some difference. When tested under inductive LDs *co arr2-4* flowered more than two weeks after the wild-type identical to its co single mutant parent. This may suggest that under LDs *CO* may be epistatic to *ARR2* or event that they work independent and because the weak phenotype of *arr2-4* under LDs this difference could not be observed. When *co arr2-4* double mutant was grown under non-inductive SD conditions it flowered earlier than the wild-type showing the identical phenotype like *arr2-4* single parent. This is because *co* mutants are only delayed in long days, and *CO* mRNA is more abundant in long than in short days (Blazquez *et al.* 1997) and CO dependent pathways are not active under non-inductive SD conditions, only under LDs where the CO protein is stabilized by light and this leads to induction of the floral activators *FT* and *SOC1* (Eriksson *et al.* 2006). The effect observed in the *co arr2-4* double mutant is more due to the lack of *ARR2* only.

Another tested mutant with *SOC1* lacking floral signal integrator, *soc1-2 arr2-4* when grown under inductive LD conditions showed the late flowering phenotype which was statistically

not different than *soc1-2* single parent suggesting epistasis of *SOC1* under *ARR2*. Nevertheless, *soc1 arr2-4* each time showed tendency to flower slight earlier than the *soc1-2* single mutant but because of the very weak flowering phenotype of *arr2-4* under inductive conditions it could not be statistically proven but still independent working *ARR2* and *SOC1* with respect to flowering time cannot be excluded. This claim was supported by fact that when the *soc1-2 arr2-4* double mutant was grown under non-inductive SD conditions it showed an intermediate flowering phenotype. The double mutant plant flowered approximately one week after *arr2-4* single parent and also one week before the *soc1-2* single parent. This all suggested that under non-inductive SD conditions, and most probably under inductive LD conditions, the *ARR2* and *SOC1* are working independent of each other with respect to flowering time which was before proven on expression level as well; *ARR2* works here as negative regulator of flowering (alternatively positive regulator of floral suppressors) and *SOC1* is known to be a positive regulator of flowering.

5.4 References for Chapter 2

Abe, M, Kobayashi, Y, *et al.* (2005). "Fd, a bzip protein mediating signals from the floral pathway integrator ft at the shoot apex." Science **309**(5737): 1052-1056.

Achard, P, Renou, JP, *et al.* (2008). "Plant dellas restrain growth and promote survival of adversity by reducing the levels of reactive oxygen species." Curr Biol **18**(9): 656-660.

Ahn, JH, Miller, D, *et al.* (2006). "A divergent external loop confers antagonistic activity on floral regulators ft and tfl1." EMBO J **25**(3): 605-614.

Alonso-Blanco, C and Koornneef, M (2000). "Naturally occurring variation in arabidopsis: An underexploited resource for plant genetics." Trends Plant Sci **5**(1): 22-29.

Amasino, R (2004). "Vernalization, competence, and the epigenetic memory of winter." Plant Cell **16**(10): 2553-2559.

An, H, Roussot, C, *et al.* (2004). "Constans acts in the phloem to regulate a systemic signal that induces photoperiodic flowering of arabidopsis." Development **131**(15): 3615-3626.

Andres, F and Coupland, G (2012). "The genetic basis of flowering responses to seasonal cues." Nat Rev Genet **13**(9): 627-639.

Argyros, RD, Mathews, DE, *et al.* (2008). "Type b response regulators of arabidopsis play key roles in cytokinin signaling and plant development." Plant Cell **20**(8): 2102-2116.

Ayre, BG and Turgeon, R (2004). "Graft transmission of a floral stimulant derived from constans." Plant Physiol **135**(4): 2271-2278.

Balasubramanian, S, Sureshkumar, S, *et al.* (2006). "Potent induction of arabidopsis thaliana flowering by elevated growth temperature." PLoS Genet **2**(7): e106.

Bari, R and Jones, JD (2009). "Role of plant hormones in plant defence responses." Plant Mol Biol **69**(4): 473-488.

Bastow, R, Mylne, JS, *et al.* (2004). "Vernalization requires epigenetic silencing of flc by histone methylation." Nature **427**(6970): 164-167.

Bernier, G (2011). "My favourite flowering image: The role of cytokinin as a flowering signal." J Exp Bot.

Bernier, G, Havelange, A, *et al.* (1993). "Physiological signals that induce flowering." Plant Cell **5**(10): 1147-1155.

Blazquez, MA, Ahn, JH, *et al.* (2003). "A thermosensory pathway controlling flowering time in arabidopsis thaliana." Nat Genet **33**(2): 168-171.

Blazquez, MA, Green, R, *et al.* (1998). "Gibberellins promote flowering of arabidopsis by activating the leafy promoter." Plant Cell **10**(5): 791-800.

Blazquez, MA, Soowal, LN, *et al.* (1997). "Leafy expression and flower initiation in arabidopsis." Development **124**(19): 3835-3844.

Bohlenius, H, Huang, T, *et al.* (2006). "Co/ft regulatory module controls timing of flowering and seasonal growth cessation in trees." Science **312**(5776): 1040-1043.

Bond, DM, Dennis, ES, *et al.* (2009). "Histone acetylation, vernalization insensitive 3, flowering locus c, and the vernalization response." Mol Plant **2**(4): 724-737.

Brenner, WG, Ramireddy, E, *et al.* (2012). "Gene regulation by cytokinin in arabidopsis." Front Plant Sci **3**: 8.

Chapman, EJ and Estelle, M (2009). "Mechanism of auxin-regulated gene expression in plants." Annu Rev Genet **43**: 265-285.

Chen, M, Chory, J, *et al.* (2004). "Light signal transduction in higher plants." Annu Rev Genet **38**: 87-117.

Choi, J, Huh, SU, *et al.* (2010). "The cytokinin-activated transcription factor arr2 promotes plant immunity via tga3/npr1-dependent salicylic acid signaling in arabidopsis." Dev Cell **19**(2): 284-295.

Choi, K, Kim, S, *et al.* (2005). "Suppressor of frigida3 encodes a nuclear actin-related protein6 required for floral repression in arabidopsis." Plant Cell **17**(10): 2647-2660.

Colasanti, J and Sundaresan, V (2000). "'Florigen' enters the molecular age: Long-distance signals that cause plants to flower." Trends Biochem Sci **25**(5): 236-240.

Corbesier, L, Vincent, C, *et al.* (2007). "Ft protein movement contributes to long-distance signaling in floral induction of arabidopsis." Science **316**(5827): 1030-1033.

Coupland, G (1995). "Genetic and environmental control of flowering time in arabidopsis." Trends Genet **11**(10): 393-397.
D'Aloia, M, Bonhomme, D*, et al.* (2011). "Cytokinin promotes flowering of arabidopsis via transcriptional activation of the ft paralogue tsf." Plant J **65**(6): 972-979.
Deal, RB, Kandasamy, MK*, et al.* (2005). "The nuclear actin-related protein arp6 is a pleiotropic developmental regulator required for the maintenance of flowering locus c expression and repression of flowering in arabidopsis." Plant Cell **17**(10): 2633-2646.
Deng, W, Ying, H*, et al.* (2011). "Flowering locus c (flc) regulates development pathways throughout the life cycle of arabidopsis." Proc Natl Acad Sci U S A **108**(16): 6680-6685.
Doi, K, Izawa, T*, et al.* (2004). "Ehd1, a b-type response regulator in rice, confers short-day promotion of flowering and controls ft-like gene expression independently of hd1." Genes Dev **18**(8): 926-936.
Endo, M, Tanigawa, Y*, et al.* (2013). "Phytochrome-dependent late-flowering accelerates flowering through physical interactions with phytochrome b and constans." Proc Natl Acad Sci U S A **110**(44): 18017-18022.
Eriksson, S, Bohlenius, H*, et al.* (2006). "Ga4 is the active gibberellin in the regulation of leafy transcription and arabidopsis floral initiation." Plant Cell **18**(9): 2172-2181.
Ferrandiz, C, Gu, Q*, et al.* (2000). "Redundant regulation of meristem identity and plant architecture by fruitfull, apetala1 and cauliflower." Development **127**(4): 725-734.
Fowler, S, Lee, K*, et al.* (1999). "Gigantea: A circadian clock-controlled gene that regulates photoperiodic flowering in arabidopsis and encodes a protein with several possible membrane-spanning domains." EMBO J **18**(17): 4679-4688.
Franklin, KA (2009). "Light and temperature signal crosstalk in plant development." Curr Opin Plant Biol **12**(1): 63-68.
Franklin, KA, Larner, VS*, et al.* (2005). "The signal transducing photoreceptors of plants." Int J Dev Biol **49**(5-6): 653-664.
Garner, WW (1933). "Comparative responses of long-day and short-day plants to relative length of day and night." Plant Physiol **8**(3): 347-356.
Genoud, T and Metraux, JP (1999). "Crosstalk in plant cell signaling: Structure and function of the genetic network." Trends Plant Sci **4**(12): 503-507.
Geraldo, N, Baurle, I*, et al.* (2009). "Frigida delays flowering in arabidopsis via a cotranscriptional mechanism involving direct interaction with the nuclear cap-binding complex." Plant Physiol **150**(3): 1611-1618.
Greenboim-Wainberg, Y, Maymon, I*, et al.* (2005). "Cross talk between gibberellin and cytokinin: The arabidopsis ga response inhibitor spindly plays a positive role in cytokinin signaling." Plant Cell **17**(1): 92-102.
Grefen, C and Harter, K (2004). "Plant two-component systems: Principles, functions, complexity and cross talk." Planta **219**(5): 733-742.
Gregis, V, Andres, F*, et al.* (2013). "Identification of pathways directly regulated by short vegetative phase during vegetative and reproductive development in arabidopsis." Genome Biol **14**(6): R56.
Griffiths, J, Murase, K*, et al.* (2006). "Genetic characterization and functional analysis of the gid1 gibberellin receptors in arabidopsis." Plant Cell **18**(12): 3399-3414.
Gupta, S and Rashotte, AM (2012). "Down-stream components of cytokinin signaling and the role of cytokinin throughout the plant." Plant Cell Rep **31**(5): 801-812.
Harmer, SL, Hogenesch, JB*, et al.* (2000). "Orchestrated transcription of key pathways in arabidopsis by the circadian clock." Science **290**(5499): 2110-2113.
Hass, C, Lohrmann, J*, et al.* (2004). "The response regulator 2 mediates ethylene signalling and hormone signal integration in arabidopsis." EMBO J **23**(16): 3290-3302.
Hayama, R and Coupland, G (2004). "The molecular basis of diversity in the photoperiodic flowering responses of arabidopsis and rice." Plant Physiol **135**(2): 677-684.
He, Y, Michaels, SD*, et al.* (2003). "Regulation of flowering time by histone acetylation in arabidopsis." Science **302**(5651): 1751-1754.

Hedden, P and Phillips, AL (2000). "Gibberellin metabolism: New insights revealed by the genes." Trends Plant Sci **5**(12): 523-530.

Helliwell, CA, Wood, CC, *et al.* (2006). "The arabidopsis flc protein interacts directly in vivo with soc1 and ft chromatin and is part of a high-molecular-weight protein complex." Plant J **46**(2): 183-192.

Heo, JB and Sung, S (2011). "Encoding memory of winter by noncoding rnas." Epigenetics **6**(5): 544-547.

Hepworth, SR, Valverde, F, *et al.* (2002). "Antagonistic regulation of flowering-time gene soc1 by constans and flc via separate promoter motifs." EMBO J **21**(16): 4327-4337.

Hicks, KA, Millar, AJ, *et al.* (1996). "Conditional circadian dysfunction of the arabidopsis early-flowering 3 mutant." Science **274**(5288): 790-792.

Hill, K, Mathews, DE, *et al.* (2013). "Functional characterization of type-b response regulators in the arabidopsis cytokinin response." Plant Physiol **162**(1): 212-224.

Hirsch, S and Oldroyd, GE (2009). "Gras-domain transcription factors that regulate plant development." Plant Signal Behav **4**(8): 698-700.

Hisamatsu, T and King, RW (2008). "The nature of floral signals in arabidopsis. Ii. Roles for flowering locus t (ft) and gibberellin." J Exp Bot **59**(14): 3821-3829.

Hsuan, HM, Salleh, B, *et al.* (2011). "Molecular identification of fusarium species in gibberella fujikuroi species complex from rice, sugarcane and maize from peninsular malaysia." Int J Mol Sci **12**(10): 6722-6732.

Hwang, I and Sheen, J (2001). "Two-component circuitry in arabidopsis cytokinin signal transduction." Nature **413**(6854): 383-389.

Imaizumi, T and Kay, SA (2006). "Photoperiodic control of flowering: Not only by coincidence." Trends Plant Sci **11**(11): 550-558.

Ishikawa, R, Aoki, M, *et al.* (2011). "Phytochrome b regulates heading date 1 (hd1)-mediated expression of rice florigen hd3a and critical day length in rice." Mol Genet Genomics **285**(6): 461-470.

Jaeger, KE and Wigge, PA (2007). "Ft protein acts as a long-range signal in arabidopsis." Curr Biol **17**(12): 1050-1054.

Jasinski, S, Piazza, P, *et al.* (2005). "Knox action in arabidopsis is mediated by coordinate regulation of cytokinin and gibberellin activities." Curr Biol **15**(17): 1560-1565.

Jeong, S and Clark, SE (2005). "Photoperiod regulates flower meristem development in arabidopsis thaliana." Genetics **169**(2): 907-915.

Johanson, U, West, J, *et al.* (2000). "Molecular analysis of frigida, a major determinant of natural variation in arabidopsis flowering time." Science **290**(5490): 344-347.

Kardailsky, I, Shukla, VK, *et al.* (1999). "Activation tagging of the floral inducer ft." Science **286**(5446): 1962-1965.

Kim, DH, Doyle, MR, *et al.* (2009). "Vernalization: Winter and the timing of flowering in plants." Annu Rev Cell Dev Biol **25**: 277-299.

Kim, HJ, Kieber, JJ, *et al.* (2012). "Overlapping and lineage-specific roles for the type-b response regulators of monocots and dicots." Plant Signal Behav **7**(9): 1110-1113.

Kim, HJ, Ryu, H, *et al.* (2006). "Cytokinin-mediated control of leaf longevity by ahk3 through phosphorylation of arr2 in arabidopsis." Proc Natl Acad Sci U S A **103**(3): 814-819.

Kim, W, Park, TI, *et al.* (2013). "Generation and analysis of a complete mutant set for the arabidopsis ft/tfl1 family shows specific effects on thermo-sensitive flowering regulation." J Exp Bot **64**(6): 1715-1729.

Kojima, S, Takahashi, Y, *et al.* (2002). "Hd3a, a rice ortholog of the arabidopsis ft gene, promotes transition to flowering downstream of hd1 under short-day conditions." Plant Cell Physiol **43**(10): 1096-1105.

Kolmos, E and Davis, SJ (2007). "Elf4 as a central gene in the circadian clock." Plant Signal Behav **2**(5): 370-372.

Koornneef, M, Alonso-Blanco, C, *et al.* (1998). "Genetic control of flowering time in arabidopsis." Annu Rev Plant Physiol Plant Mol Biol **49**: 345-370.

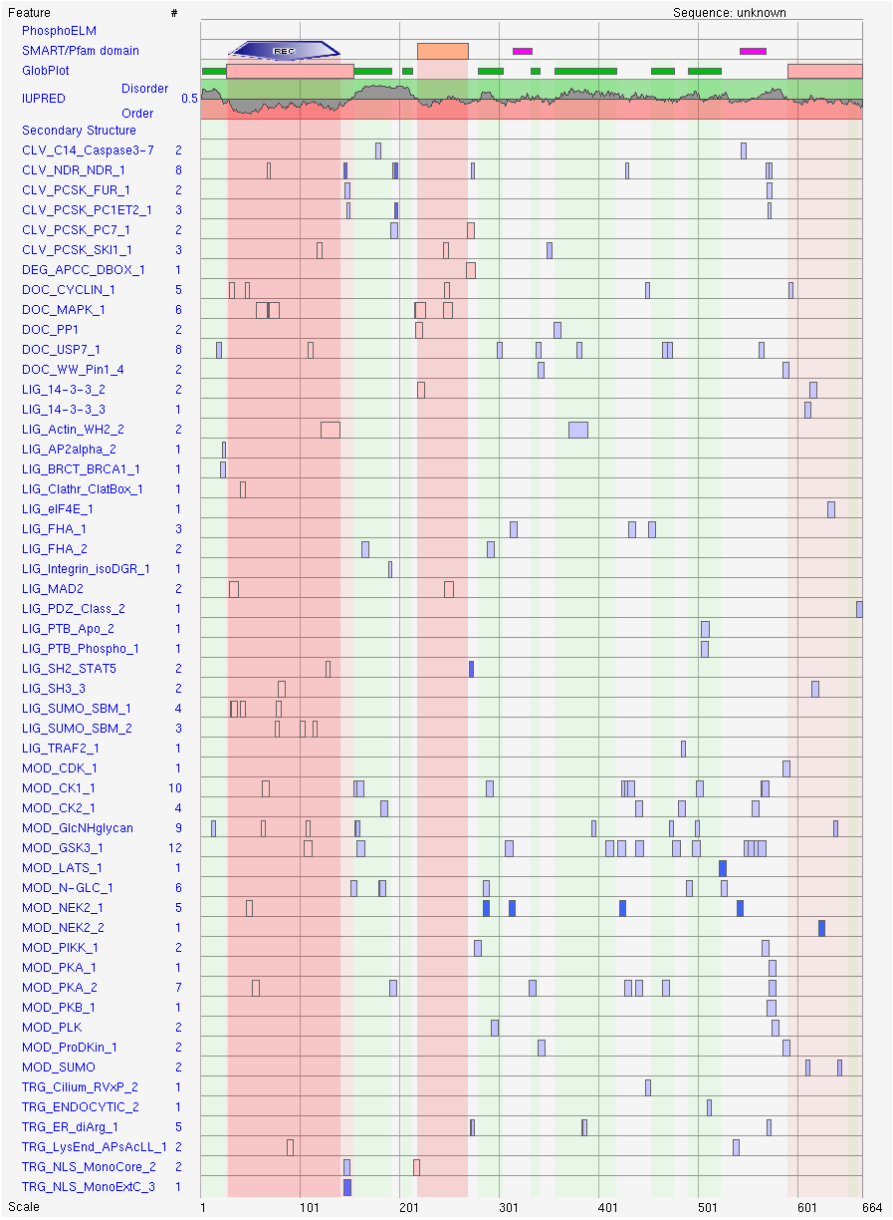
Koornneef, M, Alonso-Blanco, C*, et al.* (2004). "Naturally occurring genetic variation in arabidopsis thaliana." Annu Rev Plant Biol **55**: 141-172.
Kumar, SV and Wigge, PA (2010). "H2a.Z-containing nucleosomes mediate the thermosensory response in arabidopsis." Cell **140**(1): 136-147.
Lariguet, P and Dunand, C (2005). "Plant photoreceptors: Phylogenetic overview." J Mol Evol **61**(4): 559-569.
Lee, I and Amasino, RM (1995). "Effect of vernalization, photoperiod, and light quality on the flowering phenotype of arabidopsis plants containing the frigida gene." Plant Physiol **108**(1): 157-162.
Lee, JH, Ryu, HS*, et al.* (2013). "Regulation of temperature-responsive flowering by mads-box transcription factor repressors." Science **342**(6158): 628-632.
Li, C, Zhang, K*, et al.* (2009). "A cis element within flowering locus t mrna determines its mobility and facilitates trafficking of heterologous viral rna." J Virol **83**(8): 3540-3548.
Li, D, Liu, C*, et al.* (2008). "A repressor complex governs the integration of flowering signals in arabidopsis." Dev Cell **15**(1): 110-120.
Lifschitz, E, Eviatar, T*, et al.* (2006). "The tomato ft ortholog triggers systemic signals that regulate growth and flowering and substitute for diverse environmental stimuli." Proc Natl Acad Sci U S A **103**(16): 6398-6403.
Lin, C (2000). "Photoreceptors and regulation of flowering time." Plant Physiol **123**(1): 39-50.
Liu, C, Xi, W*, et al.* (2009). "Regulation of floral patterning by flowering time genes." Dev Cell **16**(5): 711-722.
Mason, MG, Mathews, DE*, et al.* (2005). "Multiple type-b response regulators mediate cytokinin signal transduction in arabidopsis." Plant Cell **17**(11): 3007-3018.
Mathieu, J, Warthmann, N*, et al.* (2007). "Export of ft protein from phloem companion cells is sufficient for floral induction in arabidopsis." Curr Biol **17**(12): 1055-1060.
Matsushika, A, Makino, S*, et al.* (2000). "Circadian waves of expression of the aprr1/toc1 family of pseudo-response regulators in arabidopsis thaliana: Insight into the plant circadian clock." Plant Cell Physiol **41**(9): 1002-1012.
Michaels, SD and Amasino, RM (1999). "Flowering locus c encodes a novel mads domain protein that acts as a repressor of flowering." Plant Cell **11**(5): 949-956.
Mira-Rodado, V, Sweere, U*, et al.* (2007). "Functional cross-talk between two-component and phytochrome b signal transduction in arabidopsis." J Exp Bot **58**(10): 2595-2607.
Mizoguchi, T, Wright, L*, et al.* (2005). "Distinct roles of gigantea in promoting flowering and regulating circadian rhythms in arabidopsis." Plant Cell **17**(8): 2255-2270.
Moon, J, Suh, SS*, et al.* (2003). "The soc1 mads-box gene integrates vernalization and gibberellin signals for flowering in arabidopsis." Plant J **35**(5): 613-623.
Mouradov, A, Cremer, F*, et al.* (2002). "Control of flowering time: Interacting pathways as a basis for diversity." Plant Cell **14 Suppl**: S111-130.
Notaguchi, M, Abe, M*, et al.* (2008). "Long-distance, graft-transmissible action of arabidopsis flowering locus t protein to promote flowering." Plant Cell Physiol **49**(11): 1645-1658.
Pin, PA and Nilsson, O (2012). "The multifaceted roles of flowering locus t in plant development." Plant Cell Environ **35**(10): 1742-1755.
Poethig, RS (2013). "Vegetative phase change and shoot maturation in plants." Curr Top Dev Biol **105**: 125-152.
Porri, A, Torti, S*, et al.* (2012). "Spatially distinct regulatory roles for gibberellins in the promotion of flowering of arabidopsis under long photoperiods." Development **139**(12): 2198-2209.
Pose, D, Verhage, L*, et al.* (2013). "Temperature-dependent regulation of flowering by antagonistic flm variants." Nature.
Putterill, J, Robson, F*, et al.* (1995). "The constans gene of arabidopsis promotes flowering and encodes a protein showing similarities to zinc finger transcription factors." Cell **80**(6): 847-857.
Rashotte, AM, Carson, SD*, et al.* (2003). "Expression profiling of cytokinin action in arabidopsis." Plant Physiol **132**(4): 1998-2011.
Reeves, PA, He, Y*, et al.* (2007). "Evolutionary conservation of the flowering locus c-mediated vernalization response: Evidence from the sugar beet (beta vulgaris)." Genetics **176**(1): 295-307.

Reeves, PH and Coupland, G (2000). "Response of plant development to environment: Control of flowering by daylength and temperature." Curr Opin Plant Biol **3**(1): 37-42.
Samach, A, Onouchi, H*, et al.* (2000). "Distinct roles of constans target genes in reproductive development of arabidopsis." Science **288**(5471): 1613-1616.
Sawa, M and Kay, SA (2011). "Gigantea directly activates flowering locus t in arabidopsis thaliana." Proc Natl Acad Sci U S A **108**(28): 11698-11703.
Sawa, M, Nusinow, DA*, et al.* (2007). "Fkf1 and gigantea complex formation is required for day-length measurement in arabidopsis." Science **318**(5848): 261-265.
Schluepmann, H, Pellny, T*, et al.* (2003). "Trehalose 6-phosphate is indispensable for carbohydrate utilization and growth in arabidopsis thaliana." Proc Natl Acad Sci U S A **100**(11): 6849-6854.
Schmid, M, Uhlenhaut, NH*, et al.* (2003). "Dissection of floral induction pathways using global expression analysis." Development **130**(24): 6001-6012.
Scortecci, K, Michaels, SD*, et al.* (2003). "Genetic interactions between flm and other flowering-time genes in arabidopsis thaliana." Plant Mol Biol **52**(5): 915-922.
Searle, I, He, Y*, et al.* (2006). "The transcription factor flc confers a flowering response to vernalization by repressing meristem competence and systemic signaling in arabidopsis." Genes Dev **20**(7): 898-912.
Skylar, A, Hong, F*, et al.* (2010). "Stimpy mediates cytokinin signaling during shoot meristem establishment in arabidopsis seedlings." Development **137**(4): 541-549.
Smith, HM, Ung, N*, et al.* (2011). "Specification of reproductive meristems requires the combined function of shoot meristemless and floral integrators flowering locus t and fd during arabidopsis inflorescence development." J Exp Bot **62**(2): 583-593.
Song, J, Angel, A*, et al.* (2012). "Vernalization - a cold-induced epigenetic switch." J Cell Sci **125**(Pt 16): 3723-3731.
Song, YH, Ito, S*, et al.* (2013). "Flowering time regulation: Photoperiod- and temperature-sensing in leaves." Trends Plant Sci **18**(10): 575-583.
Srikanth, A and Schmid, M (2011). "Regulation of flowering time: All roads lead to rome." Cell Mol Life Sci **68**(12): 2013-2037.
Suarez-Lopez, P, Wheatley, K*, et al.* (2001). "Constans mediates between the circadian clock and the control of flowering in arabidopsis." Nature **410**(6832): 1116-1120.
Sun, T, Goodman, HM*, et al.* (1992). "Cloning the arabidopsis ga1 locus by genomic subtraction." Plant Cell **4**(2): 119-128.
Sun, TP (2010). "Gibberellin-gid1-della: A pivotal regulatory module for plant growth and development." Plant Physiol **154**(2): 567-570.
Sung, S and Amasino, RM (2004). "Vernalization in arabidopsis thaliana is mediated by the phd finger protein vin3." Nature **427**(6970): 159-164.
Sweere, U, Eichenberg, K*, et al.* (2001). "Interaction of the response regulator arr4 with phytochrome b in modulating red light signaling." Science **294**(5544): 1108-1111.
Taniguchi, M, Sasaki, N*, et al.* (2007). "Arr1 directly activates cytokinin response genes that encode proteins with diverse regulatory functions." Plant Cell Physiol **48**(2): 263-277.
Tran, LS, Nakashima, K*, et al.* (2007). "Co-expression of the stress-inducible zinc finger homeodomain zfhd1 and nac transcription factors enhances expression of the erd1 gene in arabidopsis." Plant J **49**(1): 46-63.
Truernit, E and Sauer, N (1995). "The promoter of the arabidopsis thaliana suc2 sucrose-h+ symporter gene directs expression of beta-glucuronidase to the phloem: Evidence for phloem loading and unloading by suc2." Planta **196**(3): 564-570.
Tucker, MR and Laux, T (2007). "Connecting the paths in plant stem cell regulation." Trends Cell Biol **17**(8): 403-410.
Tudzynski, B (1999). "Biosynthesis of gibberellins in gibberella fujikuroi: Biomolecular aspects." Appl Microbiol Biotechnol **52**(3): 298-310.
Valverde, F, Mouradov, A*, et al.* (2004). "Photoreceptor regulation of constans protein in photoperiodic flowering." Science **303**(5660): 1003-1006.

Veerabagu, M, Elgass, K*, et al.* (2012). "The arabidopsis b-type response regulator 18 homomerizes and positively regulates cytokinin responses." Plant J **72**(5): 721-731.
Wahl, V, Ponnu, J*, et al.* (2013). "Regulation of flowering by trehalose-6-phosphate signaling in arabidopsis thaliana." Science **339**(6120): 704-707.
Wang, JW, Czech, B*, et al.* (2009). "Mir156-regulated spl transcription factors define an endogenous flowering pathway in arabidopsis thaliana." Cell **138**(4): 738-749.
Werner, JD, Borevitz, JO*, et al.* (2005). "Quantitative trait locus mapping and DNA array hybridization identify an flm deletion as a cause for natural flowering-time variation." Proc Natl Acad Sci U S A **102**(7): 2460-2465.
Werner, T and Schmulling, T (2009). "Cytokinin action in plant development." Curr Opin Plant Biol **12**(5): 527-538.
Wigge, PA (2011). "Ft, a mobile developmental signal in plants." Curr Biol **21**(9): R374-378.
Wigge, PA, Kim, MC*, et al.* (2005). "Integration of spatial and temporal information during floral induction in arabidopsis." Science **309**(5737): 1056-1059.
Willige, BC, Ghosh, S*, et al.* (2007). "The della domain of ga insensitive mediates the interaction with the ga insensitive dwarf1a gibberellin receptor of arabidopsis." Plant Cell **19**(4): 1209-1220.
Wilson, RN, Heckman, JW*, et al.* (1992). "Gibberellin is required for flowering in arabidopsis thaliana under short days." Plant Physiol **100**(1): 403-408.
Wippel, K and Sauer, N (2012). "Arabidopsis suc1 loads the phloem in suc2 mutants when expressed from the suc2 promoter." J Exp Bot **63**(2): 669-679.
Wu, Z, Skjelvag, AO*, et al.* (2004). "Quantification of photoperiodic effects on growth of phleum pratense." Ann Bot **94**(4): 535-543.
Yamaguchi, A, Kobayashi, Y*, et al.* (2005). "Twin sister of ft (tsf) acts as a floral pathway integrator redundantly with ft." Plant Cell Physiol **46**(8): 1175-1189.
Yano, M, Kojima, S*, et al.* (2001). "Genetic control of flowering time in rice, a short-day plant." Plant Physiol **127**(4): 1425-1429.
Yanovsky, MJ and Kay, SA (2002). "Molecular basis of seasonal time measurement in arabidopsis." Nature **419**(6904): 308-312.
Yoo, SK, Chung, KS*, et al.* (2005). "Constans activates suppressor of overexpression of constans 1 through flowering locus t to promote flowering in arabidopsis." Plant Physiol **139**(2): 770-778.
Yoshida, R, Fekih, R*, et al.* (2009). "Possible role of early flowering 3 (elf3) in clock-dependent floral regulation by short vegetative phase (svp) in arabidopsis thaliana." New Phytol **182**(4): 838-850.
Yu, H, Ito, T*, et al.* (2004). "Repression of agamous-like 24 is a crucial step in promoting flower development." Nat Genet **36**(2): 157-161.
Zwack, PJ and Rashotte, AM (2013). "Cytokinin inhibition of leaf senescence." Plant Signal Behav **8**(7).

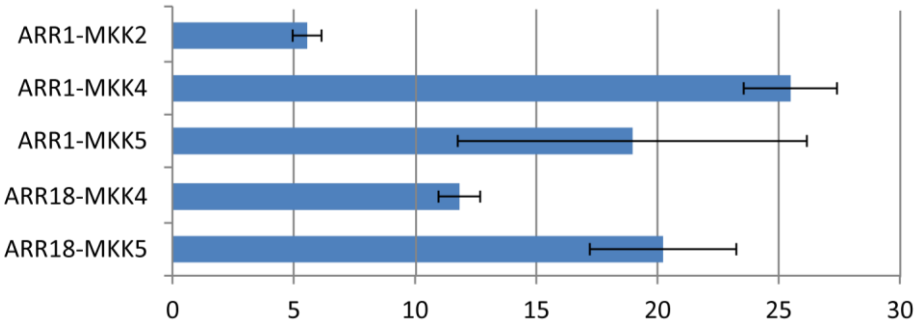
6 Appendix

6.1 Supplemental Figures



Supplemental 1 Supplement to Figure 4.6. *In silico* predictions of MKK docking motifs on ARR2 based on ELM software

The figure shows different predicted functional domains of ARR2; four docking motifs for the MKKs are marked (yellow). ELM - (the database of eukaryotic linear motifs, http://elm.eu.org). The docking motif for MKKs is called DOC_MAPK_1.



Supplemental 2 Supplement to Figure 4.8. oNPG data for interaction strength between ARR1 and ARR18 with biotic-stress-related MKKs in yeast.

The β-galactosidase activity was measured in the extracts of three independent yeast clones. Diluted yeast colonies were incubated for 2 days at 28°C on interaction selective (CSM-L, W, A) media. The experiment was repeated at least two times.

A

LSD results for experiment with Pst DC3000

Level							Mean
WT (Col-0) Day 4	A						6,8327273
arr1-4 Day 4	A						6,7877965
arr1-4xarr2-4 Day 4		B					6,3391667
WT (Col-0) Day 2		B					6,1881818
arr1-4 Day 2			C				5,9050000
arr1-4xarr2-4 Day 2			C				5,7327273
arr2-4 Day 2			C				5,6250000
arr2-4 Day 4				D			5,2648189
arr1-4xarr2-4 Day 1					E		4,0383333
arr1-4 Day 1					E		4,0081818
WT (Col-0) Day 1					E		3,9445455
arr2-4 Day 1					E		3,9416667
arr1-4xarr2-4 Day 0						F	1,8800000
arr2-4 Day 0						F	1,7900000
arr1-4 Day 0						F	1,6400000
WT (Col-0) Day 0						F	1,6400000

Levels not connected by same letter are significantly different.

B

LSD results for experiment with Pst DC3000 and cytokinin treatment

Level									Mean
arr1-4 Mock Day 4	A								7,4163636
WT (Col-0) Mock Day 4	A	B							7,0091784
arr1-4 Cytokinin Day 2	A	B							6,9850000
arr2-4 Mock Day 4	A	B	C						6,8054545
arr1-4xarr2-4 Cytokinin Day 2		B	C	D					6,6600000
arr1-4 Mock Day 2		B	C	D					6,6309091
arr1-4xarr2-4 Mock Day 2			C	D	E				6,3552786
arr1-4xarr2-4 Mock Day 4			C	D	E				6,2557137
WT (Col-0) Cytokinin Day 2			C	D	E				6,2483333
WT (Col-0) Mock Day 2				D	E				6,0954545
arr2-4 Mock Day 2					E	F			5,9818182
WT (Col-0) Cytokinin Day 4					E	F			5,8886552
arr2-4 Cytokinin Day 2					E	F			5,7925000
arr1-4 Cytokinin Day 4					E	F			5,7854545
arr1-4xarr2-4 Cytokinin Day 4						F	G		5,4081842
arr2-4 Cytokinin Day 4							G		5,0945975
arr2-4 Cytokinin Day 1								H	4,3000000
WT (Col-0) Mock Day 1								H	4,2213819
arr2-4 Mock Day 1								H	4,2109091
arr1-4 Mock Day 1								H	4,1754545
arr1-4 Cytokinin Day 1								H	4,1225000
arr1-4xarr2-4 Mock Day 1								H	4,1058333
WT (Col-0) Cytokinin Day 1								H	4,0991667
arr1-4xarr2-4 Cytokinin Day 1								H	3,9491667
arr2-4 Mock Day 0									I 2,3485714
arr1-4 Cytokinin Day 0									I 2,1800000
arr1-4 Mock Day 0									I 2,1787500
WT (Col-0) Mock Day 0									I 2,0916667
arr1-4xarr2-4 Cytokinin Day 0									I 2,0116667
arr2-4 Cytokinin Day 0									I 1,9160000
WT (Col-0) Cytokinin Day 0									I 1,8400000
arr1-4xarr2-4 Mock Day 0									I 1,8000000

Levels not connected by same letter are significantly different.

Supplemental 3 Supplement to Figure 4.13B. Results of Fisher's Least Significant Difference (LSD) test for pathogen assay with *Pseudomonas syringae Pst* DC3000

(A) LSD results of plants treated only with *Pst* DC3000. (B) LSD results of plants treated with PstDC3000 in addition of cytokinin (1 μM t-Zeatin). WT stands for Col-0. Data are presented for days: 0, 1, 2 and 4 post treatment. LSD tests were conducted at the 0.05 α level.

LSD results for stomata aperture

Level							Mean
wtDC3000	A						5,3746250
wtt-zeatin	A						5,2938941
2-4t-zeatin	A	B					5,1199891
1-4x2-4t-zeatin	A	B					5,0776780
1-4x2-4DC3000		B	C				4,7440645
wtcontrol			C				4,4587333
1-4t-zeatin				D			4,0488333
2-4control				D	E		4,0190758
1-4x2-4control				D			4,0102941
1-4control				D	E	F	3,7739500
2-4ABA				D	E	F	3,6802353
wtABA					E	F	3,6649109
1-4x2-4ABA						F	G 3,5012639
1-4ABA							G 3,1161346

Levels not connected by same letter are significantly different.

Supplemental 4 Supplement to Figure 4.15. Results of Fisher's Least Significant Difference (LSD) test for measurements of stomata aperture

Wt stands for Col-0, 1-4 for *arr1-4*, 2-4 for *arr2-4* and 1-4x2-4 for *arr1-4 arr2-4*. Control represents MOCK treatment. LSD tests were conducted at the 0.05 α level.

A

LSD results for Alternaria brassicicola experiment day 7

Level			Mean
arr2-4	A		201,38889
arr1-4Xarr2-4	A		200,00000
arr1-4		B	176,71233
WT (Col-0)		B	169,01408

Levels not connected by same letter are significantly different.

LSD results for Alternaria brassicicola experiment day 10

Level			Mean
arr1-4Xarr2-4	A		314,49275
arr2-4	A		311,11111
WT (Col-0)		B	270,42254
arr1-4		B	266,15385

Levels not connected by same letter are significantly different.

B

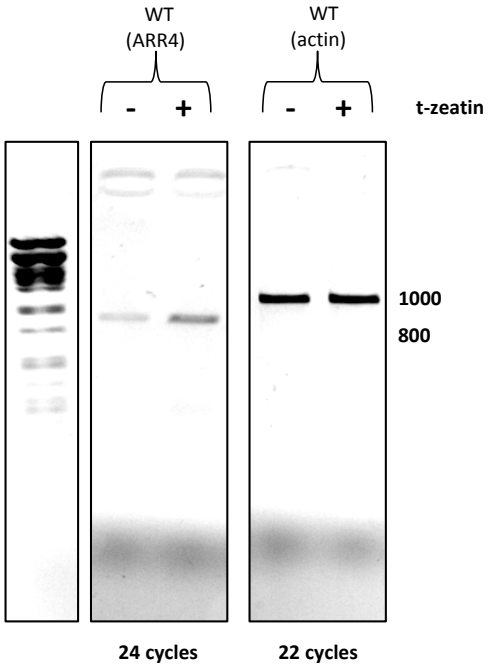
LSD results for *Alternaria brassicicola* experiment with cytokinin

Level									Mean
arr2-4 Mock Day 10	A								308,92857
arr1-4Xarr2-4 Mock Day 10	A								308,33333
WT (Col-0) Mock Day 10	A								308,33333
arr2-4 Cytokinin Day 10	A	B							303,57143
arr1-4Xarr2-4 Cytokinin Day 10	A	B							303,33333
arr1-4 Mock Day 10	A	B	C						298,21429
WT (Col-0) Cytokinin Day 10		B	C	D					290,00000
arr2-4 Mock Day 7			C	D	E				283,63636
arr1-4 Cytokinin Day 10				D	E	F			280,35714
arr1-4Xarr2-4 Mock Day 7				D	E	F	G		275,00000
arr1-4Xarr2-4 Cytokinin Day 7					E	F	G		271,18644
arr2-4 Cytokinin Day 7						F	G	H	266,07143
WT (Col-0) Mock Day 7							G	H	263,33333
arr1-4 Mock Day 7							G	H	261,81818
WT (Col-0) Cytokinin Day 7								H	I 253,33333
arr1-4 Cytokinin Day 7									I 239,28571

Levels not connected by same letter are significantly different.

Supplemental 5 Supplement to Figure 4.16 Results of Fisher's Least Significant Difference (LSD) test for pathogen assay with *Alternaria brassicicola*

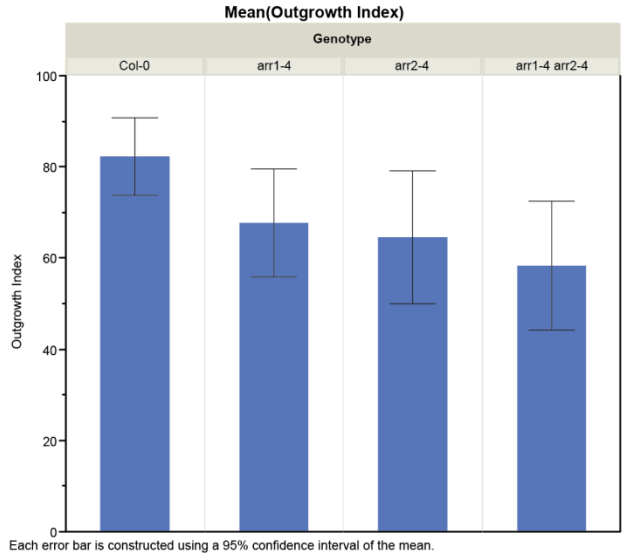
(A)LSD results for plants treated with *Alternaria* only after 7 days (top) and after 10 days (bottom). (B) LSD results for plants treated with *Alternaria* in the presence and absence of cytokinin (1μM t-Zeatin). WT stands for Col-0. Control represents MOCK treatment. LSD tests were conducted at the 0.05 α level.



Supplemental 6 Supplement to Figure 4.16B. Plants treated with cytokinin for pathogen assays with *Alternaria brassicicola*

Cytokinin application was monitored by detecting expression of *ARR4*, which is a known cytokinin responsive gene. Plants treated with 1 µM t-Zeatin showed increased expression of *ARR4* indirectly proving that cytokinin treatment was successful.

A



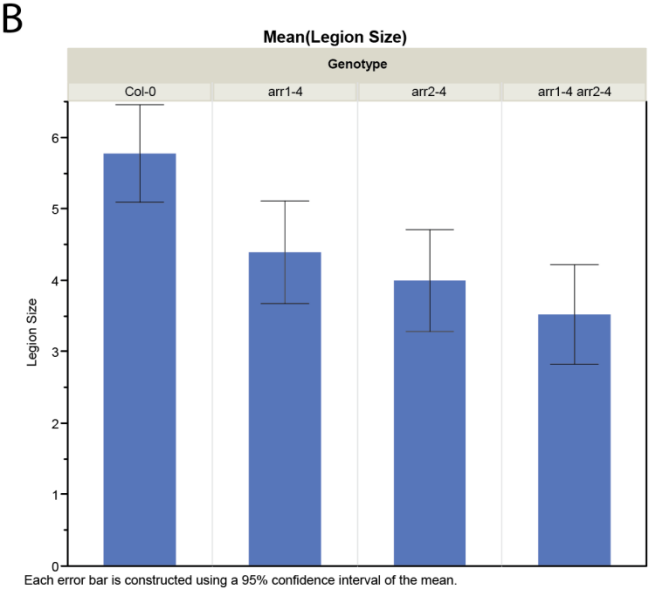
Connecting Letters Report

Level		Mean
Col-0	A	82,291667
arr1-4	B	67,708333
arr2-4	B	64,583333
arr1-4 arr2-4	B	58,333333

Levels not connected by same letter are significantly different.

Level	- Level	p-Value
Col-0	arr1-4 arr2-4	0,0062*
Col-0	arr2-4	0,0411*
Col-0	arr1-4	0,0914*
arr1-4	arr1-4 arr2-4	0,2756
arr2-4	arr1-4 arr2-4	0,4666
arr1-4	arr2-4	0,7155

B



Connecting Letters Report

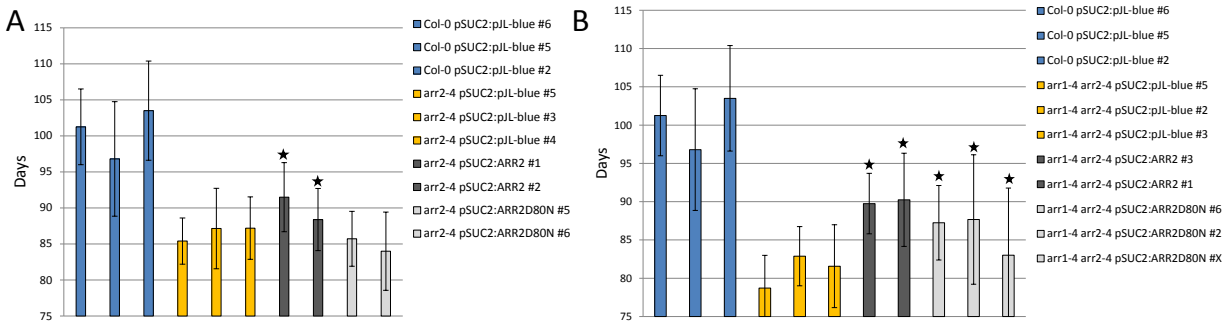
Level		Mean
Col-0	A	5,7726042
arr1-4	B	4,3903125
arr2-4	B	3,9953125
arr1-4 arr2-4	B	3,5207292

Levels not connected by same letter are significantly different.

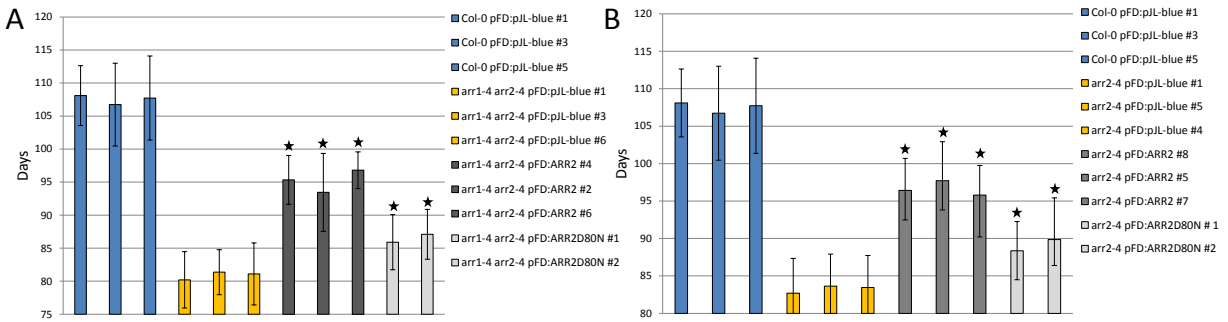
Level	- Level	p-Value
Col-0	arr1-4 arr2-4	<,0001*
Col-0	arr2-4	0,0004*
Col-0	arr1-4	0,0060*
arr1-4	arr1-4 arr2-4	0,0830
arr2-4	arr1-4 arr2-4	0,3434
arr1-4	arr2-4	0,4303

Supplemental 7 Supplement to Figure 4.17. Plants treated with cytokinin for pathogen assays with *Botrytis cinerea*.

(A) Left: Outgrowth Index data shown as mean and the 95% confidence interval. Right: LSD results for the Outgrowth Index data about the mean at the 0.1 α level. All of the mutants are significantly smaller than the wild-type at the 0.1 alpha level and those with arr2-4 at the 0.05 α level. (B) Left: Lesion Size data shown as mean and the 95% confidence interval. Right: LSD results for the Lesion Size about the mean at the 0.06 α level. All of the mutants are significantly smaller than the wild-type at the 0.06 α level.

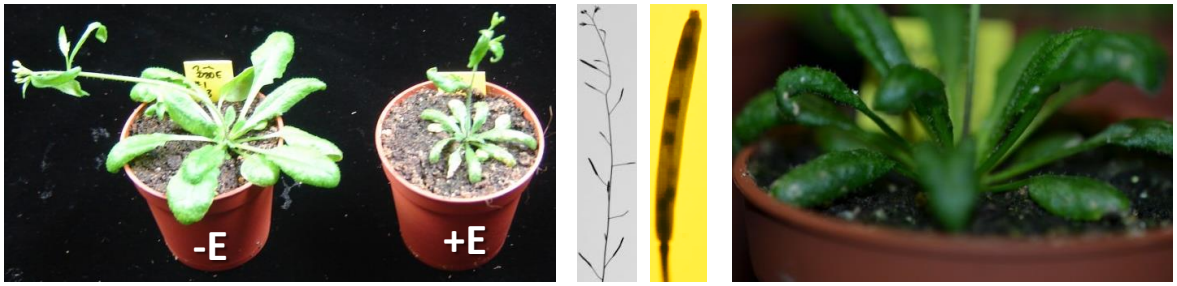


Supplemental 8 to table 5.3. Flowering time of transgenic lines driven under tissue specific *pSUC2* promoter under short day non-inductive conditions (alternative representation).
(A) Experiments where *pSUC2::ARR2* was complemented in *arr2-4* mutant background. **(B)** Experiments where *pSUC2::ARR2* was complemented in *arr1-4 arr2-4* mutant background. All independent lines show the same tendency. Lines used for this experiment were in T2 generation and preselected with BASTA. Mean values and standard deviations are shown.

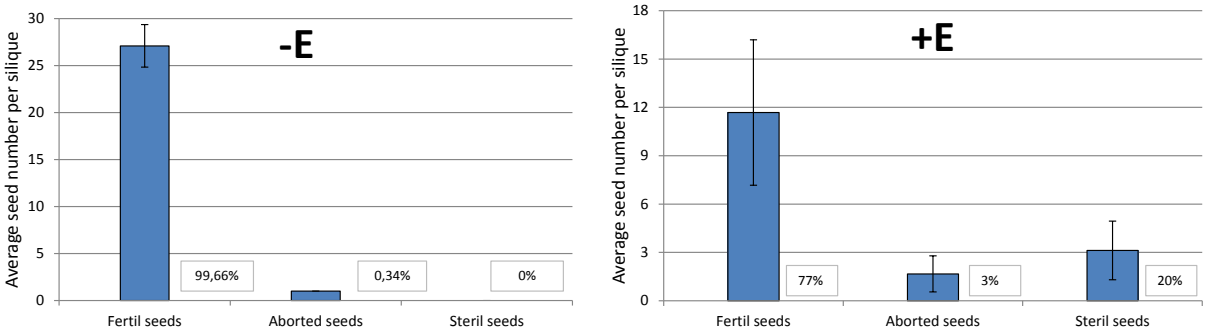


Supplemental 9 to table 5.4. Flowering time of transgenic lines driven under tissue specific *FD* promoter under short day non-inductive conditions (alternative representation).
(A) Experiments where *pFD::ARR2* was complemented in *arr2-4* mutant background. **(B)** Experiments where *pFD::ARR2* was complemented in *arr1-4 arr2-4* mutant background. All independent lines show the same tendency and stronger complementation than under *SUC2* promoter. Lines used for this experiment were in T2 generation and preselected with BASTA. Mean values and standard deviations are shown.

A

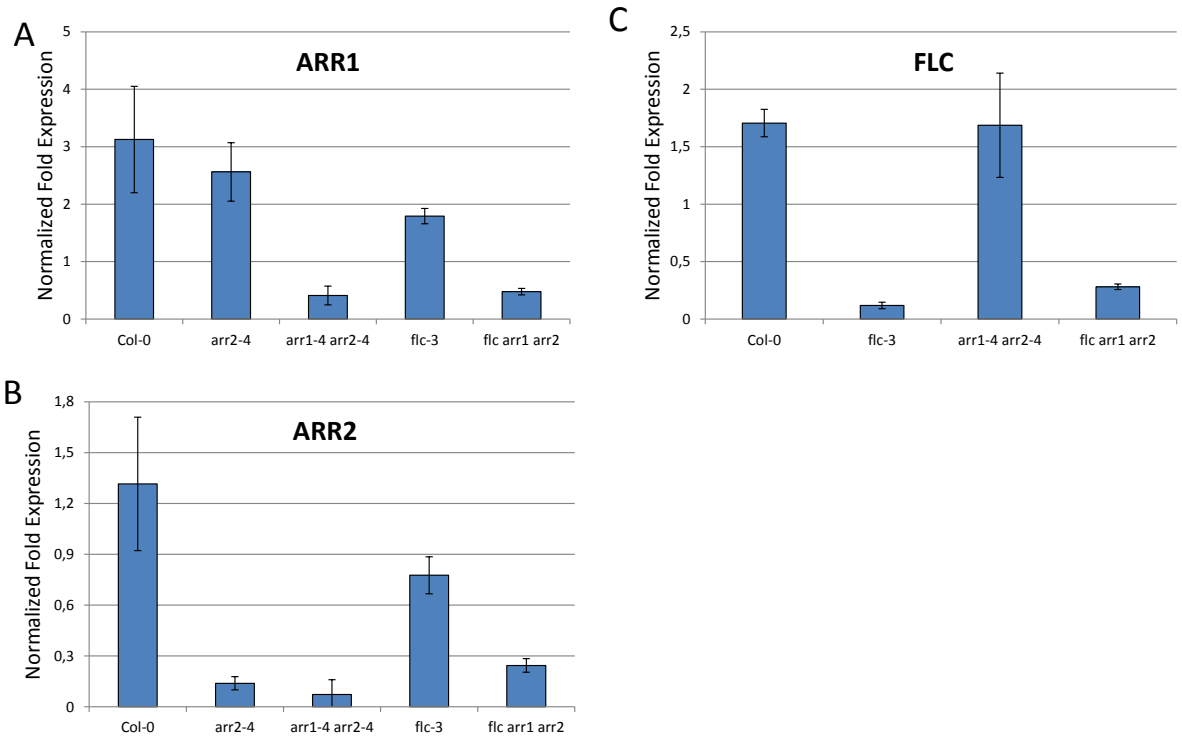


B



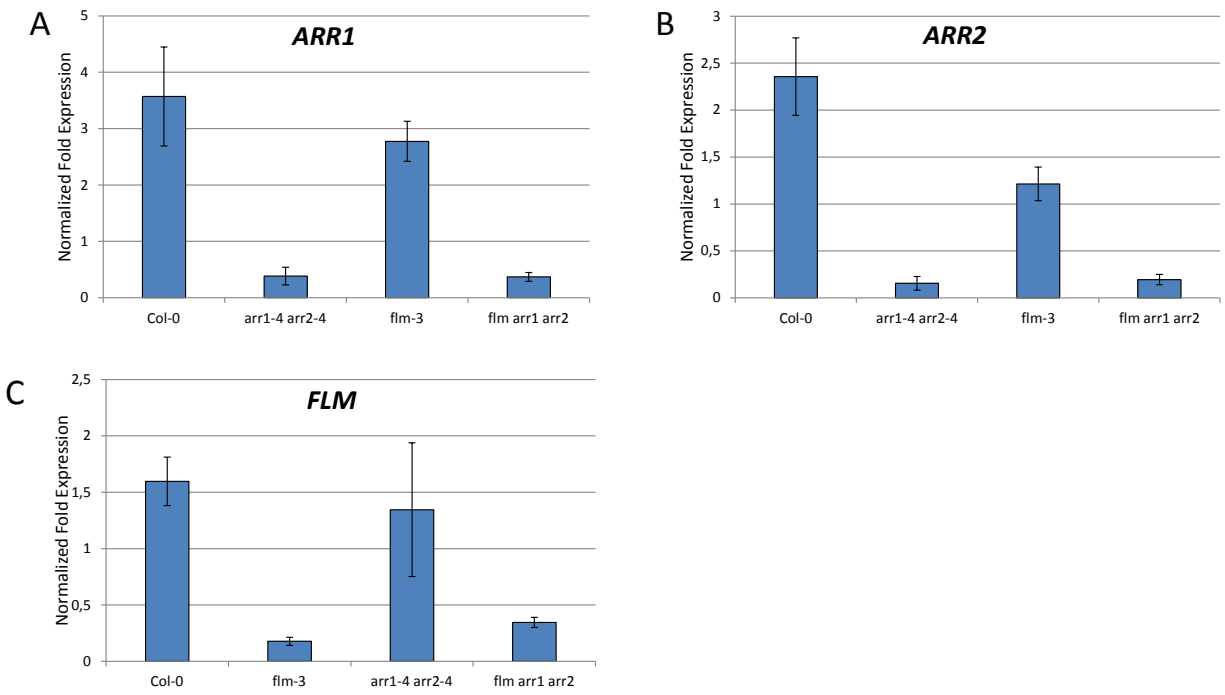
Supplemental 10 Phenotypic differences of *arr1-4 arr2-4* transgenic lines transformed with *pABind::ARR2D80E::GFP* estradiol inducible vector when treated with estradiol or Mock control

(A) *arr1-4 arr2-4* transformed with *pABind::ARR2D80E::GFP* estradiol inducible vector in T2 generation. Left photograph shows phenotypic differences of transgenic plant not induced (-E) and induced (+E) with β-estradiol. In addition photographs of disproportional silique size, reduced seed number and leaf changes, respectively, of transgenic line treated with β-estradiol are shown. (B) Difference in seed number and types of seeds between non-induced (left) and induced (right) transgenic line. For this experiment at least 10 plants per transgenic line were taken, total 3 independent lines were used, and 5 siliques per plant were used for statistical evaluation.



Supplemental 11 Transcriptional levels of *ARR1, ARR2* and *FLC* in the *triple flc-3 arr1-4 arr2-4* mutant knock out.

(A) Expression of *ARR1* in *flc-3, arr2-4, arr1-4 arr2-4* and *flc-3 arr1-4 arr2-4* mutants. **(B)** Expression of *ARR2* in *flc-3, arr2-4, arr1-4 arr2-4* and *flc-3 arr1-4 arr2-4* mutants. **(C)** Expression of *FLC* in *flc-3, arr2-4, arr1-4 arr2-4* and *flc-3 arr1-4 arr2-4* mutants. For RNA isolation leaves were taken from 3-week-old plants grown under SD. RT-qPCR was done using one biological replicate with three technical repetitions each and normalized to *ELF-1-α*. Error bars indicate SD of the mean.



Supplemental 12 Transcriptional levels of *ARR1, ARR2* and *FLM* in the *triple flm-3 arr1-4 arr2-4* mutant knock out.

(A) Expression of ARR1 in Col-0, *flm-3, arr1-4 arr2-4* and *flm-3 arr1-4 arr2-4* mutants. **(B)** Expression of ARR2 in Col-0, *flm-3, arr2-4, arr1-4 arr2-4* and *flm-3 arr1-4 arr2-4* mutants. **(C)** Expression of *FLM* in Col-0, *flm-3, arr2-4, arr1-4 arr2-4* and *flm-3 arr1-4 arr2-4* mutants. For RNA isolation leaves were taken from 3-week-old plants grown under SD. RT-qPCR was done using one biological replicate was used with three technical repetitions and normalized to *ELF-1-α*. Error bars indicate SD of the mean.

6.2 List of Figures

6.3 List of Tables

6.4 List of Supplements

7 Curriculum Vitae

Name:	Marko Vesić
Date of Birth:	April 13th 1986
Place of Birth:	Kruševac, Socialist Federal Republic of Yugoslavia *now* Republic of Serbia
Marital Status:	Unmarried

Since 2009	Doctoral studies at the Center for Plant Molecular Biology (ZMBP) University of Tübingen, Germany Supervisor: Prof. Dr. Klaus Harter Bench Supervisor: Dr. Kenneth Berendzen
2009	Diploma thesis (M.S.) at the Institute of Field and Vegetable Crops, Laboratory for Molecular Markers in Novi Sad, Serbia. Title: "Inheritance of Sunflower *Downy Mildew* resistance gene analogs"
2005-2009	Studies of Molecular Biology at the University of Novi Sad in Novi Sad, Autonomous Province Vojvodina, Serbia
2001-2005	Secondary education at the mathematical Gymnasium in Kruševac, Serbia
1993-2001	Basic education at the primary school in Kruševac, Serbia

8 Acknowledgements

Oh, I thought I will never come to this point of my PhD and therefore I did not even think about what I would write in here...

First person coming on my mind is **Klaus** (and that not because of the fact that he is one who makes important decisions about this dissertation). When I go 6 years back in the past I remember myself, young and ambitious student of molecular biology just finished 3rd year of his studies, sitting in front of computer (exactly like now) and sending an Email to some Professor from Germany whose group is working on very interesting topic and me willing to spend whole my summer holiday in his lab. The "shock "occurred only one hour after that when Klaus wrote me back (using his direct, kind and polite style) and said that he would be very happy having me in his lab for a practical course. No one was happier than me in that moment! Ever since then Klaus has always been there to give me help, support and his never-ending optimism. Klaus you were/are a real DoktorVATER to me and thank you for that!

Kenneth! Thank you for everything! Billion thanks you for correcting my thesis and critically reviewing it. Thank you for all your attempts to make me an independent scientist, I believe you made it☺.

Furthermore, I would like to thank a lot to **PD Dr. Markus Schmid** for the successful collaboration and finding time to read and correct my thesis and constantly reminding me to submit it☺. I am also very grateful to **Prof. Claudia Oecking** for finding time and accepting to be a committee member for my thesis defence making me a great honour. Many thanks to **Dr.** Gabriel Schaaf for a great time spent together at the ZMBP, constructive discussions about various topics and for accepting to read my thesis and be a member of the committee for my defence.

To **Rebecca Schwab** and **Sascha Laubinger**, extraordinary people, I thank you for all your advices, great help and support I was very lucky that I met you guys.

Of course my sincere gratitude to **Prof. Silvana Andrić** and **Prof. Snežana Radulović** (University of Novi Sad, Serbia) and to **Prof. Dejana Panković-Saftić** (EDUCONS University, Serbia) for their constant support and encourigment on the way to accomplishment of my dreams and wishes.

I would also like to thank to the DAAD, DGF, Ministry of Youth and Sport of Republic of Serbia and to Reinhold-und-Maria-Teufel-Stiftung for the financial support making my studies and stay in Germany possible.

Jochen Eisele (Mann mit einem goldenem Herzen), **Christina Chaban**, **Tante Christel** (meine zweite Mutter), **Caterina**, **Mani**, **Steven**, **Babs**, **Franzi-Schw...i** (meine größte Unterstützung), **Bea** (mein bester Kumpel), **Simon der Förschter**, **Tanja**, **Christian**, **Patty**, **Niklas**... Thank you

guys for making my time in Germany beautiful and helping me to solve all the problems I was facing with.

Meli tebi posebno hvala na SVEMU! Special thanks to my new **Meli & D´Boys** Immuno Dream Team (**Niko**, **Dominik**, **Hasan**, **Davide**, **Aki**, **Anu**) for accepting me and making me feel great in your company and integrating me within your team.

Svenito-Kurito Punišić DANKE for wonderfull vacations, sports, mutual support and teaching me how to get my car repaired all by myself. Du biSch omeglich!

Thierry mon ami merci pour tout! I am going to miss our long philosophical discussions about science, art and life early in the morning on the way home after having "successful" evenings down town! These are the things which enriched my life a lot!

To my Serbian mates and the roommates **Zorica**, **Jelena** *alias* **Baba Stamena** and **Verica** thank you for all the priceless moments we spent together in good and bad times and thank you for being entire time with me even when I left and went to Germany. Волим Вас!

Special passage samo za tebe **Honey**! Muchas gracias por todo! Por tu apoyo, comprensión y locuras! Cuando sólo pienso en nosotros en: Madrid, Barsa, Roma, Novi Sad, Cerova, Toledo... ... sólo espero que esta lista de „pichvajza" no se termina aquí!

Непроцењиву заслугу у целокупном мом животном развоју има, наравно, и мој комшилук из Церове (комшије **Снежа** и **Горан**) и касније Новог Сада (моји **Снежа** и **Иван**, **Срнче-Прасе**, **Романа**, дивни **Мира** и **Влада**). Хвала Вам на искреној и безусловној подршци, ручковима и вечерама, трпљења мојих провокација и разноразних испада, силним покушајима да ме „уразумите" и „доведете у ред" (мада ми је драго да се само делимично у томе успели!).

За крај, велико ХВАЛА мојој породици - **родитељима** и **брату**, посебно мојој **мами**, за сав труд и пожртвовање које су уложили да мог брата и мене изведу на „прави" пут.

ХВАЛА Вам **Наталија**, **Милице**, **Милане**, **Дивна**, **Светиславе** где год да сте!

Овај рад посвећујем мојој баки **Нади** и мојим малим *venturi*-јима: **Наталији**, **Иви** и **Сергеју**!

Ваш Марко

Table of Contents

Summary

Two-component system (TCS) and MAPK signalling cascades play essential roles in plant signal transduction. The *Arabidopsis* Response Regulator 2 (ARR2), a member of B-type response regulators in the two-component circuit, serves as a molecular hub integrating several incoming signals. A comparable hub function is also known for the MAPK signalling cascade. Based on preliminary experiments we have started to address the hypothesis that there exists a link between ARR2-dependent TCS and MAPK signalling cascade, *i.e.* a crosstalk of those two evolutionary divergent signal transduction systems.

Potential protein-protein interactions between ARR2 and MAPK cascade members (MKKKs, MKKs, and MPKs respectively), were tested. The results revealed that ARR2 as well as other tested B-type ARRs, but not the A-type ARRs, strongly interacted with MKKs and some MPKs where none of them interacted with MKKKs. Interestingly, despite a high sequence similarity with ARR2, ARR1 did not interact with the all those MAPK members ARR2 is interacting with. ARR2 was found to interact preferentially with MKK4 and MKK5, which play an important role in the biotic stress defence specially including responses to pathogen attacks. Pathogenesis assays applied on *ARR2* and *ARR1* mutants lines, using the necrothrophic, semi biotrophic and biotrophic pathogens differing in evolution and distinct defence strategies in plants, revealed us that ARR2 mutants showed difference only in necrotrophic stress responses but not in biotrophic stresses.

The detailed analysis of *arr1* and *arr2* single loss-of-function mutants as well as an *arr1 arr2* double mutant revealed an early flowering phenotype of these plants especially visible under short day conditions (SDs) with the double mutant having an additive effect compared to the single parents. Despite very similar sequence homology of the *ARR1* and *ARR2* genes, the loss of *ARR2* effects the flowering time was quite stronger than *ARR1* but they still function in the same signalling pathway. From the mechanistic point of view the lack of *ARR1/ARR2* activity causes down-regulation of specific floral repressor genes but not of genes that act as floral activators and this miss-regulation of expression is not due developmental defects but directly linked to floral induction. ARR2 expressed either in the shoot apical meristem (SAM) or in phloem companion cells recues the early flowering phenotype of the *arr2* single and the *arr1 arr2* double mutants. The crossing of *ARR1* and *ARR2* mutants with flowering time-related mutants revealed that *ARR1* and *ARR2* function predominantly independent of *Flowering Locus C* (*FLC*). However, this approach revealed that *Flowering Locus M* (*FLM*) acts epistatic to *ARR1* and *ARR2*.

These findings are revealing and describing special features of the TCS elements and their crosstalks with other signalling pathways and contributing to a better understanding of their physiological role.

Zusammenfassung

Zweikomponentensystem (TCS) und MAPK Signalkaskaden spielen wichtige Rollen in der Signaltransduktion der Pflanze. Der *Arabidopsis* Response Regulator 2 (ARR2), der ein Mitglied der B-Typ Response Regulatoren des Zweikomponentensystems ist, dient als molekularer Knotenpunkt, welcher viele Signale integriert. Eine ähnliche Funktion wurde bereits für die MAPK Signalkaskade gezeigt. Basierend auf früheren Experimenten stellten wir die Hypothese auf, dass eine Verbindung zwischen ARR2-abhängigen TCS und der MAPK Signalkaskade besteht, beispielsweise eine Interaktion dieser evolutionär divergenten Signaltransduktionssystemen.

Protein-Protein-Interaktionen zwischen ARR2 und Proteinen der MAPK Signalkaskade (MKKKs, MKKs und MPK) wurden untersucht. Wir konnten zeigen, dass ARR2 und auch andere ARRs aus der Gruppe der B-Typ Response Regulatoren, aber nicht der A-Typ ARRs, stark mit MKKs und einigen MPKs interagieren. Dabei gab es keine Interaktion mit den MKKKs. Trotz einer hohen Sequenzänlichkeit zwischen ARR2 und ARR1, interagiert ARR1 nicht mit allen Komponenten der MAPK Signalkaskade mit denen ARR2 wechselwirkt. Wir konnten zeigen, dass ARR2 mit MKK4 und MKK5 interagiert. Beide haben eine wichtige Rolle bei der Stressantwort der Pflanze, vor allem bei der Pathogenabwehr. Versuche an *ARR2*- und *ARR1*-Knockoutlinien mit nekrotrophen, hemibiotrophen und biotrophen Pathogenen, die jeweils evolutionär unterschiedliche Abwehrstartegien in Pflanzen hervorrufen, haben gezeigt, dass *ARR2*-Mutanten keine Unterschiede bei biotrophen, dafür aber bei nekrotrophen Stressantworten hervorrufen.

Genaue Untersuchungen der *arr1*- und *arr2*-Einzelmutanten, sowie der *arr1 arr2* Doppelmutante zeigen einen Frühblüher-Phänotyp, vor allem unter Kurztagbedingungen. Trotz der großen Homologie der *ARR1* und *ARR2* Gene, wirkt sich der Knockout des *ARR2* Gens auf den Blühzeitpunkt wesentlich stärker aus als der Verlust von *ARR1*, dennoch wirken sie beide im selben Signaltransduktionsweg. Die Abwesenheit von ARR1/ARR2 führt zur negativen Regulation von spezifischen Blüh-Repressorgenen. Gene die für die Blüh-Induktion zuständig sind, werden davon nicht betroffen. Diese Unterschiede in der Genregulation haben keinen Efekt in der Entwicklung der Pflanze, sondern wirken sich nur auf den Zeitpunkt der Blühinduktion aus. ARR2 wird im Sprossapikalmeristem (SAM) oder in den Geleitzellen im Phloem expremiert, und ist für den Frühblüher-Phänotyp in der arr2 Einzelmutante und in der *arr1 arr2* Doppelmutante verantwortlich. Kreuzungen von ARR1 und ARR2 Mutanten mit Mutanten, die eine Veränderung des Blühzeitpunkts aufweisen, zeigen, dass *ARR1* und *ARR2* überwiegend unabhängig von dem *Flowering Locus C (FLC)* wirken. Die Kreuzungslinien belegen, dass der *Flowering Locus M (FLM*) epistatisch zu *ARR1* und *ARR2* ist.

Diese Daten beschreiben spezielle Elemente des TCS und deren Interaktion mit anderen Signaltransduktionswegen, welche zu einem besseren Verständnis der physiologischen Rolle führt.

1 General Introduction

1.1 Two-component systems

Two-component transduction systems (TCS or TSCT) pathways represent principal means for coordinating responses to environmental changes in bacteria and also in plants, some fungi, *archaea* and *protozoa*, but it is absent in higher eukaryotes and metazoans (Koretke *et al.* 2000; Hwang *et al.* 2002; Capra and Laub 2012). In prokaryotes these systems are mostly composed of a receptor sensor histidine kinase (HK or SK) and its cognate response regulator (Koretke *et al.* 2000; Capra and Laub 2012). Histidine kinases sense a specific signal and generally form functional homodimers allowing kinases to autophosphorylate at an internal histidine (H, His) by the γ-phosphoryl group of ATP (Koretke *et al.* 2000). The high-energy phosphoryl group is further transferred to an aspartate (D, Asp) residue of the two-component receiver domain in the response regulator protein. Response regulators (RRs) typically contain a two-component receiver domain and an effector domain allowing the protein to modify and regulate cellular behaviour in a TCS-dependent manner (Koretke *et al.* 2000; Hwang *et al.* 2002). The name "two-component" actually originates from this fact: in its simplest case only two elements are involved in TCS signal transduction, however there are exceptions which are elaborated on below. HKs share very high homology their ATP-binding domains with Hsp90, type II topoisomerases and MutL (mismatch repair protein) (Dutta *et al.* 1999; Koretke *et al.* 2000; Capra and Laub 2012). These proteins are all members of GHKL superfamily and it is suggested that HKs originated from one of these ATPases and had further evolved by series of duplications, lineage-specific expansion and divergence (Capra and Laub 2012). Generally, HKs are bifunctional, *i.e.* they can also act as phosphatases of their response regulators (Capra and Laub 2012). In order to make dimers, autophosphorylate and then successfully transfer the γ-phosphate group, histidine kinases must contain the dimerization and histidine phosphotransfer domain (DHp) and the catalytic and ATP binding (CA) domain (Koretke *et al.* 2000). Other domains most frequent in HKs are the sensory binding domains Per Arnt Sim (PAS) and cGMP-specific phosphodiesterases Adenylyl cyclases and FhlA (GAF), a conserved "linker" domain Histidine kinase Adenyl cyclases Methyl accepting proteins and Phophatases (HAMP) (Galperin *et al.* 2001). These periplasmic sensory domains are responsible for direct signal (PAS and GAF) recognition and relaying this information to the DHp and CA domains; consequently these domains more variable than the other ones as reflected by the large diversity of detectable signalling molecules (Moglich *et al.* 2009; Parkinson 2010). Besides these domains, HKs usually have transmembrane domains and other domains which specialise and define their function. As mentioned, HK autophosphorylation appears to be dependent on forming homodimers (Ashenberg *et al.* 2011). HAMP domains are likely to be involved in dimerization in a sensory dependent manner (Parkinson 2010). Formation of functional homodimers is common for almost all HKs as there is only a single observation of a physiologically functional heterodimer in cyanobacteria *Pseudomonas aeruginosa* (Goodman *et al.* 2009). Some HKs have an additional

receiver domain fused to their C-terminus; these are called hybrid histidine kinases (also abbreviated HKs) and almost 25% of all bacterial HKs belong to this group (Cock and Whitworth 2007). Hybrid HKs might have originated by fusion of upstream encoded HKs and RRs through the mutation of stop codons in operons (Qian *et al.* 2008). After a signal is perceived and autophosphorylation occurs in hybrid HKs, the high energy phosphate group is delivered from the DHp histidine to the *cis* internal receiver domain. This phosphate is then shuttled by other elements of the TCS in a typically a four-step relay via hybrid HK to HPt (a free DHp domain protein called a histidine phosphotransfer protein) to a classical RR protein, thus the ~P group moves from His to Asp to His to Asp. This multistep phospho-transfer is commonly referred to as a phosphorelay. In eukaryotes, hybrid HKs are found in the majority of systems (Koretke *et al.* 2000) although the real reason for such selective pressure is not known. It has been proposed that the spatial arrangement within the hybrid HK enforces the specificity of phosphotransfer avoiding crosstalk with other TCS pathways (Wegener-Feldbrugge and Sogaard-Andersen 2009). In support to this claim, it has been proven that HKs missing their receiver domains can phosphorylate non-cognate RRs even better than their own internal response regulator domain (Biondi *et al.* 2006; Wegener-Feldbrugge and Sogaard-Andersen 2009). Next crucial element of two-component system signalling is RRs, as up until now, HPt proteins have only been shown to be ~P shuttles and add little specificity to TCSs with the exception of YPD1, which has been shown to stabilize the phosphoryl~RR form of SSK1 (Janiak-Spens *et al.* 2000) and the *Arabidopsis* protein AHP6 which cannot receive a TCS phosphate. AHP6 has been shown regulate developmental and hormonal processes, presumably at the level of protein-protein interaction within the TCS network (Mahonen *et al.* 2006; Moreira *et al.* 2013). All RRs have an evolutionary well conserved receiver domain which pulls the phosphate group onto its Asp residue thereby causing conformal changes to the protein which activate or even inhibit it initiating an output response (Gao *et al.* 2007; Capra and Laub 2012). Numerous RRs possess DNA-binding output domains (Galperin 2006) whereby phosphorylation of the conserved Asp promotes also dimerization of receiver domains favouring the DNA binding and direct control of target gene transcription (Capra and Laub 2012). Other domains frequent within RRs are diguanylate cyclases and methyltransferases domains (Koretke *et al.* 2000). In bacteria, RRs are signal integrators as these organisms usually have, if not an equal number, more HKs than RRs (Koretke *et al.* 2000; Capra and Laub 2012). Free RRs are therefore the last direct step of TCS signalling pathways and they are the final factor responsible ascribed for causing TCS-dependent physiological changes (Capra and Laub 2012). The diversity of in bacterial RRs is a direct consequence of gene duplications, lateral gene transfer events and point mutations (Rabin and Stewart 1993). Divergent evolution of the RRs has enabled them to specifically recognize promoters and control different genes (Price *et al.* 2008). Fifty percent of all known RRs form homodimers upon phosphorylation (Gao and Stock 2010) and there are subsets of interfacial residues that enforce homo-dimerisation and prevent hetero-dimerisation which lie within the β4-α4-β5-α5 region of the conserved RR domain (Weigt *et al.* 2009; Szurmant and Hoch 2010; Capra and Laub 2012).

In both *Archea* and eukaryotes, TCS independently originated from bacteria by lateral gene transfer (Koretke *et al.* 2000; Kim and Forst 2001). In plants, TCS plays important roles in developmental processes (Ren *et al.* 2009; El-Showk *et al.* 2013) and most likely they obtained their TCS from chloroplasts after integration of chloroplast genes into their nuclear genome (Martin *et al.* 2002). As mentinoed higher eukaryotes and metazoans do not possess TCS (Hwang *et al.* 2002; Schaller *et al.* 2011) and instead, TCS (histidine/aspartal phosphorylation) has been replaced by serine/threonine/tyrosine phosphorylation perhaps due to the intrinsic liability of phosphoryl groups on Asp compared to its stability on serine, threonine or tyrosine (Capra and Laub 2012). Thus the idea is that eukaryotes as "compartmented" organisms need more stable and longer lived outputs for signal conduction from the cell membrane to the nucleus. Supporting this claim would be the direct modulation of activity MAPK kinase signalling pathway in *Saccharomyces cerevisiae* by the SLN1-YDP1-SSK1 TCS pathway (Posas *et al.* 1996). On the other hand this is not entirely the case in plants. Here TCS is directly controlling expression of many target genes as in classical TCSs (Hwang *et al.* 2012; El-Showk *et al.* 2013). One of the most described examples in plants is cytokinin signalling where the TCS plays a crucial role, and B-Type RRs directly modulate gene activity of A-Type *RRs* and other genes (Shi and Rashotte 2012; El-Showk *et al.* 2013). The shuttling of the phosphoryl group from hybrid HKs in the plasma membrane and endoplasmic reticulum by HPts to RRs in the nucleus (Shi and Rashotte 2012) could have been selected for in plants because it is strongly believed that the histidyl~P residue is more stable than an aspartyl~P residue (Koretke *et al.* 2000; Capra and Laub 2012).

1.2 References for General Introduction

Ashenberg, O, Rozen-Gagnon, K, *et al.* (2011). "Determinants of homodimerization specificity in histidine kinases." J Mol Biol **413**(1): 222-235.

Biondi, EG, Reisinger, SJ, *et al.* (2006). "Regulation of the bacterial cell cycle by an integrated genetic circuit." Nature **444**(7121): 899-904.

Capra, EJ and Laub, MT (2012). "Evolution of two-component signal transduction systems." Annu Rev Microbiol **66**: 325-347.

Cock, PJ and Whitworth, DE (2007). "Evolution of prokaryotic two-component system signaling pathways: Gene fusions and fissions." Mol Biol Evol **24**(11): 2355-2357.

Dutta, R, Qin, L, *et al.* (1999). "Histidine kinases: Diversity of domain organization." Mol Microbiol **34**(4): 633-640.

El-Showk, S, Ruonala, R, *et al.* (2013). "Crossing paths: Cytokinin signalling and crosstalk." Development **140**(7): 1373-1383.

Galperin, MY (2006). "Structural classification of bacterial response regulators: Diversity of output domains and domain combinations." J Bacteriol **188**(12): 4169-4182.

Galperin, MY, Nikolskaya, AN, *et al.* (2001). "Novel domains of the prokaryotic two-component signal transduction systems." FEMS Microbiol Lett **203**(1): 11-21.

Gao, R, Mack, TR, *et al.* (2007). "Bacterial response regulators: Versatile regulatory strategies from common domains." Trends Biochem Sci **32**(5): 225-234.

Gao, R and Stock, AM (2010). "Molecular strategies for phosphorylation-mediated regulation of response regulator activity." Curr Opin Microbiol **13**(2): 160-167.

Goodman, AL, Merighi, M, *et al.* (2009). "Direct interaction between sensor kinase proteins mediates acute and chronic disease phenotypes in a bacterial pathogen." Genes Dev **23**(2): 249-259.

Hwang, I, Chen, HC, *et al.* (2002). "Two-component signal transduction pathways in arabidopsis." Plant Physiol **129**(2): 500-515.

Hwang, I, Sheen, J, *et al.* (2012). "Cytokinin signaling networks." Annu Rev Plant Biol **63**: 353-380.

Janiak-Spens, F, Sparling, DP, *et al.* (2000). "Novel role for an hpt domain in stabilizing the phosphorylated state of a response regulator domain." J Bacteriol **182**(23): 6673-6678.

Kim, D and Forst, S (2001). "Genomic analysis of the histidine kinase family in bacteria and archaea." Microbiology **147**(Pt 5): 1197-1212.

Koretke, KK, Lupas, AN, *et al.* (2000). "Evolution of two-component signal transduction." Mol Biol Evol **17**(12): 1956-1970.

Mahonen, AP, Bishopp, A, *et al.* (2006). "Cytokinin signaling and its inhibitor ahp6 regulate cell fate during vascular development." Science **311**(5757): 94-98.

Martin, W, Rujan, T, *et al.* (2002). "Evolutionary analysis of arabidopsis, cyanobacterial, and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus." Proc Natl Acad Sci U S A **99**(19): 12246-12251.

Moglich, A, Ayers, RA, *et al.* (2009). "Structure and signaling mechanism of per-arnt-sim domains." Structure **17**(10): 1282-1294.

Moreira, S, Bishopp, A, *et al.* (2013). "Ahp6 inhibits cytokinin signaling to regulate the orientation of pericycle cell division during lateral root initiation." PLoS One **8**(2): e56370.

Parkinson, JS (2010). "Signaling mechanisms of hamp domains in chemoreceptors and sensor kinases." Annu Rev Microbiol **64**: 101-122.

Posas, F, Wurgler-Murphy, SM, *et al.* (1996). "Yeast hog1 map kinase cascade is regulated by a multistep phosphorelay mechanism in the sln1-ypd1-ssk1 "two-component" osmosensor." Cell **86**(6): 865-875.

Price, MN, Dehal, PS, *et al.* (2008). "Horizontal gene transfer and the evolution of transcriptional regulation in escherichia coli." Genome Biol **9**(1): R4.

Qian, W, Han, ZJ, *et al.* (2008). "Two-component signal transduction systems of xanthomonas spp.: A lesson from genomics." Mol Plant Microbe Interact **21**(2): 151-161.

Rabin, RS and Stewart, V (1993). "Dual response regulators (narl and narp) interact with dual sensors (narx and narq) to control nitrate- and nitrite-regulated gene expression in escherichia coli k-12." J Bacteriol **175**(11): 3259-3268.

Ren, B, Liang, Y*, et al.* (2009). "Genome-wide comparative analysis of type-a arabidopsis response regulator genes by overexpression studies reveals their diverse roles and regulatory mechanisms in cytokinin signaling." Cell Res **19**(10): 1178-1190.

Schaller, GE, Shiu, SH*, et al.* (2011). "Two-component systems and their co-option for eukaryotic signal transduction." Curr Biol **21**(9): R320-330.

Shi, X and Rashotte, AM (2012). "Advances in upstream players of cytokinin phosphorelay: Receptors and histidine phosphotransfer proteins." Plant Cell Rep **31**(5): 789-799.

Szurmant, H and Hoch, JA (2010). "Interaction fidelity in two-component signaling." Curr Opin Microbiol **13**(2): 190-197.

Wegener-Feldbrugge, S and Sogaard-Andersen, L (2009). "The atypical hybrid histidine protein kinase rodk in myxococcus xanthus: Spatial proximity supersedes kinetic preference in phosphotransfer reactions." J Bacteriol **191**(6): 1765-1776.

Weigt, M, White, RA*, et al.* (2009). "Identification of direct residue contacts in protein-protein interaction by message passing." Proc Natl Acad Sci U S A **106**(1): 67-72.

2 Aim of this work

Besides its functions in ethylene signal transduction, cytokinin and H_2O_2 signalling *etc*., the two-component-signalling system seems to play very significant roles in crosstalk and fine-tuning between distinct signalling pathways. These additional functions of the TCS might be essential for the plant's fitness within the natural environment and also for their general survival. Based on these observations, this thesis intends to contribute to a better understanding of these special features of TCS and describe them. The specific aims of this thesis are:

- To investigate and determine the functional (physiological) role of ARR2 interactions with biotic stress-related members of MAPK cascade, obtaining new data by expanding the comprehensive ARRs/MAPKs module interaction map and determine of the roles of *ARR1* and *ARR2* in response to pathogen attack and different elements that cause biotic stress in plants (see Chapter 1).

- Characterisation of a novel flowering time phenotype in the *ARR1* and *ARR2* lacking mutants under non-inductive short day conditions discovered during this dissertation by resolving which floral pathway(s), their mechanism and nature of interaction, these two TCS elements are involved in (see Chapter 2).

3 Materials and Methods

3.1 Chemicals

All chemicals were ordered from Biorad (München), Fluka (Buchs, CH), Merck (Darmstadt), Carl Roth (Karlsruhe), Duchefa (Haarlem, NL) or Sigma (Steinheim). Organic solvents were delivered by Brenntag Chemiepartner GmbH NL (Plochingen) and Merck. BASTA pesticide was ordered from Bayer Crop Science. The ingredients used for culture media were ordered from Invitrogen (Carlsbad, USA), Merck, Sigma and Duchefa (Haarlem, NL). Restriction enzymes, ligase and DNA modification enzymes used for nucleic acids studies were ordered from Invitrogen, Stratagene (La Jolla, USA), New England Biolabs (Beverly, USA), Promega (Mannheim) and Amersham Pharmacia Biotech. Oligonucleotides were received from Eurofins MWG Operon (Ebersberg) and antibodies from the companies Sigma-Aldrich (Taufkirchen) and New England Biolabs (Beverly, USA). The synthetically generated fg22, elf18 peptides, PEN and Xac extracts were a kind gift from the Laboratory of Georg Felix.

3.1.1 Media

The different media used are presented in the following table. For media preparation deionized water was used and the media was sterilized by autoclaving for 20 minutes at 121°C. Bacto-agar 15g/L was used for solid media preparation, (BD) or 8g/L Select-Agar for MS plates (Sigma-Aldrich). Where it was needed, filter sterilized antibiotics were added to the sterilized medium at appropriate final concentrations as listed in Table 3.1

Table 3.1 List with recipes of common used media in the thesis

Medium	Ingredients per 1 liter	Species
LB	10 g Bacto-Tryptone, 5 g NaCl, 5 g Yeast extract (YE)	*Escherichia coli*
Kings's B	20 g glycerol, 40 g Proteose Pepton 3, after autoclaving addition of 0.1 % (v/v) $MgSO_4$ and KH_2PO_4	*Pseudomonas syringae*
½ MS	2.2 g MS (Duchefa), pH 5.7 (KOH)	*Arabidopsis thaliana*
YPD	20g Bactopeptone (BD #211677), 20g Glucose (monohydrate), 10g Yeast extract (BD #212750), 1000ml ddH_2O	*Saccharomyces cerevisiae*
CSM	20g Glucose (monohydrate), 6,6g Yeast nitrogen base (BD #291940), 0,64g CSM-(L-W, or L-W-A, Qbiogene #4520-012), 1000ml ddH_2O	*Saccharomyces cerevisiae*
Z buffer	10,68g Na_2HPO_4, 5,5g NaH_2PO_4, 0,75g KCl, 246mg $MgSO_4$, 1000ml ddH_2O, adjust PH to 7,0	*Saccharomyces cerevisiae*

3.1.2 Antibiotics

Media were supplemented when required with antibiotics at the final concentrations listed in the following table:

Table 3.2 List of used antibiotics and applied concentrations

Antibiotics	Concentration (µg/µl)	Solvent
Carbenicillin	100	Water
Kanamycin	50	Water
Rifampicin	50	Methanol
Spectinomycin	100	Water
Tetracyclin	50	Ethanol

3.2 Vectors and Primers

All the vectors used for this thesis are listed directly within each method text section. Primers were synthesized by Eurofins MWG Operon. Primers stocks were kept at a 100 µM concentration diluted in nuclease-free water and stored at -20°C.

Table 3.3 List of primers (primer sequences) used for genotyping and cloning

Primers used for genotyping		
Name of the mutant line	**Sequence (5´-> 3´)**	**Source**
flc-3	AAA ATA TCT GGC CCG ACG AAG	Johanne Lempe, PhD Thesis, University of Tübingen, 2007
	CGA CGA GAA GAG CGA CGG ATG	
arr2-4	GAACGGGAGGAGCTCGAG	Laboratory Harter, ZMBP, MV
	GACCTGGATATTATCGATGGAGTATCC	
arr1-4	GAAGAACAAACATGGATTCGATATAGTA	Laboratory Harter, ZMBP, MV
	CCGTCATAAACGAGTTGTTAAGATTG	
tDNA(SALK)	TGGTTCACGTAGTGGGCCATCG	Laboratory Harter, ZMBP, MV
co-9	CAACTCTATCTCCCCCGTAGC	Balasubramanian et al., PloS Gen. 2006
	GATGCTCAAGTTCACTCTGCC	
soc1-2	GGATCCATGGTGAGGGGCAAAACTC	Yoo et al., Plant Phys 2005
	CTGAAACATCTGATCAAAAGCTG	
	TTGGGTTCACGTAGTGGGCCATCG	
flm-3	GATGCGGTTTTTGGTGTTATG	Laboratory Harter, ZMBP, MV
	GCCTAGAATATGGCCTTTATCG	
Primers used for cloning		
Truncation size/position	**Sequence (5´-> 3´)**	**Source**
$ARR2^{1-300}$	ATGGTAAATCCGGGTCACGGAAG	Laboratory Harter, ZMBP, MV
	GATCAAATCCATTCAACGAAGA	
$ARR2^{300-664}$	TCTTCGTTGAATGGATTTGATC	Laboratory Harter, ZMBP, MV
	TCAGACCTGGATATTATCGATG	
$ARR2^{1-145}$	ATGGTAAATCCGGGTCACGGAAG	Laboratory Harter, ZMBP, MV
	CCACTCGTTACGCTTCTTCCG	
$ARR2^{145-664}$	CGGAAGAAGCGTAACGAGTGG	Laboratory Harter, ZMBP, MV
	TCAGACCTGGATATTATCGATG	
$ARR2^{51-81}$	CTCTACAGAGTAACTAAATGTA	Laboratory Harter, ZMBP, MV
	AACATCACTAATGACAATATC	
$ARR2^{215-260}$	AAGAAACCACGCGTGGTTTGGTC	Laboratory Harter, ZMBP, MV
	TACGTTTTCTCGCGTTAGCCCG	

Table 3.4 List of primers (primer sequences) used for RTq-PCR

Primers used for RTq-PCR				
Gene Name	**Probe**	**Sequence (5´-> 3´)**	**Size (bp)**	**Source**
SOC1	#69	CACAAACCCTTTATCCTCGAA	103	Laboratory Harter, ZMBP, MV
		TTGCCCCTCACCATATCTTC		
CO	#77	AACAATGACCGATCCAGAGAA	77	Laboratory Harter, ZMBP, MV
		CCTCCTTGGCATCCTTATCA		
FD	SYBR	GGCAGAAAATGCAAGACTCA	74	Laboratory Harter, ZMBP, MV
		TCTTTTTGGGTTGCTGAATTG		
GA4	#127	TGCCTTCCAAATCTCAAACC	67	Laboratory Harter, ZMBP, MV
		ACCGGTGAGAAACTCAATGTC		
GA5	#45	CATGGGTTTCAGCCATTTG	121	Laboratory Harter, ZMBP, MV
		CTCTAAAGTAGTCCCGTTTTACGC		
LFY	#69	TTGATGCTCTCTCCCAAGAAG	113	Laboratory Harter, ZMBP, MV
		TTGACCTGCGTCCCAGTAA		
SHY2	#9	TGATCCTTAGTCTCTTGCACGTA	77	Laboratory Harter, ZMBP, MV
		CAAAGATGGTGATTGGATGCT		
ARR7	#68	TCATCTGAGAACATCTTACCTCGT	77	Laboratory Harter, ZMBP, MV
		TTCACCGGTTTCAACAAGAAT		
WUS	#33	AACCAAGACCATCATCTCTATCATC	86	Laboratory Harter, ZMBP, MV
		TCAGTACCTGAGCTTGCATGA		
AP1	#68	AAAACAGCATGCTTTCTAAACAGA	89	Laboratory Harter, ZMBP, MV
		GTGGCCTTGGTTCTGCTG		
FDP	#112	AACTTGAGCTTGAAATTGCTCAC	87	Laboratory Harter, ZMBP, MV
		GAGTTGCTTCGGCTATTTTCA		
TFL	#140	CCTGCACTGGATCGTTACAA	79	Laboratory Harter, ZMBP, MV
		TGGCAATTCATAGCTCACCA		
TSF	#138	TGGAGGAGACGACTTCAGAAA	67	Laboratory Harter, ZMBP, MV
		GCTTGGACTCGGCACATC		
FLM	#65	CGGACAGAGCAGTCTCAAGTT	108	Laboratory Harter, ZMBP, MV
		TGAAGAACCAAATGTCGATAATGT		
SVP	#67	TGACTGCAAGTTATGCCTCTCT	68	Laboratory Harter, ZMBP, MV
		CCGGAAAACTGTTCGAGTTC		
FT	#22	TCAAAACAAGCCAAGAGTTGAG	77	Laboratory Harter, ZMBP, MV
		CATCTGGATCCACCATAACCA		
FLC	#65	GGAGAGGGCAGTCTCAAGGT	103	Laboratory Harter, ZMBP, MV
		GCTACTTGAACTTGTGGATAGCAA		
ARR1	SYBR	GCGCACTTCTTAAGCAGGAA	66	Laboratory Harter, ZMBP, MV
		TGGAGTATGCGTCAAAGTCG		
ARR2	SYBR	CGTTGATGATGATCCAACTTGT	94	Laboratory Harter, ZMBP, MV
		TCCGAAGCAGAGACAATGC		
ARR4	SYBR	GTCATCGAGAGATTGCTTCGT	66	Laboratory Harter, ZMBP, MV
		ACGCCATCCACTATCTACCG		
ARR5	SYBR	TCAGAGAACATCTTGCCTCGT	94	Laboratory Harter, ZMBP, MV
		ATTTCACAGGCTTCAATAAGAAATC		
PR1	#135	TGATCCTCGTGGGAATTATGT	76	Laboratory Harter, ZMBP, MV
		TGCATGATCACATCATTACTTCAT		
PDF1.2	#139	GTTCTCTTTGCTGCTTTCGAC	87	Laboratory Harter, ZMBP, MV
		GCAAACCCCTGACCATGT		
EF-1- α	#5	TCATGGATCAAGCGGTGA	63	Laboratory Harter, ZMBP, MV
		CGCAACCAAACCTTCCATA		

3.3 Bacterial strains

3.3.1 *Escherichia coli* strain DH5α

The *E. coli* strain DH5α [F-(Φ80*lac*ZΔM15) Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1] was used for cloning of the different constructs. The *E. coli* strain DB3.1 [F- gyrA462 endA1 glnV44 Δ(sr1-recA) mcrB mrr hsdS20(r_B^-, m_B^-) ara14 galK2 lacY1 proA2 rpsL20(Sm^r) xyl^5 Δleu mtl1] was used for the cloning and propagation of Gateway™ vectors carrying the *ccdb* gene.

3.3.2 *Agrobacterium* strain

The *Agrobacterium tumefaciens* strain GV3101 (T-DNA^- vir^+ rif^r) with helper plasmid pMP90 (gen^r) or pMP90RK (gen^r, kan^r) was used.

3.4 Organisms

3.4.1 Organisms used for pathogen assays

Two biotrophs *Pseudomonas syringae* pv. tomato Pto DC3000 (marked with Rif^r resistance) and the fungus *Peronospora parasitica* were used. Two necrothrophic fungi *Alternaria brasisicola* and *Botrytis cinerea* were used.

3.4.2 Plant lines

For all experiments Columbia-0 (Col-0) ecotype of *Arabidopsis thaliana* was used, and all transgenic lines were generated in this ecotype. For some experiments *Nicotiana benthamiana* was also used. The T-DNA insertion mutant lines used in this work include *arr1-4* (SALK 042196), *arr2-4 (SALK 016143), flm-3 (*SALK 141971), *co* (SAIL_24_H04), *soc1-2* (Lee *et al.* 2000; Yoo *et al.* 2005) and the deletion mutant *flc-3* (Michaels and Amasino 1999). The double mutant *arr1-4 arr2-4* was crossed and isolated by Dr. Katharina Caesar. Other mutants listed in the work were made by crossing; the lines obtained are: *flm-3 arr2-4*, *co arr2-4*, *soc1-2 arr2-4*, *flm-3 arr1-4 arr2-4, flc-3 arr1-4 arr2-4*. All of the crossed lines were genotyped by PCR for known insertion markers and characterised by checking mRNA expression. Single mutants *flc-3*, *flm*-3, *soc1-2* and *co* were kindly donated by Dr. Markus Schmid and Dr. Rebecca Schwab (Max-Planck Institute for Developmental Biology, Tübingen). *ARR2* and *ARR2D80N* loss-of-function genes were (both in pDONR207 Gateway vector; previously cloned in the laboratory of Prof. Dr. Klaus Harter) and pJL-Blue Gateway entry plasmid (kindly donated by Dr. Rebecca Schwab) were recombined into the respective pGREEN-IIS Gateway vectors pFK-101 (BAR_FD_rfA*) and pHW-059 (BAR_SUC2_rfA+) described by (Mathieu *et al.* 2007). These constructs were transformed into Col-0 wild-type, *arr1-4*, *arr2-4* or *arr1-4 arr2-4* mutants. Transgenic plants were selected with BASTA (Bayer) at a dilution of 1:1000 (§3.5.6.1).

3.5 Cultivation

3.5.1 Growth of *Escherichia coli*

E.coli strains were cultivated overnight at 37°C either on LB-plates or in liquid LB medium at 230 rpm. Antibiotics were added into the media according to the resistance cassettes the strains were harboring.

3.5.2 Growth of *Pseudomonas syringae*

P. syringae Pto pv. DC3000 strains were grown for 24 to 48 hours at 28°C either on King's B plates or in liquid King's B medium at 180 rpm. For the determination of bacterial growth in infection assays the *Pseudomonas* strain was re-isolated from plant material and plated on LB plates containing cycloheximide in addition to rifamycin.

3.5.3 Growth of *Agrobacterium tumefaciens*

A.tumefaciens strains were cultivated for 48 hours at 28°C on LB-plates or liquid LB medium at 230 rpm. Additional antibiotics were added into the media according to the plasmid-DNA the strains were carrying.

3.5.4 Growth of *Alternaria brassicicola*

The cultivation of *A. brassicicola* and the preparation of the spores for the infection assays were personally performed in the Laboratory of Dr. Birigit Kemmerling exactly as previously described (Kemmerling *et al.* 2007).

3.5.5 Growth of *Peronospora parasitica* and *Botrytis cinerea*

Assays with *Peronospora parasitica* and *Botrytis cinerea* were both performed by our collaboration partners. The *Peronospora parasitica* assay was performed by the research group of Professor Dr. Volker Lipka from University of Göttingen and the B*ortytis cinerea* assay was done in the laboratory of Professor Dr. Jean-Pierre Métraux at the University of Fribourg, Switzerland. *Peronospora parasitica* was grown as previously described by (Jacobs *et al.* 2003) and *Botrytis cinerea* as described by (Ferrari *et al.* 2003).

3.5.6 Growth of *Arabidopsis thaliana* and *Nicotiana benthamiana*

3.5.6.1 Growth conditions for flowering time analysis

For flowering time analysis, plants were first stratified by being kept at 4°C for 2 to 4 days and then transferred to 22°C; this was defined as day 0. *Arabidopsis thaliana* plants were grown on steam-sterilized GS90-soil (Gebr. Patzer GmbH) and for pathogen experiments soil was mixed with Vermiculite. Plants were grown in growth chambers on soil at 22°C under long days (LD) 16 hours light / 8 hours dark or short days (SD) 8 hours light / 16 hours dark (110 mEm^{-2} s^{-1}, 50-60% humidity). Plants used for pathogen experiments were grown only under SDs. Plants complemented under tissue specific promoters were used in T2 generation and therefore they were pre-selected with 1:1000 dilution of BASTA (dilution from the stock, 183 g/L glufosinate; 200 g/L ammonium salt, Bayer CropScience). The soil was directly soaked in the BASTA solution.

3.5.6.2 Growth of Nicotiana benthamiana

Nicotiana benthamiana plants were cultivated in a mixture of soil and sand containing 0.1 % (v/v) Confidor by the ZMBP Greenhouse (13 h light, 11 h darkness).

3.6 Standard molecular biology methods

General protocols were used for PCR, side directed mutagenesis, agarose gel electrophoresis, western blot, restriction digestion, ligation, transformation of bacteria and plasmid isolation (Sambrook and Russell 2001), Techniques done differently are explained directly in the text. Restriction enzymes were used according the manufacturer's protocols (Fermentas and NEB). For the generation of PCR fragments either the *Taq* DNA-Polymerase or the *Pfu* DNA polymerase (cloning purposes; Fermentas) were used. GeneRuler DNA Ladder Mix (Fermentas) was used as size marker for the agarose gel electrophoresis. DNA fragments were extracted out of agarose gels or purified out of PCR reactions by using the Qiagen Gel Extraction Kit and Qiagen PCR Purification Kit (Qiagen).

3.6.1 Yeast-two-hybrid

Yeast two-hybrid experiments were performed using the Matchmaker™System (Clontech). Plasmids were constructed by LR-reaction of corresponding Entry clones and destination vectors pGBKT7-DEST or pGADT7-DEST (Horak *et al.* 2008). Primers for clones produced during this thesis project are listed in the general primer list (§3.2), the rest of the clones were made by Achim Hahn and can be found in the plasmid repositories of Prof. Dr. Klaus Harter. Clones with *ARR18* were kindly provided by Manikandan Veerabagu and clones with *ARR4* are from the lab of Dr. Virtudes Mira-Rodado. Yeast strain PJ69-4A (James *et al.* 1996) was transformed using lithium acetate/SS-DNA/PEG method (James *et al.* 1996; Horak *et al.* 2008). After 3 days of growth on vector selective media (CSM, -L, -W), 6 independent clones were picked, resuspended in ddH_2O and 10µl were dropped on vector-selective media. Subsequently, 10µl of culture were dropped on vector- and interaction-selective media (CSM, -L, -W, -A) and incubated at 28°C. At day 3 the growth of the clones was monitored. In addition, yeasts from selective media were inoculated in selective media (CSM,-L,-W) harvested and analysed by western-blot using α-HA and α-myc antibodies to determine the correct expression of the fusion proteins (Horak *et al.* 2008).

3.6.2 Transient expression in tobacco leaves

Expression of proteins expressed transiently in tobacco leaves was performed as described previously (Marion *et al.* 2008). A single colony of *Agrobacterium tumefaciens* strain GV3101 pMP90 transformed with the desired constructs and was inoculated in 5ml of YEB-Medium (0.5% beef extract, 0.5% sucrose, 0.1% yeast extract, 0.05% $MgSO_4 \cdot 7H_2O$) containing Rif/Gent/ and vector-specific antibiotic at 28°C overnight. In the morning, 1 ml of the pre-culture was taken and re-inoculated into 5 ml of the same Medium. The same was done for *Agrobacterium* strain carrying the p19 RNAi-suppressor protein from tomato bushy stunt virus (Voinnet *et al.* 2003). Each culture was collected in a 15 ml Falcon Tube and centrifuged at 4000 rpm for 20 min. Bacteria pellets were then resuspended in AS-Medium (10 mM $MgCl_2$, 10 mM MES [pH 5.6], 150 µM acetosyringone) to an optical density at 600 nm of

about 0.7-0.8. The resuspended bacteria (two potential interaction partners and p19 strain) were mixed 600□ml each, a 1:1:1 ratio, in a 2ml Eppendorf tube and incubated for 0.5 to 1 hours at 4°C.

The bacterial solution was injected into the entire leaf area through the abaxial sides using a 1 ml syringe; two leaves per plant were inoculated. After inoculation, the plants were kept in a tray with a hood at 25°C. Two days after the bacterial inoculation, the β-estradiol responsive promoters in the *pABind* vectors (Zimmermann and Nentwig 1989) and the N-terminal *mRFP* vector pB7WGR2,0 (Plant Systems Biology, Gent) were induced by application of β-estradiol by brushing a 20 µM β-estradiol (in 0.1% Tween-20) solution onto the abaxial leaf surface. FRET measurements and localization studies were performed 24 to 48 hours after β-estradiol application. Localization studies were performed with confocal laser scanning microscopy using a Leica TCS SP2 confocal microscope (Leica Microsystems GmbH). Microscopy was carried out as previously described (Horak *et al.* 2008). Images were taken with HCX APO LW 20×/0.5 or the HCX PL APO 63×/1.2 W water-immersion objective.

3.6.3 Ethylene accumulation measurements

For ethylene measurements, leaf material of 4 week-old plants were cut into 1 mm thick strips and floated overnight in water. Afterward three leaf stripes (20 mg) were transferred in 6 ml glass vials containing 0.5 ml of an aqueous solution of the elicitor to be tested. The tubes were closed with a rubber septa and ethylene accumulating in the free air space was measured by gas chromatography (GC-14A Shimadzu) after 3h incubation at 170 rpm at room temperature. Following PAMPs and their working concentrations were used: PEN from *Penicilium schizogonium* 1 µL/500 µL, *Xanthomonas axonopodis citri* 3 µL/500 µL, flg22 (0,1 nM, 1 nM, 10 nM, 100 nM, 1 µM), elf 18(0,1 nM, 1 nM, 10 nM, 100 nM, 1 µM).

3.6.4 ROS (Reactive Oxygen Species) measurements

Leaf discs were excised from 6 week-old *Arabidopsis* plants and incubated in water overnight. The following day, the discs were transferred to a solution of 20 µM luminol L-012 (Wako Pure Chemical Industries) and 10 µg/mL (w/v) horseradish peroxidase in a 96-well-plate and treated with 100 nM flg22, 100 nM elf18, 1 µM chitin octamers or 1 mg/mL chitin (crab). The plates were analysed for a period of at least 30 min using a multiplate reader Centro LB 900 (Berthold Technologies). For each data point at least 9 replicates were measured.

3.6.5 Statistical analysis

Statistical analysis was performed using Microsoft Office Excel or JMP (SAS). The data represent the average of replicates with plus or minus standard error of the mean (SE). The significance of the differences was calculated using Student's t-test, ANOVA or Fisher's Least Significant Difference (LSD) test; which test was used is explicitly mentioned in the text, figures and tables.

3.6.6 ELM software for *in silico* predictions

ELM software (http://elm.eu.org) was used for MKK docking site identification in ARR2.

3.6.7 Cloning and site-directed mutagenesis

All the clones used in our experiments were constructed using Gateway™ technology (Invitrogen™ | Life Technologies). The Entry clones were obtained *via* BP-reaction in pDONR207. cDNA preparations derived from *Arabidopsis* leaves were used as template to clone *ARR2* (*AT4G16110*). For the generation of truncated ARR2 versions, $ARR2^{1-300,}$ $ARR2^{300-664,}$ $ARR2^{1-165}$, $ARR2^{145-664}$, $ARR2^{165-664}$ and $ARR2^{1-145}$, primers were made corresponding to the *ARR2* cDNA and the fragments were amplified by PCR using the *ARR2* Entry clone (see primer table §3.2). Site-directed mutagenesis of *ARR2* was carried out on the *ARR2* Entry clones using QuikChange® Site-Directed Mutagenesis Kit (Stratagene) and the *D80E*, *D80N ARR2* mutants were already present at the Laboratory of Klaus Harter at the time when the experiments were performed. The binary constructs for FRET-FLIM measurements (*pABind* vectors) under the control of the β-estradiol inducible promoter and for stable *Arabidopsis* transformations under the control of the *FD* and *SUC2* promoters were obtained *via* LR-reactions. LR-reactions were also done for yeast-two hybrid system destination vectors pGADT7 and pGBKT7.

3.6.8 Quantitative RT-PCR (RT-qPCR)

Total RNA from corresponding material (leaves, shoot apical meristem or whole plant) was isolated using the "Universal RNA Purification Kit" (Roboklon, Germany) including a DNA digestion step to ensure the absence of genomic DNA contamination/presence. The cDNA was synthetized using oligo-dT or random hexamer primers with H-Minus Reverse Transcriptase (Fermentas). qPCR primer were tested for doubling time by diluting 1:2 and ensuring that there was exactly a loss of 1 CT; primer efficiency was thereafter assumed to be 100%. At least two PCR reactions (in triplicate repeats each) using two independent cDNA synthesis were performed for every treatment. The amplification of cDNA was performed with PerfeCta qPCR SuperMix or PerfeCta SYBR Green Super Mix (Quanta Biosciences, distributed by VWR) using, when necessary, the corresponding Probe (Universal ProbeLibrary Single Probes, Roche) accordingly to manufacturer. The PCR reactions were ran in the Bio-Rad CFX384 Real Time PCR system (Bio-Rad). The used primers are listed in the §3.2. Expression levels of each gene were normalized according to the expression of the following housekeeping genes: *EF-1-α* (*AT5G60390*). The CFX Manager software (Version 1.1; Bio-Rad) has been used for the quantification of relative expression levels except for §4.2.3.4 for which the CT values were exported and ΔΔCT was manually processed in Excel to ensure proper normalisation.

3.6.9 ONPG assay

Before the Day One, 3 to 4 transformed yeast colonies were pooled and inoculated and incubated overnight at 28°C while shaking in 0.5ml of CSM-L/W liquid medium at 180 rpm.

Day 1: the tubes were taken from the shaker and 3 ml of YPD liquid medium was added in each one and put back to the shaker for an additional 3 hours. After that, the optical density at OD_{600} was measured. The tubes were then centrifuged for one minute at 11000 rpm and

the pellet was re-suspended in 1 ml of Z Buffer (the same procedure was repeated three times). The tubes were frozen in liquid nitrogen and stored at -80°C.

Day 2: Frozen tubes were thawed in a 37°C water bath and then returned to liquid nitrogen; this cycle freezing/thawing was repeated at least 4 times. Blank control (with all following solutions) was set and OD_{600} was measured. Immediately after adding of 500 µl of Z buffer-ME (38.6mM ß-Mercaptoethanol) and 160 µl of Z buffer/ONPG (13,3mM ß-Mercaptoethanol) the timer was started and the tubes were incubated at 30°C until yellow colouring appeared. The reaction was stopped by the addition of 400 µl 1M Na_2CO_3. The tubes were centrifuged afterwards and the supernatant was transferred to another tube. The optical density was measured at OD_{420}. Galactosidase units were calculated as follows:

$$\text{Galactosidase units} = {}^{1000 \times OD_{420}}/_{t \times V \times OD_{600}}$$

with t being elapsed time in min, V is the volume of the yeast culture used for the assay, OD_{420} and OD_{600} the optical density of the yeast cultures used for the assay.

3.6.10 MPK assay using anti-phospho antibodies

Anti-phospho antibodies p44/42 (Cell Signaling Technology, NEB #9101) were used as they are known to specifically detect phosphorylated MPK3, MPK4 and MPK6 in *Arabidopsis thaliana*. 5 week-old plants grown under short day conditions were used. Total protein amount was extracted from 50-100 mg of leaf material. For protein extraction 50mM Tris/HCl [pH 7.5], 5mM EDTA pH 8 and 2mM DTT was used. Protease inhibitor cocktail tablets (Roche) were used. Protein concentration was determined with Bradford Reagent (Biorad-System) where 10µl of protein extract was added to 990µl Bradford-Solution (Bradford Reagent diluted 1: 5 in H_2O), exposed for 5 min at RT; and then the optical density (OD) was measured at 595 nm. To estimate protein concentration the following formula was used:

$$\text{Protein concentration } \left[\frac{\text{mg}}{\text{ml}}\right] = {}^{OD_{595}}/_{(0{,}0283 \times vol.protein\ extract\ used)}$$

3.6.11 Stomata measurement assays

Leaves from 5 week-old *Arabidopsis thaliana* plants were floated for 2 h under continuous illumination (120–150 µE m^{-2} s^{-1}) in MES/KCl buffer (5mM KCl/10mM MES/50µM $CaCl_2$, pH 6,15) as previously described by (Mira-Rodado *et al.* 2012). Once the stomata were fully open, leaves were treated with either 1µM t-zeatin or 10µM ABA for further 2 h. The leaves were subsequently homogenized individually in a Waring blender for 30 sec and the epidermal fragments collected on a 100 µm nylon mesh (SpectraMesh, BDH-Merck, UK) and placed on a microscope slide and covered with a coverslip. Stomata apertures from epidermal fragments were then measured using a calibrated light microscope attached to an imaging system (Leica QWin software, Leica, UK). Lens 20X-0.75 magnification was used plus. Leaves were collected from plants also treated with *Pseudomonas syringae* DC3000 3 days after infection and directly homogenized without being exposed to additional light or being put in water. To

calculate stomata aperture, relative pixel distance values were measured in ImageJ software. For statistical analyses ANOVA was used.

3.6.12 Day length shifting experiments

This method was used for the synchronized flowering experiments. Plants were first grown at 8h light/16h dark (short days) at 23°C for 30 days and then shifted to 16h light/8h dark conditions (long days), (Maizel and Weigel 2004; Wahl *et al.* 2013). Apical centres (meristems) were harvested after removal of all visible leaves at day 0 and 5 (counting from the shifting day).

3.6.13 Generation of stable *Arabidopsis thaliana* transgenic lines

Stable transgenic plants were generated by *Agrobacterium tumefaciens*-mediated transformation using the floral-dip method (Clough and Bent 1998). For all transformations *Agrobacteria* GV3101::pMP90 or GV3101::pMP90RK strain were used. The RK strain possesses the RK2 replicase and *trf* gene required for the replication of RK plasmids. The *Agrobacterium* RK strain was co-transformed with pSoup vector which provides replication functions in trans for pGREEN-IIS destination vectors into which the different promoters used in this study (*FD*, *SUC2*) which had been cloned in front of a modified Gateway recombination cassette. Transgenic plants were selected with BASTA (Bayer) at a dilution of 1:1000.

3.6.14 Tissue fixation, embedding and sectioning of *Arabidopsis thaliana* apical meristem

3.6.14.1 Embedding

Day 1: Material was directly harvested into fixative FAA (Formaldehyde: Acetic Acid) in a little glass beaker. Fixative: FAA (Formaldehyde: Acetic Acid):

50%	Ethanol
5%	Glacial Acetic Acid
3.7%	Formaldehyde
41.3%	Water

Vacuum was applied and released (soft up and down with the vacuum) for 1 to 4 hours until the material became translucent and sank to the bottom. The FAA was afterwards changed and left in a cold-room overnight. FAA was replaced with 70% Ethanol; this step was repeated once to be sure all FAA is gone, after that material can be stored in falcon tubes for a long time.

Day 2: These steps were done at 4°C in a cold-room. 70% Ethanol was replaced with 85% Ethanol for 60 min. After one hour previous solution was replaced with 96% ethanol with Eosin and it was left overnight until a light pink colour emerged and tissue could be visualized.

Day3: These steps were done at room temperature. The previous solution was replaced by 96% ethanol with Eosin for one hour, and then two times replaced by the same solution for one hour each time. After that the tissue was treated with 25% Histoclear with75% Ethanol for 30 minutes and after each thirty minutes the percentage of Histoclear was increased by

25% where the percentage was decreased at the same amount until the percentage of Histoclear reached 100%. The tissue was further treated with the 100% Histoclear twice for 1 hour each time. After that paraplast chips were added 25% (v/v) of the solution and so left overnight at room temperature.

Day4: The tubes were placed at 42°C until the chips completely melted and one more ¼ volume of paraplast chips was constantly added until they completely melted. The tubes were afterwards moved to 60°C for several hours and parallel in another tube wax was melted overnight at 60°C to prepare for the next day.

Day5: Two wax changes approximately ¼ volume separated by several hours, were performed. The same procedure is for days 6 and 7.

Day8: The tissue was placed in sectioning moulds and stored at 4°C. The tissue was then ready for sectioning.

3.6.14.2 Sectioning

The sections made were 8µm thick. Slides were ProbeOne Plus from Fisher Biotechnology; they were pre-cleaned and charged. Moulds were trimmed in order to get as close to the tissue as possible. The ribbons were placed in 40°C water bath to remove compressions, and then they were fished out with a slide. They slides were kept on a heating plate at 42°C overnight. Thereafter, slides can be stored in a box at 4°C for a long time. Sections were scanned for clear visibility of two big air vacuoles; always the same plane was used for comparative measurements and statistics. Images were captured using a Leica DM-IRB microscope with a 20x lens.

3.6.15 Pathogen Assays with *Pseudomonas syringae* DC3000

Bacteria were inoculated from an LB plate into 50 ml LB medium with the corresponding antibiotics (rifampicin) and incubated over night at 28°C and shaken at 180 rpm in a shaker. The cells were harvested in the next morning by centrifugation in 50-ml falcon tubes at 3500 rpm at 4°C for 10 min. The pellet was resuspended in 10 mM $MgCl_2$ and the OD_{600} was set to OD_{600}=0.2 which refers to approximate concentration of 10^8 cfu/ml. The cells were afterwards diluted up to density $1x10^4$ cfu/ml and used likewise for the assays. Bacteria were inoculated on two leaves per 5-week-old plant by infiltration with a needless syringe in the middle of a leaf apoplast half (two leaves per plant) ensuring that an area was covered that was always bigger than the cork borer used for extracting leaf discs. The infected leaves were marked for easy identification.

The leaves were harvested at the time points 0 h and 1, 2 and 4 days past infection. For each time point, five plants and two leaves from each plant per line were used. One disc was made from each leaf with the cork borer. The leaf discs were surface was sterilised by washing them in 70% (v/v) ethanol first for about 1 minute and then 1 minute in water. Afterwards the leaf discs were placed into Eppendorf tubes containing 100µl 10mM $MgCl_2$. Immediately after placing the leaf in the Eppendorf tube, it was homogenized with the sterile pistil and

additional 100µl 10mM $MgCl_2$ was added on the pistil in order to wash out the remains of the leaf discs. The homogenised material was plated on LB-plates with rifampicin and 50µg/ml cycloheximide (it prevents growth of eukaryotes like fungi). Extracts were serially diluted 5 times at 10-fold per dilution and dilutions anticipated to yield evaluable data were scored by plating out 10 µl (see Table 3.3). At least two technical replicates per sample were evaluated.

For those experiments involving a cytokinin treatment, trans-zeatin in a concentration of 1µM was used. Cytokinin was given directly to the $1x10^4$ cfu/ml bacterial dilution before infiltration. Three days before the experiment was performed plants were also treated with cytokinin by mixing it with water to the working concentration and applied by watering.

Table 3.5 Predicted evaluable dilutions for PSt DC3000 in 10µl volume

Days/Dilutions	Undiluted	$1:10^1$	1.10^2	$1:10^3$	$1:10^4$	$1:10^5$
0h	*	*				
1st day	*	*	*			
2nd day		*	*	*	*	
4th day			*	*	*	*

3.6.16 Infection with *Alternaria brassicicola*

Alternaria brassicicola spores used for infection assays were obtained as previously published (Thomma *et al.* 1999). Leaves of 5 week-old *Arabidopsis* plants were drop-inoculated with two 5 µl droplets of spore solution ($5*10^5$ Spores/ml). Two leaves per plant and a minimum of 20 plants per line were infected. Plants of different lines were randomly distributed in the tray and incubated at 100% relative humidity. Fungal infection was scored using the following table (Table 3.4). The Disease Index was defined as previously published (see below and (Kemmerling *et al.* 2007)). For experiments with cytokinin, *trans*-zeatin was used at concentration of 1µM. Shortly before drop-inoculation, cytokinin was given to the $5*10^5$ Spores/ml bacterial dilution. Three days before the experiment was performed plants were also treated with cytokinin by mixing it with water to the working concentration and applied by watering.

3.6.16.1 Disease indexes assigned with their description

Scoring system was adopted from the laboratory of Dr. Birgit Kemmerling at the department of Biochemistry at ZMBP.

A

RANK	Description
1	No symptoms
2	Light brown spots at infection site
3	Dark spots brown spots at infection site
4	Spreading necrosis
5	Leaf maceration
6	Sporulation

B

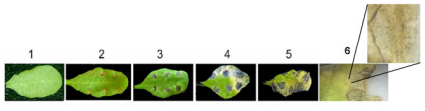


Figure 3.1 Scoring scale for determination of disease index for plants treated with *Alternaria brassicicola* (A) Description of different disease indexes **(B)** Photograph of treated *Arabidopsis* leaves with spores where is the phenotype related with appropriate disease indexes (image provided by courtesy of Thierry Halter, Dr. Birgit Kemmerling's group)

3.6.17 Infection with *Peronospora parasitica*

Treatment with the virulent *Peronospora parasitica* isolate was done on 4 to 6 week-old soil-grown plants. Spore suspensions of *Peronospora parasitica* were prepared as described (Shah *et al.* 2001). Plants were sprayed with a suspension of conidiospores diluted in water ($1x10^6$spores ml^{-1}). Inoculated plants were kept under humid conditions at 18°C for 1 week and then scored for fungal sporulation as previously published (Delaney *et al.* 1995).

3.6.18 Infection with *Botrytis cinerea*

Botrytis cinerea strain BMM was used (Zimmerli *et al.* 2000). The strain was grown on Difco potato dextrose agar (Becton Dickinson). Spores were harvested in water and then filtered and diluted in quarter-strength Difco potato dextrose broth (PDB) for inoculation as previously described (La Camera *et al.* 2011). For disease assays, 6 µL of spore solution (5×10^4 spores mL^{-1}) were deposited on three leaves (detached leaf assay) of 5 weeks old plants. Lesion diameters were measured after 3 days. Fungal growth was measured as previously described (Gachon and Saindrenan 2004). Plants were drop-inoculated and leafs discs were harvested at the indicated time points (0h, 12h, 24h, 36h, 48h post infection). The inoculated plants were kept under high humidity in a tray closed with a water-sprayed transparent lid.

3.6.19 FRET-FLIM and microscopy

Two days after the infection of Nb abaxial leaf surface with Agrobacteria carrying the desired binary plamids *pABind* vectors (Zimmermann and Nentwig 1989) were induced by application of β-estradiol by brushing a 20 µM β-estradiol (in 0.1% Tween-20) solution onto the abaxial leaf surface. FRET measurements were performed 24 to 48 hours after β-estradiol application as described in (Berendzen et al. 2012). The FRET-FLIM measurements were performed with a custom-built CSSM (confocal stage scanning microscope), based on a Zeiss Axiovert 135 TV, and equipped with a pulsed supercontinuum laser–source (SuperK™, NKT Photonics) as excitation light source operating at 471 nm and a repetition rate of 40 MHz. A microscope objective with high numerical aperture (Plan-Neofluar, 100×/1.30 oil, Zeiss) was used to focus the excitation light as well as to collect the fluorescence emission. The setup was equipped with a 500 nm dichroic mirror (FF500-Di01-25×36, Semrock) to block back-scattered excitation light and with a 527 nm bandpass filter (Semrock BrightLine BL527/20) to detect GFP-fluorescence. An avalanche photo diode (PDM series, MicroPhotonDevices (MPD), Italy) served as a spectrally integrating detector to record fluorescence intensity. Lifetime decays were recorded using a time-correlated single photon counting board for data acquisition (PicoHarp 300, Picoquant, Software: SymPhoTime, Picoquant) and the MPD as a detector (Wanke et al. 2011).

3.7 References for Materials and Methods

Berendzen, KW, Bohmer, M, *et al.* (2012). "Screening for in planta protein-protein interactions combining bimolecular fluorescence complementation with flow cytometry." Plant Methods **8**(1): 25.

Clough, SJ and Bent, AF (1998). "Floral dip: A simplified method for agrobacterium-mediated transformation of arabidopsis thaliana." Plant J **16**(6): 735-743.

Delaney, TP, Friedrich, L, *et al.* (1995). "Arabidopsis signal transduction mutant defective in chemically and biologically induced disease resistance." Proc Natl Acad Sci U S A **92**(14): 6602-6606.

Ferrari, S, Plotnikova, JM, *et al.* (2003). "Arabidopsis local resistance to botrytis cinerea involves salicylic acid and camalexin and requires eds4 and pad2, but not sid2, eds5 or pad4." Plant J **35**(2): 193-205.

Gachon, C and Saindrenan, P (2004). "Real-time pcr monitoring of fungal development in arabidopsis thaliana infected by alternaria brassicicola and botrytis cinerea." Plant Physiol Biochem **42**(5): 367-371.

Horak, J, Grefen, C, *et al.* (2008). "The arabidopsis thaliana response regulator arr22 is a putative ahp phospho-histidine phosphatase expressed in the chalaza of developing seeds." BMC Plant Biol **8**: 77.

Jacobs, AK, Lipka, V, *et al.* (2003). "An arabidopsis callose synthase, gsl5, is required for wound and papillary callose formation." Plant Cell **15**(11): 2503-2513.

James, P, Halladay, J, *et al.* (1996). "Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast." Genetics **144**(4): 1425-1436.

Kemmerling, B, Schwedt, A, *et al.* (2007). "The bri1-associated kinase 1, bak1, has a brassinolide-independent role in plant cell-death control." Curr Biol **17**(13): 1116-1122.

La Camera, S, L'Haridon, F, *et al.* (2011). "The glutaredoxin atgrxs13 is required to facilitate botrytis cinerea infection of arabidopsis thaliana plants." Plant J **68**(3): 507-519.

Lee, H, Suh, SS, *et al.* (2000). "The agamous-like 20 mads domain protein integrates floral inductive pathways in arabidopsis." Genes Dev **14**(18): 2366-2376.

Maizel, A and Weigel, D (2004). "Temporally and spatially controlled induction of gene expression in arabidopsis thaliana." Plant J **38**(1): 164-171.

Marion, J, Bach, L, *et al.* (2008). "Systematic analysis of protein subcellular localization and interaction using high-throughput transient transformation of arabidopsis seedlings." Plant J **56**(1): 169-179.

Mathieu, J, Warthmann, N, *et al.* (2007). "Export of ft protein from phloem companion cells is sufficient for floral induction in arabidopsis." Curr Biol **17**(12): 1055-1060.

Michaels, SD and Amasino, RM (1999). "Flowering locus c encodes a novel mads domain protein that acts as a repressor of flowering." Plant Cell **11**(5): 949-956.

Mira-Rodado, V, Veerabagu, M, *et al.* (2012). "Identification of two-component system elements downstream of ahk5 in the stomatal closure response of arabidopsis thaliana." Plant Signal Behav **7**(11): 1467-1476.

Sambrook, J and Russell, DW (2001). Molecular cloning: A laboratory manual, third edition (3 volume set), Cold Spring Harbor Laboratory Press.

Shah, J, Kachroo, P, *et al.* (2001). "A recessive mutation in the arabidopsis ssi2 gene confers sa- and npr1-independent expression of pr genes and resistance against bacterial and oomycete pathogens." Plant J **25**(5): 563-574.

Thomma, BP, Nelissen, I, *et al.* (1999). "Deficiency in phytoalexin production causes enhanced susceptibility of arabidopsis thaliana to the fungus alternaria brassicicola." Plant J **19**(2): 163-171.

Voinnet, O, Rivas, S, *et al.* (2003). "An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus." Plant J **33**(5): 949-956.

Wahl, V, Ponnu, J, *et al.* (2013). "Regulation of flowering by trehalose-6-phosphate signaling in arabidopsis thaliana." Science **339**(6120): 704-707.

Wanke, D, Hohenstatt, ML, *et al.* (2011). "Alanine zipper-like coiled-coil domains are necessary for homotypic dimerization of plant gaga-factors in the nucleus and nucleolus." PLoS One **6**(2): e16070.

Yoo, SK, Chung, KS, *et al.* (2005). "Constans activates suppressor of overexpression of constans 1 through flowering locus t to promote flowering in arabidopsis." Plant Physiol **139**(2): 770-778.

Zimmerli, L, Jakab, G, *et al.* (2000). "Potentiation of pathogen-specific defense mechanisms in arabidopsis by beta -aminobutyric acid." Proc Natl Acad Sci U S A **97**(23): 12920-12925.

Zimmermann, M and Nentwig, GH (1989). "[survival rate of desmodontal cells in relation to their extraoral dehydration]." Schweiz Monatsschr Zahnmed **99**(9): 1007-1010.

4 Chapter 1

Interaction of Type-B TCS components with MAPK kinase cascade members and a putative crosstalk function in pathogen response

4.1 Introduction

4.1.1 Two-component system (TCS) in *Arabidopsis thaliana*

Like in all plants, the TCS in *Arabidopsis thaliana* is also a multi-step phosphorelay system (Hwang *et al.* 2002; Heyl and Schmulling 2003; El-Showk *et al.* 2013). TCS mediates physiological response to hormones (cytokinin and ethylene, cross-talk with auxin), nutrients, light and osmotic stress. Its components have important roles in the regulation of several developmental processes such as control of stem cell number in SAM, female gametophyte development, differentiation of root vascular tissue, just to name a few (Hwang *et al.* 2012; El-Showk *et al.* 2013). This involves members of three protein families: *Arabidopsis* histidine kinases (AHKs), *Arabidopsis* histidine phosphotransferase proteins (AHPs) and *Arabidopsis* response regulators (ARRs). *Arabidopsis* possess 11 AHKs and they are hybrid kinases, with an exception of *ETHYLENE RESPONSE SENSOR 1 (ERS1)* kinase (Guo and Ecker 2004). Based on their functional and structural characteristics the AHKs can be grouped into three subfamilies (Grefen and Harter 2004). Five *AHKs* have roles as ethylene receptors (Grefen and Harter 2004): *ERS1, ERS2, ETHYLENE RESISTANT 1 (ETR1), ETR2* and *ETHYLENE INSENSITIVE 4 (EIN4*); *ERS1* and *ETR1* belong to the class I subfamily of AHKs (Grefen and Harter 2004) and are localised to the endoplasmic reticulum (Guo and Ecker 2004). *ETR2*, *ERS2* and *EIN4* belong to the class II of ethylene receptors (Grefen and Harter 2004; Guo and Ecker 2004) and have degenerated transmitter domains, *i.e.* they do not have histidine kinase activity as they lack at least one crucial amino acid to be functional (Guo and Ecker 2004). Kinase *AHK1* is playing role in stress signalling as membrane-bound osmosensor (Urao *et al.* 1999) and *CYTOKININ INSENSITIVE I (CKI1)* is enrolled in female gametophyte development (Pischke *et al.* 2002; Hejatko *et al.* 2003). *AHK5,* or also known as *CKI2,* is missing a transmembrane domain (Urao *et al.* 1999; Pischke *et al.* 2002) and therefore some authors classify it as a separate group. The *AHK5/CKI2* is playing a role in stomata closure signalling (Desikan *et al.* 2008) and it is predicted to be a cytoplasmic hybrid histidine kinase (Grefen and Harter 2004; Hass *et al.* 2004).

Members of the final subfamily respond to cytokinin: *AHK2, AHK3* and *AHK4/WOODEN LEG (WOL)/ CYTOKININ RESPONSE 1 (CRE1)* (Hwang *et al.* 2002; Grefen and Harter 2004; El-

Showk *et al*. 2013). Briefly, these AHKs contain a ligand-binding CYCLASE HISTIDINE KINASE ASSOCIATED SENSORY EXTRACELLULAR (CHASE) domain at their N-terminus which is flanked by two transmembrane domains and followed on the cytoplasmic side by the C-terminus with a histidine kinase domain and output domain (Suzuki *et al*. 2001; Ueguchi *et al*. 2001; Hwang *et al*. 2002). This sensor domain is like other bacterial histidine kinases PAS domains and their mechanism of function (Hothorn *et al*. 2011). The cytokinin receptors are localised not only on plasma membrane but also at the endoplasmic reticulum membrane highlighting that compartmentalization is important for cytokinin signal transduction (Caesar *et al*. 2011). These receptors show different sensitivity to different types of cytokinin hormones and also are expressed and functional in different tissues (Stolz *et al*. 2011; Lomin *et al*. 2012). AHK2 and AHK4/CRE1 have roughly the same high affinity to isopentenyladenine (iP) and *t*-zeatin but a lower affinity to dihydrozeatin whereas AHK3 has high affinity to dihydrozeatin compared to isopentenyladenine (Lomin *et al*. 2012). P_{ARR5}*::GUS* experiments in *ahk2*, *ahk3* and *cre1* mutant backgrounds demonstrated that loss of *cre1* affected promoter responses in the root while loss of *ahk2*, *ahk3* crippled shoot responses (Stolz *et al*. 2011). Interestingly, *AHK4* is known to be prevalent in the roots and *AHK3* in the shoots (Lomin *et al*. 2012). If correctly interpreted, the receptors are expressed and receptive to the corresponding cytokinins that are transported from the opposing tissue, *i.e*. *t*-zeatin from root to shoot and iPs from shoot to root (Lomin *et al*. 2012).

There are 6 AHPs in *Arabidopsis* that mediate phosphorelay and shuttle through the nucleus between AHKs and ARRs. *AHP1* to *AHP5* are positive regulators in cytokinin signalling (Hutchison *et al*. 2006) and are also known as "canonical" AHPs because they contain the conserved histidine HPt residue thus they can carry the TCS-phosphate group on further to ARRs (Suzuki *et al*. 1998). Despite their partial redundancy, different subsets of AHPs have been shown to trigger cytokinin responses in specific tissues and organs. For example the *ahp2/ahp3/ahp5* triple loss-of-function mutant showed increased seed size similar to that observed in the *ahk2/ahk3/ahk4* triple mutant (Hutchison *et al*. 2006; Riefler *et al*. 2006). Different combinations of *ahp1-5* mutants do not show the same responses to exogenous cytokinin and its effects on chlorophyll content, lateral root formation or hypocotyl elongation (Hutchison *et al*. 2006). Generally speaking however, the loss of *AHPs* seems to be additive, as expected due to redundancy. *AHP6* is called a "pseudo AHP" because it lacks the conserved histidine residue required for TCS-dependent phosphorylation: nevertheless, it is an active cytokinin signalling inhibitor (Suzuki *et al*. 1998; Grefen and Harter 2004; Mahonen *et al*. 2006) (Moreira *et al*. 2013). Recently it has been reported that *AHP2*, *AHP3*, and *AHP5*, also work redundantly to control responses to drought stress in a negative and redundant manner as loss of these three *AHPs* resulted in a strong drought-tolerant phenotype that was associated with the stimulation of protective mechanisms (Nishiyama *et al*. 2013).

There are 23 functional ARRs in *Arabidiopsis* and based on their protein properties and phylogeny they are divided into 3 subgroups: the type-A, type-B and pseudo-response regulators. The Type-A subfamily of *ARRs* are single-domain response regulators that

contains the members: *ARR3, ARR4, ARR5, ARR6, ARR7, ARR8, ARR9, ARR15, ARR16* and *ARR17*. Members of this subfamily only have a functional receiver domain (with functional Asp residue) and a short C-terminal tail (Grefen and Harter 2004). Members of the type-A subfamily generally act as negative regulators of cytokinin signalling based on genetic and transgenic studies (Lee *et al.* 2008; Hwang *et al.* 2012; El-Showk *et al.* 2013). So far only ARR4 has been described with a function outside of the TCS. ARR4 positively interacts with phytochrome B and stabilizes the active PhyB-Pfr form under extended red light conditions and thus functions as modulator of photomorphogenesis (Sweere *et al.* 2001; Mira-Rodado *et al.* 2007).

The type-B response regulators have additional effector domains on their extended C-termini and are therefore able to activate the transcription in a TCS-dependent manner (Sakai *et al.* 2001). Their expression is not regulated by any stimulus tested so far (Grefen and Harter 2004). This subfamily consists of 11 members: *ARR1, ARR2, ARR10, ARR11, ARR12, ARR13, ARR14, ARR18, ARR19, ARR20* and *ARR21*. The output domain of the B-Type members usually is composed of the GARP (named after Golden2 found in maize) DNA-binding domain, at least one NLS and C-terminal transactivation domain (Lohrmann and Harter 2002). B-Type response regulators work as transcription factors binding to target promoters that contain 5´-W/GAT/W-3´ motif (W represents either A or T) (Lohrmann and Harter 2002). Last group of response regulators are C-Type *ARRs* (Horak *et al.* 2008). Both type-A and type-B ARRs are now know to be regulated by the 26S proteasome (Ren *et al.* 2009; Kim *et al.* 2013; Kurepa *et al.* 2013).

Two genes (*ARR22* and *ARR24*) encode single-response domain response regulators and are structurally very similar to type-A response regulators but their expression is not regulated by cytokinin. It has been recently suggested that they function as phosphatases for phosphates on histidine moiety of AHPs, but their expression domain is restricted to reproductive organs (Kiba *et al.* 2003; Gattolin *et al.* 2006; Horak *et al.* 2008).

Beside these three groups of the *ARRs* there is one more group, composed out of 9 members, called *pseudo-response regulators* (*pseudo-RRs*). These are response regulator proteins that have a mutation in the conserved response regulator aspartate (Asp) residue (Hwang *et al.* 2002). It has already been proven that pseudo-RRs regulate flowering time in *Arabidopsis thaliana* and that *TOC1/APRR1*, an *Arabidopsis* pseudo response regulator, is part of the plant circadian clock (Farre and Liu 2013). Even though pseudo-RRs have lost their ability to accept the phosphorelay phosphate, they are constitutively active because the highly conserved Asp is mutated to a glutamate naturally mimicking a phosphorylated Asp (Grefen and Harter 2004). The pseudo-response regulators can be split into two subfamilies (Matsushika *et al.* 2000): The APRR2 family which more similar to a classical response regulator structure with an N-terminal receiver domain followed by a DNA binding domain (also Myb-like GARP) and C-terminal effector extension; the spacing between the RR domain and the GARP domain is however larger than that of the type-B ARRs (Makino *et al.* 2000; Matsushika *et al.* 2000). The APRR1 family has an "inverted" response regulator structure:

there is an N-terminal RR domain, a DNA-binding CCT domain at the C-terminal end, and, presumptuously, the effector domain in-between these two domains (Makino *et al.* 2000). PRR9, PRR7, and PRR5 function as transcriptional repressors of *CCA1* and *LHY* (Nakamichi *et al.* 2010) recently shown to bind to DNA via their C-terminal CCT domains (Gendron *et al.* 2012). The pseudo-response regulators are also targeted by the 26S proteasome (Baudry *et al.* 2010).

4.1.2 Crosstalk of TCS-related pathways in plants

Although most type-B *ARRs* have been described to only be involved in cytokinin signalling, an exception is *ARR2*. Recently it was discovered that cytokinin-activated ARR2 promotes plant immunity in *Arabidopsis* via salicylic acid signalling whereby ARR2 directly interacts with salicylic acid response factor TGA3 and increases pathogen resistance (Choi *et al.* 2010). *ARR2* is also known to be involved in ethylene signalling by. working downstream of *ETR1* (Hass *et al.* 2004). Furthermore, *ARR2* is involved in ethylene and H_2O_2-mediated stomatal closure (Desikan *et al.* 2006). The crosstalk of TCSs with other plants signalling pathways became more evident after a comparative microarray analysis was done on 30-day-old *arr2* mutant and control Landsberg erecta (Ler) plants where the results showed altered expression of about 600 genes mostly related to biotic and abiotic stresses, ethylene and auxin signalling (Hass *et al.* 2004). The expression of dominant-active, non-phosphorylatable, $ARR2^{D80E}$ (aspartate-to-glutamate mutation) caused even more dramatic changes in more than 16000 genes, and most of the disturbed genes are involved in hormone homoeostasis and its signal transduction, biotic and abiotic stress, photomorphogenesis, and others (Hass *et al.* 2004).

4.1.3 Mitogen-activated protein kinase cascade

Eukaryotic mitogen-activated protein kinase (MAPK) cascades transduce environmental and developmental signals into adaptive and programmed responses. In plants, MAPK cascades have evolved to regulate innate immunity, hormonal response, stress and developmental processes (Colcombet and Hirt 2008; Rodriguez *et al.* 2010). MAPK signalling cascade(s) are evolutionary conserved within eukaryotes (Schwartz and Madhani 2004) and have roles in abiotic stress, response to pathogens and pathogen-derived elicitors, plant hormones (ethylene, auxin), cell cycle and developmental processes (Tena *et al.* 2011; Rasmussen *et al.* 2012; Pathak *et al.* 2013; Smekalova *et al.* 2013). MAPK cascades transduce and amplify their signal by three different types of kinase members: *MAP kinase kinase kinase (MKKK), MAP kinase kinase (MKK)* and *MAP kinase (MPK)* (Rodriguez *et al.* 2010). In general, they function as a three tiered kinase cascade with each upstream member required to active the lower level eventually influencing genes expression (Rodriguez *et al.* 2010). Initially, a ligand activates plasma membrane receptors and these stimulated receptors activate MKKKs that are serine or threonine kinases that in turn phosphorylate MKKs at a conserved S/T-X_{3-5}-S/T motif (X can be any amino acid) (Chang and Karin 2001; Rodriguez *et al.* 2010). The MKKs afterwards phosphorylate MPKs on threonine and tyrosine residues at a conserved T-X-Y repeat (Chang and Karin 2001; Rodriguez *et al.* 2010). Activity of MAPKs can be regulated/deactivated by serine/threonine or tyrosine phosphatases (Luan 2003). Different cascades can share kinase components but still maintain their signalling specificity by various

mechanisms like protein-protein interactions, scaffolding, cross-inhibition and feedback control (Whitmarsh and Davis 1998; Bardwell *et al.* 2001; Takekawa *et al.* 2005). MAPK cascades are present in all eukaryotes and is evolutionarily highly conserved which confirms its essential function in these organisms (Kim *et al.* 2003).

4.1.4 Mitogen-activated protein kinase cascade and its signalling in *Arabidopsis thaliana*

Around 60 *MKKKs*, 10 *MKKs* and 20 *MPKs* are found in the genome of *Arabidopsis thaliana* (Group 2002). Besides having the largest number of members, *MKKKs* also have the greatest variety in domain composition and primary structures among all the MAPK cascade members (Group 2002). Their nomenclature is still not totally defined as some authors have different opinions about this topic. Based on amino acid sequence of the kinase catalytic domains the *Arabidopsis MKKKs* are grouped into 2 main classes which are further divided into different subclasses (Group 2002; Rodriguez *et al.* 2010): *MEKKs* and the Raf-like kinases. The *MEKK* MKKKs family comprises members who kinase domains share significant similarity to previously described *MKKKs*, for example MEKK1 in mammals or to yeast STE1 (Group 2002). Members of this family are generally active in response to abiotic stress, especially drought, touch and high salinity (Mizoguchi *et al.* 1996). The *Raf-like kinase* class is named after RAF1 mammalian kinase. Interestingly, most members of this class have a PAS domain which is the most frequent sensor domain present among TCS histidine kinases and other pathways (Zhulin *et al.* 1997; Zwerger and Hirt 2001). The most studied members of this *MKKK* class are *CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1)* and *ENHANCED DISEASE RESISTANCE 1 (EDR1)* which are both involved in ethylene-mediated signalling and defence responses (Kieber *et al.* 1993; Huang *et al.* 2003). Among the *Raf-like* class there is also the ZIK sister clade for which no phosphorylation activity has been shown on MKKs (Figure 4.1) (Rodriguez *et al.* 2010).

The small number of putative *MKKs* in *Arabidopsis* suggests that crosstalk between various signal-transduction pathways might be concentrated at this level of the MAPK signalling cascade. It is known that one MKK can activate various different MPKs (Popescu *et al.* 2009). Considering their small number, the MKKs are very well described in *Arabidopsis* and rice and both are classified into 4 groups (A-D) (Group 2002; Hamel *et al.* 2006; Rodriguez *et al.* 2010). Members of the group A are *MKK1, MKK2* and *MKK6*. Kinases MKK1 and MKK2 are involved in abiotic (cold, salinity) and biotic (especially innate immune response) stresses (Teige *et al.* 2004; Meszaros *et al.* 2006; Rodriguez *et al.* 2010), both acting upstream of MPK4 (Ichimura *et al.* 1998). Group B includes only one member: *MKK3* which is distinguishing from the other kinases by the presence of a nuclear transport factor 2 (NTF2) domain (Kiegerl *et al.* 2000; Group 2002). Group C includes *MKK4* and *MKK5* (Group 2002) and they play very important roles in biotic stresses by providing resistance to bacterial and fungal pathogens controlling converging signals initiated by diverse pathogens (Asai *et al.* 2002; Group 2002; Rodriguez *et al.* 2010). In addition, both MKK4 and MKK5 are in the pathway downstream of YODA (MAPK kinase kinase) regulating cell fate specification in stomata development (Wang *et al.* 2007) and the ethylene synthesis pathway (Babula *et al.* 2006). Members of group D are *MKK7, MKK8, MKK9* and *MPK10* whose physiological roles are not yet precisely described. It is known that the MKK9–MPK3/MPK6 cascade promotes *ETHYLENE-INSENSITIVE 3 (EIN3)*-

mediated transcription in response to ethylene signalling (Yoo *et al.* 2008; Hahn and Harter 2009) and that MKK7 repressed expression causes deficiency or enhancement in auxin transport (Dai *et al.* 2006).

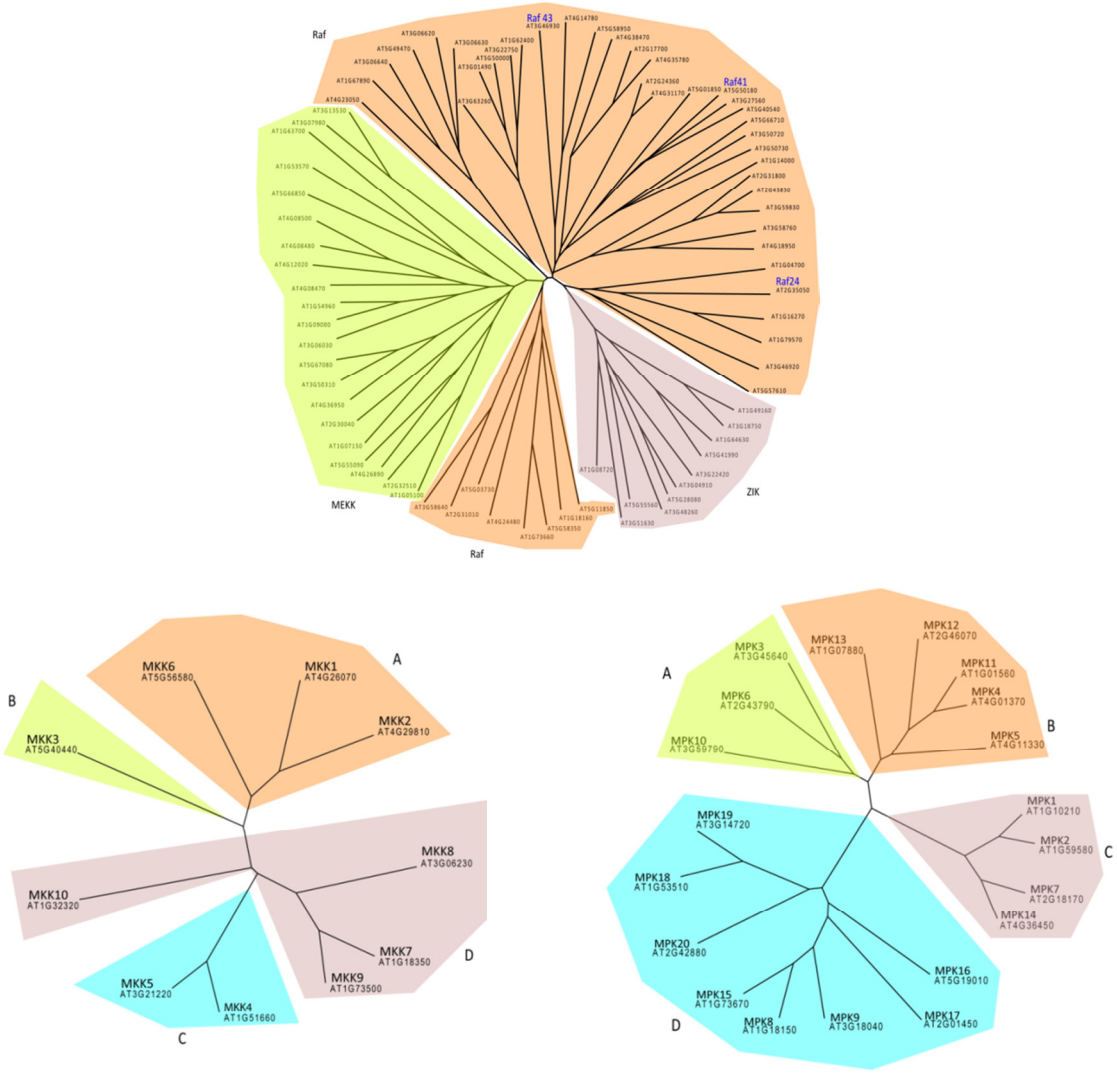


Figure 4.1 Relatedness of *Arabidopsis MKKKs* based on their protein sequence
The unrooted tree was made by aligning complete protein sequences with Clustal W (default settings) available on TAIR (http://www.arabidopsis.org/) of the ***(A)*** *AtMKKKs,* ***(B)*** *AtMKKs and* ***(C)*** *AtMPKs. S*ubfamilies are marked according to the classifications proposed in (Group 2002). The trees were draw with TreeView X.

The last members of the MAPK signalling cascade are MPKs. They are also grouped into 4 groups (A-D) based on their sequence homology (Group 2002; Rodriguez *et al.* 2010). In plants, MPKs contain either the TDY (present also in all mammals) or TEY phosphorylation motif which is phosphorylated by MKKs and causing MPK activation (Group 2002; Rodriguez *et al.* 2010). Members of groups A, B and C families contain TEY whereas only group D has the TDY phosphorylation motif. The most studied group of MPKs is Group A. Generally, members of this group are involved in environmental and hormonal responses (Group 2002). *MPK3* and *MPK6* are members of this group and they have been the most studied group

among all MAP kinases. MPK3/MPK6 play roles in ethylene signalling downstream of MKK9 (Hahn and Harter 2009) whereas MPK6 is additionally required for interaction with ETHYLENE RESPONSE FACTOR 104 (ERF104) in ethylene responses regulated by flg22 (Bethke *et al.* 2009). Findings for the group B are mostly based on physiological studies of *Arabidopsis* MPK4 involved in pathogen defence and abiotic stresses (Widmann *et al.* 1999; Yuasa *et al.* 2001; Qiu *et al.* 2008). MPK4 is known to play important role in plant immunity, it functions as a negative regulator of pathogen defence and also interferes with stress signalling pathways at several distinct steps in pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) as well as in effector-triggered immunity (ETI) (Berriri *et al.* 2012; Colcombet *et al.* 2012). Furthermore, it is also known that the *Pseudomonas syringae* HopAI1 effector irreversibly inactivates MPK4 to prevent immune responses (Rasmussen *et al.* 2012). Data on C group are very limited and based on *MPK7* whose expression is regulated by the circadian rhythm (Group 2002). Group D also has a C-terminal MKKs docking domain (Rodriguez *et al.* 2010). It was found that some members of this group are induced by fungi and wounding in rice and alfalfa (He *et al.* 1999; Schoenbeck *et al.* 1999). One described member is *MPK17*. Generally not much is known about *MPK17* but it is thought to be important, like whole group D, in plant sugar signal transduction (Sheen *et al.* 1999).

4.1.5 Plant pathogens and mechanisms of plant defence

4.1.5.1 Immunity

Plants are often attacked by a huge variety of microbial pathogens and herbivore insects. In order to response to these threats they have developed numerous defence mechanisms and many of them are directly induced by pathogen attack (Glazebrook 2005). Generally, defence often begins with gene-for-gene pathogen recognition where the production of pathogen virulence effectors leads to their recognition by plants that carry correspondent resistance *(R)* genes (Glazebrook 2005). This kind of resistance is accompanied by fast reactive oxygen species (ROS) production also known as oxidative burst which is needed for a further process known as hypersensitive cell death (HR). Reactive oxygen species (ROS) were initially recognized as toxic by-products of aerobic metabolism, removed by means of antioxidants and antioxidative enzymes (Kawano 2003) and was not suspected to provide meaningful information for the plant.

On one side, R gene-driven resistance activates salicylic acid (SA)-dependent signalling pathways which in turn lead to the activation of pathogenesis-related proteins (PR) that also contribute to resistance. This rapid activation of defence results in the limitation of pathogen growth. Innate immunity is generally initiated with recognition of conserved pathogen/microbial-associated molecular patterns (PAMPs/MAMPs) (Nishimura and Dangl 2010). Pathogen-associated molecular patterns, or PAMPs, are molecules associated with groups of pathogens that are recognized by cells of the innate immune system. These molecules can be referred to as small molecular motifs conserved within a class of microbes (Boller and He 2009) and they are typically essential components of whole classes of pathogens, such as bacterial flagellin or fungal chitin (Dodds and Rathjen 2010). PAMPs are

perceived by pattern recognition receptors (PRRs), leading to activation of a series of immune responses, including the expression of defence genes, Reactive Oxygen Species (ROS) production, nitric oxide, ethylene, jasmonic acid (JA), and salicylic acid (SA) and activation of MAP kinase cascades (Jones and Dangl 2006; Tena *et al.* 2011; Yamaguchi *et al.* 2013). Activation of PRRs leads to PAMP-triggered immunity (PTI); PTI is often the first inducible response of a plant to PAMPs (Jones and Dangl 2006; Nishimura and Dangl 2010). The largest group within the *R genes* is binding site-leucine rich repeats (NBS-LRR) (Belkhadir *et al.* 2004), now mostly called NLR-dependent effector-triggered immunity (ETI) (Dangl *et al.* 2013). ETI is a second class of perception called effector-triggered immunity. This way of perception involves recognition by molecules called effectors which present intracellular receptors of pathogen virulence presenting, in contrast to PTI, co-evolutionary dynamics between the plant and pathogen. Generally, PTI and ETI give rise to similar responses. PTI is generally effective against non-adapted pathogens in a phenomenon called non-host resistance, whereas ETI, which is stronger and faster than PTI, is mostly active against adapted pathogens (Dodds and Rathjen 2010). Plants also respond to endogenous molecules which are released by pathogen invasion, cell wall or cuticular fragments, called danger-associated molecular patterns (DAMPs) (Dodds and Rathjen 2010).

Beside defence response controlled by SA pathways, defence responses can also be controlled by ethylene and/or jasmonic acid (JA) and this responses are mostly overlapping with response to wounding (Glazebrook 2005). Nevertheless, SA, JA and ethylene interacting between each other, SA and JA are reported to mutually inhibit the expression of many target genes whereas JA and ethylene are sometimes both acquired for expression of some genes but there are also some cases of negative interaction between their signalling.

4.1.5.2 Biotrophic and necrotrophic pathogens

According to their lifestyle pathogens are divided into biotrophs and necrotrophs. Biotrophs are pathogens that live and feed on living tissue; necrotrophs are defined as organisms that live and feed on dead tissue (Mcdowell and Dangl 2000; Thaler *et al.* 2004). Besides their different life style more important is the fact that Necrotrophic and biotrophic pathogens have evolved differently leading to distinct defence strategies in plants and thus activation of different genes required for pathogenicity (Idnurm and Howlett 2001; Oliver and Ipcho 2004). SA is linked to resistance to biotrophic pathogens and is important to trigger the HR, a programmed cell death (PCD) to locally counteract pathogen attack and progression. ET and JA play a role in the control of PCD spreading (Glazebrook 2005; Colcombet and Hirt 2008) and regulate resistance against necrotrophic pathogens (Colcombet and Hirt 2008). However, some pathogens cannot be assigned as biotrophs or necrotrophs There are also hemibiotrophic pathogens: hemibiotrophic pathogens incorporate aspects of both biotrophic and necrotrophic infection strategies. Often this involves an initial biotrophic infection phase during which the pathogen spreads in host tissue, followed by a necrotrophic phase during which host cell death is induced (Dodds and Rathjen 2010). However, the interactions between SA-, JA-, and ethylene-dependent pathways do not appear to be simple.

Complications could arise from different roles of these pathways in different stages of plant-pathogen interactions (Katagiri *et al*. 2002).

In order SA to accumulate two genes encoding enzymes similar to triacyl-glycerol lipases are required *PHYTOALEXIN DEFICIENT 4 PAD4* and *ENHANCED DISEASE SUSCEPTIBILITY 1 EDS1* (Zhou *et al*. 1998; Falk *et al*. 1999), raised SA levels in turn are activating various defense effector genes including *PR-1* (Glazebrook 2005). The *PR-1* effector gene for plant defense is activated by NPR1 and TGA-type transcription factors. *NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1)* has function in regulation of systemic acquired resistance (SAR) in *Arabidopsis* (Rochon *et al*. 2006) and NPR1, as recently reported, plays the role of an SA receptor *in vivo*, in the signalling cascade leading to *PR-1* activation (Wu *et al*. 2012). Systemic acquired resistance (SAR) means a mechanism of induced defence and implies long lasting protection against a broad spectrum of microorganisms. Salicylic acid is absolutely required by SAR as signalling molecule (Durrant and Dong 2004). Based on their physical interaction *TGA* transcription factors are considered as regulators of pathogenesis-related (*PR*) genes because of their physical interaction with the known positive regulator NPR1 (Kesarwani *et al*. 2007). Using reverse genetics approach it has been characterized 10 *TGA* factors in *Arabidopsis* so far and they all belong to the bZIP transcription factor family (Jakoby *et al*. 2002). When the level of SA are low, NPR1 exists as oligomeric form in cytoplasm, the monomers are held together by disulfide linkages which are getting reduced after the level of NPR1 increases and NPR1 is now present in monomeric form which as such can enter the nucleus and interact with the nuclear TGAs and induce expression of *PR-1* (Zhang *et al*. 2003).

As response to pathogen attack the levels of JA can also increase and as a consequence to that defence effector genes are increasing as well, especially *VSP1* (Otani *et al*. 1998) and *PDF1.2*. The *VEGETATIVE STORAGE PROTEIN 1 (VSP1)* gene of *Arabidopsis thaliana* encodes a storage protein that accumulates in vegetative organs and it is jasmonate-responsive element (Guerineau *et al*. 2003). The *PLANT DEFENSIN PDF1.2* gene in *Arabidopsis* encodes a plant defensin and it is commonly used as a marker for characterization of the jasmonate-dependent defence responses (Brown *et al*. 2003). Expression of the *PDF1.2* requires both JA and ethylene (Glazebrook 2005). For known signalling activities of JA in *Arabidopsis CORONATINE INSENSITIVE 1 (COI1)* is required. *COI1* gene encodes protein from F-box group and it is believed to act in proteolysis (Xie *et al*. 1998) but it can also bind histone deacetylases (Devoto *et al*. 2002). Beside the essential COI1, JA also requires members of MAPK cascade for its activity, the MPK4. MPK4 knock-out mutant failed to express the *PD1.2*. gene (Petersen *et al*. 2000). For ethylene connection to pathogen defence *EIN2* is involved but that is not the only ethylene-related gene involved in pathogen defence (Lorenzo *et al*. 2003).

Pseudomonas syringae is a gram-negative, rod-shaped bacterium with polar flagella and it infects a wide variety of plants (Katagiri *et al*. 2002). *Pseudomonas* mostly acts as biotrophic pathogen (Zhao *et al*. 2003; Tsai *et al*. 2011). *Peronospora parasitica* is an oomyceteous

fungus from the *Peronosporaceae* family. In contrast to *Pst* DC3000, *Peronospora* is an obligate biotroph that is controlled by the salicylic acid-dependent SAR (systemic acquired resistance) pathway (Nawrath and Metraux 1999; Jambunathan *et al.* 2001). Defence responses under SA control are critical for resistance to both *Pst* DC3000 and *Peronospora parasitica (Glazebrook 2001; Van Wees et al. 2003)* (Nawrath and Metraux 1999; Jambunathan *et al.* 2001). *Alternaria brassicicola* belongs to phylum *Ascomycota* (family *Pleosporaceae*) and it known to be a very common plant pathogen. Black spot disease caused by *Alternaria brassicicola* is an important fungal disease affecting cruciferous crops (*Brassicaceae*). The interaction between *Arabidopsis thaliana* and *A. brassicicola* is a representative model system and objective estimation of disease progression is indispensable for accurate functional analyses (Su'udi *et al.* 2013). Resistance against *Alternaria* requires the *Arabidopsis* phytoalexin, camalexin and jasmonic acid (JA)-dependent signalling, respectively (Van Wees *et al.* 2003). *Botrytis cinerea* is necrotrophic fungus belonging to the phylum *Ascomycota* (family *Sclerotianiaceae*) and it is a natural pathogen of *Arabidopsis thaliana* (P *et al.* 2007). *Botrytis* belongs in top 10 fungal pathogens in molecular plant pathology because it has an impact because of its broad host range causing severe damage: *Botrytis* causes bunch rot in viticulture or grey mould in horticulture (Dean *et al.* 2012). The fungus is considered as a typical necrotroph, which co-opts programmed cell death pathways in the host to achieve infection (Amselem *et al.* 2011; Dean *et al.* 2012).

4.1.6 TCS type-B response regulator and MAPK cascade members are involved in pathogen signalling

MAPK cascade members found to be involved in pathogen signalling are *MPK3, MPK4* and *MPK6* (Colcombet and Hirt 2008). These kinases in *Arabidopsis* are activated by PAMPs, mostly by flg22 (Droillard *et al.* 2004). Very soon other MAPK cascade members, working upstream of MPK3/4/6 and downstream of the FLS2-BAK1, were found (Colcombet and Hirt 2008). These are MKKK1 and two MKKs, MKK4 and MKK5 (Colcombet and Hirt 2008). Nevertheless, in *mekk1* knock-out plants, flg22 was still able to activate MPK3 and MPK6 suggesting redundant effects at the MKKK level (Ichimura *et al.* 2006; Suarez-Rodriguez *et al.* 2007). Interestingly, biosynthesis of ethylene is also triggered by flg22 via MPK6 which in turn activates *ACS6 (1-amino-cyclopropane-1-carboxylic acid) synthase 6* a crucial element in ethylene synthesis (Liu and Zhang 2004; Joo *et al.* 2008). *Pst* DC3000 induces *MKK3* to positively regulate expression of *PR1*. On the other hand *mkk3-1* knock-out plants showed high susceptibility to *Pst* DC3000 and this effect was the opposite when the *mpk3-1* mutant was complemented by *MKK3* wild-type form or constitutive active *MKK3-EE* form (S235E and S411E, MKKK phosphorylation residues) (Doczi *et al.* 2007), that is, the downstream MPK targets are needed for a functional readout of MKK activity. Downstream elements for MKK3 thus appear to be Group C MPKs based on yeast-two-hybrid interaction studies; physiological roles were only confirmed with MPK7 (Zhang *et al.* 2007). Recently was found that ARR2 also contributes resistance against *Pst* DC3000. ARR2 cannot directly do this, but interacts with salicylic acid response factor TGA3 after it is picks up a TCS phosphate at D80, thereafter interacting with the TGA3/NPR1 dimer making ARR2/TGA3/NPR1 trimer complex

that can bind to the *PR1* promoter, activate it, and increase pathogen resistance. *arr2-4* mutant plants in that study showed susceptibility to *Pseudomonas* (compared to the wild-type Col-0 plants) and also retarded induction of *PR1* in response to SA (Choi *et al.* 2010). ARR2 was shown to pull down *PR1*, *PR2*, and *ARR6* promoter DNA via ChIP assays. The association to DNA at *PR1* however was mediated by TGA3 and not ARR2 (Choi *et al.* 2010), is suggests that a type of scaffolding role for ARR2 was discovered. Possibly other such roles for ARR2 and or type-B RRs independent of direct DNA binding could be discovered.

4.1.7 Cell-specificity effect of the MAPK cascade

Despite the fact that only couple members of the MAPK cascade control huge range of physiological responses, these signalling cascades are quite specific because they show cell specificity (Colcombet and Hirt 2008). For example, activity of *MPK4* is increased in response to osmotic shock in *Arabidopsis* but only in suspension cells not in plantlets (Droillard *et al.* 2004). Subcellular-dependent function can be very well observed on ethylene-related roles of *MPK6*. The cascade MKK4-MPK6-ACS6 is involved in ethylene production whereas MKK9-MPK6-EIN3 cascade plays role in ethylene signalling. The MKK9–MPK3/MPK6 cascade promotes *ETHYLENE-INSENSITIVE 3 (EIN3)*-mediated transcription in ethylene signalling (Yoo *et al.* 2008; Hahn and Harter 2009). Ethylene as a ligand binds and inactivates the negative regulator CTR1 (MKKK family member) to activate the positive MKK9–MPK3/6 cascade (Yoo *et al.* 2008). This signalling pathway breaks a linear model and common MAPK signalling, and opens many questions, for example, both the real connection between CTR1 and MKK9 or how CTR1 is actually inhibiting the autophosphorylation of MKK9 through direct protein-protein interaction or in some other way (Hahn and Harter 2009). Supporting cell-specificity idea, scaffolding proteins have extremely important functions because they help MPK kinases to build physiologically functional cascades. Such an example is present in yeast where the C-terminal of the SSK1 RR binds to the N-terminus of the MKKK SSK2 inducing a conformational change which induces its autophosphorylation and activation of the kinase cascade (Posas and Saito 1998). Therefore it is very important to identify and describe these proteins in their crosstalk points and this work contributes to this issue.

4.2 Results

4.2.1 Background

The TCS and MAPK signal transduction pathways are two differently evolved signalling pathways both crucial for mediating control of physiological responses and development in plants (Grefen and Harter 2004; Hass *et al.* 2004; Mira-Rodado *et al.* 2007; Pathak *et al.* 2013; Smekalova *et al.* 2013). It has been known that *ARR2* also functions in ethylene signalling as an *arr2 Ler-0* knock-out shows an hyposensitive phenotype (Hass *et al.* 2004). Transcriptomic data of *arr2 Ler-0* knock-out and *ARR2* overexpression lines revealed drastic mis-regulation of various defence-related genes some of which are well known to be controlled/activated by MAPK cascade signalling (Hass *et al.* 2004). Preliminary data suggested that ARR2 co-localises and interacts with several MAP kinase kinases in Y2H and *in vivo* BIFC (Achim Hahn, personal correspondence). These findings indicated that there might be a functional connection of ARR2-dependent TCS signalling and MAPK cascades which were further explored in this thesis.

4.2.2 Interaction of ARR2 with MAPK cascade members

4.2.2.1 Response regulators Type A or B do not interact with MKKKs

MKKKs were tested for protein-protein interaction against ARR2 using the yeast-two-hybrid system as an *in vivo* heterologous approach. The large number of MKKK proteins made it impossible to test all of them, but nevertheless, three representative members were chosen for this experiment: *Raf24, Raf43* and *Raf41* due to their distinct sequence and functions (Jouannic *et al.* 1999; Group 2002). Besides ARR2, two other members of type-B family were chosen, ARR1 and ARR18, and a well-known type-B family member, ARR4. ARR1 has very high sequence homology with ARR2 and it is proposed to be redundant to ARR2 in many physiological functions (Sakai *et al.* 2000). ARR18 on the other hand is different from its group members ARR1 and ARR2 in sequence, expression pattern and presumably function (Mason *et al.* 2004; Veerabagu *et al.* 2012) even though these three type-B response regulators are all members of the subgroup I. ARR4 was taken as a typical member of type-A group of response regulators to distinguish B-type and A-type characteristics, if there are any.

After cloning the ORFs into the yeast-two-hybrid expression vectors, yeast strain PJ69-4A (James *et al.* 1996) was transformed and colonies carrying the auxotrophic markers were selected on CSM –W-L media. MKKKs were cloned with the GAL4-BD fusion and ARRs were cloned as GAL4-AD fusions. None of tested MKKK showed transactivation in yeast (only shown for Raf41) indicating that all three MKKKs could be evaluated in yeast. Nevertheless, when the colonies were plated on interaction-selective media (-W, L, A), none of these MKKKs interacted with any of tested ARRs (Figure 4.2). This experiment was repeated three times using all interaction elements and each time the same result was obtained. Based on this experiment, if there is intercommunication between this two signaling pathways, then it is downstream of MKKKs or requires other MKKK elements.

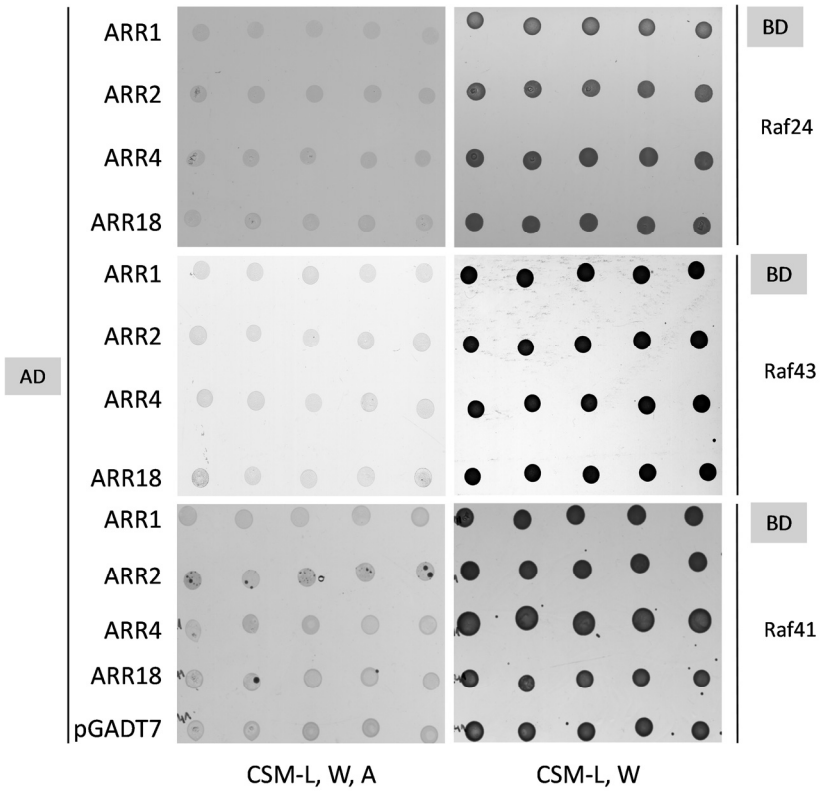


Figure 4.2 *Arabidopsis* response regulators do not interact with selected MKKKs in yeast-two-hybrid assays.

Proteins were expressed either as GAL4-AD-fusions (ARR1, ARR2, ARR4, ARR18) or as GAL4-BD-fusions (Raf24, Raf43, Raf41). Yeast cells were incubated for 4 days at 28°C on either vector selective (CSM-L, W) or interaction selective (CSM-L, W, A) media. The free AD domain was expressed along with BD-fusions for transactivation controls; only Raf41 is shown. For all interactions PJ96-4A was used. This experiment was repeated at least three times with similar results.

4.2.2.2 B-Type response regulators interact with MKK members

MKKs represent a point of signal convergence within MAPK signalling cascade (Mordret 1993). After cloning the ORFs into the yeast-two-hybrid expression vectors, yeast strain PJ69-4A was transformed and colonies carrying the auxotrophic markers were selected on CSM-W, L medium. MKK were cloned with a GAL4-BD fusion and ARRs were cloned as GAL4-AD fusions. As before ARR1, 2, 4 and 18 were tested against MKKs: MKK1, MKK2, MKK4, MKK5. MKK3 is transitive in yeast and cannot be analyzed as a full-length protein and therefore was not tested (not shown). None of other tested MKK constructs showed transactivation in yeast (Figure 4.3) indicating that all three MKKs could be evaluated in yeast. Interaction was observed for ARR1, 2 and 18 with MKK1, MKK2, MKK4, MKK5, whereas no interaction was observed for ARR4 with any MKK tested (Figure 4.3).

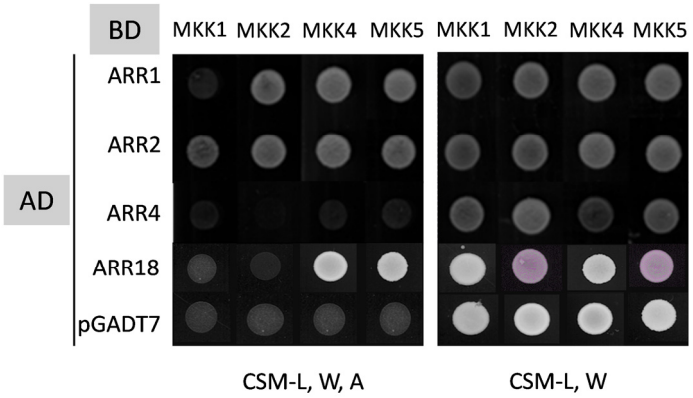


Figure 4.3 Type-B *Arabidopsis* response regulators interacted with MKKs in yeast-two-hybrid assays.
Proteins were expressed either as GAL4-AD-fusions (ARR1, ARR2, ARR4, ARR18) or as GAL4-BD-fusions (MKK1, MKK2, MKK4 and MKK5). Yeast cells were incubated for 4 days at 28°C on either vector selective (CSM-L, W) or interaction selective (CSM-L, W, A) media. The free AD domain was expressed along with BD-fusions for transactivation controls. For all interactions PJ96-4A was used. This experiment was repeated at least three times with similar results; representative colonies are shown for simplification.

4.2.2.3 ARR2 response regulator interacts also with MPKs

As the last members of MAPK signalling cascades, MPKs were also tested against ARR2. For this experiment MPK4 and MPK17 were chosen as they both have been described in the literature and have distinct evolutionally histories (Group 2002).

After cloning the ORFs into the yeast-two-hybrid expression vectors, yeast strain PJ69-4A was transformed and colonies carrying the auxotrophic markers were selected on CSM –W-L media. MPKs were cloned with a GAL4-BD fusion and ARRs were cloned as GAL4-AD fusions. ARR1, 2, 4 and 18 were tested against MPK4 and MPK17 (Figure 4.4). Neither MPK showed transactivation in yeast indicating that each MPKs could be evaluated in yeast (data for MPK17 shown).

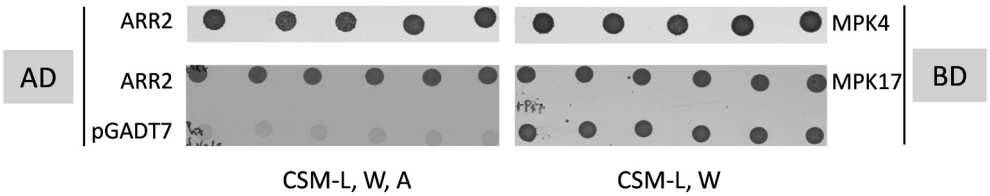


Figure 4.4 ARR2 interacted with MPKs in yeast-two-hybrid assays.
Proteins were expressed either as GAL4-AD-fusions (ARR2) or as GAL4-BD-fusions (MPK4 and MPK17). Yeast cells were incubated for 4 days at 28°C on either vector selective (CSM-L, W) or interaction selective (CSM-L, W, A) media. The free AD domain was expressed along with BD-fusions for transactivation controls; only MPK17 is shown. For all interactions PJ96-4A was used. This experiment was repeated at least three times with similar results.

Both MPK4 and MPK17 showed interaction with ARR2. It is known that MKKs and MPKs interactions can be observed in yeast. Therefore, the MKK1/2/4/5 were tested against the two MPKs, MPK4 and MPK17. Interestingly, only MPK17 interacted with MKK5. MPK4 interacted with all four MKKs but a stronger interaction was observed with MKK4 and MKK5, especially with a preference for with MKK5 (Figure 4.5).

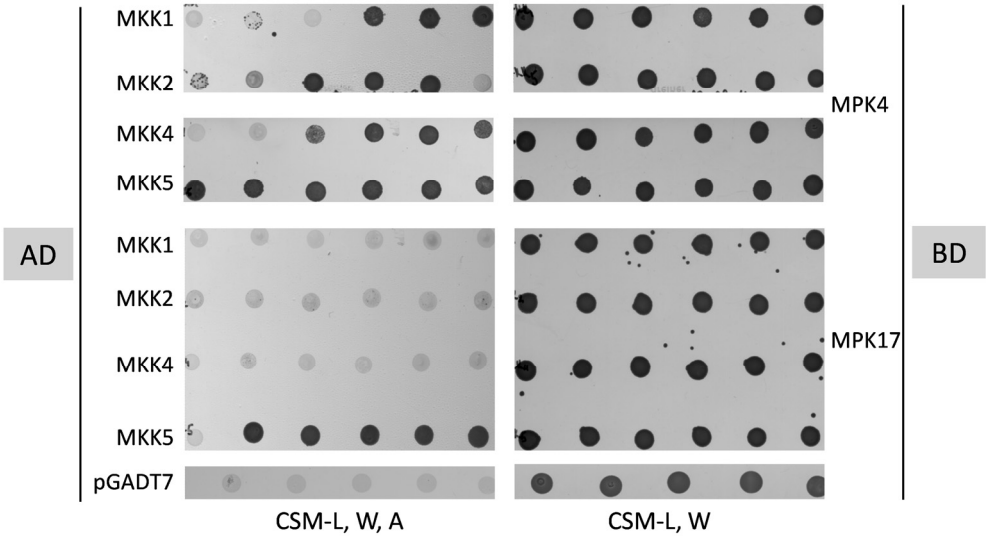


Figure 4.5 MPK4 and MPK17 interact with biotic stress-related MKKs in yeast-two-hybrid assays.

Proteins were expressed either as GAL4-AD-fusions (MKK1, MKK2, MKK4 and MKK5) or as GAL4-BD-fusions (MPK4 and MPK17). Yeast cells were incubated for 4 days at 28°C on either vector selective (CSM-L, W) or interaction selective (CSM-L, W, A) media. The free AD domain was expressed along with BD-fusions for transactivation controls; only MPK4 is shown. For all interactions PJ96-4A was used. This experiment was repeated at least three times with similar results.

4.2.2.4 MKK4 and MKK5 interacted with truncated versions of ARR2 containing only receiver or output domains

In order to get a closer look at which regions of ARR2 are essential for ARR2-MKK interactions, *ARR2* was truncated (Figure 4.6, C). Protein truncation $ARR2^{1-300}$ contains receiver domain, part of the DNA-binding GARP domain and two out of three nuclear localisation signals (NLSs) present in ARR2. $ARR2^{300-664}$ carries the other half of the DNA-binding GARP domain and the third NLS motif. $ARR2^{1-145}$ contains a receiver domain only. $ARR2^{145-664}$ contains the output domain with all three NLSs. $ARR2^{1-165}$ contains the receiver domain and one of three NLSs while the part. $ARR2^{165-664}$ contains other two NLSs and the output domain. This information is summarized in Figure 4.6C as a graphic representation of the ARR2 protein domains, the location of MKK docking sites and the truncation positions.

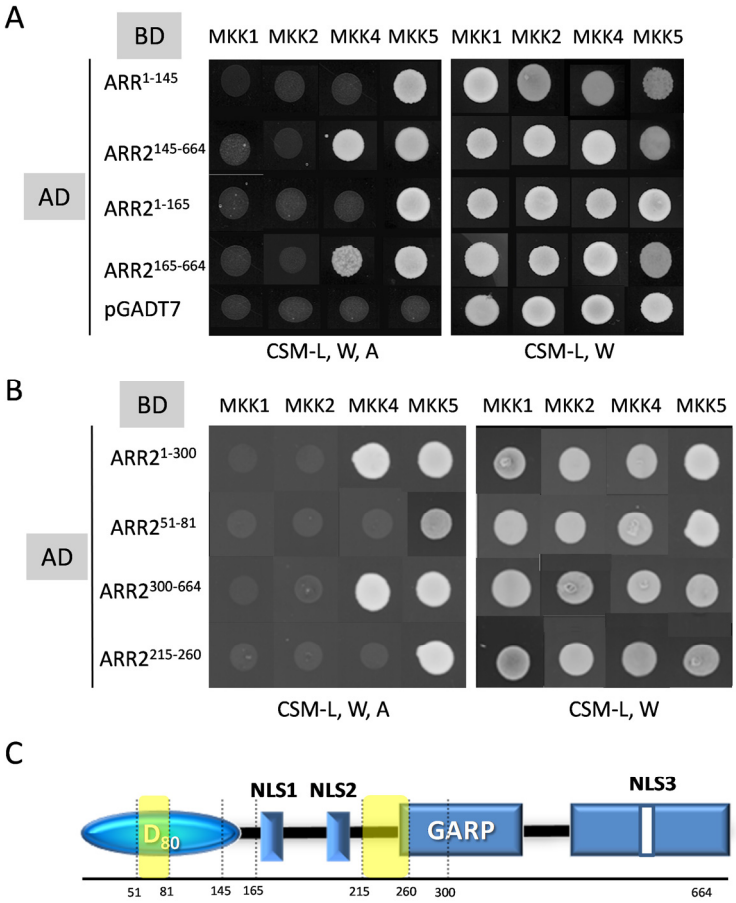


Figure 4.6 MKK docking motifs are present on both receiver and output domains in ARR2. **(A, B)** Proteins were expressed either as GAL4-AD-fusions ($ARR2^{1-145}$, $ARR2^{145-664}$, $ARR2^{1-165}$, $ARR2^{165-664}$, $ARR2^{1-300}$, $ARR2^{300-664}$, $ARR2^{51-81}$ and $ARR2^{215-260}$) or as GAL4-BD-fusions (MKK1, MKK2, MKK3, MKK4 and MKK5). **(C)** Cartoon presentation of the location of the truncated parts of ARR2 with MKK binding sites indicated in yellow. Yeast cells were incubated for 4 days at 28°C on either vector selective (CSM-L, W) or interaction selective (CSM-L, W, A) media. The free AD domain was expressed along with BD-fusions for transactivation controls shown in A. For all interactions PJ96-4A was used. This experiment was repeated at least three times with similar results; representative colonies are shown.

The various ARR2 truncations were tested against MKK1, MKK2, MKK4 and MKK5 for interaction using yeast-two-hybrid system (Figure 4.6, A,B). The receiver domain-containing truncations $ARR2^{1-145}$, $ARR2^{1-165}$ and $ARR2^{1-300}$ extended variant showed positive interaction only with MKK5; for all other tested kinase members no interaction was observed. On the other hand, the truncation containing the output domain $ARR2^{145-664}$, $ARR2^{165-664}$ and the $ARR2^{300-664}$ truncation showed very strong interaction with MKK4 and MKK5, but not with MKK1 or MKK2. These results suggested that there are a couple of different MKK docking sites within ARR2 that are widely dispersed along the protein. It is therefore possible that more than one, or maybe all, binding sites are needed. In summary, the type-B receiver domain and output domain can interact with MKKs.

4.2.2.5 MKK docking motifs are present on ARR2 in both receiver and output domains

MKK4 and MKK5 play an important role in the plant´s response to biotic stress, such as pathogen attack (Asai *et al.* 2002) and stomata development (Wang *et al.* 2007). The interaction of ARR2 with MKK4 and MKK suggested it would be useful to find out more about the number and position of MKK docking sites in ARR2 by applying an *in silco* approach. For this purpose ELM software (http://elm.eu.org) was used. ELM is a computational biology resource for investigating candidate functional sites in eukaryotic proteins reported to have high significance value (Dinkel *et al.* 2012). The docking interaction in the MAP kinase cascades is achieved through specific conserved regions on MKKs (docking groove) and MAPK-interacting molecules (the MKK docking motif). After evaluation of the raw output from ELM (Supplemental figure 1), docking motifs for MAPK cascade interacting molecules (e.g. MKKs, substrates, phosphatases) were predicted. There are at least two different MKK docking types: the classical docking motif and short peptide containing the sequence FxF (Sharrocks *et al.* 2000; Bardwell *et al.* 2001; Galanis *et al.* 2001). The classical docking motif is characterized by a cluster of at least two positively charged amino acids followed by a spacer of 2-to-6 residues from a hydrophobic-X-hydrophobic sequence, where the hydrophobic residues are long-chain aliphatics (usually Leu or Ile).

In the spacer and in the sequence immediately C-terminal to the hydrophobic-X-hydrophobic element, there is a high propensity for the presence of Pro, Asn, and/or Gly, which are residues that are both turn-forming and helix-breaking (Bardwell *et al.* 2001). The classic motif approximates (R/K)X_{2-6}ΨXΨ where Ψ is a hydrophobic residue and X any residue. Four such docking motifs were found in ARR2 (Supplemental figure 1, figure 4.6). Interestingly, two of them were found on the receiver domain of the ARR2 at 56-66 (KCNRAESALSL) and 68-78 (RKNKNGFDIVI) amino acid positions. Two other potential docking domains were located in the output domain of ARR2 at amino acid positions 215-225 (KKPRVVWSVEL) and 244-252 (KKILEMMNV) (Figure 4.6, C). In order to check if these motifs are really docking sites for MKKs two truncation peptides of ARR2 to were made: $ARR2^{51-81}$ and $ARR2^{215-260}$. Only two fragments were made because of the fact that between the potential receiver domain docking sites only a very small physical distance lies between them and such small peptides would most likely be degraded in yeast. Surprisingly, both peptides $ARR2^{51-81}$ and $ARR2^{215-260}$ interacted only with MKK5 and that very strongly; No interactions were obtained with the other MKKs (Figure 4.6, B). This suggests that for all of the other MKKs other docking sites must be present which do not fit into the typical pattern for MKK docking domains, or, an alternative type of interaction domain exists. These experiments confirmed the previous observations in yeast, where truncated versions of ARR2 containing only receiver domain sequence were used, that receiver domain may also play role in ARR2-MKKs interactions and not just the output domain only. Thus it can be concluded that the inability of the ARR4 type-A RR to interact with MKKs is not due to an absence of output domain but rather due to difference in sequence of receiver domains between type-A and the types-B ARRs.

4.2.2.6 ARR2 does not need to be phosphorylated by TCS elements in order to interact with MKKs in Y2H

The ARR2 can obtain a phosphoryl group from the AHP TCS elements on its aspartate at the 80th position (Grefen and Harter 2004). Mutation of D80 to E (glutamate, Glu) increases the transactivation activity of ARR2 (Hwang and Sheen 2001) and leads to severe developmental defects if expressed in plants (Hass *et al.* 2004). Mutation of D80 to N (asparagine, Asn) however, prevents TCS dependent phosphorylation (Kim *et al.* 2006) and impairs basal reporter-gene activation capacity. To check if the phosphorylation state of ARR2 at the D80 position influences MPK(K)-ARR2 protein interaction loss-of-function ARR2D80N mutant and gain of function ARRD80E protein variants were tested in the yeast-two-hybrid system.

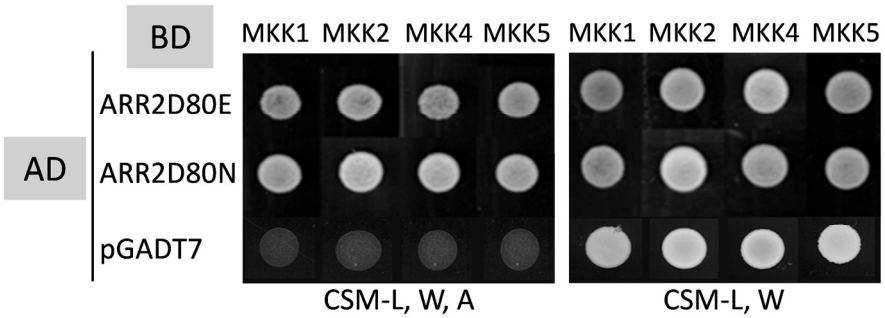


Figure 4.7 ARR2 interacts with the MKKs independent of TCS-mediated phosphorylation.
Proteins were expressed either as GAL4-AD-fusions (ARR2D80E and ARR2D80N) or as GAL4-BD-fusions (MKK1, MKK2, MKK4 and MKK5). Yeast cells were incubated for 4 days at 28°C on either vector selective (CSM-L, W) or interaction selective (CSM-L, W, A) media. The free AD domain was expressed along with BD-fusions for transactivation controls. For all interactions PJ96-4A was used. This experiment was repeated at least three times with similar results.

MKK1-5 were used for this experiment. Both mutant versions of ARR2 showed exactly the same pattern like the wild-type protein (Figure 4.7). This suggests that ARR2 does not need to be phosphorylated by TCS elements for successful interaction with MKKs and that ARR2 might serve as scaffold protein in signal transduction between MAPK cascade elements and the TCS or yet other unknown, undescribed proteins.

4.2.2.7 ARR2 shows very strong interaction with biotic-stress-related MKKs in yeast

MKK1 and MKK2 are known to be involved in response to abiotic stress (*e.g.* osmotic stress, temperature change, high salinity) (Teige *et al.* 2004) whereas MKK4 and MKK5 are known for their role in biotic stress (*e.g.* pathogen interactions) (Asai *et al.* 2002). In the previous yeast experiments some interactions appeared stronger as judged by both the growth intensity of the colonies and the number of colonies that displayed positive interactions. In particular, MKK4 and MKK5 repeatedly displayed stronger interactions with ARR2, its variants and other B-types. Therefore, the interaction strength of the MKK interactions with ARR2 was quantified in yeast using serial dilutions and quantification of reporter-gene strength by the O-NPG assay.

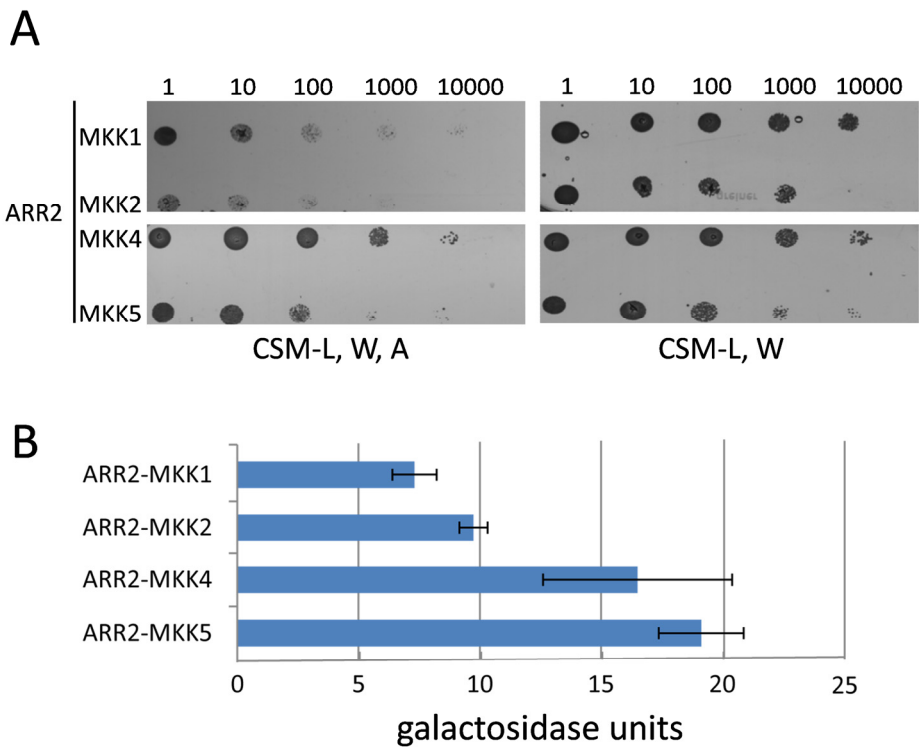


Figure 4.8 ARR2 shows strong interaction with biotic-stress-related MKKs in yeast.
(A) Dilution series of fusion proteins co-expressed from the yeast-two-hybrid expression vectors: GAL4AD-ARR2 and MKK1, MKK2, MKK4 and MKK5 fused to N-terminal GAL4BD. **(B)** The β-galactosidase assay of the same constructs used for dilution series. The β-galactosidase activity was measured in the extracts of three independent yeast clones. Diluted yeast colonies were incubated for 2 days at 28°C on interaction selective (CSM-L, W, A) media. The experiment was repeated at least two times.

Serial dilutions (1, $1:10^1$, $1:10^2$, $1:10^3$ and $1:10^4$) of the corresponding yeast culture was grown on selective medium and transformed PJ64-4A yeast cells with corresponding vectors were used for the O-NPG assay (β-galactosidase assay). Five colonies containing ARR2 and MKK1/2/4/5 were tested. As suspected, ARR2 interacted much stronger with biotic-related MKKs (MKK4 and MKK5) than the abiotic-related ones (MKK1 and MKK2) by the growth assay (Figure 4.8).

While the serial dilution method is informative, the more quantitative and less error-prone method is measuring β-galactosidase reporter-gene activity *via* the O-NPG assay. In this case, the O-NPG assay was extended to include ARR1 and ARR18. As before, ARR2 interacted stronger with MKK4/5 (~17 and ~20 O-NPG units for MKK4 and MKK5 respectively) than MKK1/2 (~5 and ~7 O-NPG units for MKK1 and MKK2 respectively). The same tendency was observed for ARR1 and ARR18 (Supplemental figure 2). It can be therefore concluded that. in general, MKK4 and MKK5 have a stronger affinity for B-type subclass I response regulators.

4.2.2.8 ARR2 shows very strong interaction with biotic-stress-related MKKs in planta

In order to see if this strong and specific interaction between ARR2 and MKK4 and MKK5 obtained in yeast is also the case in plant cells, FRET-FLIM (Förster Resonance Energy Transfer-Fluorescence Life Time Imaging) microscopy analysis was performed *in planta*. For this experiment the fluorescence lifetime of a Donor (i.e. GFP) is measured. A reduction in the lifetime is observed when an Acceptor (i.e. mCherry or mRFP) is within a distance of 10 Å or less; this distance is what is expected of protein-protein interactions (Caesar *et al.* 2011). Since MKK3 is also known to be involved in biotic stress in a JA dependent manner (Doczi *et al.* 2007) and was previously not characterized in yeast due to its strong transactivation effect, MKK3 was also tested against ARR2 *in planta* using the FRET-FLIM approach.

ARR2 was cloned in-frame with *GFP* to make an *ARR2::GFP* fusion. The *MKKs* were therefore cloned in-frame with *mCherry*. A positive readout control was created by fusing *ARR2* to a tandem *GFP::mCherry* coding frame via a short linker and subsequently called *ARR2::FRET*. After cloning, the constructs were transformed into *Agrobacteria tumefaciens* and transiently expressed in tobacco (*Nicothiana bethamiana*) epidermal cells as a heterologous plant system. The negative control was *ARR2::GFP* transformed alone.

All three tested MKKs interacted with ARR2 (Figure 4.9). Both MKK4 and MKK5 interacted with ARR2 stronger than MKK3, with MKK5 having the strongest interaction with ARR2 (Figure 4.9, B). These *in planta* data confirmed the previous data obtained in the yeast-two hybrid system and lead to more stable and concrete evidence that these interactions, especially ones with MKK4 and MKK5, might be have physiologically functional roles in *Arabidopsis* to biotic stresses.

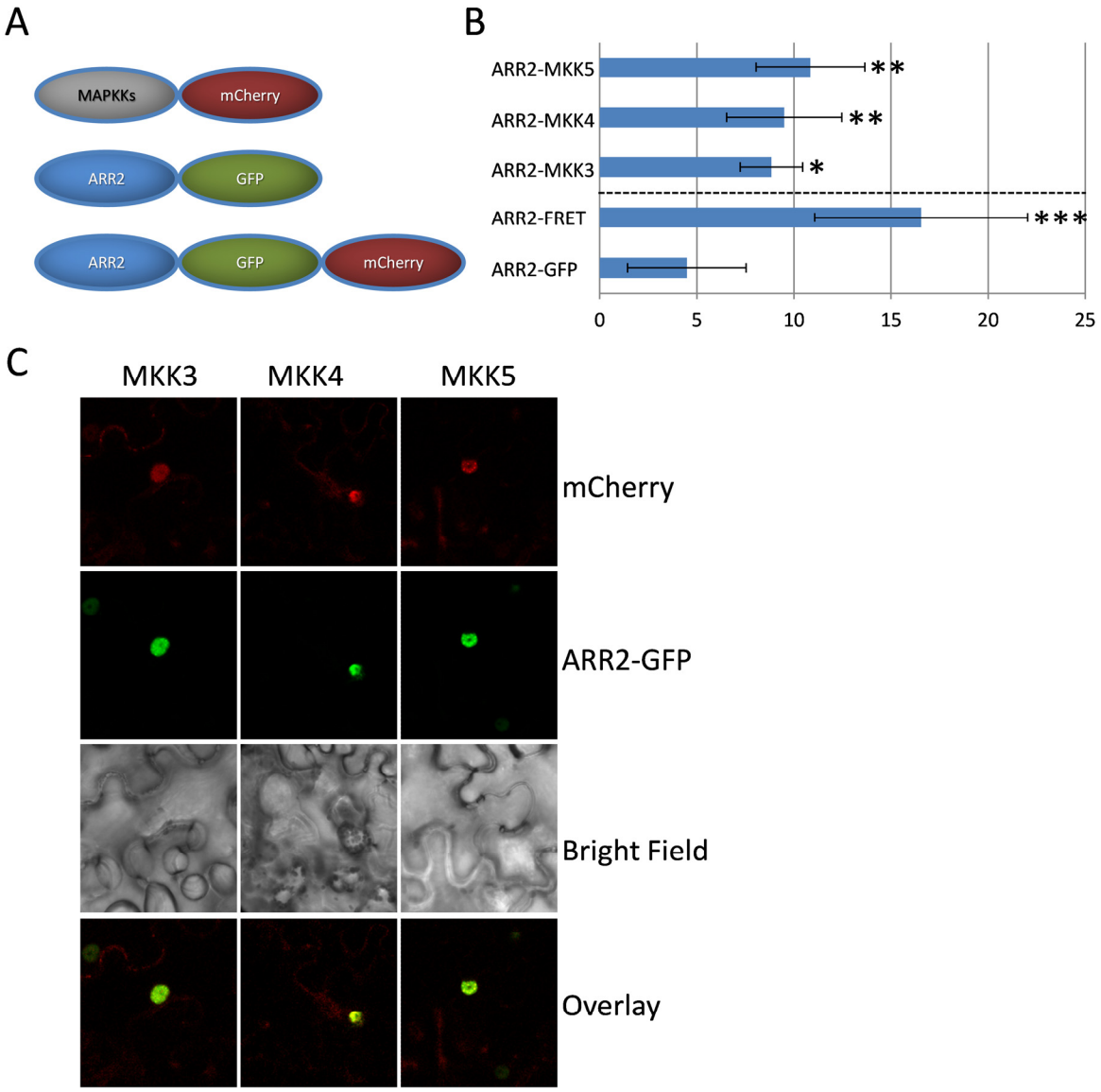


Figure 4.9 ARR2 interacts very strongly with biotic-stress-related MKKs *in planta*.
(A) Cartoon representation of ARR2 and MKKs proteins fused for fluorescent proteins for FRET-FLIM measurements. **(B)** FRET-FLIM results of ARR2-GFP interaction with MKK3, MMK4 and MKK5 fused to mCherry. **(C)** CLSM photos of tobacco leaves showing localization of expressed ARR2-GFP fusion protein with indicated MKKs fused to mCherry fluorescent protein. The fusion proteins were transiently expressed in tobacco cells and the results are given as function of Chi^2 test (measurements and evaluation courtesy of Dr. Kirstin Elgass). ARR2-GFP and ARR2-GFP-mCherry were used as negative and positive controls respectively. This experiment was repeated two times with similar results.

4.2.3 Roles of *ARR2* in pathogen-related phenomenon

As previously shown, FRET-FLIM *in planta* measurements and experiments using the yeast-two-hybrid system connect TCS type-B RRs to MKKs *via* physical interaction. ARR2 especially interacted strongly with MKK3, MKK4 and MKK5. Additionally, ARR1 and ARR2 interacted with MPK4 and MPK17 as well. MKKs play an important role in the plant´s response to biotic stress, such as pathogen attack (Asai *et al.* 2002). And interestingly, this is also the case for all three *in planta* tested MKKs: MKK4 and MKK5 (Nakagami *et al.* 2005; Popescu *et al.* 2009) as well as MKK3 (Doczi *et al.* 2007) clearly implicating the involvement of *ARR1* and *ARR2* in pathogen defence.

4.2.3.1 Reactive Oxygen Species (ROS) and ethylene measurements in ARR1 and ARR2 mutants after treatment with Pathogen-Associated Molecular Patterns (PAMPs)

As a first look into pathogen-related responses in ARR2, wild-type and mutants *arr1-4, arr2-4* and *arr1-4 arr2-4* lines were treated with PAMPs. As output, ROS and ethylene production were measured.

4.2.3.1.1 *The arr1-4 arr2-4* double mutant showed differences in ROS production after treatment with flg22 and elf18

For ROS experiments, leaves from 5 to 6 week-old plants were used. Leaves were cut in discs from the same leaf surface and incubated in water overnight.

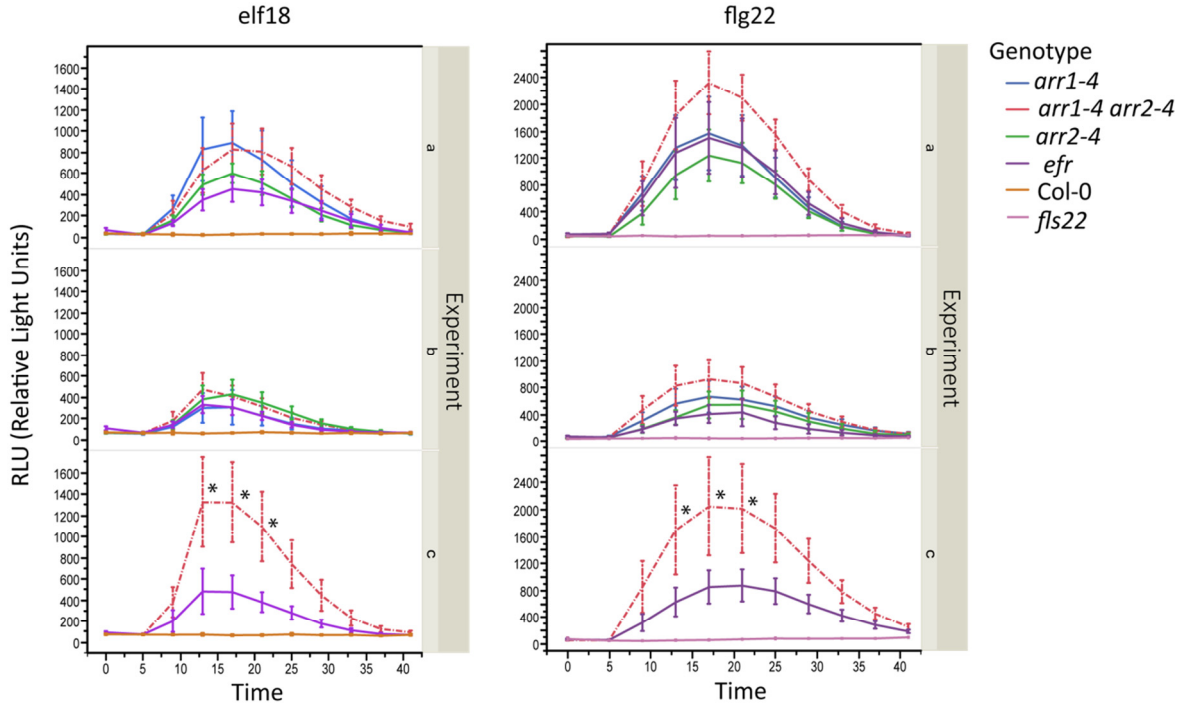


Figure 4.10 *arr1-4 arr2-4* double mutant shows differences in ROS production after treatment with PAMPs.

Arabidopsis leaves from different mutants were treated with elf18 (left) and flg22 (right). Mutants *efr* and *fls22* were used as negative response controls. Letters a, b and c stand for biological experimental repetitions whereas in the c repetition only the *arr1-4 arr2-4* double mutant was used along with control plants. Fisher's Least Significant Difference (LSD) test was used to compute significance levels at the 0.05 α level separately for the time points 13, 17 and 21 minutes.

The following day, the discs were transferred to a reaction solution and the ROS production was triggered by treatment with 100nM of flg22 or 100nM elf18. In addition to the type-B mutant lines, *fls2* (Dunning *et al.* 2007; Krol *et al.* 2010) and *efr* mutants lacking leucine rich repeat-receptor-like kinases *FLS2 (Flagellin-Sensitive 2)* and *EFR (Elongation Factor-Tu receptor*), respectively (Krol *et al.* 2010). FLS2 recognizes bacterial flagellum (active epitope flg22) and EFR recognizes elongation factor Tu (active epitopes elf 18, elf13 and elf26).

Both times the same tendency was observed: *arr1-4 arr2-4* double mutant significantly produced more ROS than the wild-type (p≤0,05 at time-points 13, 17, 21 min post treatment) in response to both applied PAMPs. Although there were differences between the single mutants, these observations were not consistent between experiments; the double mutant consistently however showed more ROS than the wild-type (Figure4.10, experiment a verses b). Therefore, a third experiment was conducted using more replicates to confirm the previous two experiments. It could be concluded that the *arr1-4 arr2-4* double mutant significantly produces more ROS in response to applied PAMPs, flg22 (p≤0,05 at time points 13, 17, 21 min post treatment) and elf18 (p≤0,05 at time-points 13, 17, 21 min post treatment) but it is unclear what the effect of the single mutants have on ROS production (Figure 4.10).

4.2.3.1.2 *The arr1-4 arr2-4* double mutant did not show any difference in ethylene production after treatment with different PAMPs

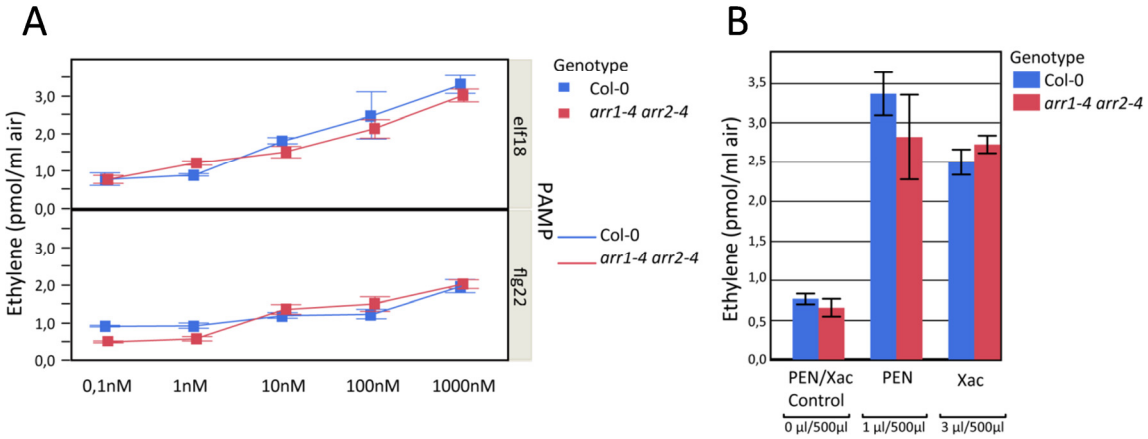


Figure 4.11 *arr1-4 arr2-4* double mutant shows no difference in ethylene production after treatment with PAMPs

Arabidopsis leaves from *arr1-4 arr2-4* double mutant and Col-0 wild-type were treated with **(A)** elf18 and flg22 using different concentrations: 0nM, 1nM, 1nM, 10nM, 100nM and 1000nM **(B)** Fungal extracts used were 1µl of PEN and 3µl of Xac in a final volume of 500µl. Fisher's Least Significant Difference (LSD) test was used to compute significance levels at the 0,05 α level. This experiment was performed as two biological replicates each containing three technical replicates.

Ethylene production was measured as a response to different PAMPs flg22 and elf18 or crude extracts PEN from *Penicilium schizogonium* and Xac from *Xanthomonas axonopodis citri*. A dose response curve was obtained for Flg22 and Elf18 (0.1nM, 1nM, 10nM, 100nM, 1µM) but only single concentrations 1µL/500µL PEN and Xac 3µL/500µL for the crude extracts was

conducted. Based on results from obtained the previous ROS measurements and due to lack of growth space, only Col-0 wild-type and *arr1-4 arr2-4* double mutant lines were used. The double mutant did not show any statistically significant difference in ethylene production to any of the tested PAMPs compared to the wild-type (Figure 4.11, A, B). Based on this experiment, it can be concluded that either there is no significant difference in ethylene production. Alternatively, perhaps the analytical system was not sensitive enough to detect subtler changes in ethylene production compared to that of the ROS system.

4.2.3.1.3 *The arr1-4 arr2-4* double mutant did not show any difference in activation pattern of MPK3, MPK4 and MPK6 after treatment with flg22

The MPK kinases MPK3, MPK4 and MPK6 are known to be activated by phosphorylation in a flg22 dependent manner (Droillard *et al.* 2004). Activation of these MPKs can be analysed by using the phospho-p44/p42 MPK antibody (Brock *et al.* 2010; Montillet *et al.* 2013). As previously observed, flg22 was able to elicit a higher ROS response in the *arr1-4 arr2-4* mutant background (§4.2.3.1.1). Thus by conclusion, it is possible that these MPK kinases are differentially activated in the *arr1-4 arr2-4* mutant background compared to the wild-type. For this experiment 4 week-old plants were used and their leaves were infected by dropping of 1µM solution of flg22 or mock onto a leaf surface. Samples were collected at three time points: immediately after infection, 15 minutes and 30 minutes after treatment (Figure 4.12).

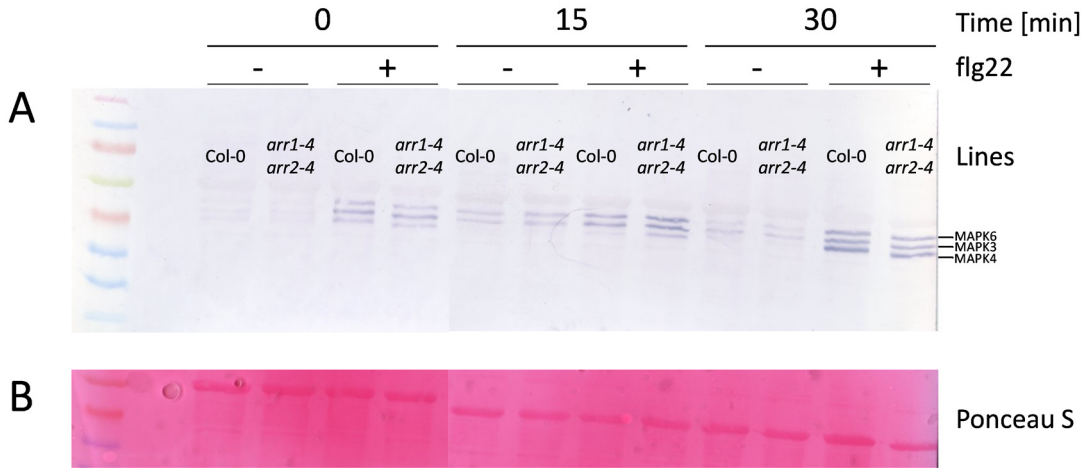


Figure 4.12 No differences in MPK3/4/6 phosphorylation pattern induced by flg22 elicitor are observed between *arr1-4 arr2-4* double mutant and Col-0 wild-type.
(A) Western blot of protein extracts from *Arabidopsis* leaves taken from *arr1-4 arr2-4* double mutant and Col-0 treated with flg22. The phospho-p44/p42 MPK Antibody was used to detect phosphorylated MPK3/4/6. **(B)** Membrane stained with Ponceau S shows equal loading quantity for all samples. PAMP flg22 was used in 1µM concentration and the samples were collected at 0 min, 15min, and 30 min after treatment. At least five different plants per line were used and two leaves per plant were treated with flg22 or MOCK and the material pooled. This experiment was repeated three times with similar results.

Before flg22 treatment only weak signals were obtained for MPK6 and MPK3 showing that these two kinases are phosphorylated at some basal level. After 15 minutes of flg22 treatment, the signals for MPK3/6 became much stronger and a third band representing MPK4 appeared (Figure 4.12). Half an hour after infection the phosphorylation pattern stayed

the same as the 15 minute time point, indicating that the peak flg22-depdenent MPK activation level was already reached at the 15th minute or before. Mock treated leaves at 15 and 30 minutes after treatment maintained the same basal MPK3/6 pattering as the zero time point. In conclusion, no dramatic differences were observed between the wild-type and the double mutant in activation pattern or intensity with any of these three kinases.

4.2.3.2 Pathogen assays using biotrophs and necrotrophs

A broad spectrum of different pathogens was used in this work to cover both necrotrophic and biotrophic types of infection strategies. These pathogens were challenged on Col-0, *arr1-4, arr2-4* and *arr1-4 arr2-4* plant lines. It was very important to obtain various measurements of a given interaction with high spatial and temporal resolution. For necrotrophs, *Alternaria brasisicola* and *Botrytis cinerea* were tested. For biotrophs, the obligate biotrophic fungus *Peronospora parasitica* was tested as well as the biotrophic/hemibiotrophic bacterium *Pseudomonas syringae pv. tomato* DC3000 *(Pto* DC3000*).*

4.2.3.2.1 Pathogen assays using biotrophs

Pathogen assays with biotrophs were always performed using one bacterial and one fungal pathogen: the bacterium *Pto* DC3000 and fungus *Peronospora parasitica*.

4.2.3.2.1.1 There is no difference in response of ARR1 and ARR2 mutants to biotrophic Pst DC3000

This pathogenic assay was done using five-week old plants and treating them with *Pto* DC3000 bacterial strain. Bacterial cells were infiltrated into two leaves per plant. Bacterial cells at an approximate concentration of 10^4 cfu/ml were infiltrated with a needless syringe into the middle of the leaf apoplastic space (two leaves per plant) to an area was always bigger than the cork borer used for cutting the leaf discs with which the experiment was further performed. Afterwards the leaves were harvested, at the time point 0h and 1st, 2nd and 4th days past infection, material was plated on LB-plates with antibiotics and number of colonies per plate/mutant formed was counted.

Based on number of grown colonies per plate the difference in resistance between the mutants was determined. Although this experiment was repeated six times none of them consistently showed any pattern that could be attributed to the loss of *ARR1* or *ARR2* or both. That is, any differences that could be observed in the second and fourth day were never reproduced in any other experiment (all repetitions are not shown but compare 4.13A with the 4.13B mock for an example.

Knowing that ARR2 has been implemented in *Pto* DC3000 pathogen resistance and that this response required both cytokinin and SA (Choi *et al.* 2010), a cytokinin treatment was added to the experimental design. *t*-zeatin was used at a working concentration of 1μM. Before infiltration cytokinin was given to the $1x10^4$ cfu/ml bacterial dilution. The plants were also treated with cytokinin 3 days before the experiment by adding it to the water used for watering. No clear trend could be observed that could be ascribed to a combined effect of exogenous cytokinin and loss of *ARR1* or *ARR2* could be found. In fact, in this experiment, the cytokinin treatment resulted in reduced bacterial growth in all plant lines which was highly

statistically significant by day 4 (Supplement figure 3). This effect of cytokinin on *Pto* DC3000 growth has been observed before (Choi *et al.* 2010; Choi *et al.* 2011).

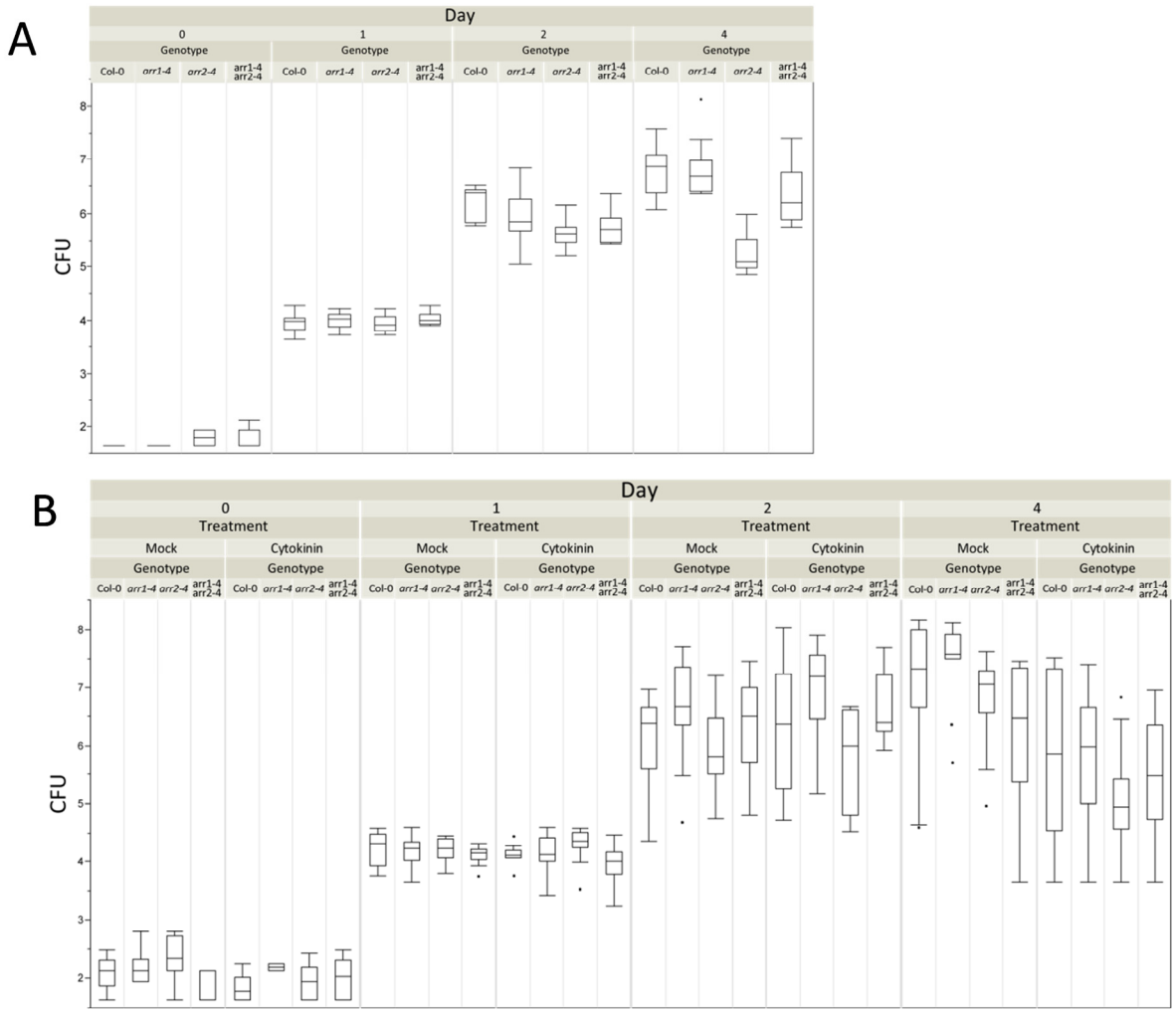


Figure 4.13 *ARR1* and *ARR2* mutant plants do not show reproducible differences in response to biotrophic *Pseudomonas syringae* pv. tomato DC3000.

Leaves of *Arabidopsis ARR1* and *ARR2* single and double mutants and corresponding wild-type (Col-0) were treated with *Pseudomonas*. The evaluation of infection was done at day 0, 1, and 4. **(A)** Plant lines treated only with *Pseudomonas syringae* pv. tomato DC3000. **(B)** Plant lines treated either with *Pseudomonas syringae pv. tomato* DC3000 only (Mock) and also with 1μM t-zeatin (cytokinin). Fisher's Least Significant Difference (LSD) test was used to compute significance levels at the 0.05 α level (Supplemental figure 3). These experiments were repeated at least three times each with similar results. CFU stands for Colony Forming Units. Data are represented as box-and-whisker plots: the central horizontal line is the median (2nd quartile) and the boxed region extends from the beginning of the 1st quartile to the end of the 3rd quartile. Lines extending outside of the boxed region are 1.5 x (the respective interquartile range). Data points outside this region are shown as dots and are called outliers.

4.2.3.2.1.2 *There is no difference in response of ARR1 and ARR2 mutants to Peronospora parasitica*

Next *arr1-4 arr2-4* plants were challenged with *Peronospora parasitica*. This experiment was performed in collaboration with the research group of Prof. Dr. Volker Lipka from University of Göttingen. Four to six-week old plants were inoculated by spraying the leaves with a *Peronospora parasitica* conidial suspension and the infected plants were observed after seven days.

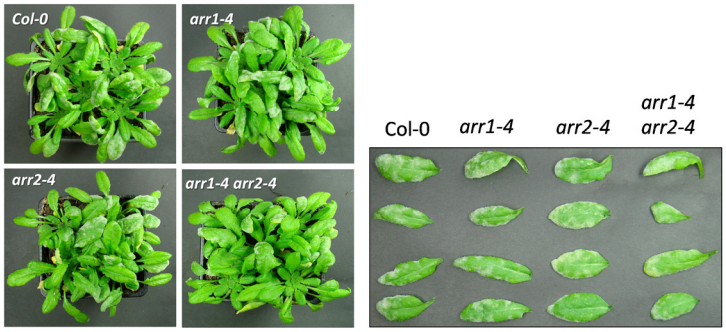


Figure 4.14 *ARR1* and *ARR2* mutant plants do not show differences in response to biotroph *Peronospora parasitica*.
Leaves of *Arabidopsis* wild-type (Col-0), *ARR1* and *ARR2* single and double mutants were sprayed with *Peronospora*. After seven days there were no visible differences among the mutant plants and the wild-type. This experiment was repeated once with similar results. Data were produced by the research group of Prof. Dr. Volker Lipka.

If there is any difference in resistance to *Peronospora*, a clear phenotypic difference would have been visible. However, since there was no visual difference obtained between the wild-type and the mutant plants there was not any need for counting of sporangiophores on the leaves (pers. communication V. Lipka). It was concluded that the mutant plants do not show any difference in resistance to *P. parasitica* compared to the wild-type (Figure 4.14).

4.2.3.2.1.3 *Lack of differential response to biotrophs Pst DC 3000 and Peronospora parasitica is not due to perturbation of stomata aperture regulation*

It was proposed that a larger stomata aperture would make it easier for bacteria to enter into the plant, infect cells and form a stabile microfilm in vascular bundles allowing it to further multiply and invade the plant. It is also known that cytokinin can cause stomata to open (Tanaka *et al.* 2006) and abscisic acid (ABA) causes them to close (Desikan *et al.* 2006). Initially, *Pst* DC3000 also causes stomata to close (Zeng *et al.* 2010; Desclos-Theveniau *et al.* 2012), however after 3 hrs, the bacteria produce diffusible phytotoxin COR that reopens closed stomata to increase the number of sites for bacterial invasion (Melotto *et al.* 2008). Therefore an experiment was designed to test this hypothesis.

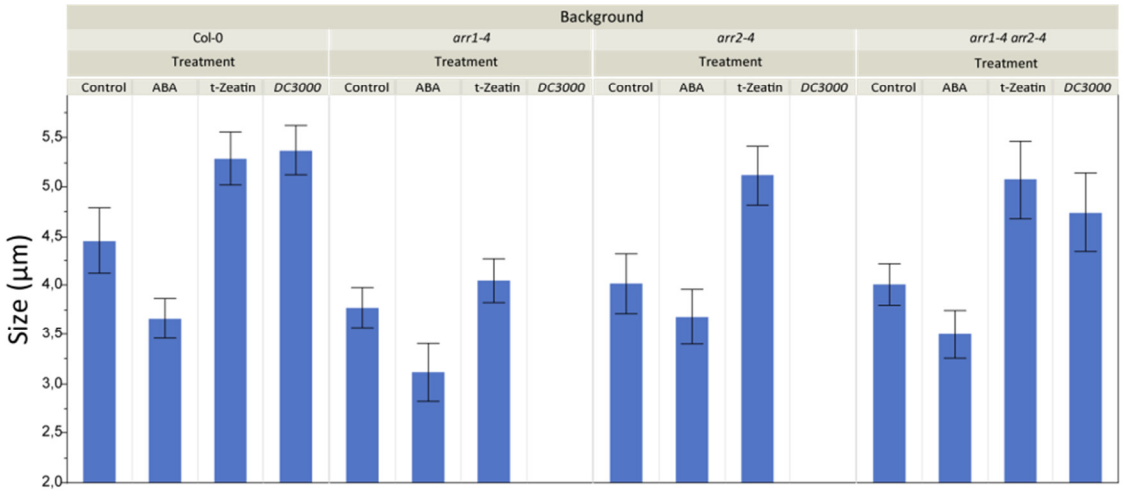


Figure 4.15 Regulation of stomata aperture in *ARR1* and *ARR2* lacking mutants in response to hormonal treatment with ABA and *t*-zeatin and *Pst DC 3000 Pseudomonas* strain

For treatments with hormones at least five plants per line were used and from those 2 leaves per plant were treated with the hormones. Experiments with *Pst DC 3000* were done only for *arr1-4 arr2-4* double mutant and Col-0 whereby five plants per line were used and the stomata aperture size was measured 3 days after infection. This experiment was repeated once with similar results. The mean and SE is shown. Leaves were floated on water or water containing 10µM ABA or 1µM *t*-zeatin (see §3.6.11 for details). For statistical results see Supplemental Figure 4.

Leaves were treated with water (Control), ABA, *t*-zeatin for 2 hours or three days after *Pst* DC3000 infection. The results are displayed as bar graphs (Figure 4.15) and the significance tests results are given in Supplemental Figure 4. Importantly for this work, it is known that ABA-dependent stomata closure is not dependent on ARR2, as the *arr2-4* responds like wild-type (Desikan *et al.* 2006). This response was replicated; that is, the stomata apertures were significantly closed in response to ABA treatment. On the contrary, treatment with *t*-zeatin resulted in a significant increase in stomata aperture for all plant lines. Plants sprayed with *Pst* DC3000 also had stomata apertures wider than the non-treated controls (data only for Col-0 and *arr1-4 arr2-4* were obtained). The data showed that there was no difference in stomata aperture response in the mutant backgrounds compared to the wild-type. Furthermore, all plant lines responded the same way after treatment with different hormones and after application of *Pst* DC3000 by spraying (Figure 4.15).

In conclusion, based on the preceding pathogen experiments and this stomata aperture measurement, there is no difference in the resistance of *arr1-4, arr2-4* or *arr1-4 arr2-4* plants to *Pseudomonas syringae Pst DC3000* under my experimental conditions.

4.2.3.2.2 Pathogen assays using necrotrophs

Two necrotrophic fungi *Alternaria brassicola* and *Botrytis cinerea* were challenged with Col-0 wild-type and the *arr1/2 mutants*. Resistance against *Alternaria* requires the *Arabidopsis* phytoalexin, camalexin and jasmonic acid (JA)-dependent signalling pathways, respectively (Van Wees *et al.* 2003). *Botrytis cinerea* is a natural necrotrophic pathogen of *Arabidopsis thaliana* (P *et al.* 2007) and is considered as a typical necrotroph that co-opts programmed cell death pathways in the host to achieve infection (Amselem *et al.* 2011; Dean *et al.* 2012).

4.2.3.2.2.1 arr2-4 single and arr1-4 arr2-4 double mutants are more susceptible to Alternaria brassicicola

Alternaria brassicicola was applied as a sporal suspension dropped (5µl droplets of spore solution of $5*10^5$ Spores/ml) onto *Arabidopsis* leaves (two leaves per plant) and scored on the 7^{th} and 10^{th} day after infection using a disease progression index as in (Kemmerling *et al.* 2007) and summarized in Methods (§3.6.16). Statistically significant differences were obtained between *arr2-4* single, and *arr1-4 arr2-4* double mutants compared to the WT (Col-0) were computed using the mean. Single *arr2-4* ($p \leq 0.001$) and the double *arr1-4 arr2-4* mutants ($p \leq 0.001$) showed statistically significant susceptibility compared to the wild-type where the difference in *arr1-4* single mutant ($p \leq 0.01$) was not statistically significant from wild-type (Figure 4.16, A).

The same experiment was therefore repeated in presence of 1 µM exogenous cytokinin, *t*-zeatin. Similar to the previous experiment with *Pst* DC3000, before the assay was performed, cytokinin was added to the fungal dilution. The plants were also treated with cytokinin by mixing it with water used for watering 3 days before the experiment was performed. Cytokinin induced some kind of resistance in all of the plants (Figure 4.16, B) and this resistance was statistically significant (see Supplementary Figures 5 and 6, Figure 4.16B). At the day 7 after treatment the disease index of cytokinin treated plants decreased from 270.8 by mock treated plants to 257.5 ($p \leq 0.0029$, LSD (Fisher's Least Squared Difference Test). The same tendency was observed 10^{th} day after treatment where the disease index of treated plants decreased from 306.0 to 294.4 ($p \leq 0.0066$ LSD (Fishers Least Squared Difference Test).

The *arr2-4* and the double mutant are more sensitive irrespective of the cytokinin treatment to *Alternaria* which was statistically confirmed (see Supplemental Figure 5 and 6). In contrast, wild-type plants and the *arr1-4* single mutant did not show differences in their disease indices. This means that *arr2-4* is responsible for susceptibility phenotype observed in the double mutant (Figure 4.16, B).

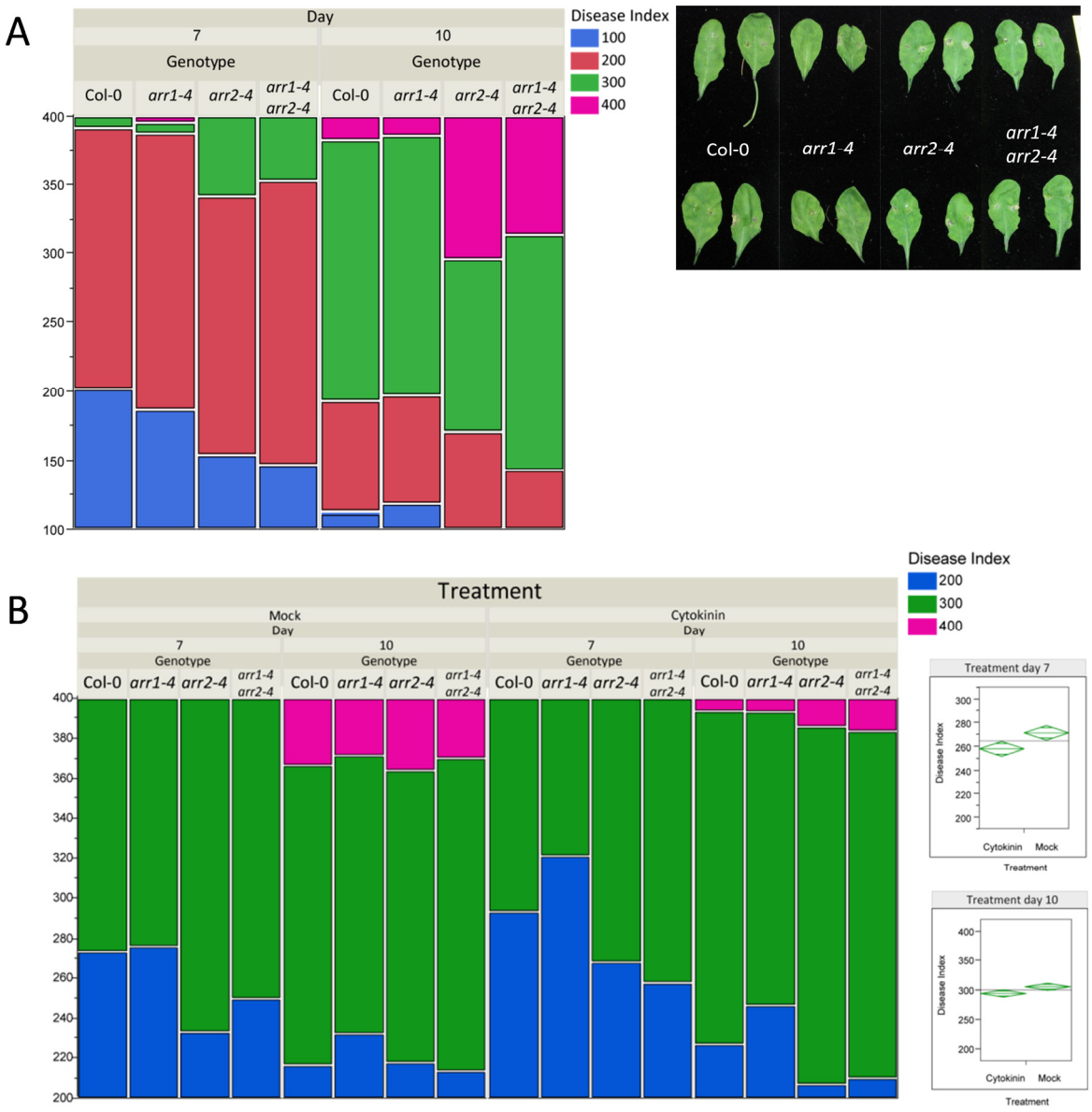


Figure 4.16 *Arabidopsis thaliana arr2-4* single and *arr1-4 arr2-4* double mutants are susceptible to necrotroph *Alternaria brassicicola*

Leaves of *arr1-4, arr2-4, arr1-4 arr2-4* and Col-0 were treated with *Alternaria* spores. The infection was evaluated at day 7 and 10. The data are shown as mosaic plots which are a graphical representation of an automatically computed two-way frequency table (also called a Contingency Table) (A) Plant lines only treated with *Alternaria brassicicola* spores. Image insert to the right shows a representative leaf composition at Day 7 (B) Plant lines treated either with *Alternaria brassicicola* spores only (Mock) and also with 1µM t-zeatin. To the right, the pooled mean and ANOVA 95% confidence interval for all samples either treated or not treated with cytokinin is shown for Day 7 (ANOVA $p \leq 0.0029$) and Day 10 (ANOVA $p \leq 0.0066$); the cytokinin treated samples showed less disease symptoms. LSD (Fisher's Least Squared Difference Test) was also used to compute significance levels at the $\alpha=0,05$ level based on the mean (Supplemental Figures 5 and 6). For this experiment at least 20 plants per line were used and two leaves per plant were infected with *Alternaria* spores. This experiment was repeated at least three times with similar results.

4.2.3.2.2.2 arr1-4 arr2-4 double mutant is resistant to Botrytis cinerea

Botrytis cinerea is especially interesting as it is known to be a natural pathogen of *Arabidopsis thaliana*. *Botrytis cinerea was* applied to the single mutants, double mutant and wild-type by dropping a fungal suspension on the leaves. Susceptibility to *B. cinerea* was determined using a detached leaf assay and a visually identified by a lesion disease index. Lesion size has been used as an indicator of susceptibility to *B. cinerea* (Ferrari *et al.* 2003; Denby *et al.* 2004) and this has been shown to correlate with whole plant susceptibility and pathogen growth within the plant (Govrin and Levine 2000; Denby *et al.* 2004).

This experiment was performed in collaboration with Floriane L'Haridon from research group of Prof. Dr. Jean-Pierre Métraux from University of Fribourg, Switzerland. Hyphae and conidiophores were visually identified throughout the lesions and the infection sites were categorized according to their lesion sizes which are divided into three groups: Group 1 are lesions less than or equal to 2mm, group 2 lesions between 2 mm and 6 mm and the group 3 are lesions bigger than 6 mm. To reflect different severities of infection (Figure 4.17, A) the outgrowing lesion size was also determined (Figure 4.17, B) as well as the average lesion size (Figure 4.17, C). The lesions were measured three days after application of the pathogen.

As the data are categorical, they are presented in Figure 4.17 A and B as mosaic plots; alternative plots and statistics can be found in Supplemental Figure 7. The categorized lesion size is shown in Figure 4.17A and average lesion size is shown as a bar graph of the mean (Figure 4.17, C). As can be seen in Figure 4.17 A and B the severity of in infection was reduced in all mutant lines compared to Col-0. Col-0 had the majority of its lesions in Category 3 and its overall outgrowth index at 75% or more (87.5% of all lesions). In contrast, each *ARR* mutant had more lesions in Category 1 and 2, less in 3 (Figure 4.17, A). In fact looking only at Category 3 results, Col-0 was infected better with over 58.3% its lesions bigger than 6 mm. In contrast, the double mutant had only 31.3% of its lesions bigger than 6 mm. The single mutants showed in infection levels in-between wild-type and the double mutant: *arr2-4* (40.6% ≥6 mm) and *arr1-4* single mutant (43.8% ≥6 mm). This difference in infection efficiency is also evident from inspection of the outgrowth index, whereby only 58.3% of *arr1-4*, 54.2% of *arr2-4* and 50.0% of *arr1-4 arr2-4* leaves had an outgrowth index at 75% or more.

The average lesion size (Figure 4.17, C) again showed a clear picture of mutant resistance. Average lesion size for the wild-type was about 5.77 mm whereas the single mutants this value decreased to 4.39 mm for *arr1-4* and 3.99 mm for *arr2-4*. In the double mutant the average lesion size was 3.52 mm only. Leaves treated with inoculation media alone did not develop any lesions or lesion-like symptoms (data not shown).

All of these parameters very clearly show that *arr1-4 arr2-4* double mutant is more resistant to *Botrytis* compared to Col-0 wild-type. Interestingly the same tendency was observed in the single mutants as well, but only the difference by the double mutant was statistically significant when ascertained for average lesion size. These results clearly show the redundancy (additive effect) of the *ARR1* and *ARR2* in respect to defense against *Botrytis*.

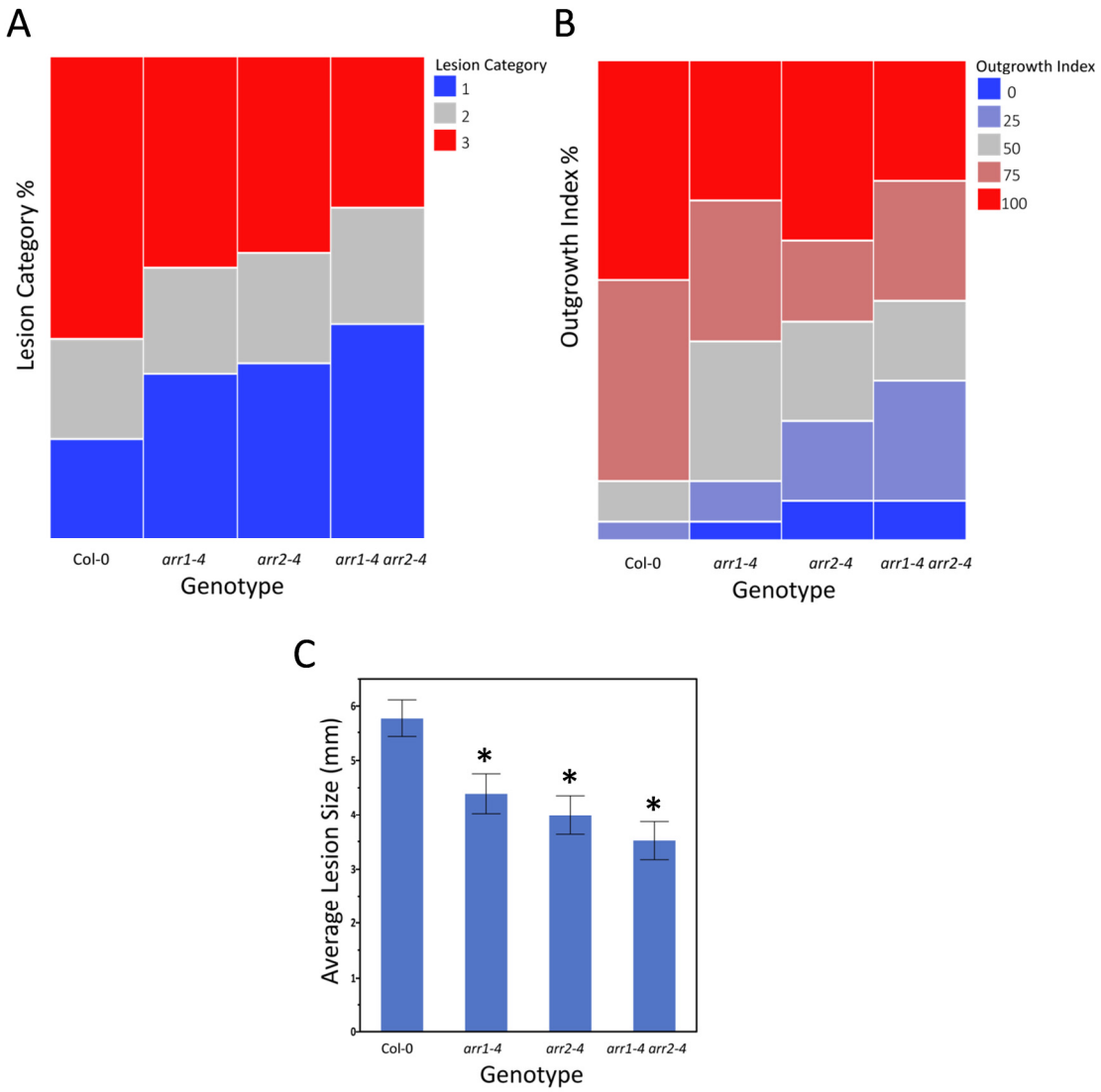


Figure 4.17 *Arabidopsis thaliana arr1-4 arr2-4* double mutants is susceptible to necrotroph *Botrytis cinerea*

Leaves of *Arabidopsis ARR1* and *ARR2* single and double mutants and corresponding wild-type (Col-0) were treated with *Botrytis cinerea*. The evaluation of infection was done 48hrs after the treatment. Data for (A,B) are shown as mosaic plots which are a graphical representation of an automatically computed two-way frequency table (also called a Contingency Table). Data in C is the mean and SE. **(A)** Susceptibility of plant lines to *Botrytis cinerea* are visually presented in a mosaic plot by lesion disease index (Category 1: ≤ 2mm, Category 2: 2-6mm, Category 3: >6mm). **(B)** Susceptibility of plant lines to *Botrytis cinerea* are visually presented in a mosaic plot by outgrowth index in % **(C)** Average lesion size of the tested plant lines in mm. LSD (Fishers Least Squared Difference Test) was used to compute significance levels at the α ≤ 0.05 level using the mean; asterisk shows a significance difference compared to Col-0 only. For this experiment at least 20 plants per line were used. This experiment was repeated two times with similar results.

4.2.3.3 Response of common marker genes PR-1 and PDF1.2 with respect to Botrytis cinerea

Knowing that the double mutant was more resistant to *Botrytis* compared to the wild-type, leaf material of treated and not treated plants was harvested and the transcript levels of *PR-1* and *PDF 1.2* were quantified by RT-qPCR. *PR-1* and *PDF1.2* are induced in response to a variety of pathogens (Penninckx *et al.* 1996; Schenk *et al.* 2000). The *PR-1* gene has been used to elucidate transcriptional control mechanisms regulating SAR (Pape *et al.* 2010) and expression of *PR-1* is salicylic-acid responsive (Metraux *et al.* 1990; Zhang *et al.* 2013).

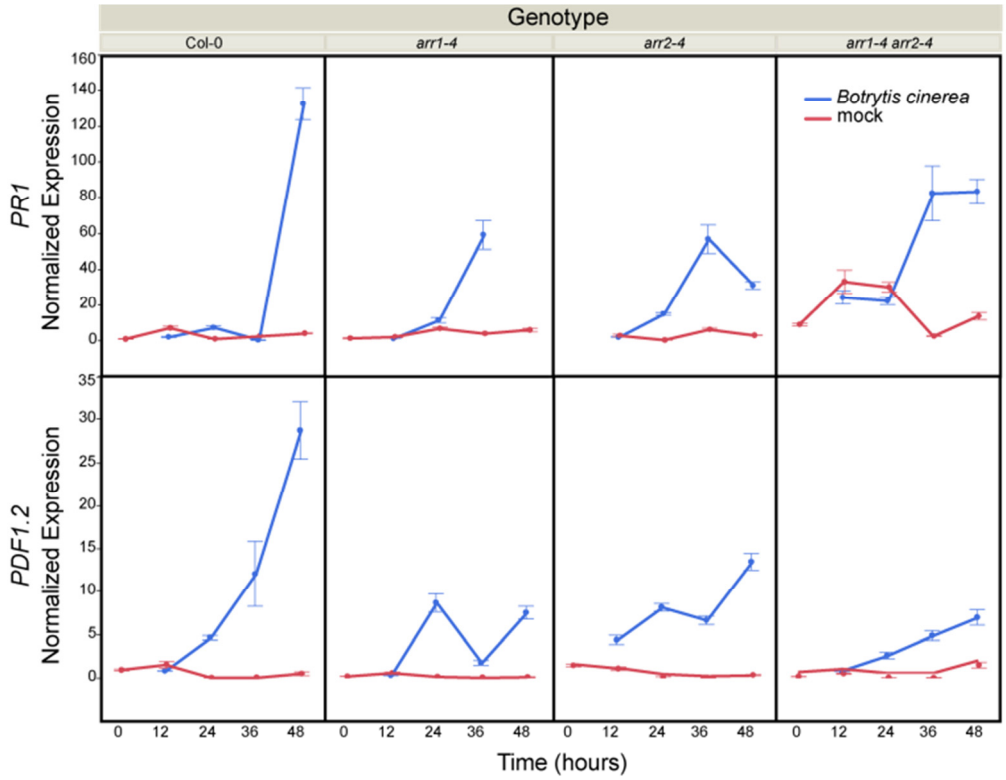


Figure 4.18 Response of common marker genes *PR-1* and *PDF1.2* after treatment with *Botrytis cinerea*

Relative expression levels of *PR-1* and *PDF1.2* genes in *arr1-4, arr2-4, arr1-4 arr2-4* and Col-0. Gene expression was measured after 0, 12, 24, 36 and 48 hrs after treatment with *Botrytis*. For this experiment all of the treated material for each plant line from both biological experiments (§4.2.3.3) was pooled together. This mRNA extraction and RT-qPCR was repeated two times with similar results. For each gene, time point 0 for Col-0 was used as reference and *EF-1-α* was used as the internal control to calculate ΔΔCT values.

PDF1.2 belongs to the plant *defensin (PDF)* family encodes an ethylene- and jasmonate-responsive plant defensin protein (Penninckx *et al.* 1998; Ferrari *et al.* 2003). *PDF1.2* mRNA levels are not responsive to salicylic acid treatments (Manners *et al.* 1998) although jasmonate and salicylic acid can act synergistically to enhance the expression of this gene (Mur *et al.* 2006; Koornneef and Pieterse 2008).

For this experiment all of the treated material for each plant line from both biological experiments (§4.2.3.3) was pooled together. Infected leaves from both treated and non-treated plants were harvested after 0 hrs, 12 hrs, 24 hrs, 36 hrs and 48 hrs post inoculation

and the mRNA expression levels for *PR-1* and *PDF1.2* were quantified. In non-treated plants *PR-1* was, as expected, very low expressed in all harvested time points. The first significant up-regulation of the *PR-1* was after 24 h where the mRNA level was higher in all mutants compared to the wild-type. This trend in rising expression continued until 36 hrs after inoculation and the levels of *PR-1* were still higher in the mutants. However, there was no difference in expression among the mutants themselves (Figure 4.18). Two days after inoculation (48 hrs) the expression of *PR-1* in all mutants maintained the same level as seen at 36 hrs while expression in the wild-type was increased. Thus, although the basal levels of *PR-1* was higher in the mutant plants, the pattern of induction with respect to *Botrytis* was not altered in any way.

Expression level of *PDF1.2* was very low in non-treated plants independently of the time point and the first increase of expression among the inoculated plants was visible 24 hrs after inoculation. The same tendency in all mutants and the wild-type plants was observed as an up-regulation at 24 hrs and 36 hrs post inoculation. Up until the 36 hrs time point, no difference was observed between either single mutants or the wild-type. However a difference was obtained by the double mutant with the level of *PDF1.2* down-regulated. After 48 hrs, the expression level of *PDF1.2* in the wild-type strongly went up compared to any of the mutants.

Based on these observations it must be concluded that even though regulation of *PR-1* and *PDF1.2* is slightly perturbed in *arr1/2* plants, it does not appear reflect the resistance of *arr1-4 arr2-4* to *Botrytis*. Thus even though *PR-1* and *PDF 1.2* are commonly used marker genes for these kind of studies, in this case, the genes themselves do not correlate directly to the resistance phenotype and are more a reflection of the current molecular status in the plants after infection.

4.3 Discussion

4.3.1 ARR2 interacts with MKK members

For the first time a connection has been shown between MAPK signalling cascades and the two-component signalling system in plants. These findings supported earlier presumptions and previous data (Hass *et al.* 2004) that *ARR2* might play role in response in biotic stresses, especially in response to pathogens. This crosstalk was observed strictly downstream of MKKKs, at the level of MKKs and MPKs. This is because none of the tested response regulators (A- and B-type) interacted with any of tested MKKKs. In comparison, only type-B RRs interacted with MKKs and MPKs.

Nevertheless, although only type-B interacted with MKKs, they did not interact with all of them. All MAPK kinase kinase members, except MKK3, were tested against: ARR1, ARR2, ARR10, ARR14, ARR18 and ARR4 (type-A) using yeast-two-hybrid method (studies with MKK6-10, and with ARR10 and ARR14 were done in our laboratory in collaboration with Niklas Wallmeroth (Wallmeroth, Diplom, Tübingen). Besides MKK3, MKK9 showed also transactivation in yeast, but its growth on control medium still allowed for evaluation of protein-protein interactions. ARR10 interacted only with MKK4, MKK5 and MKK9 while ARR14 interacted only with MKK5. ARR1, ARR2, and ARR18 interacted with more MKKs whereas the A-type ARR ARR4 did not interact with any. This information has been summarized below (Table 4.1).

Table 4.1 Summary table of protein-protein interactions between MKKs and RRs

+ means positive scored interaction, - stands for negative scored interaction, (+) stands for slightly transactive protein but a scorable interaction could still be observed, and blank means interaction was not tested. All interactions, except the one with MKK3 (grey labelled), were observed based on yeast two-hybrid system. Fields in blue represent my own results and the rest of data were produced in our laboratory by Niklas Wallmeroth during his Diploma thesis.

	ARR1	ARR2	ARR10	ARR14	ARR18	ARR4
MKK1	-	+	-	-	-	-
MKK2	+	+	-	-	-	-
MKK3		+				
MKK4	+	+	+	-	+	-
MKK5	+	+	+	+	+	-
MKK6	-	-	-	-	-	-
MKK7	-	+	-	-	+	-
MKK8	-	-	-	-	-	-
MKK9	(+)	(+)	(+)	-	(+)	-
MKK10	-	-	-	-	-	-

Despite very high sequence homology between ARR1 and ARR2, they did not interact with the same MKK members suggesting that the interactions could be rather specific. Interestingly, ARR2 interacted with the largest number of MKKs. On the converse side, MKK4, MKK5 and MKK9 interacted with the largest number of type-B ARRs.

MKK7 and MKK9 both are class D MKKs and also play important roles in biotic stress responses. Only ARR2 and ARR18 interacted with MKK7. MKK7 is very well known as a negative regulator of polar auxin transport, defence response to bacterium and salicylic acid mediated signalling pathways (Dai *et al.* 2006). ARR1 is known to control polar auxin transport by directly controlling *SHY2/IAA3*, an Aux/IAA transcriptional repressor, expression, which in turn controls expression of *PIN1*, *PIN3* and *PIN7* in the vascular tissue (Taniguchi *et al.* 2007). As ARR1 did not interact with MKK7 in the yeast-two-hybrid, it is unclear if this rules out a role for MAPK cascades *in planta*. The high homology between *ARR2* and *ARR1*, plus the fact that *ARR12*, the closest homolog to *ARR10*, also has been shown to have a role in regulating *SHY2* (Moubayidin *et al.* 2010) leaves this possibility still open. ARR1, ARR2, ARR10, and ARR12 all complement the root cytokinin response phenotype of the *arr1 arr12* mutant and therefore can functionally replace each other in root elongation assays (Hill *et al.* 2013). Taken together, it is worth speculating that ARR2 also has a role in polar auxin transport by controlling *SHY2* expression and integrating signal information from MKK7. This hypothesis of course must be tested experimentally.

MKK9 is known to be enrolled in phosphorylation between MPK3 and MPK6 and it is independently involved in ethylene and calmalexin biosynthesis, response to salt stress and wounding (Xu *et al.* 2008). In addition, both MKK7 and MKK9 are thought to be involved in the innate immunity cell death response (Popescu *et al.* 2009). ARR1, ARR2, ARR10 and ARR18 all interacted with MKK9. That ARR2 has been described to also function downstream of ethylene signalling (Popescu *et al.* 2009) this implies that these B-types could mediate allosteric functions. Expression of constitutive active version $MKK9^{EE}$ lead to enhanced cell death in *N. benthamiana* transfection assays (Popescu *et al.* 2009). Whether or not type-B ARRs could be part of this process is not yet known.

As mentioned, ARR2 interacted with the most MKKs and of those most of them are hormonal/stress-related MKKs (MKK3, MKK4, MKK5, MKK7 and MKK9). Moreover, ARR2 strongly interacted with MKK4 and MKK5 compared to the abiotic-stress related MKKs MKK1 and MKK2. This difference of interaction strength was conferred by O-NPG assay and by making serial dilution of transformed yeast cells. Therefore, MKK4, MKK5, MKK3 were tested *in planta* by quantifying protein-protein interaction strength using FRET-FLIM. The FRET-FLIM results revealed strong interaction between ARR2 and MKK4, MKK5, MKK3 *in planta*. Thus, there is little doubt that these two protein classes do interact in living plant cells.

Two MPKs were tested against ARR2 and MKKs, the biotic stress-related MPKs MPK4 and MPK17 (Group 2002). Both MPK4 and MPK17 interacted with ARR2, ARR18 and ARR1. MPK4 is especially known to play very important roles in pathogen response (Rasmussen *et al.* 2012). It has been known that *mpk4* knockout mutant plants show constitutive activation of SA-mediated defences (Petersen *et al.* 2000). Furthermore, MPK4 activity inhibits basal defences to virulent *Pst* DC3000 and pathogen-induced SA accumulation, MPK4 also plays role in ETI as compromising it and its activity negatively regulate ROS production induced by PAMPs (Berriri et al. 2012). The interaction of MPK4 with both biotic stress-related MKKs

MKK4 and MKK5 could be confirmed in this thesis. MPK17, whose physiological function(s) are not described yet, only interacted with MKK5. Recall however that MKK5 interacted with all of the type-B ARRs. MKK9 also interacted with the majority of type-B ARRs, albeit over its yeast-two-hybrid transactivation capacity. However, only MKKs 1 to 5 were tested against MPK17 in this thesis. Nevertheless, it is reported that MPK17 interacts with MKK9 and MKK10 in yeast-two-hybrid assays (Lee *et al.* 2008). Thus, although the interaction of MPK17 with MKK9 was not confirmed in this thesis, it suggests that a *bona fide* interaction network has been documented. Furthermore, expounding on the knowledge of roles for MKK5 and MKK9, the type-B ARRs are somehow involved in physiological responses related to biotic stress and/or hormonal regulation. With regards to MKK9 which is known to be involved in ethylene signalling downstream of CTR1 (Yoo *et al.* 2008) as a MKK9–MPK3/6 module. Haß et al. 2004 from our laboratory showed that *ARR2* also responds to ethylene in an ETR1-TCS dependent manner. In addition, MKK4/5 have also been placed in a MKK4/5/9-MPK6 ethylene biosynthesis model (Hahn and Harter 2009; Zhao and Guo 2011). Interestingly, the most type-B ARR interactive MKKs MKK4/5/7/9 were shown to activate signalling cascades controlling cell death pathways in plants (Popescu *et al.* 2009). How these two signalling pathways converge on ethylene signalling is still a mystery, yet it suggests that part of the signal integration can occur at a protein-protein interaction level.

The FRET-FLIM analysis revealed that MKK3 and ARR2 interact in living plant cells. MKK3 is an activator of group C MPKs (MPK1, MPK2, MPK7, and MPK14) of which MKK3 also activates MPK7 in response to plant pathogen *Pst* DC3000 and ROS (Doczi *et al.* 2007). MKK3 is interesting as it is shown to play a physiological role in response to biotic stresses as a MKK3-MPK6 cascade important for JA signal transduction in *Arabidopisis* (Takahashi et al. 2007) and the same cascade plays crucial role in resistance to *Salmonella* infections in *Arabidopsis* (Schikora *et al.* 2008). Additionally, ARR2 showed very strong interaction with MKK4 and MKK5 compared to MKK1 and MKK2 and this interaction was also confirmed *in planta*. The MKK1 and MKK2 are mostly involved in abiotic stresses (Asai *et al.* 2002). For example MKK2 is specially activated by cold and salt stress and by stress-induced MKK1 (Teige *et al.* 2004). They have also been described with a role in biotic stress as a part of MKK1/2-MPK4 cascade functioning as negative regulator of SA-dependent systemic required resistance (Petersen *et al.* 2000) and positive regulator of ET- and JA-mediated defences (Brodersen *et al.* 2006; Qiu *et al.* 2008). Here, it is also possible the MKK1/2-MPK4 module is allosterically influenced by ARR2 and ARR1.

The presumptuously stronger interaction of MKK4 and MKK5 over the other MKKs with B-types was confirmed in yeast-serial dilution and quantitative O-NPG assays. MKK4/5 are known to be active in response to different biotic stresses but especially active and described in pathogen defence (Asai *et al.* 2002) where MKK4 is strongly induced when plants treated with *Pseudomonas syringae* or together with MKK9 against *Phytophthora infestans* (Menges *et al.* 2008). Even newly characterised homologue of *MKK5* in cotton *(Gossypium hirsutum) GhMKK5* affects pathogen resistance to the bacterial pathogen *Ralstonia solanacearum* by

elevating the expression of pathogen resistance *(PR)* genes (*PR1a, PR2, PR4, PR5,* and *NPR1*) (Zhang *et al.* 2012). Interestingly, the interaction capability of ARR2 was not due to the presence of the ARR2 type-B output domain, since a truncated version of ARR2 containing only its receiver domain was able to interact with MKK4 and MKK5. Furthermore, using an *in silico* approach, it was shown that MKK docking domains are dispersed throughout ARR2 and not restricted to its output domain. In total, four different MKK binding domains were predicted to be present in ARR2. All of these regions seem to be functional when tested by yeast-two-hybrid system based on the larger protein truncations. When tested as peptides, the receiver domain-based and output domain-based MKK docking motifs showed interaction only with MKK4 and MKK5. This observation was taken to confirm previous observations that these two MKKs interact much stronger with ARR2 than the others. Furthermore, the interaction of ARR2 with MKKs does not require phosphorylation by TCS elements on its D80 position in order to interact with any of tested MPK(K) cascade elements. This suggests that ARR2 might interact with MAPK cascade members independent of cytokinin or ethylene, *i.e.* TCS signalling. In fact, this would put *ARR2* in novel signalling pathways which may or may not require its transcription factor roles. This potential function of *ARR2* might make signalling pathways and protein-protein interaction more specific. One way to unravel TCS dependent signalling and the physiological role of MAPK cascades would be possible with constitutive expression of non-phosphorylatable $ARR2^{D80N}$ (Hwang and Sheen 2001; Choi *et al.* 2010). This was not performed at this time, but similar experiments with the constitutive TCS active form $ARR2^{D80E}$ were attempted. Readout however was not possible as mutant lines complemented with $ARR2^{D80E}$ driven under a constitutively active promoter were lethal in embryonic stage (Hass *et al.* 2004). In this work, attempts to bypass constitutive expression of $ARR2^{D80E}$ using a β-estradiol inducible promoter only for a few days after treatment still showed huge developmental defects, early necrosis and most likely embryonic lethality (see Supplemental Figure 10).

Taken together, the protein-protein interaction data suggest involvement of ARR2 in a biotic stress direction concerning its association with biotic stress-related MKKs and MPKs, and in particular pathways mediated by MKK4/5/7/9. This crosstalk of ARR2 with MKKs and MPKs could be a conformation of the early presumptions that TCS and B-type response regulators *per se* play an important role in fine-tuning of different signals and in mediating crosstalk between different signalling pathways in aim of improving the fitness of plants for better adaptation within their natural ecosystems. Therefore, a connection to biotic stress and *ARR1* and/or *ARR2* was pursued in this thesis to provide a physiological basis for which a functional output could be eventually be assigned to. Provided that this is successful, cross-talk relationships between ARR1/2 and MKKs could be pursued.

4.3.2 *arr2-4* can be shown to be involved in pathogen responses to necrotrophs

Innate immunity is generally initiated with recognition of conserved pathogen-associated molecular patterns (PAMPs). As a first look into pathogen-related responses in *ARR2*, wild-type and *arr1-4*, *arr2-4* and *arr1-4 arr2-4* mutant lines were treated with PAMPs and as output, ROS and ethylene production was measured.

Indeed, ROS measurement triggered by flg22 and elf18 showed that *arr1-4 arr2-4* double mutant significantly produced more ROS than the wild-type or single mutants in response to both applied PAMPs. Although there were differences between the single mutants, these observations were not consistent between experiments but the double mutant however consistently showed more ROS than the wild-type. This was first hint that *ARR1* and *ARR2* are enrolled in response to pathogen defence. Since the double mutant showed the strongest effect in ROS production only the *arr1-4 arr2-4* double mutant and Col-0 wild-type were taken and tested against PAMPs with fungal origin (PEN, Xac) for ethylene production.

Double mutant here did not show any statistically significant difference in ethylene production to any of tested PAMPs compared to the wild-type. This approach only showed that there is no significant difference in ethylene production between the mutant and the wild-type but it doesn't necessarily mean that the signalling pathways controlled by ethylene are not affected in the double mutant since differences in ROS production were observed. It could be that the differences in ethylene production between the double mutant and the wild-type are below the detection range of the machine used for ethylene measurements. Similarly, it could be that the ROS measurements were much more sensitive than those of ethylene.

Changes in ROS have been correlated with MKK and MPK activity (Pitzschke and Hirt 2009). Unfortunately, the lack of *ARR1* and *ARR2* did not cause any dramatic differences between the wild-type and the double mutant in MPK phosphorylation status in response to flg22 of pathogen-response related MPK4, MPK3 and MPK6. This does not exclude that actually ARR1 and ARR2 may serve here as MKK scaffold proteins which redirect and specify pathogen-related signalling pathways, but it does suggest the function of ARR1 and ARR2 does not regulate MKK kinase activity.

The data up to this point suggested that ARR2 (and other B-types) interact with MKKs and MPKs. The interaction with ARR2 in yeast-two-hybrid and *in planta* was preferentially with MKK4 and MKK5, which are known to be involved in biotic stress. Furthermore, changes in ROS production with respect to flg22 were also observed although this could not be corroborated with ethylene production or MPK phosphorylation status. Recent publications about *ARR2* and cytokinin have indicated a growing connection to pathogen stress (Choi *et al.* 2010). Therefore, pathogen assays were performed on *ARR1* and *ARR2* single and double mutants. For these assays necrotrophic and biotrophic pathogens were taken since each has evolved differently leading to distinct defence strategies in plants and thus activation of different genes required for pathogenicity (Idnurm and Howlett 2001; Oliver and Ipcho 2004).

No any statistically significant differences between wild-type (Col-0) and the mutant lines were obtained using *Pst* DC3000 *when* applying it by leaf infiltration at 3 x 10^6 CFU. The same mutant lines were also twice challenged with *Pst* DC3000 in presence of 1µM exogenous cytokinin (*t-zeatin*). No biologically significant differences were found either. These results are in contrast to data who published a weak *arr2-4* phenotype for resistance to *Pst* DC3000 at one log difference (Choi *et al.* 2010). The majority of their assays worked with overexpressor lines to show the effects of cytokinin and *ARR2* variants however. Considering that our data are of very high quality as they were reproduced at least 5 times, we conclude that *arr1-4*, *arr2-4* and *arr1- arr2-4* do not confer any significant resistance to *Pst* DC3000 infection. *Peronospora* is known to activate defence pathways via salicylic acid (SA) accumulation, which is a general characteristic of biotrophic pathogens (Glazebrook 2005), and independently of SA via *RPPs* (*RECOGNITION OF PERONOSPORA PARASITICA*) genes, especially via *RPP7*. *RPPs* represent R (resistance) genes, genes responsible for gene-dependent defence activation in *Arabidopsis* (Mcdowell *et al.* 2000). Here seems that *ARR1* and *ARR2* are not involved in these signalling pathways or that the other B-types work in a redundant fashion no evident from the yeast-two-hybrid data.

It could be postulated that the defence response could depend on stomata developmental/response defects. This is because *ARR2* has been described to be involved in mediating stomatal closure in an H_2O_2-depdenent manner (Desikan *et al.* 2006; Mira-Rodado *et al.* 2012) and that *MKK4* and *MKK5* are known to be involved in stomatal patterning (Lampard *et al.* 2009). Such same effects should be seen by spraying the bacteria onto plants. Stomata aperture was measured in mutant backgrounds and the wild-type treated with hormones (ABA and cytokinin) and after *Pst* DC3000 application. Although the wild-type and mutants closed in response to ABA as previously described (Desikan *et al.* 2006), wild-type and mutants also responded equally to cytokinin application or *Pst* DC3000 challenge. In fact, stomata in all the plant lines treated with *Pst* DC3000 were more open than non-treated control consistent with the release of diffusible phytotoxin COR by the bacteria which reopen closed stomata to increase the number of bacterial invasion sites (Melotto *et al.* 2008). Stomata aperture measurements were done 3 days after pathogen application which was more than enough to cause reopening of stomata. Here was shown that *ARR1* and *ARR2* are not any how involved in mechanism for recognition of phytotoxin COR and in this case they are not part of apparatus for regulation of stomata aperture. Therefore under our conditions and experimental approach we could not confirm the previously published observation that cytokinin modulates SA signalling by augmenting resistance against *Pst* DC3000, a process in which the SA/cytokinin-dependent interaction TGA3 and ARR2 is important (Choi *et al.* 2010). This discrepancy, despite the performed experiment where stomata aperture was measured and also no difference obtained, may still be due to differences in infection conditions.

Interestingly, cytokinin caused the stomata to open in the wild-type and the *arr1/2* mutant lines. This means, that although there is a lack of two cytokinin responsive (Hill *et al.* 2013), type-B they are not crucial for stomatal aperture responses. This indicates that this response

is more than likely redundantly controlled by other B-types. On the other hand, the strong effects of previously reported for *arr2-4* on H_2O_2-dependent closure point towards explaining MKK4/5/9 cross-talk by the fact that ARR2 interacts with more MKKs than does all the other type-B ARRs.

Generally speaking, JA-dependent signalling pathways play a crucial role in defence against necrotrophic pathogens (Glazebrook 2005). Therefore assays done with nectrotrophic pathogens were performed in order to see if there could be a defect in regulation of jasmonic acid (JA)-dependent signalling pathways. The *arr2-4* single mutant and *arr1-4 arr2-4* double mutant but not *arr1-4* single mutant, showed enhanced susceptibility to the necrotrophic fungus *Alternaria brassicicola* compared to the wild-type. This experiment strongly suggested that, despite their high homology, *ARR2* but not *ARR1,* is the only one of the two involved in signalling pathways responsible for defence against *Alternaria*. It is known that *COI1* (CORONATINE INSENSITIVE 1), *COI1*-dependent genes and camalexin biosynthesis-dependent genes are primarily responsible for its defence against *Alternaria brassicicola* (Van Wees *et al*. 2003). In almost all *Arabidosis* mutants that show susceptibility to *Alternaria*, a perfect correlation has been observed between camalexin deficiency after *A. brassicicola* infection and susceptibility (Van Wees *et al*. 2003). However, it is known that even though infection by *A. brassicicola* and infection by *P. syringae* are quite different stimuli, genes under *COI1* control are common for plant resistance against both pathogens but this is not the case with camalexin biosynthesis-dependent genes (Van Wees *et al*. 2003; Jones and Dangl 2006). Based on the data obtained with *arr2-4* mutants, *ARR2* is more likely involved in camalexin biosynthesis pathways where it might positively regulate its synthesis. Furthermore, ARR2 interacted with MKK3 further strengenthing a role of ARR2 in the MKK3-MPK6 JA signal transduction. How these two are intertwined is yet unclear at this point.

The effects of the necrotrophic fungus *Botrytis cinerea* was challenged on *arr1/2*. This pathogen is important for two reasons: *Botrytis cinerea* is known to activate distinct signalling pathways from *Alternaria* and more importantly, *Arabidopsis thaliana* is natural host of *Botrytis cinerea*. Based on measured disease index parameters, the *arr1-4 arr2-4* double mutant clearly showed resistance to *Botrytis* compared to the wild-type and an additive effect compared to the single mutant parents. That is, the double mutant twice as less lesions (~30%) than the wild-type (~60%) lesions greater for equal to 6 mm (≥6 mm). Both single mutants showed the same resistance tendency and were statistically different from the wild-type. It was also observed that *arr2-4* single mutant had a slight, non-significant tendency to be more resistant than the *arr1-4* single mutant: it had 40% of lesions ≥6 mm whereas the *arr1-4* had only 45%. Again this favours the role of *ARR2* compared to *ARR1* with respect to resistance against necrotrophic pathogens.

The common marker genes *PR-1* and *PDF1.2* were tested from the pooled material from the *Botrytis cinerea* challenged *Arabidopsis* plants. Despite the fact that *PR-1* is rather SA marker and not suitable for infection with necrotrophs, expression of *PR-1* was induced 36 hrs post infection, 12 hrs earlier than the wild-type. This expression was maintained at the same level

in the mutants one day later whereas *PR-1* expression in the wild-type was strictly upregulated in the wild-type at 48 hrs. This would imply that the *arr1/2* caused early induction of *PR-1* in response to *Botrytis* infection. Based on the current models, the expression level of *PDF1.2* should be increased in the mutant plants compared to the wild-type knowing the role of *PDF1.2* as JA marker and is upregulated in response to necrotroph infection (Seo *et al.* 2001; Qi *et al.* 2012; Lu *et al.* 2013). Although *PDF1.2* was induced in response to *Botrytis* infection as expected in all samples, it was not as highly induced in the mutants compared to the wild-type. Thus, the expression of this gene cannot be correlated with the mutant resistance phenotype. These results present a conundrum as to the mechanism of resistance to *Botrytis*. On one hand, we expected that *PDF1.2* expression to be increased in the mutant plants, however dampened expression kinetics were observed. On the other hand, *PR-1* was upregulated at an earlier time in the mutant plants but did not reach the same maximum as the wild-type at 48 hrs. Therefore, it is unclear if these two markers really can be correlated with the resistance phenotype of *arr1/2*. It is known that despite the wide use of *PR-1* and *PDF1.2* as markers for the induction SA- and JA-dependent pathogen pathways, the levels of both *PR-1* and *PDF1.2* expression do not clearly correlate with the level of susceptibility to *Botrytis cinerea* (Ferrari *et al.* 2003). So the observed differences in expression of these genes in the *arr1/2* mutants are more a reflection of the current molecular status in the plants after infection, that is, it definitely showed that the current status was of plants under pathogen attack.

The dramatic difference in resistance between *Botrytis cinerea* and *Alternaria brassisicola* could be explained by the very wide, naturally variable resistance to necrotrophic plant pathogens which appears to be quantitative and polygenic (Micic *et al.* 2004; Rowe and Kliebenstein 2008). This tendency is exactly visible in the *arr1-4 arr2-4* double mutant when the same mutants tested against two different necrotrophic pathogens showed completely opposite phenotypes. Furthermore, all of the parameters very clearly showed the redundancy (additive effect) of the *ARR1* and *ARR2* in respect to defence against *Botrytis* but not *Alternaria*, of which *arr2-4* played the major role. In addition, the nature and extent of isolate-specific interaction between plants and necrotrophic pathogens is relatively unknown, and no qualitative naturally variable resistance genes effective against necrotrophic pathogens have been yet described (Jones and Dangl 2006; Rowe and Kliebenstein 2008). Provocatively, infection by the fungal pathogen *Phytophthora infestans* led to the rapid transcriptional induction of *MKKK19, MKK9 and MKK4*, while *Botrytis cinerea* infection led to the rapid transcriptional induction of completely different genes *MKKK18, 19* and *20, Raf43, ZIK2, 8* (Menges *et al.* 2008). Although no direct genetic or transgenic connection has been shown between ARR1/2 and MKKs, a role for ARR1/2 is more than likely evaluable in necrotrophic but not biotrophic pathogen resistance as neither *Pst* DC3000 nor *Peronospora parasitica* differentially infected *arr1/2* compared to their wild-type control. There are also reports that have shown signalling responses to bacterial and fungal pathogen attack are distinct, *i.e.* different genes are induced, especially with regard to MAPK cascades. It is still open if differences in resistance of *arr1/2* mutants when tested against distinct pathogens is

correlated with MKK/type-B ARR crosstalk. If valid, the role of TCS-dependent phosphorylation, at least for protein-protein interaction is not required (so far judged by yeast-two-hybrid assays). In addition, *arr1/2* mutants did not change the phosphorylation level of MPK3/4/6 in response to flg22. Therefore, regulation of MKK activity also does not appear to be mediated by type-B ARRs, although it was not tested if modulating the TCS-dependent phosphorylation state of ARR2 could influence this. It is therefore proposed that the proposed protein-protein crosstalk mediates protein target networks dynamics, *i.e.* their interaction affects binding/signalling kinetics of the two systems without actually controlling the physical TCS or MAPK cascade phosphorylation mechanism. As both the TCS and MAPK cascades target gene expression, the interaction of the two networks would modify their own information flow leading to changes in output gene expression similar to what has been observed for a TCS and MAPK cascade in yeast (Xu and West 1999).

4.4 References for Chapter 1

Amselem, J, Cuomo, CA, *et al.* (2011). "Genomic analysis of the necrotrophic fungal pathogens sclerotinia sclerotiorum and botrytis cinerea." PLoS Genet **7**(8): e1002230.

Asai, T, Tena, G, *et al.* (2002). "Map kinase signalling cascade in arabidopsis innate immunity." Nature **415**(6875): 977-983.

Babula, D, Misztal, LH, *et al.* (2006). "Genes involved in biosynthesis and signalisation of ethylene in brassica oleracea and arabidopsis thaliana: Identification and genome comparative mapping of specific gene homologues." Theor Appl Genet **112**(3): 410-420.

Bardwell, AJ, Flatauer, LJ, *et al.* (2001). "A conserved docking site in meks mediates high-affinity binding to map kinases and cooperates with a scaffold protein to enhance signal transmission." J Biol Chem **276**(13): 10374-10386.

Baudry, A, Ito, S, *et al.* (2010). "F-box proteins fkf1 and lkp2 act in concert with zeitlupe to control arabidopsis clock progression." Plant Cell **22**(3): 606-622.

Belkhadir, Y, Subramaniam, R, *et al.* (2004). Curr. Opin. Plant Biol. **7**: 391.

Berriri, S, Garcia, AV, *et al.* (2012). "Constitutively active mitogen-activated protein kinase versions reveal functions of arabidopsis mpk4 in pathogen defense signaling." Plant Cell **24**(10): 4281-4293.

Bethke, G, Unthan, T, *et al.* (2009). "Flg22 regulates the release of an ethylene response factor substrate from map kinase 6 in arabidopsis thaliana via ethylene signaling." Proc Natl Acad Sci U S A **106**(19): 8067-8072.

Boller, T and He, SY (2009). "Innate immunity in plants: An arms race between pattern recognition receptors in plants and effectors in microbial pathogens." Science **324**(5928): 742-744.

Brock, AK, Willmann, R, *et al.* (2010). "The arabidopsis mitogen-activated protein kinase phosphatase pp2c5 affects seed germination, stomatal aperture, and abscisic acid-inducible gene expression." Plant Physiol **153**(3): 1098-1111.

Brodersen, P, Petersen, M, *et al.* (2006). "Arabidopsis map kinase 4 regulates salicylic acid- and jasmonic acid/ethylene-dependent responses via eds1 and pad4." Plant J **47**(4): 532-546.

Brown, RL, Kazan, K, *et al.* (2003). "A role for the gcc-box in jasmonate-mediated activation of the pdf1.2 gene of arabidopsis." Plant Physiol **132**(2): 1020-1032.

Caesar, K, Elgass, K, *et al.* (2011). "A fast brassinolide-regulated response pathway in the plasma membrane of arabidopsis thaliana." Plant J **66**(3): 528-540.

Caesar, K, Thamm, AM, *et al.* (2011). "Evidence for the localization of the arabidopsis cytokinin receptors ahk3 and ahk4 in the endoplasmic reticulum." J Exp Bot **62**(15): 5571-5580.

Chang, L and Karin, M (2001). "Mammalian map kinase signalling cascades." Nature **410**(6824): 37-40.

Choi, J, Choi, D, *et al.* (2011). "Cytokinins and plant immunity: Old foes or new friends?" Trends Plant Sci **16**(7): 388-394.

Choi, J, Huh, SU, *et al.* (2010). "The cytokinin-activated transcription factor arr2 promotes plant immunity via tga3/npr1-dependent salicylic acid signaling in arabidopsis." Dev Cell **19**(2): 284-295.

Colcombet, J, Berriri, S, *et al.* (2012). "Constitutively active mpk4 helps to clarify its role in plant immunity." Plant Signal Behav **8**(2).

Colcombet, J and Hirt, H (2008). "Arabidopsis mapks: A complex signalling network involved in multiple biological processes." Biochem J **413**(2): 217-226.

Dai, Y, Wang, H, *et al.* (2006). "Increased expression of map kinase kinase7 causes deficiency in polar auxin transport and leads to plant architectural abnormality in arabidopsis." Plant Cell **18**(2): 308-320.

Dangl, JL, Horvath, DM, *et al.* (2013). "Pivoting the plant immune system from dissection to deployment." Science **341**(6147): 746-751.

Dean, R, Van Kan, JA, *et al.* (2012). "The top 10 fungal pathogens in molecular plant pathology." Mol Plant Pathol **13**(4): 414-430.

Denby, KJ, Kumar, P, *et al.* (2004). "Identification of botrytis cinerea susceptibility loci in arabidopsis thaliana." Plant J **38**(3): 473-486.

Desclos-Theveniau, M, Arnaud, D*, et al.* (2012). "The arabidopsis lectin receptor kinase lecrk-v.5 represses stomatal immunity induced by pseudomonas syringae pv. Tomato dc3000." PLoS Pathog **8**(2): e1002513.

Desikan, R, Horak, J*, et al.* (2008). "The histidine kinase ahk5 integrates endogenous and environmental signals in arabidopsis guard cells." PLoS One **3**(6): e2491.

Desikan, R, Last, K*, et al.* (2006). "Ethylene-induced stomatal closure in arabidopsis occurs via atrbohf-mediated hydrogen peroxide synthesis." Plant J **47**(6): 907-916.

Devoto, A, Nieto-Rostro, M*, et al.* (2002). Plant J. **32**: 457.

Dinkel, H, Michael, S*, et al.* (2012). "Elm--the database of eukaryotic linear motifs." Nucleic Acids Res **40**(Database issue): D242-251.

Doczi, R, Brader, G*, et al.* (2007). "The arabidopsis mitogen-activated protein kinase kinase mkk3 is upstream of group c mitogen-activated protein kinases and participates in pathogen signaling." Plant Cell **19**(10): 3266-3279.

Dodds, PN and Rathjen, JP (2010). "Plant immunity: Towards an integrated view of plant-pathogen interactions." Nat Rev Genet **11**(8): 539-548.

Droillard, MJ, Boudsocq, M*, et al.* (2004). "Involvement of mpk4 in osmotic stress response pathways in cell suspensions and plantlets of arabidopsis thaliana: Activation by hypoosmolarity and negative role in hyperosmolarity tolerance." FEBS Lett **574**(1-3): 42-48.

Dunning, FM, Sun, W*, et al.* (2007). "Identification and mutational analysis of arabidopsis fls2 leucine-rich repeat domain residues that contribute to flagellin perception." Plant Cell **19**(10): 3297-3313.

Durrant, WE and Dong, X (2004). "Systemic acquired resistance." Annu Rev Phytopathol **42**: 185-209.

El-Showk, S, Ruonala, R*, et al.* (2013). "Crossing paths: Cytokinin signalling and crosstalk." Development **140**(7): 1373-1383.

Falk, A, Feys, BJ*, et al.* (1999). Proc. Natl. Acad. Sci. USA **96**: 3292.

Farre, EM and Liu, T (2013). "The prr family of transcriptional regulators reflects the complexity and evolution of plant circadian clocks." Curr Opin Plant Biol **16**(5): 621-629.

Ferrari, S, Plotnikova, JM*, et al.* (2003). "Arabidopsis local resistance to botrytis cinerea involves salicylic acid and camalexin and requires eds4 and pad2, but not sid2, eds5 or pad4." Plant J **35**(2): 193-205.

Galanis, A, Yang, SH*, et al.* (2001). "Selective targeting of mapks to the ets domain transcription factor sap-1." J Biol Chem **276**(2): 965-973.

Gattolin, S, Alandete-Saez, M*, et al.* (2006). "Spatial and temporal expression of the response regulators arr22 and arr24 in arabidopsis thaliana." J Exp Bot **57**(15): 4225-4233.

Gendron, JM, Pruneda-Paz, JL*, et al.* (2012). "Arabidopsis circadian clock protein, toc1, is a DNA-binding transcription factor." Proc Natl Acad Sci U S A **109**(8): 3167-3172.

Glazebrook, J (2001). "Genes controlling expression of defense responses in arabidopsis--2001 status." Curr Opin Plant Biol **4**(4): 301-308.

Glazebrook, J (2005). "Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens." Annu Rev Phytopathol **43**: 205-227.

Glazebrook, J (2005). "Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens." Annu Rev Phytopathol **43**(1): 205-227.

Govrin, EM and Levine, A (2000). "The hypersensitive response facilitates plant infection by the necrotrophic pathogen botrytis cinerea." Curr Biol **10**(13): 751-757.

Grefen, C and Harter, K (2004). "Plant two-component systems: Principles, functions, complexity and cross talk." Planta **219**(5): 733-742.

Group, M (2002). "Mitogen-activated protein kinase cascades in plants: A new nomenclature." Trends Plant Sci **7**(7): 301-308.

Guerineau, F, Benjdia, M*, et al.* (2003). "A jasmonate-responsive element within the a. Thaliana vsp1 promoter." J Exp Bot **54**(385): 1153-1162.

Guo, H and Ecker, JR (2004). "The ethylene signaling pathway: New insights." Curr Opin Plant Biol **7**(1): 40-49.

Hahn, A and Harter, K (2009). "Mitogen-activated protein kinase cascades and ethylene: Signaling, biosynthesis, or both?" Plant Physiol **149**(3): 1207-1210.
Hamel, LP, Nicole, MC*, et al.* (2006). "Ancient signals: Comparative genomics of plant mapk and mapkk gene families." Trends Plant Sci **11**(4): 192-198.
Hass, C, Lohrmann, J*, et al.* (2004). "The response regulator 2 mediates ethylene signalling and hormone signal integration in arabidopsis." EMBO J **23**(16): 3290-3302.
He, C, Fong, SH*, et al.* (1999). "Bwmk1, a novel map kinase induced by fungal infection and mechanical wounding in rice." Mol Plant Microbe Interact **12**(12): 1064-1073.
Hejatko, J, Pernisova, M*, et al.* (2003). "The putative sensor histidine kinase cki1 is involved in female gametophyte development in arabidopsis." Mol Genet Genomics **269**(4): 443-453.
Heyl, A and Schmulling, T (2003). "Cytokinin signal perception and transduction." Curr Opin Plant Biol **6**(5): 480-488.
Hill, K, Mathews, DE*, et al.* (2013). "Functional characterization of type-b response regulators in the arabidopsis cytokinin response." Plant Physiol **162**(1): 212-224.
Horak, J, Grefen, C*, et al.* (2008). "The arabidopsis thaliana response regulator arr22 is a putative ahp phospho-histidine phosphatase expressed in the chalaza of developing seeds." BMC Plant Biol **8**: 77.
Hothorn, M, Dabi, T*, et al.* (2011). "Structural basis for cytokinin recognition by arabidopsis thaliana histidine kinase 4." Nat Chem Biol **7**(11): 766-768.
Huang, Y, Li, H*, et al.* (2003). "Biochemical and functional analysis of ctr1, a protein kinase that negatively regulates ethylene signaling in arabidopsis." Plant J **33**(2): 221-233.
Hutchison, CE, Li, J*, et al.* (2006). "The arabidopsis histidine phosphotransfer proteins are redundant positive regulators of cytokinin signaling." Plant Cell **18**(11): 3073-3087.
Hwang, I, Chen, HC*, et al.* (2002). "Two-component signal transduction pathways in arabidopsis." Plant Physiol **129**(2): 500-515.
Hwang, I and Sheen, J (2001). "Two-component circuitry in arabidopsis cytokinin signal transduction." Nature **413**(6854): 383-389.
Hwang, I, Sheen, J*, et al.* (2012). "Cytokinin signaling networks." Annu Rev Plant Biol **63**: 353-380.
Ichimura, K, Casais, C*, et al.* (2006). "Mekk1 is required for mpk4 activation and regulates tissue-specific and temperature-dependent cell death in arabidopsis." J Biol Chem **281**(48): 36969-36976.
Ichimura, K, Mizoguchi, T*, et al.* (1998). "Isolation of atmekk1 (a map kinase kinase kinase)-interacting proteins and analysis of a map kinase cascade in arabidopsis." Biochem Biophys Res Commun **253**(2): 532-543.
Idnurm, A and Howlett, BJ (2001). "Pathogenicity genes of phytopathogenic fungi." Mol Plant Pathol **2**(4): 241-255.
Jakoby, M, Weisshaar, B*, et al.* (2002). "Bzip transcription factors in arabidopsis." Trends Plant Sci **7**(3): 106-111.
Jambunathan, N, Siani, JM*, et al.* (2001). "A humidity-sensitive arabidopsis copine mutant exhibits precocious cell death and increased disease resistance." Plant Cell **13**(10): 2225-2240.
James, P, Halladay, J*, et al.* (1996). "Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast." Genetics **144**(4): 1425-1436.
Jones, JD and Dangl, JL (2006). "The plant immune system." Nature **444**(7117): 323-329.
Joo, S, Liu, Y*, et al.* (2008). "Mapk phosphorylation-induced stabilization of acs6 protein is mediated by the non-catalytic c-terminal domain, which also contains the cis-determinant for rapid degradation by the 26s proteasome pathway." Plant J **54**(1): 129-140.
Jouannic, S, Hamal, A*, et al.* (1999). "Characterisation of novel plant genes encoding mekk/ste11 and raf-related protein kinases." Gene **229**(1-2): 171-181.
Katagiri, F, Thilmony, R*, et al.* (2002). "The arabidopsis thaliana-pseudomonas syringae interaction." Arabidopsis Book **1**: e0039.
Kawano, T (2003). "Roles of the reactive oxygen species-generating peroxidase reactions in plant defense and growth induction." Plant Cell Rep **21**(9): 829-837.
Kemmerling, B, Schwedt, A*, et al.* (2007). "The bri1-associated kinase 1, bak1, has a brassinolide-independent role in plant cell-death control." Curr Biol **17**(13): 1116-1122.

Kesarwani, M, Yoo, J, *et al.* (2007). "Genetic interactions of tga transcription factors in the regulation of pathogenesis-related genes and disease resistance in arabidopsis." Plant Physiol **144**(1): 336-346.

Kiba, T, Yamada, H, *et al.* (2003). "The type-a response regulator, arr15, acts as a negative regulator in the cytokinin-mediated signal transduction in arabidopsis thaliana." Plant Cell Physiol **44**(8): 868-874.

Kieber, JJ, Rothenberg, M, *et al.* (1993). "Ctr1, a negative regulator of the ethylene response pathway in arabidopsis, encodes a member of the raf family of protein kinases." Cell **72**(3): 427-441.

Kiegerl, S, Cardinale, F, *et al.* (2000). "Simkk, a mitogen-activated protein kinase (mapk) kinase, is a specific activator of the salt stress-induced mapk, simk." Plant Cell **12**(11): 2247-2258.

Kim, CY, Liu, Y, *et al.* (2003). "Activation of a stress-responsive mitogen-activated protein kinase cascade induces the biosynthesis of ethylene in plants." Plant Cell **15**(11): 2707-2718.

Kim, HJ, Chiang, YH, *et al.* (2013). "Scf(kmd) controls cytokinin signaling by regulating the degradation of type-b response regulators." Proc Natl Acad Sci U S A **110**(24): 10028-10033.

Kim, HJ, Ryu, H, *et al.* (2006). "Cytokinin-mediated control of leaf longevity by ahk3 through phosphorylation of arr2 in arabidopsis." Proc Natl Acad Sci U S A **103**(3): 814-819.

Koornneef, A and Pieterse, CM (2008). "Cross talk in defense signaling." Plant Physiol **146**(3): 839-844.

Krol, E, Mentzel, T, *et al.* (2010). "Perception of the arabidopsis danger signal peptide 1 involves the pattern recognition receptor atpepr1 and its close homologue atpepr2." J Biol Chem **285**(18): 13471-13479.

Kurepa, J, Li, Y, *et al.* (2013). "Proteasome-dependent proteolysis has a critical role in fine-tuning the feedback inhibition of cytokinin signaling." Plant Signal Behav **8**(3): e23474.

Lampard, GR, Lukowitz, W, *et al.* (2009). "Novel and expanded roles for mapk signaling in arabidopsis stomatal cell fate revealed by cell type-specific manipulations." Plant Cell **21**(11): 3506-3517.

Lee, DJ, Kim, S, *et al.* (2008). "Phosphorylation of arabidopsis response regulator 7 (arr7) at the putative phospho-accepting site is required for arr7 to act as a negative regulator of cytokinin signaling." Planta **227**(3): 577-587.

Lee, JS, Huh, KW, *et al.* (2008). "Comprehensive analysis of protein-protein interactions between arabidopsis mapks and mapk kinases helps define potential mapk signalling modules." Plant Signal Behav **3**(12): 1037-1041.

Liu, Y and Zhang, S (2004). "Phosphorylation of 1-aminocyclopropane-1-carboxylic acid synthase by mpk6, a stress-responsive mitogen-activated protein kinase, induces ethylene biosynthesis in arabidopsis." Plant Cell **16**(12): 3386-3399.

Lohrmann, J and Harter, K (2002). "Plant two-component signaling systems and the role of response regulators." Plant Physiol **128**(2): 363-369.

Lomin, SN, Krivosheev, DM, *et al.* (2012). "Receptor properties and features of cytokinin signaling." Acta Naturae **4**(3): 31-45.

Lorenzo, O, Piqueras, R, *et al.* (2003). Plant Cell **15**: 165.

Lu, X, Jiang, W, *et al.* (2013). "Aaerf1 positively regulates the resistance to botrytis cinerea in artemisia annua." PLoS One **8**(2): e57657.

Luan, S (2003). "Protein phosphatases in plants." Annu Rev Plant Biol **54**: 63-92.

Mahonen, AP, Bishopp, A, *et al.* (2006). "Cytokinin signaling and its inhibitor ahp6 regulate cell fate during vascular development." Science **311**(5757): 94-98.

Makino, S, Kiba, T, *et al.* (2000). "Genes encoding pseudo-response regulators: Insight into his-to-asp phosphorelay and circadian rhythm in arabidopsis thaliana." Plant Cell Physiol **41**(6): 791-803.

Manners, JM, Penninckx, IA, *et al.* (1998). "The promoter of the plant defensin gene pdf1.2 from arabidopsis is systemically activated by fungal pathogens and responds to methyl jasmonate but not to salicylic acid." Plant Mol Biol **38**(6): 1071-1080.

Mason, MG, Li, J, *et al.* (2004). "Type-b response regulators display overlapping expression patterns in arabidopsis." Plant Physiol **135**(2): 927-937.

Matsushika, A, Makino, S, *et al.* (2000). "Circadian waves of expression of the aprr1/toc1 family of pseudo-response regulators in arabidopsis thaliana: Insight into the plant circadian clock." Plant Cell Physiol **41**(9): 1002-1012.

McDowell, JM, Cuzick, A*, et al.* (2000). "Downy mildew (peronospora parasitica) resistance genes in arabidopsis vary in functional requirements for ndr1, eds1, npr1 and salicylic acid accumulation." Plant J **22**(6): 523-529.

McDowell, JM and Dangl, JL (2000). "Signal transduction in the plant immune response." Trends Biochem Sci **25**(2): 79-82.

Melotto, M, Underwood, W*, et al.* (2008). "Role of stomata in plant innate immunity and foliar bacterial diseases." Annu Rev Phytopathol **46**: 101-122.

Menges, M, Doczi, R*, et al.* (2008). "Comprehensive gene expression atlas for the arabidopsis map kinase signalling pathways." New Phytol **179**(3): 643-662.

Meszaros, T, Helfer, A*, et al.* (2006). "The arabidopsis map kinase kinase mkk1 participates in defence responses to the bacterial elicitor flagellin." Plant J **48**(4): 485-498.

Metraux, JP, Signer, H*, et al.* (1990). "Increase in salicylic acid at the onset of systemic acquired resistance in cucumber." Science **250**(4983): 1004-1006.

Micic, Z, Hahn, V*, et al.* (2004). "Qtl mapping of sclerotinia midstalk-rot resistance in sunflower." Theor Appl Genet **109**(7): 1474-1484.

Mira-Rodado, V, Sweere, U*, et al.* (2007). "Functional cross-talk between two-component and phytochrome b signal transduction in arabidopsis." J Exp Bot **58**(10): 2595-2607.

Mira-Rodado, V, Veerabagu, M*, et al.* (2012). "Identification of two-component system elements downstream of ahk5 in the stomatal closure response of arabidopsis thaliana." Plant Signal Behav **7**(11): 1467-1476.

Mizoguchi, T, Irie, K*, et al.* (1996). "A gene encoding a mitogen-activated protein kinase kinase kinase is induced simultaneously with genes for a mitogen-activated protein kinase and an s6 ribosomal protein kinase by touch, cold, and water stress in arabidopsis thaliana." Proc Natl Acad Sci U S A **93**(2): 765-769.

Montillet, JL, Leonhardt, N*, et al.* (2013). "An abscisic acid-independent oxylipin pathway controls stomatal closure and immune defense in arabidopsis." PLoS Biol **11**(3): e1001513.

Mordret, G (1993). "Map kinase kinase: A node connecting multiple pathways." Biol Cell **79**(3): 193-207.

Moreira, S, Bishopp, A*, et al.* (2013). "Ahp6 inhibits cytokinin signaling to regulate the orientation of pericycle cell division during lateral root initiation." PLoS One **8**(2): e56370.

Moubayidin, L, Perilli, S*, et al.* (2010). "The rate of cell differentiation controls the arabidopsis root meristem growth phase." Curr Biol **20**(12): 1138-1143.

Mur, LA, Kenton, P*, et al.* (2006). "The outcomes of concentration-specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death." Plant Physiol **140**(1): 249-262.

Nakagami, H, Pitzschke, A*, et al.* (2005). "Emerging map kinase pathways in plant stress signalling." Trends Plant Sci **10**(7): 339-346.

Nakamichi, N, Kiba, T*, et al.* (2010). "Pseudo-response regulators 9, 7, and 5 are transcriptional repressors in the arabidopsis circadian clock." Plant Cell **22**(3): 594-605.

Nawrath, C and Metraux, JP (1999). "Salicylic acid induction-deficient mutants of arabidopsis express pr-2 and pr-5 and accumulate high levels of camalexin after pathogen inoculation." Plant Cell **11**(8): 1393-1404.

Nishimura, MT and Dangl, JL (2010). "Arabidopsis and the plant immune system." Plant J **61**(6): 1053-1066.

Nishiyama, R, Watanabe, Y*, et al.* (2013). "Arabidopsis ahp2, ahp3, and ahp5 histidine phosphotransfer proteins function as redundant negative regulators of drought stress response." Proc Natl Acad Sci U S A **110**(12): 4840-4845.

Oliver, RP and Ipcho, SV (2004). "Arabidopsis pathology breathes new life into the necrotrophs-vs.-biotrophs classification of fungal pathogens." Mol Plant Pathol **5**(4): 347-352.

Otani, H, Kohnobe, A*, et al.* (1998). Physiol. Mol. Plant Pathol. **52**: 285.

P, VANB, Woltering, EJ*, et al.* (2007). "Histochemical and genetic analysis of host and non-host interactions of arabidopsis with three botrytis species: An important role for cell death control." Mol Plant Pathol **8**(1): 41-54.

Pape, S, Thurow, C, *et al.* (2010). "The arabidopsis pr-1 promoter contains multiple integration sites for the coactivator npr1 and the repressor sni1." Plant Physiol **154**(4): 1805-1818.

Pathak, RK, Taj, G, *et al.* (2013). "Modeling of the mapk machinery activation in response to various abiotic and biotic stresses in plants by a system biology approach." Bioinformation **9**(9): 443-449.

Penninckx, IA, Eggermont, K, *et al.* (1996). "Pathogen-induced systemic activation of a plant defensin gene in arabidopsis follows a salicylic acid-independent pathway." Plant Cell **8**(12): 2309-2323.

Penninckx, IA, Thomma, BP, *et al.* (1998). "Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in arabidopsis." Plant Cell **10**(12): 2103-2113.

Petersen, M, Brodersen, P, *et al.* (2000). Cell **103**: 1111.

Petersen, M, Brodersen, P, *et al.* (2000). "Arabidopsis map kinase 4 negatively regulates systemic acquired resistance." Cell **103**(7): 1111-1120.

Pischke, MS, Jones, LG, *et al.* (2002). "An arabidopsis histidine kinase is essential for megagametogenesis." Proc Natl Acad Sci U S A **99**(24): 15800-15805.

Pitzschke, A and Hirt, H (2009). "Disentangling the complexity of mitogen-activated protein kinases and reactive oxygen species signaling." Plant Physiol **149**(2): 606-615.

Popescu, SC, Popescu, GV, *et al.* (2009). "Mapk target networks in arabidopsis thaliana revealed using functional protein microarrays." Genes Dev **23**(1): 80-92.

Posas, F and Saito, H (1998). "Activation of the yeast ssk2 map kinase kinase kinase by the ssk1 two-component response regulator." EMBO J **17**(5): 1385-1394.

Qi, L, Yan, J, *et al.* (2012). "Arabidopsis thaliana plants differentially modulate auxin biosynthesis and transport during defense responses to the necrotrophic pathogen alternaria brassicicola." New Phytol **195**(4): 872-882.

Qiu, JL, Fiil, BK, *et al.* (2008). "Arabidopsis map kinase 4 regulates gene expression through transcription factor release in the nucleus." EMBO J **27**(16): 2214-2221.

Qiu, JL, Zhou, L, *et al.* (2008). "Arabidopsis mitogen-activated protein kinase kinases mkk1 and mkk2 have overlapping functions in defense signaling mediated by mekk1, mpk4, and mks1." Plant Physiol **148**(1): 212-222.

Rasmussen, MW, Roux, M, *et al.* (2012). "Map kinase cascades in arabidopsis innate immunity." Front Plant Sci **3**: 169.

Ren, B, Liang, Y, *et al.* (2009). "Genome-wide comparative analysis of type-a arabidopsis response regulator genes by overexpression studies reveals their diverse roles and regulatory mechanisms in cytokinin signaling." Cell Res **19**(10): 1178-1190.

Riefler, M, Novak, O, *et al.* (2006). "Arabidopsis cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism." Plant Cell **18**(1): 40-54.

Rochon, A, Boyle, P, *et al.* (2006). "The coactivator function of arabidopsis npr1 requires the core of its btb/poz domain and the oxidation of c-terminal cysteines." Plant Cell **18**(12): 3670-3685.

Rodriguez, MC, Petersen, M, *et al.* (2010). "Mitogen-activated protein kinase signaling in plants." Annu Rev Plant Biol **61**: 621-649.

Rowe, HC and Kliebenstein, DJ (2008). "Complex genetics control natural variation in arabidopsis thaliana resistance to botrytis cinerea." Genetics **180**(4): 2237-2250.

Sakai, H, Aoyama, T, *et al.* (2000). "Arabidopsis arr1 and arr2 response regulators operate as transcriptional activators." Plant J **24**(6): 703-711.

Sakai, H, Honma, T, *et al.* (2001). "Arr1, a transcription factor for genes immediately responsive to cytokinins." Science **294**(5546): 1519-1521.

Schenk, PM, Kazan, K, *et al.* (2000). "Coordinated plant defense responses in arabidopsis revealed by microarray analysis." Proc Natl Acad Sci U S A **97**(21): 11655-11660.

Schikora, A, Carreri, A, *et al.* (2008). "The dark side of the salad: Salmonella typhimurium overcomes the innate immune response of arabidopsis thaliana and shows an endopathogenic lifestyle." PLoS One **3**(5): e2279.

Schoenbeck, MA, Samac, DA, *et al.* (1999). "The alfalfa (medicago sativa) tdy1 gene encodes a mitogen-activated protein kinase homolog." Mol Plant Microbe Interact **12**(10): 882-893.

Schwartz, MA and Madhani, HD (2004). "Principles of map kinase signaling specificity in saccharomyces cerevisiae." Annu Rev Genet **38**: 725-748.

Seo, HS, Song, JT, *et al.* (2001). "Jasmonic acid carboxyl methyltransferase: A key enzyme for jasmonate-regulated plant responses." Proc Natl Acad Sci U S A **98**(8): 4788-4793.

Sharrocks, AD, Yang, S-H, *et al.* (2000). "Docking domains and substrate-specificity determination for map kinases." Trends Biochem Sci **25**(9): 448-453.

Sheen, J, Zhou, L, *et al.* (1999). "Sugars as signaling molecules." Curr Opin Plant Biol **2**(5): 410-418.

Smekalova, V, Doskocilova, A, *et al.* (2013). "Crosstalk between secondary messengers, hormones and mapk modules during abiotic stress signalling in plants." Biotechnol Adv.

Stolz, A, Riefler, M, *et al.* (2011). "The specificity of cytokinin signalling in arabidopsis thaliana is mediated by differing ligand affinities and expression profiles of the receptors." Plant J **67**(1): 157-168.

Su'udi, M, Park, JM, *et al.* (2013). "Quantification of alternaria brassicicola infection in the arabidopsis thaliana and brassica rapa subsp. Pekinensis." Microbiology.

Suarez-Rodriguez, MC, Adams-Phillips, L, *et al.* (2007). "Mekk1 is required for flg22-induced mpk4 activation in arabidopsis plants." Plant Physiol **143**(2): 661-669.

Suzuki, T, Imamura, A, *et al.* (1998). "Histidine-containing phosphotransfer (hpt) signal transducers implicated in his-to-asp phosphorelay in arabidopsis." Plant Cell Physiol **39**(12): 1258-1268.

Suzuki, T, Sakurai, K, *et al.* (2001). "Two types of putative nuclear factors that physically interact with histidine-containing phosphotransfer (hpt) domains, signaling mediators in his-to-asp phosphorelay, in arabidopsis thaliana." Plant Cell Physiol **42**(1): 37-45.

Sweere, U, Eichenberg, K, *et al.* (2001). "Interaction of the response regulator arr4 with phytochrome b in modulating red light signaling." Science **294**(5544): 1108-1111.

Takahashi, F, Yoshida, R, *et al.* (2007). "The mitogen-activated protein kinase cascade mkk3-mpk6 is an important part of the jasmonate signal transduction pathway in arabidopsis." Plant Cell **19**(3): 805-818.

Takekawa, M, Tatebayashi, K, *et al.* (2005). "Conserved docking site is essential for activation of mammalian map kinase kinases by specific map kinase kinase kinases." Mol Cell **18**(3): 295-306.

Tanaka, Y, Sano, T, *et al.* (2006). "Cytokinin and auxin inhibit abscisic acid-induced stomatal closure by enhancing ethylene production in arabidopsis." J Exp Bot **57**(10): 2259-2266.

Taniguchi, M, Sasaki, N, *et al.* (2007). "Arr1 directly activates cytokinin response genes that encode proteins with diverse regulatory functions." Plant Cell Physiol **48**(2): 263-277.

Teige, M, Scheikl, E, *et al.* (2004). "The mkk2 pathway mediates cold and salt stress signaling in arabidopsis." Mol Cell **15**(1): 141-152.

Tena, G, Boudsocq, M, *et al.* (2011). "Protein kinase signaling networks in plant innate immunity." Curr Opin Plant Biol **14**(5): 519-529.

Thaler, JS, Owen, B, *et al.* (2004). "The role of the jasmonate response in plant susceptibility to diverse pathogens with a range of lifestyles." Plant Physiol **135**(1): 530-538.

Tsai, CH, Singh, P, *et al.* (2011). "Priming for enhanced defence responses by specific inhibition of the arabidopsis response to coronatine." Plant J **65**(3): 469-479.

Ueguchi, C, Koizumi, H, *et al.* (2001). "Novel family of sensor histidine kinase genes in arabidopsis thaliana." Plant Cell Physiol **42**(2): 231-235.

Urao, T, Yakubov, B, *et al.* (1999). "A transmembrane hybrid-type histidine kinase in arabidopsis functions as an osmosensor." Plant Cell **11**(9): 1743-1754.

van Wees, SC, Chang, HS, *et al.* (2003). "Characterization of the early response of arabidopsis to alternaria brassicicola infection using expression profiling." Plant Physiol **132**(2): 606-617.

Veerabagu, M, Elgass, K, *et al.* (2012). "The arabidopsis b-type response regulator 18 homomerizes and positively regulates cytokinin responses." Plant J **72**(5): 721-731.

Wang, H, Ngwenyama, N, *et al.* (2007). "Stomatal development and patterning are regulated by environmentally responsive mitogen-activated protein kinases in arabidopsis." Plant Cell **19**(1): 63-73.

Whitmarsh, AJ and Davis, RJ (1998). "Structural organization of map-kinase signaling modules by scaffold proteins in yeast and mammals." Trends Biochem Sci **23**(12): 481-485.
Widmann, C, Gibson, S*, et al.* (1999). "Mitogen-activated protein kinase: Conservation of a three-kinase module from yeast to human." Physiol Rev **79**(1): 143-180.
Wu, Y, Zhang, D*, et al.* (2012). "The arabidopsis npr1 protein is a receptor for the plant defense hormone salicylic acid." Cell Rep **1**(6): 639-647.
Xie, DX, Feys, BF*, et al.* (1998). Science **280**: 1091.
Xu, J, Li, Y*, et al.* (2008). "Activation of mapk kinase 9 induces ethylene and camalexin biosynthesis and enhances sensitivity to salt stress in arabidopsis." J Biol Chem **283**(40): 26996-27006.
Xu, Q and West, AH (1999). "Conservation of structure and function among histidine-containing phosphotransfer (hpt) domains as revealed by the crystal structure of ypd1." J Mol Biol **292**(5): 1039-1050.
Yamaguchi, K, Yamada, K*, et al.* (2013). "Receptor-like cytoplasmic kinases are pivotal components in pattern recognition receptor-mediated signaling in plant immunity." Plant Signal Behav **8**(10).
Yoo, SD, Cho, YH*, et al.* (2008). "Dual control of nuclear ein3 by bifurcate mapk cascades in c2h4 signalling." Nature **451**(7180): 789-795.
Yuasa, T, Ichimura, K*, et al.* (2001). "Oxidative stress activates atmpk6, an arabidopsis homologue of map kinase." Plant Cell Physiol **42**(9): 1012-1016.
Zeng, W, Melotto, M*, et al.* (2010). "Plant stomata: A checkpoint of host immunity and pathogen virulence." Curr Opin Biotechnol **21**(5): 599-603.
Zhang, J, Shao, F*, et al.* (2007). "A pseudomonas syringae effector inactivates mapks to suppress pamp-induced immunity in plants." Cell Host Microbe **1**(3): 175-185.
Zhang, L, Li, Y*, et al.* (2012). "Cotton ghmkk5 affects disease resistance, induces hr-like cell death, and reduces the tolerance to salt and drought stress in transgenic nicotiana benthamiana." J Exp Bot **63**(10): 3935-3951.
Zhang, PJ, Li, WD*, et al.* (2013). "Feeding by whiteflies suppresses downstream jasmonic acid signaling by eliciting salicylic acid signaling." J Chem Ecol **39**(5): 612-619.
Zhang, Y, Tessaro, MJ*, et al.* (2003). Plant Cell **15**: 2647.
Zhao, Q and Guo, HW (2011). "Paradigms and paradox in the ethylene signaling pathway and interaction network." Mol Plant **4**(4): 626-634.
Zhao, Y, Thilmony, R*, et al.* (2003). "Virulence systems of pseudomonas syringae pv. Tomato promote bacterial speck disease in tomato by targeting the jasmonate signaling pathway." Plant J **36**(4): 485-499.
Zhou, N, Tootle, TL*, et al.* (1998). Plant Cell **10**: 1021.
Zhulin, IB, Taylor, BL*, et al.* (1997). "Pas domain s-boxes in archaea, bacteria and sensors for oxygen and redox." Trends Biochem Sci **22**(9): 331-333.
Zwerger, K and Hirt, H (2001). "Recent advances in plant map kinase signalling." Biol Chem **382**(8): 1123-1131.

5 Chapter 2

ARR1 and *ARR2* are involved in flowering time regulation of *Arabidopsis thaliana* under short days

5.1 Introduction

Flowering is a crucial step in plant development and it is characterised as a transition from vegetative to reproductive phase (Koornneef *et al.* 1998). Due to its importance of ensuring reproductive success, flowering is controlled by complex networks of genetic regulatory pathways (Coupland 1995; Srikanth and Schmid 2011; Poethig 2013). In the end, successful reproduction not only includes seed formation but also dispersion, germination and growth and complex regulatory mechanisms need to integrate both environmental and endogenous signals to ensure the next generation can also reproduce (Bernier *et al.* 1993; Koornneef *et al.* 1998; Srikanth and Schmid 2011). However, it is clear today that all these signalling pathways that control flowering are not strictly separated but that they work together, *i.e.* crosstalk in the regulation of flowering (Genoud and Metraux 1999; Franklin 2009). Surprisingly the number of common targets regulated by huge number of signalling pathways is quite small and they present central floral pathway integrator or "integrator genes" (Li *et al.* 2008; Srikanth and Schmid 2011). Even in self-fertile plants, flowering time is still strictly controlled by environmental and endogenous factors (Srikanth and Schmid 2011). Exogenous factors that strongly influence the flowering are day length, light quality and temperature (Coupland 1995; Reeves and Coupland 2000; Chen *et al.* 2004). On the other side, common endogenous factors that are regulating flowering are hormones (hormonal status of the plant), sugars and plant age (Blazquez *et al.* 1998; Wahl *et al.* 2013). Five genetic pathways have been identified that play role in control of flowering: the vernalization (originally jarovization) pathway, the photoperiod pathway (related to the day length and quality of perceived light), the gibberellin (GA) pathway, the autonomous pathway (endogenously controlled independent of photoperiod or gibberellin pathways), and plant age (also an endogenous pathway) (Srikanth and Schmid 2011).

5.1.1 Photoperiod-dependent flowering control

Photoperiod pathways present a cascade of events in charge for measurement day length and consequently initiating flowering (Valverde *et al.* 2004; Sawa *et al.* 2007). The organ for photoperiod perception is located exclusively in the leaves. Light perception in leaves is enabled by the presence of three main classes of photoreceptors specialized for different wavelengths: phototropins (blue light), phytochromes (red/far red light) and cryptochromes (blue light) (Franklin *et al.* 2005; Lariguet and Dunand 2005). Once a particular type of light-signal is perceived (varies between species) the flower-triggered substance called "florigen" is

produced and afterwards transmitted to the shoot apex to induce flowering (Colasanti and Sundaresan 2000). Even before the era of molecular biology had begun, it was very well known that plants measure and respond to the day length (Srikanth and Schmid 2011). This is due to the presence of the internal oscillators, genes that are regulated by the circadian clock (Harmer *et al.* 2000). The circadian clock is explained as a timekeeping mechanism in photoperiodism (Mizoguchi *et al.* 2005).

5.1.2 Photoperiod-dependent flowering control in *Arabidopsis thaliana*

In *Arabidopsis thaliana*, a circadian clock controlled flowering pathway includes the following gene members *GIGANTEA (GI), CONSTANS (CO)* and *FLOWERING LOCUS T (FT)* that promote flowering specifically under long days (Fowler *et al.* 1999; Mizoguchi *et al.* 2005). Briefly, the circadian clock in *Arabidopsis thaliana* is composed and works as follows: The central oscillator generates a self-sustaining rhythm driven by two interacting feedback loops that are active at different times of day. The first one so called "morning loop" consists of *CCA1 (CIRCADIAN AND CLOCK ASSOCIATED 1)* and *LHY (LATE ELONGATED HYPOCOTYL)*, which encode closely related MYB transcription factors that regulate circadian rhythms in *Arabidopsis*, as well as *PRR 7* and *9 (Pseudo-Response Regulators)*. The second "evening loop" consists of *GI (GIGANTEA)* and *ELF4 (EARLY FLOWERING 4)*, both are involved in regulation of flowering time genes (Fowler *et al.* 1999; Mizoguchi *et al.* 2005; Kolmos and Davis 2007).

Classification of plants due to their photoperiodic responses is usually based on flowering. The two main photoperiodic response categories are short-day and long day-plants (Garner 1933). Short-day plants (SDPs) flower in short days (qualitative SDPs) or their flowering is accelerated by short days (quantitative SDPs). Long-day plants (LDPs) flower only in long days (qualitative LDPs) or their flowering is accelerated by long days (quantitative LDPs). The essential distinction between long-day and short-day plants is that flowering in LDP is promoted only when the day length exceeds a certain duration. This time course is called critical day length. To flower SDPs require a day length less than a critical day length (Yano *et al.* 2001; Kojima *et al.* 2002). Plants that flower under any photoperiodic condition are referred to as day-neutral plants (DNPs) (Wu *et al.* 2004; Lifschitz *et al.* 2006); examples are most of the desert annuals that flower quickly whenever the water is available. *Arabidopsis thaliana* is a facultative LDP, *i.e.* quantitative LDP means it flowers also under SDs but its flowering is accelerated by inductive long days (Hicks *et al.* 1996). Important to photoperiodic flowering is the so-called phenomenon "coincidence model" proposed in the mid-1930s by the German botanist Professor Erwin Bünning which brings together endogenous and exogenous signals. The circadian oscillator controls the timing of light-sensitive and light-insensitive phases only when the light signal is coincident with the appropriate phase of the circadian rhythm thereby allowing flowering induction under both non-inductive and inductive days (Srikanth and Schmid 2011). The first mutant discovered in *Arabidopsis thaliana* incapable of a photoperiodic flowering response was *co* mutant: a mutant lacking in *CO (CONSTANS)* which encodes a zinc finger transcription factor family protein which is controlling expression of other floral regulators (Putterill *et al.* 1995; Tran *et al.* 2007). The *co*

mutant has a very strong late flowering phenotype under inductive LD conditions only whereas at non-inductive SD, *co* flowers identical like the wild-type plant suggesting the CO to be a flower activator (Putterill *et al.* 1995). It was later discovered that the expression *CO* is regulated by the circadian clock with pick expression of *CO* around dusk (Suarez-Lopez *et al.* 2001). Expression pattern of *CO* represents an example of "coincidence model". The CO proteins levels are also controlled however and CO only accumulates enough to promote flowering under LD when the light phase overlaps/coincides with the maximal pick of the *CO* mRNA (Valverde *et al.* 2004; Bohlenius *et al.* 2006). Research has shown that CO is posttranscriptional regulated and that during the dark phase the protein is tagged for degradation by ubiquitin and afterwards degraded in 26s proteasome and part of this is controlled by different photoreceptors (Valverde *et al.* 2004). It has been shown that PhyB signalling in the morning negatively regulates *CO* whereas PhyA and cryptochromes positively regulate the accumulation of CO protein in the evening (Lin 2000; Endo *et al.* 2013). This entire regulation of *CO* happens only in leaves (An *et al.* 2004; Ayre and Turgeon 2004), *i.e.* it does not transfer signal from the leaves to the shoots. Further downstream CO activates expression of other flowering-related genes which are transferring the signal information to the apical meristem and initiating flowering. One of them is *FT (FLOWERING LOCUS T)* gene (Kardailsky *et al.* 1999; Hisamatsu and King 2008). *FT* belongs to a small group of proteins that show structural similarities to mammalian phosphatidylethanolamine-binding protein (Ahn *et al.* 2006; Kim *et al.* 2013). Five more members belong to this family: *TSF (TWIN SISTER OF FT), MFT (MOTHER OF FT AND TFL1), BFT (BROTHER OF FT AND TFL1), ATC (ARABIDOPSIS THALIANA CENTRORADIALIS HOMOLOGUES)* and their major role is the regulation of photoperiodic flowering (Wigge 2011; Pin and Nilsson 2012; Kim *et al.* 2013). In the shoot apical meristem of *Arabidopsis thaliana* it seems that the FT protein, but not *FT* mRNA (Notaguchi *et al.* 2008), is needed to induce flowering, although there are some indications that *FT* mRNA could also be contributing flower induction but only together with its protein form (Li *et al.* 2009). In the shoot apex FT makes a complex with FD (Abe *et al.* 2005; Wigge *et al.* 2005). *FD* belongs to the bZIP transcription factor family and it is preferentially expressed in the shoot apex and required there for FT to promote flowering (Abe *et al.* 2005). This complex afterwards activates downstream targets such as *SOC1 (SUPPRESSOR OF OVEREXPRESSION OF CO 1)* (Searle *et al.* 2006), APETELA 1 (*AP1)* (Wigge *et al.* 2005) and indirectly *LFY (LEAFY)* (Abe *et al.* 2005; Smith *et al.* 2011). *SOC1* is floral activator required for *CO* to promote flowering, it acts downstream of *FT* (Yoo *et al.* 2005), *AP1* and *LFY* are meristem identity genes which generally promote the transition to flowering by further induction of floral homeotic genes (Yu *et al.* 2004).

5.1.3 Photoperiod-dependent flowering control in rice and other plants

The coincidence model is also present within neutral and short day plants like in tomato (*Solanum lycopersicum*) or rice (*Oryza sativa*), respectively. In tomato, the orthologue of *FT* is *SFT (SINGLE-FLOWER TRUSS)* and it is a floral activator just like in *Arabidopsis* (Lifschitz *et al.* 2006). Besides its commercial value and, in contrast to *Arabidopsis*, *Oryza sativa* belongs to the group of short day plants (Yano *et al.* 2001; Kojima *et al.* 2002). The rice genes *Hd1*

(Heading-date 1) and *Hd3a (Heading-date 3a)* encode homologs to *Arabidopsis CO* and *FT* respectively. Like *CO* in *Arabidopsis, Hd1* has an identical expression pattern in rice (Kojima *et al.* 2002) with only difference between rice and *Arabidopsis* is that under short day conditions *Hd1* acts as repressor of *Hd3a* expression. That is the reason why is the flowering in rice, in contrast to *Arabidopsis*, actually inhibited by coincidence of light and the *Hd1* expression; when the light overlaps the maximum expression pick of *Hd1* mRNA it blocks afterwards expression of *Hd3a* and inhibits the flowering. These three examples showed that *CO-FT* regulatory module is conserved among plants pointing its importance and differences in flowering time are most likely mediated through different strategies for regulating this module (Srikanth and Schmid 2011). Summed together, photoperiodism is a very complex mechanism and it is highly controlled and fine-tuned by different regulatory elements which impend on key, evolutionarily conserved genetic networks (Hayama and Coupland 2004; Franklin *et al.* 2005; Imaizumi and Kay 2006).

5.1.4 Connection between two-component system and flowering regulation in rice and *Arabidopsis*

Interestingly, in rice, FT-like gene expression can be controlled independently of *Hd1* by the so-called *Ehd1 (Early heading Date 1)* gene encoding a B-type response regulator, part of two-component signal (TCS) transduction systems (Doi *et al.* 2004) suggesting an important role of TCS in regulating flowering. *Ehd1* functions as a floral inducer and it induces *FT-like* and MADS box genes (*e.g.* the *AP1* ortholog in *Arabidopsis*) (Doi *et al.* 2004).

TCS systems typically transduce a receptor-mediated signal into a phosphorelay from a histidine-kinase onto a conserved aspartate residue of a response regulator (RR) protein. In *Arabidopsis*, there are response regulator proteins that have a mutation in this conserved aspartate (Asp) residue: these proteins are called pseudo response regulators (pseudo-RRs) (Doi *et al.* 2004). It has been already proven that pseudo-RRs regulate flowering time in *Arabidopsis thaliana* and that *TOC1/APRR1*, an *Arabidopsis* pseudo response regulator, is part of the plant circadian clock. *TOC1/APRR1* belongs to a small gene family in *Arabidopsis* (Matsushika *et al.* 2000) and it influences flowering time via the phase setting of *CO* expression (Yanovsky and Kay 2002). Even though pseudo-RRs have lost their ability to accept the phosphorelay phosphate as they are constitutively active because the highly conserved Asp is mutated to a glutamate naturally mimicking a phosphorylated Asp (Doi *et al.* 2004).

In *Arabidopsis thaliana*, ARR4 (an A-type response regulator) plays a very important role in stabilization of active PhyB-Pfr under extended red light conditions and thus functions as modulator of photomorphogenesis (Sweere *et al.* 2001; Mira-Rodado *et al.* 2007). PhyB, as above mentioned, is actively involved in expression control of *CO* in *Arabidopsis thaliana* and also *Hd3a* in *Oryza sativa*.

On the other hand it has been proven in various ways that B-Type response regulators are directly, positively regulating expression of the A-Type response regulators in *Arabidopsis*

(Grefen and Harter 2004; Brenner *et al.* 2012; Veerabagu *et al.* 2012). In addition to this, it was recently shown that floral regulator *SVP (SHORT VEGETATIVE PHASE)* is directly regulating A-type response regulators as well as *CRFs (CYTOKININ RESPONSE FACTORS)* (Gregis *et al.* 2013) was well as directly binding to *STIP (STYMPY)* newly reported component of cytokinin signalling pathway mediationg cytokinin signalling during shoot meristem establishment in *Arabidospsis thaliana* (Skylar *et al.* 2010). Furthermore, it was also recently reported that cytokinin is influencing flowering time in *Arabidopsis*, especially under non inductive short days (Bernier 2011; D'aloia *et al.* 2011). B-type response regulators are being discovered to be involved in fine-tuning and crosstalk of a multitude of signalling pathways in higher plants (Veerabagu *et al.* 2012; Zwack and Rashotte 2013). For example, *ARR2,* a member of B-type response regulators, is part of TCS signalling important for signal transmission of cytokinin and ethylene (detail about TCS signalling and members can be found in the introduction of the first chapter) (Rashotte *et al.* 2003; Grefen and Harter 2004; Hass *et al.* 2004; Mason *et al.* 2005; Zwack and Rashotte 2013). Despite the great sequence similarity of the B-types within the same subgroup they still show very high specificity among each other (Hill *et al.* 2013). The *arr2* loss-of-function mutant plant in Landsberg displayed retarded growth and development including early flowering (Hass *et al.* 2004). *ARR2* was also reported to function in ethylene signalling (Hass *et al.* 2004) and in response to pathogen attack (Choi *et al.* 2010) further demonstrating that B-types can be involved in multiple signalling pathways.

It was discovered that, when applied in hydroponic culture from the roots, in SDs, cytokinin bypassies *FT* and activates transcription of *TSF* in leaves to induce flowering (D'aloia *et al.* 2011). Furthermore, TSF interacts with FD and regulates expression of *SOC1* which also seems to be controlled by cytokinin (D'aloia *et al.* 2011). On the other hand *TSF* and *SOC1* also work independently of *FD* suggesting that *FD* and its paralogue *FDP (FD PARALOG)* (Abe *et al.* 2005) might share this function of *SOC1* controle (Bernier 2011; D'aloia *et al.* 2011). Cytokinin itself cannot induce flowering (Bernier 2011) but the fact that when cytokinin was added to roots of hydroponic-grown *Arabidopsis* plant was sufficient to induce flowering in SDs and that the cytokinin contents of leaves, phloem sap, and SAM (SHOOT APICAL MERISTEM) increased in response to LDs induction in *Arabidopsis* (Bernier 2011; D'aloia *et al.* 2011). In *Sinapis cytokinin* level is also increased in response to LDs induction (Corbesier et al., 2003) and it was reported co-dependent transportation of *SaFT* and cytokinin to the meristem suggests that cytokinin may play a part to regulatory effects attributed to "florigen" (Bernier 2011). However, biological functions of *Arabidopsis* B-type response regulators with respect to flowering have not yet been identified.

5.1.5 Temperature as floral regulator

Another factor, beside photoperiod, that is important in the regulation of flowering is temperature (Blazquez *et al.* 2003). Flowering can be controlled by temperature in two manners: by the process called vernalisation and also by ambient temperatures under which plants exposed to during their vegetative phase (Alonso-Blanco and Koornneef 2000; Koornneef *et al.* 2004).

5.1.5.1 Vernalisation

Vernalization is a process by which flowering is promoted when plants sense exposure to the prolonged cold temperatures (≤7°C). This is an adaptive trait that helps prevent flowering before winter is over thus permitting flowering in the favourable conditions of spring (Kim *et al.* 2009). In winter annual biennial and perennial plants, vernalization is an obligate process, whereas for summer annuals vernalization is facultative. For vernalization to work the temperature has to be a in range between 1 and 7°C for a time of 1 to 3 months depending on the plant species (Srikanth and Schmid 2011). In *Arabidopsis,* vernalization involves the recruitment of chromatin-modifying complexes to a clade of flowering repressors that are silenced epigenetically via histone modifications (Kim *et al.* 2009). This effect is known as "memory of winter" and it does not pass to the progeny but is reset during meiosis (Amasino 2004; Heo and Sung 2011). In *Arabidopsis* and cereals, vernalization results in the suppression of genes that repress flowering (Kim *et al.* 2009). Interestingly, in *Arabidopsis*, the most commonly used laboratory strains do not need to be vernalized, but some do flower very late if they are not vernalized first (Reeves *et al.* 2007; Srikanth and Schmid 2011). There are two dominant genes for vernalization in *Arabidopsis thaliana*: *FRI (FRIGIDA)* and *FLC (FLOWERING LOCUS C)* (Lee and Amasino 1995; Song *et al.* 2012) wehreby FRI up-regulates the expression of *FLC* (Geraldo *et al.* 2009). *FRI* encodes a nuclear protein found only in plants (Johanson *et al.* 2000; Bari and Jones 2009) and *FLC* encodes a MADS-box DNA binding protein that functions as a repressor of flowering, *i.e.* flowering-time related genes (Deng *et al.* 2011). FLC represses expression of *FT*, *FD*, and *SOC1* floral activators by direct interaction with their promoters (Hepworth *et al.* 2002; Helliwell *et al.* 2006). It has been proven that FLC also directly interacts with another MAD-box member, *SVP* which is also floral repressor that functions within the thermosensory pathway (Li *et al.* 2008). This interaction makes physiological and biological sense because it partially supresses *FLC*-mediated delay in flowering (Li *et al.* 2008; Bari and Jones 2009; Yoshida *et al.* 2009). Briefly, *FLC* is silenced in response to vernalization, after *FLC* been silenced VIN3 (VERNALIZATION INSENITIVE 3) initially repressing *FLC* during the cold exposure by remodelling the chromation, *i.e.* by methylating lysine residues of histone H3 (Sung and Amasino 2004; Bond *et al.* 2009) than at least two crucial genes *VRN1* and *VRN2 (VERNALIZATION 1 and 2)* maintain the epigenetic state of *FLC* (Bastow *et al.* 2004). Vernalization as a phenomenon is probably result of convergent evolution as outside of *Brassicaceae* no clear orthologs of *FLC* has been identified, making this a complicated research field (Srikanth and Schmid 2011).

5.1.5.1.1 Ambient temperature

Another factor that controls flowering is ambient temperature during its vegetative stage. Higher temperatures (27°C) accelerate flowering under non-inductive SDs for different *Arabidopsis* accessions serving as a substitute to inductive LDs (Balasubramanian *et al.* 2006). Ambient temperature effects on flowering are very diverse among different species, and even between different accessions of *Arabidopsis thaliana*. Many flowering time mutants also show temperature dependence, *i.e.* when exposed to higher temperatures they flowered earlier (e.g. photoreceptor mutants *phyB*, *cry2* and most *Arabidopsis fri/flc* natural accessions)

(Blazquez *et al.* 2003). Nevertheless, this is not the case when *Nd-1* or *svp* were tested. *Nd-1* accession mutant showed masked effect when grown at 27°C (Balasubramanian *et al.* 2006) as this mutant was shown to have a deletion of the floral repressor *FLM (FLOWERING LOCUS/MADS AFFECTING FLOWERING1))* as a major cause for early flowering of *Nd-1* in short days at 23 °C (Werner *et al.* 2005; Balasubramanian *et al.* 2006).

FLM is like FLC a MADS-box protein and shares strong sequence similarity with *FLC* but despite this their physiological roles seem to be different (Scortecci *et al.* 2003). Microarray data showed that genes associated with alternative splice site selection are affected by thermal induction and it has been also proven that *FLM* has temperature-dependent alternative splicing and this splicing is an important regulator of flowering. (Werner *et al.* 2005; Srikanth and Schmid 2011) In the Waissilewskija ecotype *FLM* gene has four splice variants (α, β, γ and δ) whereas in Columbia (Col-0) accession *FLM* has two splice variants (β and δ) and they are both translated (Lee *et al.* 2013; Pose *et al.* 2013). FLM- β is the prevalent splice variant at lower temperatures (16°C) whereas FLM- δ dominates at higher temperatures (27°C) (Lee *et al.* 2013; Pose *et al.* 2013). Both splice variants are interact and make heterodimers with SVP independent of the transcriptional level which does not change with variation in temperature. Interestingly, they can also interact among each other making FLM-β x FLM-δ heterodimers but only FLM-β x FLM-β homodimers are possible (Lee *et al.* 2013; Pose *et al.* 2013). Proposed model of activity says that dependent of which splice variant β or δ is making heterodimers with SVP determinates later the activity of SVP-FLM heterocomplex (Lee *et al.* 2013). At low ambient temperatures formation of SVP-SVP and SVP-FLM-β complexes is favoured and they both repressing flowering. As the temperature is rising the FLM-β splice form is down-regulated and therefore SVP-FLM- δ complex formation is increased and it now accelerates flowering. Based on this model the role of temperature-dependent mRNA splicing in adaptation to climate change is observed and explained (Pose *et al.* 2013). Other known genes which are involved in regulating flowering in response to ambient temperatures are *HSP70 (HEAT SHOCK PROTEIN 70)* found to be highly correlated with an increase in temperature (Balasubramanian *et al.* 2006; Kumar and Wigge 2010), and also *ARP6 (ACTIN RELATED PROTEIN 6)* which is known to be a nuclear protein that represses flowering as maintaining the expression of *FLC* (Choi *et al.* 2005; Deal *et al.* 2005).

5.1.6 The autonomous pathway

Beside the endogenous hormonal factors that are influencing flowering it is important to mention that autonomous pathways are defined as those that delay flowering irrespective of day length (Mouradov *et al.* 2002). Genes involved in autonomous pathway can be grouped into two broad functional categories of genes that act by repressing *FLC*; these are: general remodelling and maintenance factors of *FLC*, and proteins that effect RNA processing (He *et al.* 2003; Srikanth and Schmid 2011).

5.1.7 Gibberellic acid pathway as a regulator of flowering

One of most potent endogenous control of flowering is hormonal control regulated by the gibberellic acid (GA) pathway (Blazquez *et al.* 1998). The effect of gibberellic acid on plant

growth and development has been known since the beginning of the last century when the rice plants infected with fungus *Gibberella fujikuroi* were growing so fast that they tipped over (Tudzynski 1999; Hsuan *et al.* 2011). Other developmental roles of GA, like its influence on flowering time, became familiar much later. Numerous GAs were discovered in plants but they are not all of them are biologically active; the active ones are GA1, GA3, GA4 and GA7 named according the order of their discovery (Hedden and Phillips 2000). After huge mutagenesis screenings for mutants that affect GA biosynthesis were detected. These mutants showed, beside various developmental defects, delay of flowering by *Arabidopsis* mostly under the non-inductive SDs (Wilson *et al.* 1992). The *ga1-3* mutant, lacking the gene for synthesis of ent-kaurene needed for GA1 formation (Sun *et al.* 1992) showed under inductive LDs almost normal flowering pattern whereas it was not able to flower at all at SDs, even when previously treated with exogenous GAs. Nevertheless, this flowering effect of GA is not strictly limited to SDs as demonstrated by the triple *gid1* mutant (lacking in all three *GID-GIBBERELLIC INSENSITIVE DWARF* receptors for GA). The *gid1* mutant flowers extremely late or not even at all under inductive LDs (Griffiths *et al.* 2006; Willige *et al.* 2007). Interestingly, GA works in parallel (*i.e.* independently) of *CO* as the *ga1-3 co* double mutant showed an additive late-flowering phenotype. of *co* under LD but on the other hand the levels of *FT* mRNA expression were increased after addition of exogenous GAs suggesting that GA are on some other way independently controlling expression of *FT* (Hisamatsu and King 2008). It was later shown that *FT* expression is controlled by GA through interaction of the GID1 receptor with members of the DELLA protein family, totally independent of *CO* (Sun 2010). DELLA proteins belong to the GRAS family of transcriptional regulators and work as repressors of plant growth and development (Hirsch and Oldroyd 2009).

5.1.8 Other factors affecting flowering

Other important endogenous factors that are promoting flowering are sugars (Bernier *et al.* 1993). It is known that sucrose can promote flowering in some plant species but this effect might be rather species-specific (Srikanth and Schmid 2011). Trehalose is another sugar that promotes flowering (Schluepmann *et al.* 2003). Furthermore, T6P (trehalose-6-phosphate) was proven to be a proxy for carbohydrate status in plants and that it is absolutely essential for expression of *FT* and *TSF* in the phloem companion cells (Wahl *et al.* 2013). The age of the plant was recently reported to play a role in the regulation in flowering time independent of photoperiod, vernalization or GA pathways, and instead works via the *miR156* microRNA level which decreases with increasing age of the plant (Wang *et al.* 2009). In this work an early flowering phenotype of *ARR1* and *ARR2* lacking mutants (*arr1-4, arr2-4* single and *arr1-4 arr2-4* double mutants) under SD was further investigated. This is the first time that B-type response regulators in *Arabidopsis* (LD facultative plant) have been shown to regulate flowering. TCS and probably cytokinin were suggested to play roles as regulators of flowering. Further *ARR1* and *ARR2* are acting independent of *FLC* floral repressor but therefore *FLM* floral repressor seems to be epistatic to both *ARR1* and *ARR2* response regulators.

5.2 Results

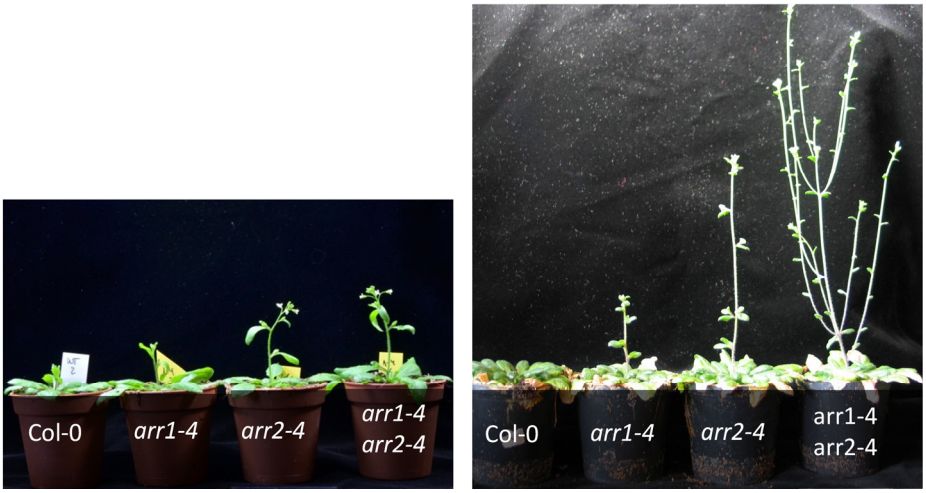
5.2.1 *ARR2* mutants show early flowering phenotype under short day (SD) conditions

Previous work on *ARR2* had been conducted with the *arr2-1 to arr2-4* mutant allele in the Ler-0 ecotype (Hass *et al.* 2004). Work on this mutant showed connections between the two-component signalling pathway, genes related to defence and abiotic stress signalling and adaptation and ethylene signalling (Hass *et al.* 2004). In order to investigate further roles of *ARR2* with its closest homolog *ARR1*, a mutant of *ARR2* was obtained in the Col-0 background. This mutant, and a mutant of *ARR1*, *arr1-4,* also in the Col-0 background, were shown to be full knock-outs. Interestingly, *arr1-4*, *arr2-4* single mutants and the *arr1-4 arr2-4* double mutant displayed precocious flowering under non-inductive short day (SD) conditions, with the double mutant having an additive effect (Figure 5.1).

Despite the high sequence similarity and reported functional redundancy of *ARR1* and *ARR2* in regulating expression of certain common targets in response to cytokinin signal (Mason *et al.* 2005), the loss of *ARR1* and *ARR2* effects flowering time to a different degree.

The single *arr1-4* and arr2-4 mutants flower one and two weeks earlier than wild-type control plants, respectively (Figure 5.1, B). In contrast, the *arr1-4 arr2-4* double mutant flowers three weeks before the Col-0 control (Figure 5.1, B). The early flowering phenotype can be also observed as a reduction of rosette leaf number. The *arr1-4* mutant induces flowering by more than 4 leaves, *arr2-4* mutant by more than 7 leaves, while *arr1-4 arr2-4* produces 15 leaves less than Col-0 (Figure 5.1, B). A trend towards early flowering was also observed when the *arr1/2* mutants were grown under inductive long day (LD) conditions. However, only the *arr1-4 arr2-4* double mutant flowered significantly earlier than wild-type ($p= 0{,}0052$; $\alpha=0{,}05$) and produced 3 leaves less than the wild-type (Figure 5.1, C). In conclusion, the loss of *ARR1*, *ARR2*, or both genes simultaneously, resulted in early flowering under SD conditions. As ARR1 and ARR2 are known B-Type transcription factors, some of known flowering time-related genes (*CO, SOC1, AP1, FT, FLC, FLM, TSF, TFL, LFY* and *FD)* were profiled for alterations in expression patterns in the various mutants under short day and long day conditions.

A



B

	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Short Day							
Col-0 (wild type)	112,8	51,5	±11,8	±4,6	102-129	43-60	20
arr1-4	108,9	48,0	±10,3	±5,6	95-119	39-56	20
arr2-4	103,8	46,2 ‡	±10,6	±6,7	85-116	36-54	20
arr1-4 arr2-4	91,7 ‡	33,7 ‡	±12,4	±7,4	79-110	20-45	20

C

	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Long Day							
Col-0 (wild type)	31,2	13,4	±3,7	±1,4	26-36	12-16	20
arr1-4	29,0	12,9	±4,6	±1,6	24-36	10-15	20
arr2-4	27,7 ‡	12,5	±4,8	±1,2	21-35	10-15	20
arr1-4 arr2-4	26,9 ‡	11,0 ‡	±2,9	±1,1	23-31	9-13	20

Figure 5.1 Novel early flowering phenotype of *arr1/2* mutants.
Photographs illustrating the phenotypes of wild-type (Col-0) and *arr1-4, arr2-4* single mutants and *arr1-4 arr2-4* double mutant grown under LDs (left panel) and SDs (right panel). Differences in Days Until Flowering (DUF) and number of Rosette Leaf Number (RLN). ‡Significance differences (α=0,05) with the wild-type were determined by LSD (Fischer's Least Significant Differences) after the data passed one-way ANOVA (α=0,05). Each day length experiment was repeated twice with similar results. Flowering time data of the mutants grown under SDs **(B)** and LDs **(C)**. DUF, days until flowering; RLN, rosette leaf number; n, number of individuals.

5.2.2 Lack of the activity of *ARR1/ARR2* genes causes down-regulation of specific floral repressors

In order to identify which flowering time pathways are disturbed in the *arr1-4*, *arr2-4* and *arr1-4 arr2-4* mutant lines, key flowering loci were tested for changes in expression with respect to Col-0. Gene expression data quantified by RT-qPCR was firstly obtained from leaves of 70-days-old SD-grown plants shortly before the *arr1-4 arr2-4* double mutant, which displays the strongest acceleration in flowering, entered the reproductive phase. In agreement with the early flowering, expression of the floral repressors *FLC* and *FLM* were 2-fold and 4-fold reduced in leaves, respectively (Figure 5.2, A). In contrast, expression of the known floral repressor *SVP* was not affected. Similarly, expression if the floral activators *CO*, *SOC1* and *FT* were also not affected with only *FD* showing a slight up-regulation (Figure 5.2,

B). Based on these observations the *arr1-4 arr2-4* double mutant causes a depression of floral repressors in leaf and the key LD inductive-pathway genes are not perturbed. Genes that are predominantly expressed at the meristem are also known to have very important roles in flowering initiation, especially in early flowering under short days (Koornneef *et al.* 1998). Therefore, some key meristem-localized genes were examined with the aim of profiling the state of the SAM from 70-day-old SD-grown plants.

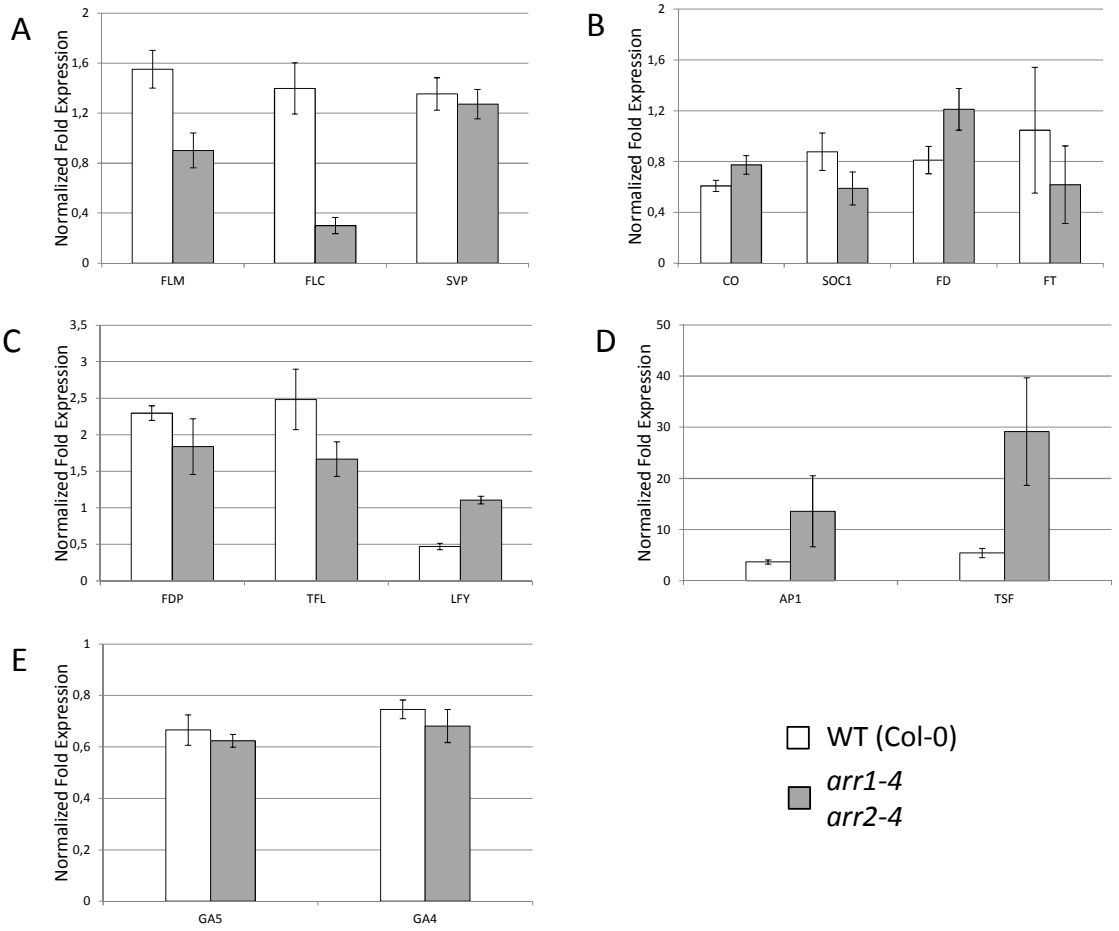


Figure 5.2 The transcript levels of the flowering time pathway-specific marker genes in the wild-type and a*rr1-4 arr2-4* double mutant at DUF 70.

(A) Expression of floral repressor genes *FLM*, *FLC* and *SVP* in the wild-type and double mutant plants. The expression of *FLM* and *FLC* in double mutant plants is down-regulated compared to the wild-type which is not the case with *SVP*. **(B)** Expression of floral activators CO, *SOC1, FD* and *FT*. *FT* levels are very low under the short days and it was taken as a control (*i.e.* levels are qPCR noise). **(C** and **D)** The transcript levels of the flowering time pathway-specific marker genes *FDP, TFL, LFY, AP1* and *TSF*. **(E)** Expression of *GA4* and *GA5*, marker genes for gibberellic acid pathway. RNA was isolated from SD plants short before flowering of the double mutants, tissue from leaves and/or shoot apical meristem was taken. For each line 15 plants were taken and pooled together, RT-qPCR was done using three biological replicates were used with three technical repetitions each and normalized by *ELF-1-α*. Error bars indicate SD of the mean. Leaf material was used for **(A, B)** and for **(C-E)** material was taken from SAM.

In the SAM, the expression of the floral repressor *TERMINAL FLOWER 1* (*TFL1*) was reduced 1.7-fold, whereas expression of the floral activator *FD PARALOG* (*FDP*) was not significantly changed (Figure 5.2, C). In contrast, the meristem identity genes *LEAFY* (*LFY*) was 2-fold increased (Figure 5.2, B) and *APETALA* (*AP1*) and *TWIN SISTER OF FT* (*TSF*) were up-regulated

in the double mutant, 3-fold and 6-fold respectively. The meristem identity genes like *AP1* and *LFY* play an important role in early flower initiation (Ferrandiz *et al.* 2000) and their higher expression confirms the early flowering state of the *arr1-4 arr2-4* double mutant compared to Col-0.

Flower initiation in *Arabidopsis thaliana* under non-inductive short day conditions (Eriksson *et al.* 2006) is dependent on the biosynthesis of the plant hormone gibberellin (GA) and cross-talk between cytokinin and GA has been described (Greenboim-Wainberg *et al.* 2005). This dependency can be explained, at least partly, by GA regulation of the flower meristem identity gene *LFY* and the flowering time gene *SOC1* (Eriksson *et al.* 2006). Gibberellic acid flowering pathway is mostly typical for non-inductive conditions, based on the observations of *ga1* mutant (Wilson *et al.* 1992). GA4 and GA5 *(*gibberellin 3 ß-hydroxylase 1 and 2) are involved in later steps of the gibberellic acid biosynthetic pathway (Hedden and Phillips 2000) and their mRNA levels were shown to somewhat parallel active GA levels *in vivo* (Achard *et al.* 2008). Therefore, the mRNA levels of *GA4* and *GA5* were examined in the SAM at DUF 70. Both *GA4* and *GA5* were unaffected in the double mutant in the SAM (Figure 5.2, E), presumptively indicating that *ARR1* and *ARR2* are not involved in the gibberellic acid flowering pathway.

In conclusion, the expression data obtained by RT-qPCR suggests that *ARR1* and *ARR2* are mostly involved in short-day, non-inductive flowering pathways. This is supported by the fact that the expression of floral repressors *FLC* and *FLM* was lower in the double mutant, whereas known floral activators were not dramatically altered in leaf. The SAM at DUF70 in the double mutant was clearly committed to flowering compared to the wild-type as evident by the high expression levels of *AP1*, *LFY*, and *TSF*. Double mutant was clearly, based on *AP1* expression, in different developmental stage than the wild-type. In order to investigate the previous observations experiments with different approach were further done.

5.2.3 Change in expression of floral repressors is strictly due to early flowering and not developmental effects

To ensure that the data reported above reflect true expression differences rather than differences in developmental stage, *arr1-4 arr2-4* plants were grown for 30 days under non-inductive SD conditions and then shifted to the inductive photoperiodic long days. It has previously been shown that plants grown for 30 days under SDs are large and developed enough to rapidly enter the reproductive phase after a shift to LDs (Schmid *et al.* 2003). For *Arabidopsis*, plants are irreversibly committed to flowering after 3 days in LDs as indicated by the expression of the floral homeotic gene *AP1* (Schmid *et al.* 2003). Therefore flowering was induced in *arr1-4 arr2-4* and wild-type plants at 30 days after germination by shifting them LD and plant material was harvested 0 (control) and 5 days after the shift.

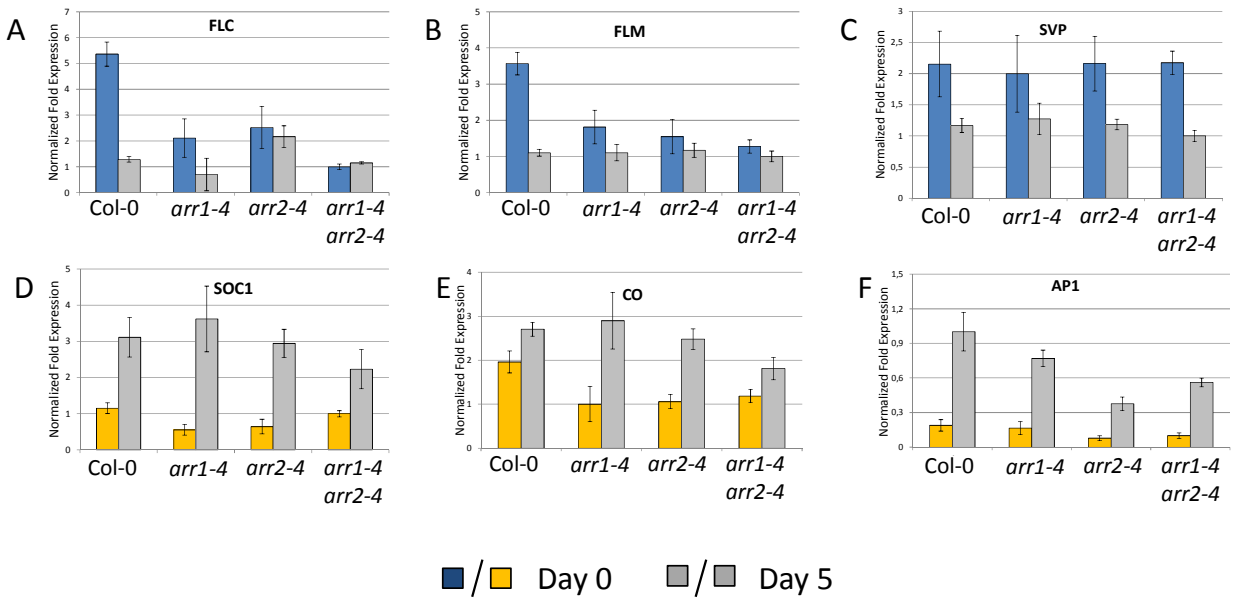


Figure 5.3 Expression of floral regulators in SAM before and after photoperiodic induction.

Transcription levels of genes known floral repressors (**A-C**), activators/integrators (**D,E**) or meristem identity genes (**F**) in wild-type Col-0, *arr1-4, arr2-4* and *arr1-4 arr2-4*. RNA was isolated from the shoot apical meristems (SAM) of 30-day-old plants grown in SD at DUF 30 (Day 0) and after being shifted to LD (Day 5). For each line at least 15 plants were taken and pooled together, RT-qPCR was performed using two biological replicates and three technical replicates each and normalized to *ELF-1-α*. Error bars indicate SD of the mean.

After shifting, the expression of the floral repressors *FLM*, *FLC* and *SVP* was reduced in Col-0 while the expression of floral activator *CO* and floral pathway integrator *SOC1* increased (Figure 5.3) indicating that SAM to the shift in photperiod. *AP1* expression (Figure 5.3, F) is suggests that plants were still in vegetative state before the shift and first after the shift under the inductive long days flowering was induced (the experiment as such was working).

Thus a vegetative phase was replaced by a reproductive phase. The expression level of the floral repressor *FLC* in *arr1-4*, *arr2-4* and *arr1-4 arr2-4* is decreased (about 2-fold) before the shift relative to Col-0 and stays reduced after the shift (Figure 5.3, A). *FLM* also follows this pattern, in that it is already reduced in expression before the shift and maintains this level after the shift (Figure 5.3, B). In contrast, the expression of floral repressor *SVP* decreased after the shift from SDs to LDs independently of the loss of *ARR1* or *ARR2* (Figure 5.3, C). Similarly to *SVP*, the expression of *SOC1* is unchanged in the mutant backgrounds and *SOC1* reached induction levels like that in Col-0 (Figure 5.3, D). *CO* also follows a similar pattern as *SOC1*, in that after the flowering shift induction, the expression of *CO* is increased in response to the shift to LD (Figure 5.3, E).

Taken together, these results suggest that the reduction of *FLC* and *FLM* expression might be causal for the early flowering observed in *arr1-4* and *arr2-4* single mutants and the *arr1-4 arr2-4* double mutant.

5.2.4 Changes in expression of floral repressors are not due to an increase in size of the apical meristem

ARR1 and *ARR2* are known to have roles in cytokinin signalling (Grefen and Harter 2004; Zwack and Rashotte 2013) and it is known that cytokinin has a positive role in regulating SAM size and activity (Tucker and Laux 2007; Werner and Schmulling 2009; Gupta and Rashotte 2012). Cytokinin is also required in the SAM for maintenance of cell division and prevention of cell differentiation (Jasinski *et al.* 2005; Gupta and Rashotte 2012). Therefore, the size of shoot apical meristem SAM was measured in mutant plants and compared to its wild-type in order to see if the early flowering phenotype could be ascribed to a difference size or in morphology. For this experiment SAM of 30-day-old plants grown under SDs were examined (Figure 5.4).

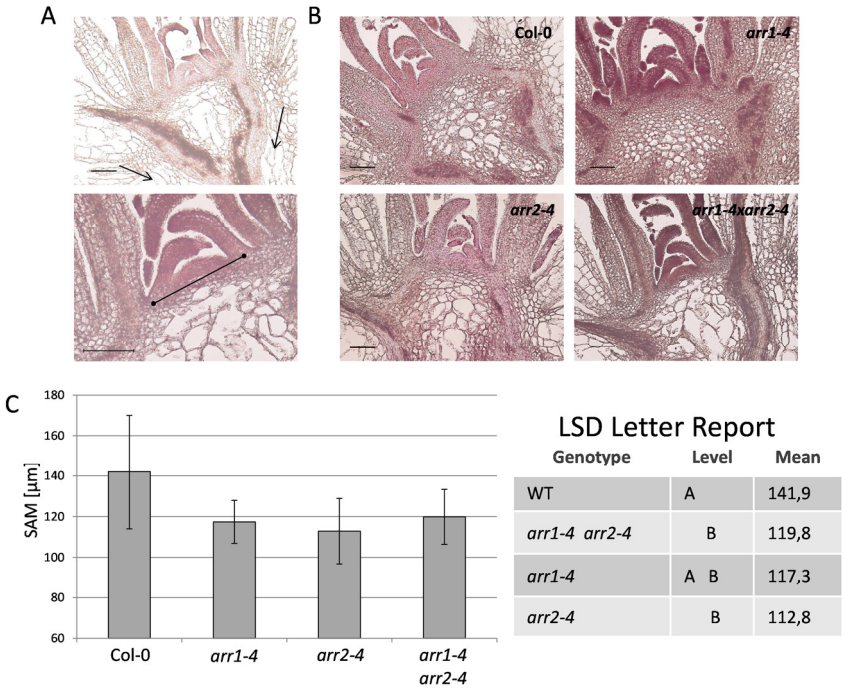


Figure 5.4 Size of shoot apical meristem of Col-0 *arr1-4* and *arr2-4* single mutants and *arr1-4 arr2-4* double mutant.

(A) Examples of the tissue taken and how measurements were made. Sections were scanned for clear visibility of the two large air vacuoles (arrows, upper image). Once found then the SAM width was measured (solid line, lower image). **(B)** Representative sections of shoot apical meristems of Col-0, *arr1-4, arr2-4* and *arr1-4 arr2-4* respectively. **(C)** Size of shoot apical meristems in single mutants and the double mutants from 30 day-old plants grown under SDs. At least 10 plants per line were used for statistical evaluation. Significance classes were computed using the Fisher's Least Significant Difference (LSD, α=0,05) test after passing one-way ANOVA (α=0.05 , p ≥0.0339). Classes *not connected* by a letter are significantly different. All of the mutants have means that are smaller than Col-0, but only *arr2-4* and *arr1-4 arr2-4* are statistically smaller than the wild-type. Error bars indicate SD of the mean. Scale bars, 100µm

SAMs of Col-0 and *arr1-4* single mutant did not show a statistical significant difference in their size (LSD, α=0.05) even though the mean of the *arr1-4* similar to the *arr2-4* and *arr1-4 arr2-4* and are not statistically different (Figure 4, C). On the other hand, the SAMs of *arr2-4* single mutant and the *arr1-4 arr2-4* double mutant are statistically significantly smaller than in the wild-type (LSD, α=0.05). The SAM of the *arr1-4 arr2-4* double mutant is not significantly different in size from *arr2-4* or *arr1-4* single mutants however (Figure 5.4, C). This

suggests that the difference in SAM size is only due to lack of *ARR2* and not *ARR1*, however one should keep in mind that the *arr1-4* SAM was never observed to be in the same range as that of Col-0 (Figure 5.4, C) and was not statistically different from *arr2-4* or the double mutant. It is possible that with a larger sample size the *arr1-4* would have also been statistically smaller. For now, it is evident that the SAMs of *arr1-4*, *arr2-4* and *arr1-4 arr2-4* are not larger than Col-0. Thus, despite of even smaller size of SAM in the mutant plants they manage to flower earlier than the wild-type. Differences in anatomy or morphology of SAMs in mutant plants and the wild-type were not observed (Figure 5.4, A, B).

5.2.5 *ARR2* expressed either in the SAM or from phloem companion cells recues the early flowering phenotype of *arr2-4* and *arr1-4 arr2-4*

The RT-qPCR results from the previous experiments indicated that there were changes in floral regulator expression in both leaf and the SAM. Investigation of the SAM indicated the early flowering phenotype could not be attributed to an increase in meristem size. Therefore as the action of *ARR2* with respect to flowering time was unclear, the double mutant was complemented with two different tissue specific promoters, *pSUC2* and *pFD*. *pSUC2* is a vasculature-specific promoter expressed only in phloem (companion cells) of all green tissues of *Arabidopsis* such as rosette leaves, stems, and sepals. *SUC2* encodes a plasma-membrane sucrose-H+ symporter (Truernit and Sauer 1995; Wippel and Sauer 2012). *pFD* is a meristem-specific promoter expressed in the shoot apex (Abe *et al.* 2005). FD is bZIP protein required for positive regulation of flowering (Abe *et al.* 2005; Wigge *et al.* 2005). Furthermore, it is know that ARR2 can be activated by phosphorylation of an aspartate (D80) in a Two-Component System (TCS) dependent manner (Grefen and Harter 2004). Mutation of D80 to E (Glutamate) mimics the phosphorylated state, increases the transactivation capacity of ARR2 (Hwang and Sheen 2001) and leads to severe developmental defects (Hass *et al.* 2004). Mutation of D80 to N (Asparagine), however, prevents TCS dependent phosphorylation (Kim *et al.* 2006) and impairs basal gene activation capacity, yet D80N still appears to respond to cytokinin according to reporter gene assays (Hwang and Sheen 2001; Kim *et al.* 2006). Therefore, the *arr2-4* and *arr1-4 arr2-4* mutants were complemented using wild-type *ARR2* and $ARR2^{D80N}$ cDNA versions under the control of the *pSUC2* and *pFD* promoters (Tables 5.1 to5. 4). Due to the stronger flowering time effect observed by the loss of *ARR2*, *arr1-4* plants were included as controls only and not complemented with the *ARR2* at this time, thus this complementation assay focuses on the effect of the *ARR2* gene.

All plants used for this experiment were in examined in the T2 generation and therefore preselected with BASTA. For each construct two or three independent lines were chosen and a minimum of 15 plants were used for the experiment. To rule out any secondary effects from the BASTA application and ensure that the vector backbones did not disturb the early flowering phenotype, all the mutant lines as well as the wild-type plants were transformed with empty vectors. This was done by using the "pJL blue" gateway compatible vector which only has a MCS (multiple cloning site (MCS) sequence between the gateway cassette. The results will be presented in the same order as they are given in Tables 5.1 to 5.4.

Table 5.1 shows the complementation results for *ARR2* and *ARR2D80N* driven under the *pSUC2* promoter in LDs. Two to three transgenic lines were chosen for each construct and genotype pair. The table presents the data for each line analysed; statistical analyses were performed by pooling the results from independent lines for each transgene. With regards to the two parameters DUF and rosette leaf number, DUF is more predictive and has a higher resolution as the number of rosette leaves was nearly invariant under our LD condition (total range 2 leaves). Therefore, only the results with respect to DUF will be presented for LDs. The control vector construct, here called "pJL-blue", did not complement the flowering time phenotype. The wild-type flowered the latest (mean: 29 DUF), the double mutant the earliest (mean: 24 DUF) and the two single mutants in-between (means: 26 DUF for *arr1-4* and 25 DUF for *arr2-4*). Thus even though the early flowering phenotype is comparably weak in LDs, the flowering time trend previously observed was not disturbed by the vector backbone nor the BASTA treatment. *ARR2* driven by *pSUC2* was able to complement the flowering defect to near wild-type levels in the *arr2-4* single mutant (mean: DUF 28 same significance class as Col-0). Most remarkably, *ARR2 D80N* loss-off-function version driven under the *pSUC2* was also able to partially complement (mean: 27 DUF) the early flowering phenotype of the single mutant under LDs. Neither construct was able to complement the double mutant however.

The complementation results for *ARR2* and *ARR2 D80N* driven under the *pFD* promoter in LDs are given in Table 5.2. Like the *pSUC2* experiment in LDs, the DUF parameter is more informative as the rosette number again is nearly invariant and has a small range (2 leaves). The control pJL-blue constructs lead to the same tendency with the wild-type flowering the latest (mean: 29 DUF), the double mutant earliest (mean: 25 DUF) and the single mutants in-between as previously observed. This again demonstrated that neither the BASTA application nor vector backbone disturbed the flowering phenotype of the respective genotypes. Expression of *ARR2* from the *pFD* promoter was able to complement the single and double mutant (means: 28.5 and 29 DUF, respectively). A partial complementation was also observed with *ARR2 D80N* in the *arr2-4* mutant but not the *arr1-4 arr2-4* double mutant. Table 5.3 summarises the results for complementation of the various mutant lines for *ARR2* and *ARR2 D80N* driven under the *pSUC2* promoter in SDs. The control pJL-blue constructs once again did not disturb the flowering time with the wild-type flowering the latest (mean: 101 DUF with 53 leaves), the double mutant earliest (mean: 81 DUF with 41 leaves) and the single mutants in-between as previously observed. All the complemented lines used for these experiments showed within the same complementation same tendency (supplemental figures 8 and 9). The rosette leaf range is larger this time (range of 13 leaves) and overall reflects the DUF parameter but not only for the double mutant. The best complementation was observed in the double mutant (mean: 90 DUF with 48 leaves) with *ARR2 D80N* also partially, but significantly, complementing the mutant (mean: 86 DUF with 45 leaves). In contrast to the *pSUC2* under LD conditions, neither *ARR2* nor *ARR2 D80N* was able to even partially complement *arr2-4* based on the significance classes. Nevertheless, this complementation of *ARR2* did have a mean DUF of 89, nearly identical to that of the partially complemented double mutant.

Table 5.1 Flowering time of transgenic lines driven under tissue specific *SUC2* promoter (*pSUC2*) under long day inductive conditions.

DUF, days until flowering; RLN, rosette leaf number; n, number of individuals; #, identifier for individual transgenic line. All transgenic lines used for this experiment were in T2 generation and preselected with BASTA. Significance levels were computed after passing ANOVA (α=0.05) by using Fischer's Least Significance Difference (LSD) with alpha levels α=0.01 for DUF and α=0.05 or RLN. Classes *not connected* by a letter are significantly different.

	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Long Day							
pSUC2:pJL-blue #6	29,4	10,3	±1,6	±1,1	27-32	9-12	15
pSUC2:pJL-blue #5	29,0	11,7	±1,6	±1,4	27-32	10-13	15
pSUC2:pJL-blue #2	28,6	11,0	±2,5	±2,2	26-33	7-13	15
arr1-4 pSUC2:pJL-blue #1	26,6	11,0	±1,0	±1,0	25-28	10-12	15
arr1-4 pSUC2:pJL-blue #5	25,6	10,2	±1,3	±0,9	24-28	9-11	15
arr1-4 pSUC2:pJL-blue #4	26,3	10,7	±2,8	±1,4	24-32	9-14	15
arr2-4 pSUC2:pJL-blue #5	25,2	9,0	±1,9	±1,2	25-29	7-10	15
arr2-4 pSUC2:pJL-blue #3	25,2	10,1	±1,7	±0,9	23-26	9-12	15
arr2-4 pSUC2:pJL-blue #4	26,3	10,1	±2,5	±1,1	23-31	8-12	15
arr1-4 arr2-4 pSUC2:pJL-blue #5	25,9	9,4	±0,8	±1,3	25-27	7-11	15
arr1-4 arr2-4 pSUC2:pJL-blue #2	25,3	9,6	±1,3	±1,3	24-28	7-12	15
arr1-4 arr2-4 pSUC2:pJL-blue #3	24,9	9,5	±1,7	±0,8	23-27	8-10	15
arr2-4 pSUC2:ARR2 #1	28,1	10,9	±1,8	±1,0	25-30	10-12	15
arr2-4 pSUC2:ARR2 #2	28,1	10,7	±1,8	±1,0	25-31	9-12	10
arr2-4 pSUC2:ARR2D80N #5	27,4	11,3	±3,2	±2,3	24-33	8-14	20
arr2-4 pSUC2:ARR2D80N #6	26,3	10,4	±1,9	±1,8	24-29	8-13	10
arr1-4 arr2-4 pSUC2:ARR2 #3	25,7	10,7	±1,5	±0,5	24-29	9-14	15
arr1-4 arr2-4 pSUC2:ARR2 #1	25,0	10,3	±1,4	±1,1	23-28	9-12	15
arr1-4 arr2-4 pSUC2:ARR2D80N #6	23,8	10,8	±2,0	±1,0	22-29	9-12	15
arr1-4 arr2-4 pSUC2:ARR2D80N #2	23,7	10,9	±1,3	±0,9	21-25	10-12	15
arr1-4 arr2-4 pSUC2:ARR2D80N #X	24,4	10,6	±1,4	±1,4	23-27	9-14	15

Genotype	LSD DUF	Mean
WT (Col-0) pSUC2::Jlblue	A	29,0
arr2-4 (Col-0) pSUC2::ARR2	A	28,1
arr2-4 (Col-0) pSUC2::ARR2D80N	B	26,8
arr1-4 (Col-0) pSUC2::Jlblue	B C	26,1
arr2-4 (Col-0) pSUC2::Jlblue	C	25,7
arr1-4Xarr2-4 (Col-0) pSUC2::Jlblue	C	25,4
arr1-4Xarr2-4 (Col-0) pSUC2::ARR2	C	25,3
arr1-4Xarr2-4 (Col-0) pSUC2::ARR2D80N	D	24,0

Genotype	LSD Rosette	Mean
WT (Col-0) pSUC2::Jlblue	A	11,0
arr2-4 (Col-0) pSUC2::ARR2D80N	A	10,8
arr1-4Xarr2-4 (Col-0) pSUC2::ARR2D80N	A	10,8
arr2-4 (Col-0) pSUC2::ARR2	A	10,8
arr1-4 (Col-0) pSUC2::Jlblue	A B	10,5
arr1-4Xarr2-4 (Col-0) pSUC2::ARR2	A B	10,3
arr2-4 (Col-0) pSUC2::Jlblue	B C	9,9
arr1-4Xarr2-4 (Col-0) pSUC2::Jlblue	C	9,5

Table 5.2 Flowering time of transgenic lines driven under tissue specific *FD* promoter under long day inductive conditions.

DUF, days until flowering; RLN, rosette leaf number; n, number of individuals; #, identifier for individual transgenic line. All transgenic lines used for this experiment were in T2 generation and preselected with BASTA. Significance levels were computed after passing ANOVA (α=0.05) by using Fischer's Least Significance Difference (LSD) with alpha levels α=0.01 for DUF and α=0.05 or RLN. Classes *not connected* by a letter are significantly different.

	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Long Day							
pFD:pJL-blue #1	29,3	11,5	±1,7	±1,7	27-32	8-13	15
pFD:pJL-blue #3	29,5	11,0	±1,7	±1,7	27-32	8-13	15
pFD:pJL-blue #5	29,3	11,4	±1,7	±1,4	27-32	9-13	15
arr1-4 pFD:pJL-blue #1	26,8	10,0	±1,6	±1,9	24-29	7-12	15
arr1-4 pFD:pJL-blue #2	26,5	10,5	±1,5	±2,1	24-28	7-14	15
arr1-4 pFD:pJL-blue #3	27,2	10,2	±1,2	±1,4	25-28	7-12	15
arr2-4 pFD:pJL-blue #1	25,1	9,6	±1,0	±2,2	23-26	7-11	15
arr2-4 pFD:pJL-blue #5	24,8	10,1	±1,2	±1,2	23-26	8-11	15
arr2-4 pFD:pJL-blue #4	25,0	10,3	±1,2	±1,5	23-26	8-13	15
arr1-4 arr2-4 pFD:pJL-blue #1	24,6	9,4	±1,3	±1,5	23-27	7-12	15
arr1-4 arr2-4 pFD:pJL-blue #3	25,1	9,6	±1,1	±1,7	24-27	7-13	15
arr1-4 arr2-4 pFD:pJL-blue #6	24,8	9,6	±1,4	±1,5	23-27	7-11	15
arr2-4 pFD:ARR2 #8	28,0	10,5	±1,6	±1,1	27-30	9-13	15
arr2-4 pFD:ARR2 #5	28,0	11,1	±1,2	±1,6	27-30	8-13	15
arr2-4 pFD:ARR2 #7	29,8	11,8	±2,3	±1,2	27-30	10-13	15
arr2-4 pFD:ARR2D80N # 1	26,2	11,4	±2,0	±1,4	24-30	9-14	10
arr2-4 pFD:ARR2D80N #2	26,6	11,4	±2,0	±1,4	24-30	9-13	15
arr1-4 arr2-4 pFD:ARR2 #4	29,4	12,1	±2,6	±2,0	26-33	9-15	15
arr1-4 arr2-4 pFD:ARR2 #2	28,1	11,6	±1,6	±1,3	26-31	10-13	15
arr1-4 arr2-4 pFD:ARR2 #6	30,0	12,0	±2,0	±1,7	28-32	10-13	15
arr1-4 arr2-4 pFD:ARR2D80N #1	25,4	10,9	±1,6	±1,7	24-29	7-12	15
arr1-4 arr2-4 pFD:ARR2D80N #2	25,6	10,7	±1,5	±1,5	24-29	8-12	15

Genotype	LSD DUF	Mean
WT (Col-0) pFD::Jlblue	A	29,3
arr1-4Xarr2-4 (Col-0) pFD::ARR2	A	28,9
arr2-4 (Col-0) pFD::ARR2	A	28,4
arr1-4 (Col-0) pFD::Jlblue	B	26,8
arr2-4 (Col-0) pFD::ARR2D80N	B C	26,4
arr1-4Xarr2-4 (Col-0) pFD::ARR2D80N	C D	25,5
arr2-4 (Col-0) pFD::Jlblue	D	25,0
arr1-4Xarr2-4 (Col-0) pFD::Jlblue	D	24,8

Genotype	LSD Rosette	Mean
arr1-4Xarr2-4 (Col-0) pFD::ARR2	A	11,9
arr2-4 (Col-0) pFD::ARR2D80N	A B	11,4
WT (Col-0) pFD::Jlblue	A B	11,3
arr2-4 (Col-0) pFD::ARR2	A B	11,0
arr1-4Xarr2-4 (Col-0) pFD::ARR2D80N	B C	10,8
arr1-4 (Col-0) pFD::Jlblue	C D	10,2
arr2-4 (Col-0) pFD::Jlblue	C D	10,0
arr1-4Xarr2-4 (Col-0) pFD::Jlblue	D	9,6

Finally, the complementation results for *ARR2* and *ARR2 D80N* driven under the *pFD* promoter under SDs are in Table 5.4. As observed in the other three experiments, the control pJL-blue constructs did not disturb the flowering time: wild-type flowered the latest (mean: 108 DUF with 54 leaves), *arr1-4* (mean: 88 DUF with 50 leaves), *arr2-4* (mean: 84 DUF with 48 leaves) and the double mutant the earliest (mean: 81 DUF with 41 leaves). In contrast to the previous experiments *ARR2* driven by the *pFD* promoter was rescued both the *arr2-4* (mean: 97 DUF with 50 leaves) and the *arr1-4 arr2-4* (mean: 95 DUF with 50 leaves) mutants almost completely. Most remarkably, the $ARR2^{D80N}$ was also able to, partially, and significantly, complement both the single *arr2-4* (mean: 89 DUF with 47 leaves) and the double mutant (mean: 86.5 DUF with 45.5 leaves).

Taken together, even though full complementation was only observed under LDs, the complementation of the early flowering of *arr2-4* under SDs was also partial, yet statistically significant, suppressed. This suggests that lack of *ARR2* is indeed causal for the observed early flowering phenotype. *ARR2* driven under *pFD* showed the strongest effect and can almost completely complement the flowering phenotype of both the single and the double mutant in SDs where the *arr2-4* and *arr1-4 arr2-4* mutation effects strongest. The rescue is slightly weaker when *ARR2* is driven under *pSUC2* but still significant in the double mutant. In LD conditions, *ARR2* expressed from either promoter was able to complement the *arr2-4* mutation, but only *ARR2* expressed under the *pFD* promoter was able to partially rescue the *arr1-4 arr2-4* mutant. Mutant plants were also slightly complemented with loss-of-function *ARR2 D80N* gene in the single and double mutants in SDs when driven under the *pFD* promoter or by *pSUC2* in *arr1-4 arr2-4*. This effect of *ARR2 D80N* was also observed in LDs but only in the *arr2-4* mutant background. The *ARR2D80N*-cDNA mutant version cannot be phosphorylated by the TCS anymore, and is less responsive to cytokinin than the wild-type *ARR2*-cDNA (Hwang and Sheen 2001; Choi *et al.* 2010; Veerabagu *et al.* 2012). The reason for this is most likely due to an incomplete inactivation of the *ARR2* by the D80N mutation. Considering this fact, it can be still concluded that ARR2 needs to be phosphorylated by the TCS (Two-Component System) in order to best rescue the early flowering phenotype as only partial complementation was observed for the *ARR2D80N* loss-of-function transgene. This strongly suggests that ARR2 may be a point of integration for the TCS and other flowering time pathways under SDs.

Table 5.3 Flowering time of transgenic lines driven under tissue specific *SUC2* promoter under short day non-inductive conditions.

DUF, days until flowering; RLN, rosette leaf number; n, number of individuals; #, identifier for individual transgenic line. All transgenic lines used for this experiment were in T2 generation and preselected with BASTA. Significance levels were computed after passing ANOVA (α=0.05) by using Fischer's Least Significance Difference (LSD) with alpha levels α=0.01 for DUF and α=0.05 or RLN. Classes *not connected* by a letter are significantly different.

	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Short Day							
pSUC2:pJL-blue #6	101,3	54,3	±5,3	±4,4	90-109	47-60	15
pSUC2:pJL-blue #5	96,8	52,0	±7,9	±4,3	90-108	48-57	15
pSUC2:pJL-blue #2	103,5	53,2	±6,9	±2,9	94-115	50-58	15
arr1-4 pSUC2:pJL-blue #1	86,6	49,4	±6,3	±2,3	84-97	46-52	15
arr1-4 pSUC2:pJL-blue #5	92,7	49,6	±4,0	±2,0	89-97	45-52	15
arr1-4 pSUC2:pJL-blue #4	90,7	48,3	±4,0	±1,5	86-93	47-50	15
arr2-4 pSUC2:pJL-blue #5	85,4	47,9	±3,2	±3,1	82-90	44-53	15
arr2-4 pSUC2:pJL-blue #3	87,1	47,6	±5,6	±2,6	81-96	45-52	15
arr2-4 pSUC2:pJL-blue #4	87,2	46,8	±4,3	±3,5	82-93	43-51	15
arr1-4 arr2-4 pSUC2:pJL-blue #5	78,7	39,8	±4,3	±3,5	74-85	35-45	15
arr1-4 arr2-4 pSUC2:pJL-blue #2	82,9	41,5	±3,9	±3,7	78-90	35-47	15
arr1-4 arr2-4 pSUC2:pJL-blue #3	81,6	42,3	±5,4	±3,7	75-89	38-47	15
arr2-4 pSUC2:ARR2 #1	91,5	48,0	±4,8	±3,5	87-98	43-52	15
arr2-4 pSUC2:ARR2 #2	88,4	47,6	±4,3	±4,4	79-93	38-54	10
arr2-4 pSUC2:ARR2D80N #5	85,7	46,4	±3,8	±3,0	79-89	39-49	20
arr2-4 pSUC2:ARR2D80N #6	84,0	45,6	±5,4	±3,6	77-90	39-49	10
arr1-4 arr2-4 pSUC2:ARR2 #3	89,8	46,9	±4,0	±2,7	84-93	43-51	15
arr1-4 arr2-4 pSUC2:ARR2 #1	90,3	47,9	±6,1	±2,2	80-100	45-52	15
arr1-4 arr2-4 pSUC2:ARR2D80N #6	87,3	45,3	±4,9	±2,5	78-93	42-51	15
arr1-4 arr2-4 pSUC2:ARR2D80N #2	87,7	45,0	±8,	±3,0	75-100	39-49	15
arr1-4 arr2-4 pSUC2:ARR2D80N #X	83,0	44,3	±8,8	±1,8	75-100	42-47	15

Genotype	LSD DUF	Mean
WT (Col-0) pSUC2::Jlblue	A	100,8
arr1-4Xarr2-4 (Col-0) pSUC2::ARR2	B	90,0
arr2-4 (Col-0) pSUC2::ARR2	B C	89,1
arr1-4 (Col-0) pSUC2::Jlblue	B C	88,9
arr2-4 (Col-0) pSUC2::Jlblue	B C	86,6
arr1-4Xarr2-4 (Col-0) pSUC2::ARR2D80N	C	86,0
arr2-4 (Col-0) pSUC2::ARR2D80N	C D	85,0
arr1-4Xarr2-4 (Col-0) pSUC2::Jlblue	D	81,1

Genotype	LSD Rosette	Mean
WT (Col-0) pSUC2::Jlblue	A	53,1
arr1-4 (Col-0) pSUC2::Jlblue	B	49,1
arr2-4 (Col-0) pSUC2::ARR2	B C	47,8
arr2-4 (Col-0) pSUC2::Jlblue	B C	47,4
arr1-4Xarr2-4 (Col-0) pSUC2::ARR2	B C	47,4
arr2-4 (Col-0) pSUC2::ARR2D80N	C D	46,0
arr1-4Xarr2-4 (Col-0) pSUC2::ARR2D80N	D	44,9
arr1-4Xarr2-4 (Col-0) pSUC2::Jlblue	E	41,3

Table 5.4 Flowering time of transgenic lines driven under tissue specific *FD* promoter under short day non-inductive conditions.

DUF, days until flowering; RLN, rosette leaf number; n, number of individuals; #, identifier for individual transgenic line. All transgenic lines used for this experiment were in T2 generation and preselected with BASTA. Significance levels were computed after passing ANOVA (α=0.05) by using Fischer's Least Significance Difference (LSD) with alpha levels α=0.01 for DUF and α=0.05 or RLN. Classes *not connected* by a letter are significantly different.

	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Short Day							
pFD:pJL-blue #1	108,1	54,6	±4,5	±3,3	102-115	49-58	15
pFD:pJL-blue #3	106,7	54,1	±6,3	±4,3	98-115	47-59	15
pFD:pJL-blue #5	107,7	52,5	±6,4	±4,5	99-118	45-56	15
arr1-4 pFD:pJL-blue #1	86,9	49,4	±4,1	±5,7	78-93	40-57	15
arr1-4 pFD:pJL-blue #2	86,9	50,5	±6,4	±4,3	79-97	41-53	15
arr1-4 pFD:pJL-blue #3	89,0	50,8	±5,1	±4,0	79-97	47-55	15
arr2-4 pFD:pJL-blue #1	82,7	48,7	±5,2	±4,8	74-89	39-53	15
arr2-4 pFD:pJL-blue #5	83,6	48,3	±4,6	±3,2	75-89	47-53	15
arr2-4 pFD:pJL-blue #4	83,5	46,7	±4,3	±4,3	74-89	39-54	15
arr1-4 arr2-4 pFD:pJL-blue #1	80,2	39,3	±4,3	±3,9	73-86	35-47	15
arr1-4 arr2-4 pFD:pJL-blue #3	81,4	41,8	±3,4	±2,9	75-87	39-46	15
arr1-4 arr2-4 pFD:pJL-blue #6	81,1	41,3	±4,7	±2,8	74-89	37-45	15
arr2-4 pFD:ARR2 #8	96,4	49,6	±4,0	±3,7	95-102	42-53	15
arr2-4 pFD:ARR2 #5	97,7	50,5	±3,9	±2,8	93-105	45-53	15
arr2-4 pFD:ARR2 #7	95,8	48,0	±5,6	±1,9	90-100	46-50	15
arr2-4 pFD:ARR2D80N # 1	88,4	47,3	±3,9	±2,3	83-94	41-50	18
arr2-4 pFD:ARR2D80N #2	89,8	46,2	±3,5	±3,1	82-94	41-53	10
arr1-4 arr2-4 pFD:ARR2 #4	95,3	49,8	±3,7	±3,0	90-100	47-54	15
arr1-4 arr2-4 pFD:ARR2 #2	93,4	48,3	±5,9	±4,0	88-106	40-54	15
arr1-4 arr2-4 pFD:ARR2 #6	96,8	51,0	±2,8	±3,1	94-101	46-55	15
arr1-4 arr2-4 pFD:ARR2D80N #1	85,9	45,3	±4,2	±2,7	79-91	39-48	15
arr1-4 arr2-4 pFD:ARR2D80N #2	87,1	45,7	±3,8	±2,7	79-91	39-49	15

Genotype	LSD DUF	Mean
WT (Col-0) pFD::Jlblue	A	107,6
arr2-4 (Col-0) pFD::ARR2	B	96,7
arr1-4Xarr2-4 (Col-0) pFD::ARR2	B	95,4
arr2-4 (Col-0) pFD::ARR2D80N	C	89,0
arr1-4 (Col-0) pFD::Jlblue	C	87,6
arr1-4Xarr2-4 (Col-0) pFD::ARR2D80N	C D	86,5
arr2-4 (Col-0) pFD::Jlblue	D E	83,3
arr1-4Xarr2-4 (Col-0) pFD::Jlblue	E	80,9

Genotype	LSD Rosette	Mean
WT (Col-0) pFD::Jlblue	A	53,5
arr1-4 (Col-0) pFD::Jlblue	B	50,3
arr1-4Xarr2-4 (Col-0) pFD::ARR2	B C	49,7
arr2-4 (Col-0) pFD::ARR2	B C	49,6
arr2-4 (Col-0) pFD::Jlblue	C D	47,9
arr2-4 (Col-0) pFD::ARR2D80N	D E	46,8
arr1-4Xarr2-4 (Col-0) pFD::ARR2D80N	E	45,5
arr1-4Xarr2-4 (Col-0) pFD::Jlblue	F	40,8

5.2.6 *ARR1* and *ARR2* work mostly independent of *FLC*

FLOWERING LOCUS C (FLC) is a transcription factor that functions as a repressor of the floral transition. It is the main target of the vernalization pathway in *Arabidopsis thaliana* and is epigenetically silenced by the prolonged exposure of plants to cold. FLC was shown to bind to and directly block the transcriptional activation of *SOC1* and *FT* (Helliwell *et al.* 2006; Srikanth and Schmid 2011).

Based on RT-qPCR data obtained from *ARR1* and *ARR2* knockouts, the *arr1-4, arr2-4* and *arr1-4 arr2-4* double mutants showed reduced levels of *FLC* compared to wild-type. In order to find out more about the nature of this interaction the *arr1-4 arr2-4* double mutant was crossed with a deletion mutant for *FLC, flc-3* (Michaels and Amasino 1999). The triple mutant was identified by PCR (see Methods) and confirmed at the expression level (supplemental figure 11).

The *flc-3 arr1-4 arr2-4* (mean: 28 DUF) triple mutant under inductive LD conditions behaved like the *flc-3* single mutant (mean: 28 DUF), flowering about 6 days earlier (mean: 34 DUF) and producing 2 leaves less than the Col-0. The *arr1-4 arr2-4* double mutant displayed an intermediate phenotype (mean: 32 DUF) (Figure 5.5, A (left panel), B). Under LDs, *FLC* appears to be epistatic to *ARR1* and *ARR2*. Under non-photoperiodic SD conditions the *flc-3 arr1-4 arr2-4* triple mutant (mean: 78 DUF) very clearly shows an additive effect. It flowers earlier than both parents, 8 days earlier than the *flc-3* (mean: 87 DUF) about 2 weeks before *arr1-4 arr2-4* (mean: 92 DUF) and more than 3 weeks before the Col-0 (mean: 111 DUF). The same flowering time was observed when this experiment was repeated under SD conditions but under a different light quality(. These results confirm the consistency of an additive early flowering phenotype of the *flc-3 arr1-4 arr2-4* triple mutant in SDs (Figure 5.5, A (right panel), D).

The *flc-3 arr1-4 arr2-4* triple mutant repeatedly showed a clear tendency towards early flowering when compared to the *flc-3* single mutant. However, the differences were not statistically significant. Therefore, the same experiment was repeated again under LDs but at 16°C temperature, which results in a mild delay in flowering. Interestingly, under these conditions, *flc-3 arr1-4 arr2-4* flowered earlier than *flc-3*. The triple mutant *flc-3 arr1-4 arr2-4* flowered over a week earlier that the wild-type (mean: 34 DUF) which was almost five days before *arr1-4 arr2-4* double mutant (mean: 39 DUF) and two days earlier than *flc-3* single mutant (mean: 36 DUF) (Figure 5.5, C), suggesting an additive effect on of *FLC* and *ARR1* and *ARR2* under inductive LD conditions.

Based on these experiments, the *flc-3 arr1-4 arr2-4* triple mutant shows very consistent additive effect in early flowering under SDs and LDs (statistically significant only at 16°C). Based on this result *ARR2* seems to work mainly independent of *FLC*. If there is any intercommunication between these two signalling pathways then it is rather small.

A

B

	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Long Day							
Col-0 (wild type) A	33,9	13,9	±1,5	±1,2	32-37	11-15	15
arr1-4 arr2-4 B	32,3	13,6	±1,4	±1,0	31-35	12-15	15
flc-3 C	28,2	11,4	±0,4	±0,7	28-29	10-12	15
flc-3 arr1-4 arr2-4 C	27,6	11,8	±0,5	±0,4	27-28	11-12	15

C

	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Long Day (16°C)							
Col-0 (wild type) A	42,05	16,74	±2,3	±1,9	38-45	14-22	19
arr1-4 arr2-4 B	39,12	15,82	±2,2	±1,4	35-42	13-18	17
flc-3 C	36,75	14,31	±2,1	±1,9	33-40	12-18	16
flc-3 arr1-4 arr2-4 D	34,75	12,95	±1,6	±1,5	32-37	10-16	20
Long Day (23°C)							
Col-0 (wild type) A	29,00	11,65	±2,7	±0,8	22-32	10-13	20
arr1-4 arr2-4 B	27,21	11,00	±2,6	±0,8	23-31	9-12	19
flc-3 C	22,43	9,67	±2,1	±0,9	20-28	8-11	21
flc-3 arr1-4 arr2-4 C	22,06	9,47	±2,3	±0,8	19-26	8-11	17

D

	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Short Day (Chamber 1)							
Col-0 (wild type) A	111,2	34,7	±6,4	±4,6	100-118	28-43	15
arr1-4 arr2-4 B	92,1	27,7	±4,7	±2,6	84-102	24-32	15
flc-3 C	86,6	27,3	±4,7	±4,4	78-93	21-36	15
flc-3 arr1-4 arr2-4 D	77,9	24,0	±4,6	±2,4	72-85	21-28	15
Short Day (Chamber2)							
Col-0 (wild type) A	113,0	47,6	±4,2	±4,2	107-120	43-51	15
arr1-4 arr2-4 B	91,4	37,9	±6,3	±1,7	81-99	40-45	15
flc-3 C	83,8	35,7	±3,0	±2,2	78-87	37-41	15
flc-3 arr1-4 arr2-4 D	76,8	27,8	±4,2	±1,8	71-83	27-32	15

Figure 5.5 Flowering phenotype of *arr1-4 arr2-4 flc-3* triple mutant.
(A) Photographs showing early flowering phenotype of *arr1-4 arr2-4* double mutant, *flc-3* single mutant and *arr1-4 arr2-4 flc-3* triple mutant under LDs (left panel) and SDs (right panel) compared to wild-type. The arrow indicates the position of floral bud of Col-0. **(B)** Flowering times of mutant plants grown under long day conditions. **(B)** Flowering times of mutant plants grown at 16°C and 23° C under LD conditions. **(C)** Flowering times of mutant plants under SD conditions. Additive effect on flowering time by the triple mutant is very visible and consistent even under different light conditions (*i.e.* Chamber 1 and Chamber 2). DUF, days until flowering; RLN, rosette leaf number; n, number of individuals. Significance tests with LSD (α=0.05) were performed on DUF after passing one-way ANOVA (α=0.05). LSD classes are given as letters in superscript. Classes *not connected* by a letter are significantly different.

5.2.7 *ARR1* and *ARR2* work in the same pathway upstream of *FLM*

FLOWERING LOCUS M (FLM) is a MADS-domain gene that acts as an inhibitor of flowering in *Arabidopsis*. Although the sequence of *FLM* is similar to that of *FLC*, *FLM* and *FLC* interact with different flowering pathways (Scortecci *et al.* 2003). The *FLM* levels were also strongly down-regulated in both *ARR1* and *ARR2* knockouts and in the double mutant. Therefore, to get more insight into the potential interaction with FLM in respect to flowering time, the *arr1-4 arr2-4* double mutant was crossed with *flm-3* single knock-out mutant for *FLM* (Balasubramanian *et al.* 2006). The triple mutant was identified by PCR (see Methods) and confirmed at the expression level (supplemental figure 12).

A

B

	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Long Day							
Col-0 (wild type) A	30,1	10,9	±2,2	±1,7	27-34	8-15	20
arr1-4 arr2-4 B	26,9	9,8	±1,4	±1,4	25-30	8-12	20
flm-3 C	23,8	7,9	±0,9	±0,9	23-25	7-9	20
flm-3 arr1-4 arr2-4 C	24,3	8,4	±1,3	±0,9	23-27	7-10	20

C

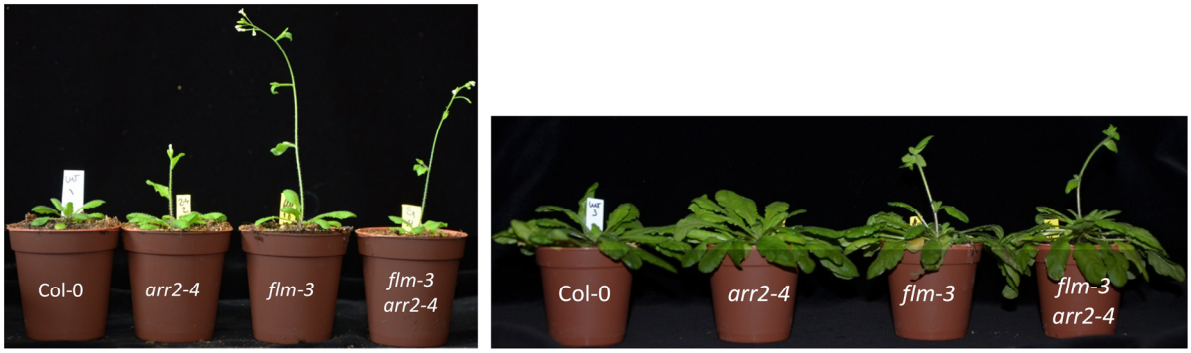
	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Short Day							
Col-0 (wild type) A	110,0	50,3	±6,4	±4,8	100-121	40-56	20
arr1-4 arr2-4 B	87,6	40,1	±5	±2,8	83-95	36-45	20
flm-3 C	77,4	28,8	±5,1	±4,3	69-85	27-34	20
flm-3 arr1-4 arr2-4 C	77,3	28,8	±7,3	±5,8	68-95	20-39	20

Figure 5.6 Flowering phenotype of *flm-3 arr1-4 arr2-4* triple mutant.
(A) Photographs showing early flowering phenotype of *arr1-4 arr2-4* double mutant, *flm-3* single mutant and *flm-3 arr1-4 arr2-4* triple mutant under LDs (left panel) and SDs (right panel) compared to wild-type. Epistatic effect of *FLM* on *ARR1* and *ARR2* on early flowering is very good visible in both long-and short days. **(B)** Flowering time of mutant plants grown under long day conditions. **(C)** Flowering time of mutant plants grown under short day conditions. DUF, days until flowering; RLN, rosette leaf number; n, number of individuals. Significance tests with LSD (α=0.05) were performed on DUF after passing one-way ANOVA (α=0.05). LSD classes are given as letters in superscript. Classes *not connected* by a letter are significantly different.

The flowering time was first determined for the *flm-3 arr1-4 arr2-4* triple mutant under LD conditions. The triple mutant showed very early flowering (compared to the Col-0) and was indistinguishable from the *flm-3* single mutant. The triple mutant *flm-3 arr1-4 arr2-4* flowered in average 6 days earlier (mean: 24 DUF) which was the time same as *flm-3* (mean:

24 DUF), and both developed 3 rosette leaves less than the Col-0 (mean: 30 DUF). The double mutant *arrr1-4 arr2-4* (mean: 27 DUF) flowered somewhat later than the *flm-3* mutants and produced one leaf less than Col-0 (Figure 5.6, A left panel ,B). Clearly *FLM* is epistatic to *ARR1* and *ARR2*.

A



B

	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Long Day							
Col-0 (wild type) A	30,1	10,9	±2,2	±1,7	27-34	8-15	20
arr2-4 A	28,8	10,9	±1,9	±1,6	27-34	8-15	20
flm-3 B	23,8	7,9	±0,9	±0,9	23-25	7-9	20
flm-3 arr2-4 B	24,7	8,2	±1,3	±1,0	22-27	6-9	7

C

	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Short Day							
Col-0 (wild type) A	110,0	50,3	±6,4	±4,8	100-121	40-56	20
arr2-4 B	95,8	45,3	±4,5	±2,7	87-105	40-48	20
flm-3 C	77,4	28,8	±5,1	±4,3	69-85	27-34	20
flm-3 arr2-4 C	87,4	28,3	±5,4	±3,1	81-99	22-31	9

Figure 5.7 Flowering phenotype of *flm-3 arr2-4* double mutant.
(A) Photographs showing early flowering phenotype of *arr2-4* and *flm-3* single mutants and *flm-3 arr2-4* double mutant under LDs (left panel) and SDs (right panel) compared to wild-type. **(B)** Flowering time of mutant plants grown under long day conditions. **(C)** Flowering time of mutant plants grown under SD conditions. DUF, days until flowering; RLN, rosette leaf number; n, number of individuals. Significance tests with LSD (α=0.05) were performed on DUF after passing one-way ANOVA (α=0.05). LSD classes are given as letters in superscript. Classes *not connected* by a letter are significantly different.

The *flm-3 arr1-4 arr2-4* triple mutant and the *flm-3 single* mutant grown under non-inductive SD condition showed epistasis to *flm-3* single mutant as well (Figure 5.6, A(right panel), C). The *flm-3* and *flm-3 arr1-4 arr2-4* triple mutant mutants flowered approximately at the same time, i.e. more than 30 days earlier (mean: 77 DUF) than the wild-type (mean: 110 DUF) and over 10 days earlier than the *arr1-4 arr2-4* double mutant (mean: 87 DUF). Both *flm-3* containing mutants developed 20 rosette leaves less (mean: RLN 29) than Col-0 (mean: RLN 50). In contrast the *arr1-4 arr2-4* double mutant made 10 leaves less than Col-0 (mean: RLN 40). Based on these results *FLM* is epistatic to *ARR1* and *ARR2* in LDs and SDs, and therefore *ARR1* and *ARR2* appear to be genetically involved within the same flowering pathway as *FLM*. *ARR1* and *ARR2* are hypostatic to *FLM*, i.e. they both probably act upstream of *FLM*). Epistasis of *ARR2* to *FLM* was one more time confirmed on *flm-3 arr2-4* double mutant. Under long

day conditions *flm-3 arr2-4* (mean: 87 DUF) double mutant flowers at the same time like the *flm* (mean: 77 DUF) *parent* which is almost a week before the wild-type and 5 days before the *arr2-4* single mutant. Single mutant *flm-3 and* the *flm-3 arr2-4* double mutant also have the same number of rosette leaves (Figure 5.7, A (left panel) and B).

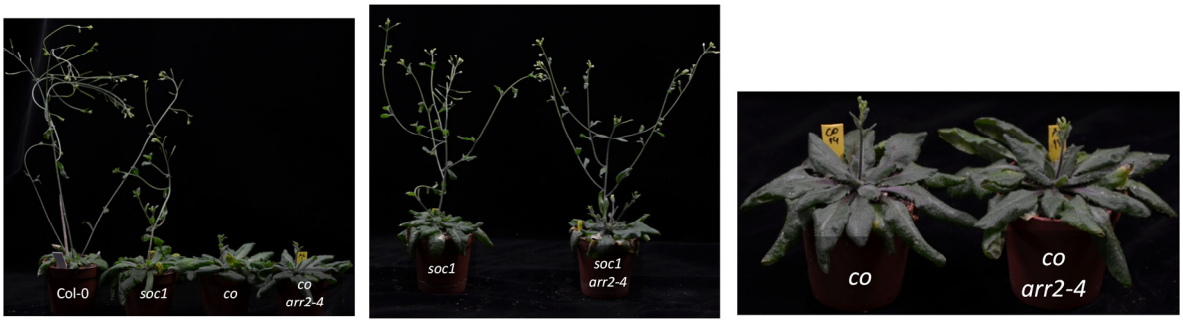
5.2.8 Initial experiments with crossings between *arr2-4* with *soc1-2* and *co* reveal unanticipated interactions

During this work it was possible to generate and analyse double mutants with *co* and *soc1-2* lines for placing *ARR2* within other known flowering time pathways. Recall that *arr2-4* had the stronger flowering time phenotype compared to *arr1-4*. Floral activators *SOC1* and *CO*, especially SOC1, are floral integrators converting most of the flowering pathways. CONSTANS (CO) promotes flowering of *Arabidopsis* in response to day length. Under LD conditios CO activates transcription of *FLOWERING LOCUS T* (*FT*) in the vascular tissue of leaves and initiates flowering (Corbesier *et al.* 2007). *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) is required for CO to promote flowering and integrates the long-day and vernalization/autonomous pathways (Samach *et al.* 2000). On the other hand *SOC1* also integrates signalling from the GA-dependent pathway, a major flowering pathway under non-inductive short days (Moon *et al.* 2003). The *SOC1* and *FT* genes are also regulated by a different flowering-time pathway that acts independently of CO and which is active also at the non-inductive SD conditions (Samach *et al.* 2000). As previously described, beside the similar role of *CO* and *SOC1* as floral activators, they can also act independently of each other. Therefore, both *arr2-4 soc1* and *arr2-4 co* double mutants were tested for flowering time under LD and SD conditions.

Under inductive LD conditions both double mutants were indistinguishable from their *soc1* and *co* single mutant parents. The *arr2-4 soc1* double mutant flowered 5 days later than the Col-0 and more than a week after its *arr2-4* parent developing 7 rosette leaves more than Col-0 and *arr2-4*. Late flowering phenotype is especially remarkable by *arr2-4 co* (mean: 45 DUF) double mutant where flowering was delayed for more than two weeks compared to Col-0 (mean: 30 DUF) and more than 17 days than by *arr2-4* (mean: 28 DUF), *arr2-4 co* double mutant developed 13 rosette leaves more than Col-0 and *arr2-4* (Figure 5.8, A and B).

Under non-inducible SD conditions CO is unable to activate the *FT* flowering-pathway. When grown under SD conditions *co* single mutant and *arr2-4 co* double mutant show very similar flowering phenotype. They flower around 10 days (*arr2-4 co* double mutant) and 15 days (*arr2-4* single mutant) (mean: 100 DUF) before the Col-0 (mean: 110 DUF) but make even 15 rosette leaves less than the wild-type). On the other hand, *co* and *arr2-4 co* double mutant flower only a couple of days later than the *arr2-4* (mean: 96 DUF although they both made 10 leaves less than *arr2-4* single parent (Figure 5.8, C).

A



B

	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Long Day							
Col-0 (wild type) C	30,1	10,9	±2,2	±1,7	27-34	8-15	20
arr2-4 C	28,8	10,9	±1,9	±1,6	27-34	8-15	20
co A	45,8	23,3	±2,5	±3,0	40-49	17-26	20
soc1 B	34,7	18,2	±2,1	±1,8	30-37	17-22	20
arr2-4 co A	45,7	23,8	±3,0	±2,3	37-48	20-28	20
arr2-4 soc1 B	33,5	17,6	±3,6	±2,5	27-57	12-22	6

C

	DUF	RLN	DUF SD	RLN SD	DUF range	RLN range	n
Short Day							
Col-0 (wild type) B	110,0	50,3	±6,4	±4,8	100-121	40-56	20
arr2-4 E	95,8	45,3	±4,5	±2,7	87-105	40-48	20
co C D	102,6	35,1	±6,9	±4,1	93-112	30-43	20
soc1 A	116,4	45,1	±8,4	±4,1	105-125	42-55	20
arr2-4 co D E	99,9	35,3	±7,2	±6,0	90-111	25-46	20
arr2-4 soc1 B C	107,3	40,8	±5,6	±6,1	101-117	33-47	6

Figure 5.8 Flowering phenotype of *soc1 arr2-4* and co arr2-4 double mutants.
(A) Photographs showing late flowering phenotype of *soc1 arr2-4* (middle panel) and *co arr2-4* (left and right panel) double mutants under LDs compared to wild-type. **(B)** *ARR2* is hypostatic to *CO* and *SOC1*. **(C)** Flowering phenotype of *soc1 arr2-4 and co arr2-4* under SD conditions . DUF, days until flowering; RLN, rosette leaf number; n, number of individuals. Significance tests with LSD (α=0.05) were performed on DUF after passing one-way ANOVA (α=0.05). LSD classes are given as letters in superscript. Classes *not* connected by a letter are significantly different.

Double mutant *arr2-4 soc1* shows intermediate flowering phenotype compared to its parents. The *arr2-4 soc1* (mean: 107 DUF) double mutant flowers around 9 days earlier than *soc1* (mean: 116 DUF) single mutant and makes 5 rosette leaves less, the same double mutant flowers around 12 days later than *arr2-4* single mutant and makes around 5 rosette leaves more than the *arr2-4* single mutant (Figure 5.8, C).

Taken together, under inductive long days *ARR2* seems to be hypostatic or even works independently of *CO* and *SOC1*. Under non-inductive short days CO-related-flowering pathways are not very active and *arr2-4 co* double mutant shows very similar phenotype like *arr2-4* parent showing again indications that *ARR2* might be hypostatic or independent to *CO* under SDs as well. Another *arr2-4 soc1* double mutant shows similar phenotype like the wild-type which is in between of both single parents suggesting that under SD conditions *ARR2* works partially independent and partially dependent of SOC1-flowering pathways.

5.3 Discussion

During this work, a novel early flowering phenotype was observed and confirmed for single mutants *arr1-4* and *arr2-4* and the double mutant *arr1-4 arr2-4* with respect to flowering time. The early flowering phenotype is especially visible under non-inductive SD conditions for all three mutant lines. The *arr1-4 arr2-4* double mutant flowers even earlier than either single mutant or the wild-type and this tendency was statistically significant between wild-type and the both single mutants. In contrast, under LD conditions, only the *arr2-4* as single mutant showed slightly statistically significant differences as early flowering on the other hand was also observed for the *arr1-4* single mutant but this tendency could not be statistically confirmed in LD. Nevertheless, these differences under inductive long days are from a physiological/functional point of view very subtle and were only revealed when plants were exposed to cool temperatures (16°C). In this case, the double mutant clearly flowered earlier than wild-type. Thus, although *arr2-4*, and presumably *arr1-4*, also affect flowering in LDs, the loss of these two genes clearly points much stronger roles of *ARR2* as a flowering repressor under SD conditions.

Flowering of *arr1-4* mutant in SDs is approximately one week earlier, *arr2-4* flowers two weeks earlier, the *arr1-4 arr2-4* double mutant flowers three weeks before the Col-0. This effect on days until flowering (DUF) was also reflected in rosette leaf number. It has been already proven that generally B-types from the subgroup I (Kim *et al.* 2012) can complement the *arr10 arr12* cytokinin response-deficient mutant (Hill *et al.* 2013). This could be also the case with early flowering phenotype. Yet interestingly, despite redundancy and very similar sequence homology of *ARR1* and *ARR2* genes, we managed to prove that under the loss of *ARR2* effects flowering more strongly than *ARR1* suggesting specificity and affinity (dominance) of *ARR2* over *ARR1* with respect to flowering time despite their high sequence homology. Interestingly, overexpression of ARR2 was shown to have higher transactivation potential and response with and without the addition of cytokinin on the *ARR6* promoter compared to ARR1 (Hwang and Sheen 2001). In addition, even though *ARR2* contributes to primary root-elongation responses to exogenous cytokinin, ARR1 is the predominate cytokinin information carrier in this tissue (Mason *et al.* 2005). Thus, even though these two homologues can have different roles in different tissues, *ARR1* and *ARR2* showed an additive effect on flowering time, which is visible in the *arr1-4 arr2-4* double mutant under both inductive- and non-inductive conditions. One can therefore conclude that both genes are involved in the same flowering time pathway and their role is especially crucial in the signalling pathways which are dominantly active under non-inductive SD conditions.

To check which genes are miss-regulated in the double mutant RT-qPCR analysis of familiar and well described floral regulators was performed. One scenario that would explain the early flowering phenotype is that *ARR1* and *ARR2* genes/pathway (cytokinin) positively regulates the expression of certain floral repressors (such as *FLM*, *FLC*, *SVP*). Alternatively, early flowering could be induced by activation of positive regulators of flowering (*TSF, FD, CO)* or floral integrators (*SOC1*). The chosen markers were applied in order to profile the state of

SAM at this chosen time point. For the first hints, leaf material and material from shoot apical meristem (SAM) from the wild-type and the double mutant was taken depending on where the tested gene is best expressed. The material was taken from plants grown under SDs and it harvested shortly before the double mutant entered in the reproductive phase (based on previous results).

Gene expression of floral activators *CO*, *SOC1*, *FT* and *FDP* did not show any statistically significant difference in the double mutant compared to the wild-type. *FT* is known to be expressed in LDs conditions and acts as a long-range signal in *Arabidopsis* (Valverde *et al*. 2004; Jaeger and Wigge 2007; Sawa and Kay 2011) and here this marker gene was used as a "control" to make sure that the plants were strictly grown under SD conditions and this is confirmed by its very low expression. Floral activators *FD* and *TSF* showed slight up-regulation, although only *TSF* was statistically significantly different compared to the wild-type. Interestingly, it was shown that under non inductive SDs exogenous cytokinin promotes early flowering via activation of the *TSF* (D'aloia *et al*. 2011). The exact mechanism of *TSF* activation is not known and it could be that *ARR1* and *ARR2* play role in its regulation knowing that both *ARR1* and *ARR2* are part of TCS and react to cytokinin treatment (Brenner *et al*. 2012). It might be that non phosphorylated formof *ARR1* and *ARR2* by TCS are somehow disturbing transcription of *TSF* and control flowering on that way. Unfortunately we have failed to produce the *tsf-1 arr1-4 arr2-4* triple knock out mutant and make a closer look how these three genes are interacting on genetic level. In general this data clearly indicated that the expression profile of floral activators is not significantly changed in the double mutant and that these genes are mainly not responsible for the early flowering phenotype in the double mutant.

On the other hand, tested floral repressors showed quite a different expression pattern in the double mutant plants. The floral repressors *FLC, FLM, SVP* and *TFL1* cover most of the crucial signalling pathways responsible for the flowering regulation in *Arabidopsis* (Coupland 1995; Koornneef *et al*. 1998; Song *et al*. 2013). Interestingly, three of tested floral repressors showed significant down-regulation of their expression in double mutant: *FLC* and *FLM* were quite reduced whereas *TFL1* showed only slight reduction. These data show the importance of *FLC* and *FLM* among the rest floral repressors with respect to early flowering in *arr1-4 arr2-4*. On the other hand, the *SVP* floral repressor seems not to be miss-regulated by the lack of *ARR1* or *ARR2*. This observation is of paramount importance and will be address later.

GA promotes flowering transition most strongly under environmental conditions such as SDs, especially when other regulatory pathways that promote flowering in *Arabidopsis thaliana* are not active (Moon *et al*. 2003; Porri *et al*. 2012). In an attempt to address this, showed that *GA4* and *GA5* reflected the state of active GA signalling in healthy plants (Achard *et al*. 2008). Therefore the expression of *GA4* and *GA5* were also monitored. Based on these two genes, we conclude that the GA flowering pathway is not disturbed in the double mutant as both *GA4* and *GA5* marker genes for this pathway are unaffected in the SAM in the double mutant.

This presumptively indicates that *ARR1* and *ARR2* are not involved in the GA flowering pathway but in other flowering signalling pathways active under non-inductive conditions.

Since meristem identity genes and some other genes predominately expressed in meristem play very important role in the initiation of the early flowering they were applied in order to profile the state of SAM at the chosen time point. For this purpose especially *AP1* but also and *LFY* expression was tested. Expression of *LFY* and *AP1* was 2-fold and 3-fold increased with respect to the wild-type. These markers confirmed the early flowering state of the *arr1-4 arr2-4* double mutant compared to Col-0 at the time point where this process could not be visually obtained. Although the low expression of FT suggested that the plants were monitored before they were flowering, it could still have been possible that the shift to inflorescence meristem had already occurred. Therefore, to eliminate the possibility of unseen developmental effects, the flowering transition induced with 30-day old plants grown under non-inductive conditions and then shifted to inductive LDs. The material collected from SAM before and after the shift guaranteed that differences in expression profiles of floral regulators among the mutant plants were due to the miss-regulation of the flowering time only. Once more, it was demonstrated that the early flowering phenotype in both single parents and the wild-type comes from the miss-regulation, i.e. down-regulation of specific floral repressors, primarily *FLC* and *FLM*, as that their level was low even under non-inductive conditions and it remained unchanged after the shifting. Expression pattern of *SVP* floral regulator remained unaffected in any of mutant plants before the shift and responded as expected after the shift (was down-regulated) confirming the previous results and suggesting that *ARR1* and *ARR2* are specifically regulating only certain floral repressors under non-inductive SD conditions. Consistently, all of the tested floral activators showed their regular expression profile like wild-type,: they were up-regulated after the shift from vegetative into reproductive state and showing that neither *ARR1* nor *ARR2* are not playing any role in transcriptional regulators of these floral activators at the not expression level. In conclusion, the early flowering phenotype was most probably a consequence of down-regulation of floral repressors but not of miss-regulation of floral activators.

Considering roles of *ARR1* and *ARR2* in cytokinin signalling and knowing the fact that cytokinin has a positive-correlative role in regulating SAM size (Werner and Schmulling 2009; Gupta and Rashotte 2012) and that SAM size has been correlated with flowering time (Jeong and Clark 2005), the SAM size of *arr1-4*, *arr2-4*, and *arr1-4 arr2-4* warranted examination. Such information could be used to deduce if changes in expression of floral repressors were due to an increase in size of the apical meristem in the mutant plants. Therefore, plants were grown under the same conditions used in the shifting experiment. Both the *arr2-4* single and *arr1-4 arr2-4* double mutants had surprisingly statistically significant smaller SAMs than the wild-type and although the measurement population also showed a smaller size for *arr1-4*, its SAM did not pass the significance threshold (α=0.05). Thus, the smaller SAM fits more with a loss of cytokinin signal, which would be expected knowing that both ARR1 and ARR2 are active in cytokinin singling and despite the smaller SAM size in the mutant plants they

manage to flower earlier than the wild-type. Differences in anatomy or morphology of SAMs in mutant plants and the wild-type were not observed either. In conclusion, the changes in expression of floral repressors were not due to an increase in size of the apical meristem.

Although the *arr2-4* and *arr1-4* mutants have been previously described (Mason *et al*. 2005) and presumed free of any other background mutations, we wanted to be sure that the early-flowering phenotype was due to the lack of *ARR1* and *ARR2*. In order to check this presumption and to determine the origin/location of acting of the *ARR2* with respect to early flowering phenotype the *arr2-4* and *arr1-4 arr2-4* mutants which had the strongest phenotype were complemented with *ARR2*-cDNA and an *ARR2D80N*-cDNA mutant. The *ARR2D80N*-cDNA mutant version cannot be phosphorylated by the TCS anymore, and is less responsive to cytokinin than the wild-type *ARR2*-cDNA (Hwang and Sheen 2001; Choi *et al*. 2010; Veerabagu *et al*. 2012). Thus although it is not completely inert, *ARR2D80N* can be used to see if the wild-type protein, and therefore TCS phosphorylation-dependency, is necessary to rescue the early flowering phenotype.

The cDNA variants were driven under tissue-specific promoters *pFD* and *pSUC2*. For this purpose following vectors with promoter expressed in specific tissues were used: *pSUC2* vasculature-specific promoter expressed in phloem (companion cells) only (Truernit and Sauer 1995; Wippel and Sauer 2012)and *pFD*-meristem-specific promoter expressed in shoot apical meristem only (Mathieu *et al*. 2007). Based on their expression pattern one can determine the most likely location where ARR2 protein activity is needed to complement the flowering time phenotype. Only T2 plants were able to be analysed, therefore a pre-selection was needed to remove any non-transgenic plants using BASTA; secondary effects were controlled by using an empty-vector backbone transformed into all of the mutant lines as well as the wild-type.

ARR2 driven under *pFD* shows the strongest effect and can almost completely complement the flowering phenotype of both the single and the double mutant in SDs where the *arr2-4* and *arr1-4 arr2-4* mutation effects were most strongly observed. This effect in SDs is slightly weaker (partial) when *ARR2* is driven under *pSUC2* but still significant in the double mutant. In LDs conditions, *ARR2* expressed from either promoter was able to complement the *arr2-4* mutation, but only *ARR2* expressed under the *pFD* promoter was able to partially rescue the *arr1-4 arr2-4* mutant. Considered that both tissue-specific promoters are equally strong and having in mind that *pSUC2* expression is on whole plant level one can conclude that *ARR2* is most potently working in the SAM with respect to flowering time.

Some remarks are warranted: first, both promoters can lead to complementation (even if partial) meaning that function of ARR2 is deliverable (or needed) at the meristem but also throughout the plant. Taken together; the observed early flowering phenotype is truly a consequence of the lack of *ARR2* as it can be rescued when it is expressed directly in the SAM by the *pFD* promoter or when it is expressed in phloem companion cells by *pSUC2*. Although not directly shown in the work if the lack of *ARR2* and *ARR1* means that cytokinin signalling

per-se is the culprit for the early-flowering phenotype, the idea is still passed around, albeit lightly, that cytokinin(s) in some form or fashion also could works in a "florigen" type function (Bernier 2011). These results are based on work in *Sinapis alba* (*Sa*) where connections to a simultaneous, but not necessarily, co-dependent transportation of *SaFT* and cytokinin to the meristem. Bernier proposed that some aspect of cytokinin, possibly on the organizing centre, contributes to flowering, but does not in itself cause flowering in *Sinapis* (Bernier 2011). This idea is interesting in lieu of the results here where a stronger effect is seen when *ARR2* is expressed in the meristem, yet *ARR2* still expressed in the phloem (distally) can also lead to complementation. Conflicting with these ideas is the observation that *Arabidopsis* can be induced to flower in SD with an 8 hour hydroponic treatment with BAP (D'aloia *et al.* 2011). This effect requires *TSF* and not *FT* (D'aloia *et al.* 2011). Recall that expression of *TSF* was altered in *arr1-4 arr2-4*.

FLC is known to interact with directly interacts with another MAD-box member SVP, *SVP* which is also floral repressor that functions within the thermosensory pathway (Li *et al.* 2008). SVP and FLC also target *CYTOKININ RESPONSE 1 (CRE1)* and other genes in cytokinin signalling *(Gregis et al. 2013)*. SVP has been further connected to cytokinin via *SVP* regulation of STIMPY (*STIP)*, but this occurs independently and downstream of *FT* and *TSF* (Gregis *et al.* 2013). *STIP* works downstream of cytokinin in establishing the SAM (Liu *et al.* 2009). Interestingly, *FLC* has been shown to be active in the SAM and in the vascular tissue directly regulating *SOC1* and *FT* (reviewed in (Andres and Coupland 2012). *TSF* was shown not to be bound by SVP (Gregis *et al.* 2013). In this work, *arr1-4* and *arr2-4* were also crossed to *tsf-1* (Yamaguchi *et al.* 2005), but triple mutants were not identified, therefore it was not possible to determine the genetic interaction of *arr1-4* or *arr2-4* with *tsf-1*.

Knowing that the loss of *ARR1* and *ARR2* impair cytokinin signalling, it is tempting to speculate that this would explain the early flowering phenotype. In this regard, *SVP* has recently been uncovered to be involved in regulation of some cytokinin genes (Gregis *et al.* 2013). Considering that the expression data and mutant analysis (discussed below) suggest that *ARR1* and *ARR2* are not in the *SVP-FLC* pathway, it is still an open question if the early flowering phenotype can only be attributed to a loss of cytokinin single transmission. Second, other possible reasons for partial complementation could be that the plants used for this experiment were preselected in the T2 generation with most of them in the hemizygous state and thus not enough ARR2 was produced. Alternatively, the gene was not driven under its native promoter and perhaps ARR2 is also needed in other tissues. For example, it is known that *ARR1* is active in controlling auxin flux by regulation of the auxin-response repressor protein SHY2 (SHORT HYPOCOTYL2) (Taniguchi *et al.* 2007; Chapman and Estelle 2009) and the functional characterisation of *ARR1* and *ARR2* have mostly been done with regards to cytokinin responses in the root (Mason *et al.* 2005; Argyros *et al.* 2008; Hill *et al.* 2013).

As said before, the connection to cytokinin, is compelling but not conclusive. In rice, it is known that a cytokinin-dependent TCS pathway exists that requires a B-type (*EHD1*) for aviation of *FT* homologues *HD3A* and *RICE FLOWERING LOCUS T1 (RFT1)* under SDs (Andres

and Coupland 2012). Provided that more evidence is mounted that connects more TCS components to this flowering time phenotype, then (although highly speculative at this point) it could be proposed that a TCS network also works in *Arabidopsis* to modulate flowering which may be analogous to that of rice (as it is not likely it is the same considering no orthologues can be found) or more indicative to the observations made in *Sinapis*. Nevertheless, this regulation mechanism in rice only supports our observation that TCS, or at least its B-Type response regulators *ARR1* and *ARR2*, might also play role in flowering regulation of *Arabidopsis thaliana* under short day conditions as mutant plants lacking in *ARR1* and *ARR2* genes showed the early flowering phenotype under non-inductive short days in *Arabidopsis*. In support to this claim is a fact that *ARR4* (A-type response regulator) in *Arabidopsis thaliana* plays very important role in stabilization of active PhyB-Pfr under extended red light conditions and thus functions as modulator of photomorphogenesis (Sweere *et al*. 2001; Mira-Rodado *et al*. 2007) and PhyB, as above mentioned, actively involved in expression control of *CO* in *Arabidopsis thaliana* and *Hd3a* in *Oryza sativa (Ishikawa et al. 2011)*.

That said, is there any evidence that TCS signalling is required for the early-flowering time phenotype? Examination of the mutant plants showed that they were also slightly complemented with loss-of-function *ARR2D80N* in the single and double mutants under SDs when driven under the *pFD* promoter or by *pSUC2* (albeit only in the *arr1-4 arr2-4*). This slight effect of *ARR2D80N* was also observed in LDs but only in the *arr2-4* mutant background. As mentioned earlier, the reason for this is most likely due to an incomplete inactivation of the *ARR2* by the D80N mutation which was proven by other groups (Hwang and Sheen 2001). ARR2 has a strong basal transactivation capacity unlike the other B-types without the presence of exogenous cytokinin; yet, it still can strongly activate promoter-reporters in a cytokinin-dependent manner (Hwang and Sheen 2001; Veerabagu *et al*. 2012). The ARR2-D80N mutation impairs, sometimes strongly, this basal transactivation capacity without destroying the cytokinin-dependent response, which is highly enhanced compared to mock controls (Hwang and Sheen 2001). Considering this fact, it can be still concluded that ARR2 needs to be phosphorylated by the TCS in order to best rescue the early flowering phenotype as only weak partial complementation were observed for the *ARR2D80N* loss-of-function gene. Type-B response regulators mediate most, if not all, of the immediate-early changes of gene expression induced by cytokinin. This is also the case with *ARR1* and *ARR2* member of the largest sub-class of response regulators expressed in almost all tissues which consists of seven members (*ARR1, ARR2, ARR10, ARR12, ARR11, ARR14,* and *ARR18*) (Brenner *et al*. 2012). This additionally supports the claim that the early flowering phenotype in the *ARR1* and *ARR2* lacking plants is originating due to the impaired signalling of cytokinin. Furthermore, the expression pattern of the *ARR1* and *ARR2* in almost all tissues is showing the importance of cytokinin in every tissue of the plant, and also explains the observation that the early flowering phenotype was rescued also when *ARR2* expressed from phloem. On the other hand mutant complementation with *ARR2D80E* gain-of-function gene under constitutive active promoter is not possible while the plants showed embryo lethality (Hass *et*

al. 2004). Based on this fact the complementation with *pSUC2* and *pFD* tissue specific promoters could not possible while due to their high potency. Therefore *arr1-4 arr2-4* was complemented with ARR2D80E cloned into estradiol inducible *pABind::GFP* vector. As expected the effect of the gain-of-function *ARR2*D80E was very strong and lethal even for plants in T2 generation (supplemental figure 10) and was clearly visible 3 days after the hormonal treatment. Plants were getting various necrotic spots and dying very fast, whereas some of them managed to flower showing early flowering phenotype caused by stress. The formed siliques showed great variation in size and the seed yield was generally more than double lower compared with the wild-type containing certain number of aborted and sterile seed as well. This strongly suggests that *ARR2* may be a "crosstalk point" between the TCS and some flowering time pathways under the short day conditions. Expression analysis, via RT-qPCR, revealed which genes were miss-regulated in the single and double mutants compared to the wild-type and after conducting the flowering time shift, it could be confirmed that the repressors *FLC* and *FLM* are down-regulated in *arr1-4 arr2-4*. In order to determine which floral pathways *ARR1* and *ARR2* are involved in, *arr1-4 arr2-4* or *arr2-4* mutants were crossed to mutants in key floral regulatory pathways: *flc-3, flm-3, tsf-1, co, and soc1*. Triple mutants were obtained and evaluated with *flc-3* and *flm-3*, whereas *arr2-4* double mutants with *co* and *soc1* were also obtained and evaluated. Crosses with *tsf* unfortunately were not evaluable. For these experiments *arr2-4* single and *arr1-4 arr2-4* double mutants were crossed against *flc-3, flm-3* single mutants lacking in these floral repressors as they showed the highest miss-regulation pattern in *ARR1* and *ARR2* lacking mutants. On the other hand, despite the fact that all tested floral activators did not show any expression miss-regulation in *arr1-4 arr2-4*, *co* and *soc1* single mutants were crossed in order to see how and if these genes are interacting on genetic level.

When grown under inductive LD conditions at standard 22/23°C temperature the *flc-3 arr1-4 arr2-4* triple mutant behaved like its *flc-3* parent suggesting that FLC and the ARR1/2 are involved in the same pathway that controls flowering, *i.e*. *FLC* is epistatic to both *ARR1* and *ARR2*. Nevertheless, early flowering phenotype is under LDs is by all the mutants quite weak and the temporal resolution of flowering was very low. In addition to this *flc-3 arr1-4 arr2-4* triple mutant after each repeat under the LDs showed clear tendency to flower even earlier than the *flc-3* single parent but none of the time this tendency could be statistically proven. Therefore, the same experiment was repeated again under LDs but at 16°C temperature. Lower temperature should generally slow down the flowering time and the discrete differences in flowering time between the different mutant lines should be observed in higher resolution. This was indeed the case at 16°C, plants generally flowered later than at 23°C. Interestingly, this time *flc-3 arr1-4 arr2-4* triple mutant flowered even earlier that *flc-3* parent suggesting the additive effect of *FLC* under *ARR1* and *ARR2* under inductive LD conditions. Surprisingly, *flc-3* deletion mutant was in all this conditions showing the same ratio of flowering compared to the wild-type suggesting that FLC is probably active at the same level on both temperatures with respect to flowering time regulation, *i.e*. it is active at 16°C as a repressor. It have been published that that there are some natural accessions that are

unresponsive to thermal induction despite having non-functional *fri/flc* alleles (Srikanth and Schmid 2011). Recently was also reported that *flc-3* single mutant restores its temperature sensitivity fist below 16°C and not at higher temperatures (Lee *et al*. 2013). Under non-inductive SD conditions the *flc-3 arr1-4 arr2-4* triple mutant very clearly shows consistent additive effect in early flowering. The same pattern was observed also when this experiment was repeated under SD conditions in other growth chamber with different light intensity.

In conclusion, *FLC* appears to work partially independent of *ARR1* and *ARR2* which lack of expression has the same outcome with respect to early flowering phenotype making the triple mutant to flower even earlier then its parents in additive manner. This additive effect is especially good visible under SD conditions due to the enhanced activity of ARR1 and ARR2 under non-inductive short days with respect to early flowering. Under LD conditions at normal ambient temperature of 23°C additive effect could not be statistically proven, but there was each time the tendency present, due to very low involvement of ARR1 and ARR2 with respect to flowering time and therefore weak flowering time phenotype with the mutants. When plants grown under inductive LD conditions but at 16°C temperature additive effect could be observed and statistically proven. On the other hand, in *flm-3 arr2-4* double and *flm-3 arr1-4 arr2-4* triple mutants obtained early flowering phenotype was very clear. When grown on both inductive LD and non-inductive SD conditions both *flm-3 arr2-4* double and *flm-3 arr1-4 arr2-4* triple mutants showed identical phenotype like *flm-3* single parent supporting previous expression data obtained by RT-qPCR. Single *flm-3* mutant has very strong early flowering effect on both LD and SD conditions so it was very easy to proof and describe the obtained phenotype. Based on obtained results both ARR1 and ARR2 seem to work in the same pathway like FLM with respect to early flowering. Genetically observed *FLM* is epistatic to *ARR1* and *ARR2*.

Despite the fact that all tested floral activators and signal integrators did not show any miss-regulation of their expression in tested *ARR1* and *ARR2* knock outs, *CO* and *SOC1* lacking mutants were crossed with *arr2-4* single mutant in order to examine their genetic interaction/hierarchy and see if there is some difference. When tested under inductive LDs *co arr2-4* flowered more than two weeks after the wild-type identical to its co single mutant parent. This may suggest that under LDs *CO* may be epistatic to *ARR2* or event that they work independent and because the weak phenotype of *arr2-4* under LDs this difference could not be observed. When *co arr2-4* double mutant was grown under non-inductive SD conditions it flowered earlier than the wild-type showing the identical phenotype like *arr2-4* single parent. This is because *co* mutants are only delayed in long days, and *CO* mRNA is more abundant in long than in short days (Blazquez *et al*. 1997) and CO dependent pathways are not active under non-inductive SD conditions, only under LDs where the CO protein is stabilized by light and this leads to induction of the floral activators *FT* and *SOC1* (Eriksson *et al*. 2006). The effect observed in the *co arr2-4* double mutant is more due to the lack of *ARR2* only.

Another tested mutant with *SOC1* lacking floral signal integrator, *soc1-2 arr2-4* when grown under inductive LD conditions showed the late flowering phenotype which was statistically

not different than *soc1-2* single parent suggesting epistasis of *SOC1* under *ARR2*. Nevertheless, *soc1 arr2-4* each time showed tendency to flower slight earlier than the *soc1-2* single mutant but because of the very weak flowering phenotype of *arr2-4* under inductive conditions it could not be statistically proven but still independent working *ARR2* and *SOC1* with respect to flowering time cannot be excluded. This claim was supported by fact that when the *soc1-2 arr2-4* double mutant was grown under non-inductive SD conditions it showed an intermediate flowering phenotype. The double mutant plant flowered approximately one week after *arr2-4* single parent and also one week before the *soc1-2* single parent. This all suggested that under non-inductive SD conditions, and most probably under inductive LD conditions, the *ARR2* and *SOC1* are working independent of each other with respect to flowering time which was before proven on expression level as well; *ARR2* works here as negative regulator of flowering (alternatively positive regulator of floral suppressors) and *SOC1* is known to be a positive regulator of flowering.

5.4 References for Chapter 2

Abe, M, Kobayashi, Y, *et al.* (2005). "Fd, a bzip protein mediating signals from the floral pathway integrator ft at the shoot apex." Science **309**(5737): 1052-1056.

Achard, P, Renou, JP, *et al.* (2008). "Plant dellas restrain growth and promote survival of adversity by reducing the levels of reactive oxygen species." Curr Biol **18**(9): 656-660.

Ahn, JH, Miller, D, *et al.* (2006). "A divergent external loop confers antagonistic activity on floral regulators ft and tfl1." EMBO J **25**(3): 605-614.

Alonso-Blanco, C and Koornneef, M (2000). "Naturally occurring variation in arabidopsis: An underexploited resource for plant genetics." Trends Plant Sci **5**(1): 22-29.

Amasino, R (2004). "Vernalization, competence, and the epigenetic memory of winter." Plant Cell **16**(10): 2553-2559.

An, H, Roussot, C, *et al.* (2004). "Constans acts in the phloem to regulate a systemic signal that induces photoperiodic flowering of arabidopsis." Development **131**(15): 3615-3626.

Andres, F and Coupland, G (2012). "The genetic basis of flowering responses to seasonal cues." Nat Rev Genet **13**(9): 627-639.

Argyros, RD, Mathews, DE, *et al.* (2008). "Type b response regulators of arabidopsis play key roles in cytokinin signaling and plant development." Plant Cell **20**(8): 2102-2116.

Ayre, BG and Turgeon, R (2004). "Graft transmission of a floral stimulant derived from constans." Plant Physiol **135**(4): 2271-2278.

Balasubramanian, S, Sureshkumar, S, *et al.* (2006). "Potent induction of arabidopsis thaliana flowering by elevated growth temperature." PLoS Genet **2**(7): e106.

Bari, R and Jones, JD (2009). "Role of plant hormones in plant defence responses." Plant Mol Biol **69**(4): 473-488.

Bastow, R, Mylne, JS, *et al.* (2004). "Vernalization requires epigenetic silencing of flc by histone methylation." Nature **427**(6970): 164-167.

Bernier, G (2011). "My favourite flowering image: The role of cytokinin as a flowering signal." J Exp Bot.

Bernier, G, Havelange, A, *et al.* (1993). "Physiological signals that induce flowering." Plant Cell **5**(10): 1147-1155.

Blazquez, MA, Ahn, JH, *et al.* (2003). "A thermosensory pathway controlling flowering time in arabidopsis thaliana." Nat Genet **33**(2): 168-171.

Blazquez, MA, Green, R, *et al.* (1998). "Gibberellins promote flowering of arabidopsis by activating the leafy promoter." Plant Cell **10**(5): 791-800.

Blazquez, MA, Soowal, LN, *et al.* (1997). "Leafy expression and flower initiation in arabidopsis." Development **124**(19): 3835-3844.

Bohlenius, H, Huang, T, *et al.* (2006). "Co/ft regulatory module controls timing of flowering and seasonal growth cessation in trees." Science **312**(5776): 1040-1043.

Bond, DM, Dennis, ES, *et al.* (2009). "Histone acetylation, vernalization insensitive 3, flowering locus c, and the vernalization response." Mol Plant **2**(4): 724-737.

Brenner, WG, Ramireddy, E, *et al.* (2012). "Gene regulation by cytokinin in arabidopsis." Front Plant Sci **3**: 8.

Chapman, EJ and Estelle, M (2009). "Mechanism of auxin-regulated gene expression in plants." Annu Rev Genet **43**: 265-285.

Chen, M, Chory, J, *et al.* (2004). "Light signal transduction in higher plants." Annu Rev Genet **38**: 87-117.

Choi, J, Huh, SU, *et al.* (2010). "The cytokinin-activated transcription factor arr2 promotes plant immunity via tga3/npr1-dependent salicylic acid signaling in arabidopsis." Dev Cell **19**(2): 284-295.

Choi, K, Kim, S, *et al.* (2005). "Suppressor of frigida3 encodes a nuclear actin-related protein6 required for floral repression in arabidopsis." Plant Cell **17**(10): 2647-2660.

Colasanti, J and Sundaresan, V (2000). "'Florigen' enters the molecular age: Long-distance signals that cause plants to flower." Trends Biochem Sci **25**(5): 236-240.

Corbesier, L, Vincent, C, *et al.* (2007). "Ft protein movement contributes to long-distance signaling in floral induction of arabidopsis." Science **316**(5827): 1030-1033.

Coupland, G (1995). "Genetic and environmental control of flowering time in arabidopsis." Trends Genet **11**(10): 393-397.
D'Aloia, M, Bonhomme, D*, et al.* (2011). "Cytokinin promotes flowering of arabidopsis via transcriptional activation of the ft paralogue tsf." Plant J **65**(6): 972-979.
Deal, RB, Kandasamy, MK*, et al.* (2005). "The nuclear actin-related protein arp6 is a pleiotropic developmental regulator required for the maintenance of flowering locus c expression and repression of flowering in arabidopsis." Plant Cell **17**(10): 2633-2646.
Deng, W, Ying, H*, et al.* (2011). "Flowering locus c (flc) regulates development pathways throughout the life cycle of arabidopsis." Proc Natl Acad Sci U S A **108**(16): 6680-6685.
Doi, K, Izawa, T*, et al.* (2004). "Ehd1, a b-type response regulator in rice, confers short-day promotion of flowering and controls ft-like gene expression independently of hd1." Genes Dev **18**(8): 926-936.
Endo, M, Tanigawa, Y*, et al.* (2013). "Phytochrome-dependent late-flowering accelerates flowering through physical interactions with phytochrome b and constans." Proc Natl Acad Sci U S A **110**(44): 18017-18022.
Eriksson, S, Bohlenius, H*, et al.* (2006). "Ga4 is the active gibberellin in the regulation of leafy transcription and arabidopsis floral initiation." Plant Cell **18**(9): 2172-2181.
Ferrandiz, C, Gu, Q*, et al.* (2000). "Redundant regulation of meristem identity and plant architecture by fruitfull, apetala1 and cauliflower." Development **127**(4): 725-734.
Fowler, S, Lee, K*, et al.* (1999). "Gigantea: A circadian clock-controlled gene that regulates photoperiodic flowering in arabidopsis and encodes a protein with several possible membrane-spanning domains." EMBO J **18**(17): 4679-4688.
Franklin, KA (2009). "Light and temperature signal crosstalk in plant development." Curr Opin Plant Biol **12**(1): 63-68.
Franklin, KA, Larner, VS*, et al.* (2005). "The signal transducing photoreceptors of plants." Int J Dev Biol **49**(5-6): 653-664.
Garner, WW (1933). "Comparative responses of long-day and short-day plants to relative length of day and night." Plant Physiol **8**(3): 347-356.
Genoud, T and Metraux, JP (1999). "Crosstalk in plant cell signaling: Structure and function of the genetic network." Trends Plant Sci **4**(12): 503-507.
Geraldo, N, Baurle, I*, et al.* (2009). "Frigida delays flowering in arabidopsis via a cotranscriptional mechanism involving direct interaction with the nuclear cap-binding complex." Plant Physiol **150**(3): 1611-1618.
Greenboim-Wainberg, Y, Maymon, I*, et al.* (2005). "Cross talk between gibberellin and cytokinin: The arabidopsis ga response inhibitor spindly plays a positive role in cytokinin signaling." Plant Cell **17**(1): 92-102.
Grefen, C and Harter, K (2004). "Plant two-component systems: Principles, functions, complexity and cross talk." Planta **219**(5): 733-742.
Gregis, V, Andres, F*, et al.* (2013). "Identification of pathways directly regulated by short vegetative phase during vegetative and reproductive development in arabidopsis." Genome Biol **14**(6): R56.
Griffiths, J, Murase, K*, et al.* (2006). "Genetic characterization and functional analysis of the gid1 gibberellin receptors in arabidopsis." Plant Cell **18**(12): 3399-3414.
Gupta, S and Rashotte, AM (2012). "Down-stream components of cytokinin signaling and the role of cytokinin throughout the plant." Plant Cell Rep **31**(5): 801-812.
Harmer, SL, Hogenesch, JB*, et al.* (2000). "Orchestrated transcription of key pathways in arabidopsis by the circadian clock." Science **290**(5499): 2110-2113.
Hass, C, Lohrmann, J*, et al.* (2004). "The response regulator 2 mediates ethylene signalling and hormone signal integration in arabidopsis." EMBO J **23**(16): 3290-3302.
Hayama, R and Coupland, G (2004). "The molecular basis of diversity in the photoperiodic flowering responses of arabidopsis and rice." Plant Physiol **135**(2): 677-684.
He, Y, Michaels, SD*, et al.* (2003). "Regulation of flowering time by histone acetylation in arabidopsis." Science **302**(5651): 1751-1754.

Hedden, P and Phillips, AL (2000). "Gibberellin metabolism: New insights revealed by the genes." Trends Plant Sci **5**(12): 523-530.

Helliwell, CA, Wood, CC*, et al.* (2006). "The arabidopsis flc protein interacts directly in vivo with soc1 and ft chromatin and is part of a high-molecular-weight protein complex." Plant J **46**(2): 183-192.

Heo, JB and Sung, S (2011). "Encoding memory of winter by noncoding rnas." Epigenetics **6**(5): 544-547.

Hepworth, SR, Valverde, F*, et al.* (2002). "Antagonistic regulation of flowering-time gene soc1 by constans and flc via separate promoter motifs." EMBO J **21**(16): 4327-4337.

Hicks, KA, Millar, AJ*, et al.* (1996). "Conditional circadian dysfunction of the arabidopsis early-flowering 3 mutant." Science **274**(5288): 790-792.

Hill, K, Mathews, DE*, et al.* (2013). "Functional characterization of type-b response regulators in the arabidopsis cytokinin response." Plant Physiol **162**(1): 212-224.

Hirsch, S and Oldroyd, GE (2009). "Gras-domain transcription factors that regulate plant development." Plant Signal Behav **4**(8): 698-700.

Hisamatsu, T and King, RW (2008). "The nature of floral signals in arabidopsis. Ii. Roles for flowering locus t (ft) and gibberellin." J Exp Bot **59**(14): 3821-3829.

Hsuan, HM, Salleh, B*, et al.* (2011). "Molecular identification of fusarium species in gibberella fujikuroi species complex from rice, sugarcane and maize from peninsular malaysia." Int J Mol Sci **12**(10): 6722-6732.

Hwang, I and Sheen, J (2001). "Two-component circuitry in arabidopsis cytokinin signal transduction." Nature **413**(6854): 383-389.

Imaizumi, T and Kay, SA (2006). "Photoperiodic control of flowering: Not only by coincidence." Trends Plant Sci **11**(11): 550-558.

Ishikawa, R, Aoki, M*, et al.* (2011). "Phytochrome b regulates heading date 1 (hd1)-mediated expression of rice florigen hd3a and critical day length in rice." Mol Genet Genomics **285**(6): 461-470.

Jaeger, KE and Wigge, PA (2007). "Ft protein acts as a long-range signal in arabidopsis." Curr Biol **17**(12): 1050-1054.

Jasinski, S, Piazza, P*, et al.* (2005). "Knox action in arabidopsis is mediated by coordinate regulation of cytokinin and gibberellin activities." Curr Biol **15**(17): 1560-1565.

Jeong, S and Clark, SE (2005). "Photoperiod regulates flower meristem development in arabidopsis thaliana." Genetics **169**(2): 907-915.

Johanson, U, West, J*, et al.* (2000). "Molecular analysis of frigida, a major determinant of natural variation in arabidopsis flowering time." Science **290**(5490): 344-347.

Kardailsky, I, Shukla, VK*, et al.* (1999). "Activation tagging of the floral inducer ft." Science **286**(5446): 1962-1965.

Kim, DH, Doyle, MR*, et al.* (2009). "Vernalization: Winter and the timing of flowering in plants." Annu Rev Cell Dev Biol **25**: 277-299.

Kim, HJ, Kieber, JJ*, et al.* (2012). "Overlapping and lineage-specific roles for the type-b response regulators of monocots and dicots." Plant Signal Behav **7**(9): 1110-1113.

Kim, HJ, Ryu, H*, et al.* (2006). "Cytokinin-mediated control of leaf longevity by ahk3 through phosphorylation of arr2 in arabidopsis." Proc Natl Acad Sci U S A **103**(3): 814-819.

Kim, W, Park, TI*, et al.* (2013). "Generation and analysis of a complete mutant set for the arabidopsis ft/tfl1 family shows specific effects on thermo-sensitive flowering regulation." J Exp Bot **64**(6): 1715-1729.

Kojima, S, Takahashi, Y*, et al.* (2002). "Hd3a, a rice ortholog of the arabidopsis ft gene, promotes transition to flowering downstream of hd1 under short-day conditions." Plant Cell Physiol **43**(10): 1096-1105.

Kolmos, E and Davis, SJ (2007). "Elf4 as a central gene in the circadian clock." Plant Signal Behav **2**(5): 370-372.

Koornneef, M, Alonso-Blanco, C*, et al.* (1998). "Genetic control of flowering time in arabidopsis." Annu Rev Plant Physiol Plant Mol Biol **49**: 345-370.

Koornneef, M, Alonso-Blanco, C*, et al.* (2004). "Naturally occurring genetic variation in arabidopsis thaliana." Annu Rev Plant Biol **55**: 141-172.

Kumar, SV and Wigge, PA (2010). "H2a.Z-containing nucleosomes mediate the thermosensory response in arabidopsis." Cell **140**(1): 136-147.

Lariguet, P and Dunand, C (2005). "Plant photoreceptors: Phylogenetic overview." J Mol Evol **61**(4): 559-569.

Lee, I and Amasino, RM (1995). "Effect of vernalization, photoperiod, and light quality on the flowering phenotype of arabidopsis plants containing the frigida gene." Plant Physiol **108**(1): 157-162.

Lee, JH, Ryu, HS*, et al.* (2013). "Regulation of temperature-responsive flowering by mads-box transcription factor repressors." Science **342**(6158): 628-632.

Li, C, Zhang, K*, et al.* (2009). "A cis element within flowering locus t mrna determines its mobility and facilitates trafficking of heterologous viral rna." J Virol **83**(8): 3540-3548.

Li, D, Liu, C*, et al.* (2008). "A repressor complex governs the integration of flowering signals in arabidopsis." Dev Cell **15**(1): 110-120.

Lifschitz, E, Eviatar, T*, et al.* (2006). "The tomato ft ortholog triggers systemic signals that regulate growth and flowering and substitute for diverse environmental stimuli." Proc Natl Acad Sci U S A **103**(16): 6398-6403.

Lin, C (2000). "Photoreceptors and regulation of flowering time." Plant Physiol **123**(1): 39-50.

Liu, C, Xi, W*, et al.* (2009). "Regulation of floral patterning by flowering time genes." Dev Cell **16**(5): 711-722.

Mason, MG, Mathews, DE*, et al.* (2005). "Multiple type-b response regulators mediate cytokinin signal transduction in arabidopsis." Plant Cell **17**(11): 3007-3018.

Mathieu, J, Warthmann, N*, et al.* (2007). "Export of ft protein from phloem companion cells is sufficient for floral induction in arabidopsis." Curr Biol **17**(12): 1055-1060.

Matsushika, A, Makino, S*, et al.* (2000). "Circadian waves of expression of the aprr1/toc1 family of pseudo-response regulators in arabidopsis thaliana: Insight into the plant circadian clock." Plant Cell Physiol **41**(9): 1002-1012.

Michaels, SD and Amasino, RM (1999). "Flowering locus c encodes a novel mads domain protein that acts as a repressor of flowering." Plant Cell **11**(5): 949-956.

Mira-Rodado, V, Sweere, U*, et al.* (2007). "Functional cross-talk between two-component and phytochrome b signal transduction in arabidopsis." J Exp Bot **58**(10): 2595-2607.

Mizoguchi, T, Wright, L*, et al.* (2005). "Distinct roles of gigantea in promoting flowering and regulating circadian rhythms in arabidopsis." Plant Cell **17**(8): 2255-2270.

Moon, J, Suh, SS*, et al.* (2003). "The soc1 mads-box gene integrates vernalization and gibberellin signals for flowering in arabidopsis." Plant J **35**(5): 613-623.

Mouradov, A, Cremer, F*, et al.* (2002). "Control of flowering time: Interacting pathways as a basis for diversity." Plant Cell **14 Suppl**: S111-130.

Notaguchi, M, Abe, M*, et al.* (2008). "Long-distance, graft-transmissible action of arabidopsis flowering locus t protein to promote flowering." Plant Cell Physiol **49**(11): 1645-1658.

Pin, PA and Nilsson, O (2012). "The multifaceted roles of flowering locus t in plant development." Plant Cell Environ **35**(10): 1742-1755.

Poethig, RS (2013). "Vegetative phase change and shoot maturation in plants." Curr Top Dev Biol **105**: 125-152.

Porri, A, Torti, S*, et al.* (2012). "Spatially distinct regulatory roles for gibberellins in the promotion of flowering of arabidopsis under long photoperiods." Development **139**(12): 2198-2209.

Pose, D, Verhage, L*, et al.* (2013). "Temperature-dependent regulation of flowering by antagonistic flm variants." Nature.

Putterill, J, Robson, F*, et al.* (1995). "The constans gene of arabidopsis promotes flowering and encodes a protein showing similarities to zinc finger transcription factors." Cell **80**(6): 847-857.

Rashotte, AM, Carson, SD*, et al.* (2003). "Expression profiling of cytokinin action in arabidopsis." Plant Physiol **132**(4): 1998-2011.

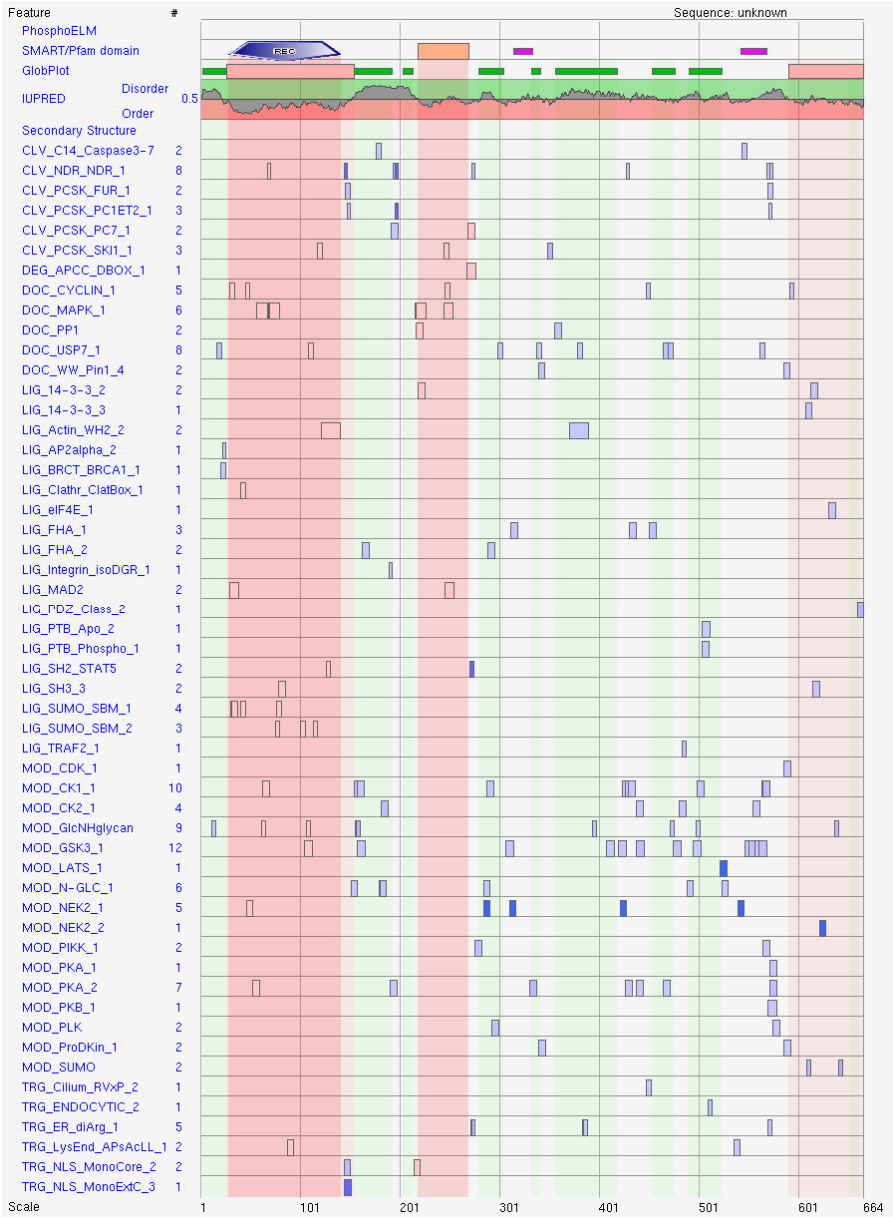
Reeves, PA, He, Y*, et al.* (2007). "Evolutionary conservation of the flowering locus c-mediated vernalization response: Evidence from the sugar beet (beta vulgaris)." Genetics **176**(1): 295-307.

Reeves, PH and Coupland, G (2000). "Response of plant development to environment: Control of flowering by daylength and temperature." Curr Opin Plant Biol **3**(1): 37-42.
Samach, A, Onouchi, H*, et al.* (2000). "Distinct roles of constans target genes in reproductive development of arabidopsis." Science **288**(5471): 1613-1616.
Sawa, M and Kay, SA (2011). "Gigantea directly activates flowering locus t in arabidopsis thaliana." Proc Natl Acad Sci U S A **108**(28): 11698-11703.
Sawa, M, Nusinow, DA*, et al.* (2007). "Fkf1 and gigantea complex formation is required for day-length measurement in arabidopsis." Science **318**(5848): 261-265.
Schluepmann, H, Pellny, T*, et al.* (2003). "Trehalose 6-phosphate is indispensable for carbohydrate utilization and growth in arabidopsis thaliana." Proc Natl Acad Sci U S A **100**(11): 6849-6854.
Schmid, M, Uhlenhaut, NH*, et al.* (2003). "Dissection of floral induction pathways using global expression analysis." Development **130**(24): 6001-6012.
Scortecci, K, Michaels, SD*, et al.* (2003). "Genetic interactions between flm and other flowering-time genes in arabidopsis thaliana." Plant Mol Biol **52**(5): 915-922.
Searle, I, He, Y*, et al.* (2006). "The transcription factor flc confers a flowering response to vernalization by repressing meristem competence and systemic signaling in arabidopsis." Genes Dev **20**(7): 898-912.
Skylar, A, Hong, F*, et al.* (2010). "Stimpy mediates cytokinin signaling during shoot meristem establishment in arabidopsis seedlings." Development **137**(4): 541-549.
Smith, HM, Ung, N*, et al.* (2011). "Specification of reproductive meristems requires the combined function of shoot meristemless and floral integrators flowering locus t and fd during arabidopsis inflorescence development." J Exp Bot **62**(2): 583-593.
Song, J, Angel, A*, et al.* (2012). "Vernalization - a cold-induced epigenetic switch." J Cell Sci **125**(Pt 16): 3723-3731.
Song, YH, Ito, S*, et al.* (2013). "Flowering time regulation: Photoperiod- and temperature-sensing in leaves." Trends Plant Sci **18**(10): 575-583.
Srikanth, A and Schmid, M (2011). "Regulation of flowering time: All roads lead to rome." Cell Mol Life Sci **68**(12): 2013-2037.
Suarez-Lopez, P, Wheatley, K*, et al.* (2001). "Constans mediates between the circadian clock and the control of flowering in arabidopsis." Nature **410**(6832): 1116-1120.
Sun, T, Goodman, HM*, et al.* (1992). "Cloning the arabidopsis ga1 locus by genomic subtraction." Plant Cell **4**(2): 119-128.
Sun, TP (2010). "Gibberellin-gid1-della: A pivotal regulatory module for plant growth and development." Plant Physiol **154**(2): 567-570.
Sung, S and Amasino, RM (2004). "Vernalization in arabidopsis thaliana is mediated by the phd finger protein vin3." Nature **427**(6970): 159-164.
Sweere, U, Eichenberg, K*, et al.* (2001). "Interaction of the response regulator arr4 with phytochrome b in modulating red light signaling." Science **294**(5544): 1108-1111.
Taniguchi, M, Sasaki, N*, et al.* (2007). "Arr1 directly activates cytokinin response genes that encode proteins with diverse regulatory functions." Plant Cell Physiol **48**(2): 263-277.
Tran, LS, Nakashima, K*, et al.* (2007). "Co-expression of the stress-inducible zinc finger homeodomain zfhd1 and nac transcription factors enhances expression of the erd1 gene in arabidopsis." Plant J **49**(1): 46-63.
Truernit, E and Sauer, N (1995). "The promoter of the arabidopsis thaliana suc2 sucrose-h+ symporter gene directs expression of beta-glucuronidase to the phloem: Evidence for phloem loading and unloading by suc2." Planta **196**(3): 564-570.
Tucker, MR and Laux, T (2007). "Connecting the paths in plant stem cell regulation." Trends Cell Biol **17**(8): 403-410.
Tudzynski, B (1999). "Biosynthesis of gibberellins in gibberella fujikuroi: Biomolecular aspects." Appl Microbiol Biotechnol **52**(3): 298-310.
Valverde, F, Mouradov, A*, et al.* (2004). "Photoreceptor regulation of constans protein in photoperiodic flowering." Science **303**(5660): 1003-1006.

Veerabagu, M, Elgass, K*, et al.* (2012). "The arabidopsis b-type response regulator 18 homomerizes and positively regulates cytokinin responses." Plant J **72**(5): 721-731.
Wahl, V, Ponnu, J*, et al.* (2013). "Regulation of flowering by trehalose-6-phosphate signaling in arabidopsis thaliana." Science **339**(6120): 704-707.
Wang, JW, Czech, B*, et al.* (2009). "Mir156-regulated spl transcription factors define an endogenous flowering pathway in arabidopsis thaliana." Cell **138**(4): 738-749.
Werner, JD, Borevitz, JO*, et al.* (2005). "Quantitative trait locus mapping and DNA array hybridization identify an flm deletion as a cause for natural flowering-time variation." Proc Natl Acad Sci U S A **102**(7): 2460-2465.
Werner, T and Schmulling, T (2009). "Cytokinin action in plant development." Curr Opin Plant Biol **12**(5): 527-538.
Wigge, PA (2011). "Ft, a mobile developmental signal in plants." Curr Biol **21**(9): R374-378.
Wigge, PA, Kim, MC*, et al.* (2005). "Integration of spatial and temporal information during floral induction in arabidopsis." Science **309**(5737): 1056-1059.
Willige, BC, Ghosh, S*, et al.* (2007). "The della domain of ga insensitive mediates the interaction with the ga insensitive dwarf1a gibberellin receptor of arabidopsis." Plant Cell **19**(4): 1209-1220.
Wilson, RN, Heckman, JW*, et al.* (1992). "Gibberellin is required for flowering in arabidopsis thaliana under short days." Plant Physiol **100**(1): 403-408.
Wippel, K and Sauer, N (2012). "Arabidopsis suc1 loads the phloem in suc2 mutants when expressed from the suc2 promoter." J Exp Bot **63**(2): 669-679.
Wu, Z, Skjelvag, AO*, et al.* (2004). "Quantification of photoperiodic effects on growth of phleum pratense." Ann Bot **94**(4): 535-543.
Yamaguchi, A, Kobayashi, Y*, et al.* (2005). "Twin sister of ft (tsf) acts as a floral pathway integrator redundantly with ft." Plant Cell Physiol **46**(8): 1175-1189.
Yano, M, Kojima, S*, et al.* (2001). "Genetic control of flowering time in rice, a short-day plant." Plant Physiol **127**(4): 1425-1429.
Yanovsky, MJ and Kay, SA (2002). "Molecular basis of seasonal time measurement in arabidopsis." Nature **419**(6904): 308-312.
Yoo, SK, Chung, KS*, et al.* (2005). "Constans activates suppressor of overexpression of constans 1 through flowering locus t to promote flowering in arabidopsis." Plant Physiol **139**(2): 770-778.
Yoshida, R, Fekih, R*, et al.* (2009). "Possible role of early flowering 3 (elf3) in clock-dependent floral regulation by short vegetative phase (svp) in arabidopsis thaliana." New Phytol **182**(4): 838-850.
Yu, H, Ito, T*, et al.* (2004). "Repression of agamous-like 24 is a crucial step in promoting flower development." Nat Genet **36**(2): 157-161.
Zwack, PJ and Rashotte, AM (2013). "Cytokinin inhibition of leaf senescence." Plant Signal Behav **8**(7).

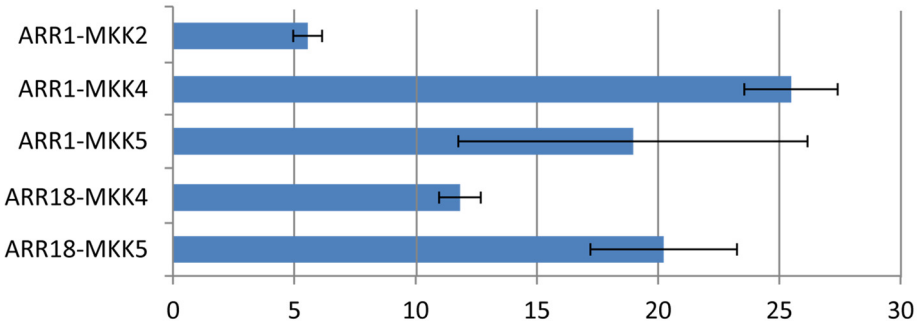
6 Appendix

6.1 Supplemental Figures



Supplemental 1 Supplement to Figure 4.6. *In silico* predictions of MKK docking motifs on ARR2 based on ELM software

The figure shows different predicted functional domains of ARR2; four docking motifs for the MKKs are marked (yellow). ELM - (the database of eukaryotic linear motifs, http://elm.eu.org). The docking motif for MKKs is called DOC_MAPK_1.



Supplemental 2 Supplement to Figure 4.8. oNPG data for interaction strength between ARR1 and ARR18 with biotic-stress-related MKKs in yeast.

The β-galactosidase activity was measured in the extracts of three independent yeast clones. Diluted yeast colonies were incubated for 2 days at 28°C on interaction selective (CSM-L, W, A) media. The experiment was repeated at least two times.

LSD results for experiment with Pst DC3000

Level						Mean
WT (Col-0) Day 4	A					6,8327273
arr1-4 Day 4	A					6,7877965
arr1-4xarr2-4 Day 4		B				6,3391667
WT (Col-0) Day 2		B				6,1881818
arr1-4 Day 2			C			5,9050000
arr1-4xarr2-4 Day 2			C			5,7327273
arr2-4 Day 2			C			5,6250000
arr2-4 Day 4				D		5,2648189
arr1-4xarr2-4 Day 1					E	4,0383333
arr1-4 Day 1					E	4,0081818
WT (Col-0) Day 1					E	3,9445455
arr2-4 Day 1					E	3,9416667
arr1-4xarr2-4 Day 0						F 1,8800000
arr2-4 Day 0						F 1,7900000
arr1-4 Day 0						F 1,6400000
WT (Col-0) Day 0						F 1,6400000

Levels not connected by same letter are significantly different.

LSD results for experiment with Pst DC3000 and cytokinin treatment

Level									Mean
arr1-4 Mock Day 4	A								7,4163636
WT (Col-0) Mock Day 4	A	B							7,0091784
arr1-4 Cytokinin Day 2	A	B							6,9850000
arr2-4 Mock Day 4	A	B	C						6,8054545
arr1-4xarr2-4 Cytokinin Day 2		B	C	D					6,6600000
arr1-4 Mock Day 2		B	C	D					6,6309091
arr1-4xarr2-4 Mock Day 2			C	D	E				6,3552786
arr1-4xarr2-4 Mock Day 4			C	D	E				6,2557137
WT (Col-0) Cytokinin Day 2			C	D	E				6,2483333
WT (Col-0) Mock Day 2				D	E				6,0954545
arr2-4 Mock Day 2					E	F			5,9818182
WT (Col-0) Cytokinin Day 4					E	F			5,8886552
arr2-4 Cytokinin Day 2					E	F			5,7925000
arr1-4 Cytokinin Day 4					E	F			5,7854545
arr1-4xarr2-4 Cytokinin Day 4						F	G		5,4081842
arr2-4 Cytokinin Day 4							G		5,0945975
arr2-4 Cytokinin Day 1								H	4,3000000
WT (Col-0) Mock Day 1								H	4,2213819
arr2-4 Mock Day 1								H	4,2109091
arr1-4 Mock Day 1								H	4,1754545
arr1-4 Cytokinin Day 1								H	4,1225000
arr1-4xarr2-4 Mock Day 1								H	4,1058333
WT (Col-0) Cytokinin Day 1								H	4,0991667
arr1-4xarr2-4 Cytokinin Day 1								H	3,9491667
arr2-4 Mock Day 0									I 2,3485714
arr1-4 Cytokinin Day 0									I 2,1800000
arr1-4 Mock Day 0									I 2,1787500
WT (Col-0) Mock Day 0									I 2,0916667
arr1-4xarr2-4 Cytokinin Day 0									I 2,0116667
arr2-4 Cytokinin Day 0									I 1,9160000
WT (Col-0) Cytokinin Day 0									I 1,8400000
arr1-4xarr2-4 Mock Day 0									I 1,8000000

Levels not connected by same letter are significantly different.

Supplemental 3 Supplement to Figure 4.13B. Results of Fisher's Least Significant Difference (LSD) test for pathogen assay with *Pseudomonas syringae Pst* DC3000

(A) LSD results of plants treated only with *Pst* DC3000. (B) LSD results of plants treated with PstDC3000 in addition of cytokinin (1 μM t-Zeatin). WT stands for Col-0. Data are presented for days: 0, 1, 2 and 4 post treatment. LSD tests were conducted at the 0.05 α level.

LSD results for stomata aperture

Level								Mean
wtDC3000	A							5,3746250
wtt-zeatin	A							5,2938941
2-4t-zeatin	A	B						5,1199891
1-4x2-4t-zeatin	A	B						5,0776780
1-4x2-4DC3000		B	C					4,7440645
wtcontrol			C					4,4587333
1-4t-zeatin				D				4,0488333
2-4control				D	E			4,0190758
1-4x2-4control				D				4,0102941
1-4control				D	E	F		3,7739500
2-4ABA				D	E	F		3,6802353
wtABA					E	F		3,6649109
1-4x2-4ABA						F	G	3,5012639
1-4ABA							G	3,1161346

Levels not connected by same letter are significantly different.

Supplemental 4 Supplement to Figure 4.15. Results of Fisher's Least Significant Difference (LSD) test for measurements of stomata aperture

Wt stands for Col-0, 1-4 for *arr1-4*, 2-4 for *arr2-4* and 1-4x2-4 for *arr1-4 arr2-4*. Control represents MOCK treatment. LSD tests were conducted at the 0.05 α level.

A

LSD results for Alternaria brassicicola experiment day 7

Level			Mean
arr2-4	A		201,38889
arr1-4Xarr2-4	A		200,00000
arr1-4		B	176,71233
WT (Col-0)		B	169,01408

Levels not connected by same letter are significantly different.

LSD results for Alternaria brassicicola experiment day 10

Level			Mean
arr1-4Xarr2-4	A		314,49275
arr2-4	A		311,11111
WT (Col-0)		B	270,42254
arr1-4		B	266,15385

Levels not connected by same letter are significantly different.

B

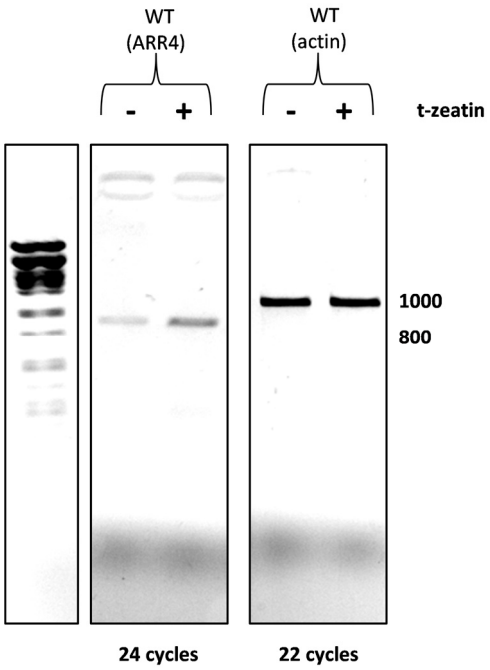
LSD results for *Alternaria brassicicola* experiment with cytokinin

Level									Mean
arr2-4 Mock Day 10	A								308,92857
arr1-4Xarr2-4 Mock Day 10	A								308,33333
WT (Col-0) Mock Day 10	A								308,33333
arr2-4 Cytokinin Day 10	A	B							303,57143
arr1-4Xarr2-4 Cytokinin Day 10	A	B							303,33333
arr1-4 Mock Day 10	A	B	C						298,21429
WT (Col-0) Cytokinin Day 10		B	C	D					290,00000
arr2-4 Mock Day 7			C	D	E				283,63636
arr1-4 Cytokinin Day 10				D	E	F			280,35714
arr1-4Xarr2-4 Mock Day 7				D	E	F	G		275,00000
arr1-4Xarr2-4 Cytokinin Day 7					E	F	G		271,18644
arr2-4 Cytokinin Day 7						F	G	H	266,07143
WT (Col-0) Mock Day 7							G	H	263,33333
arr1-4 Mock Day 7							G	H	261,81818
WT (Col-0) Cytokinin Day 7								H	I 253,33333
arr1-4 Cytokinin Day 7									I 239,28571

Levels not connected by same letter are significantly different.

Supplemental 5 Supplement to Figure 4.16 Results of Fisher's Least Significant Difference (LSD) test for pathogen assay with *Alternaria brassicicola*

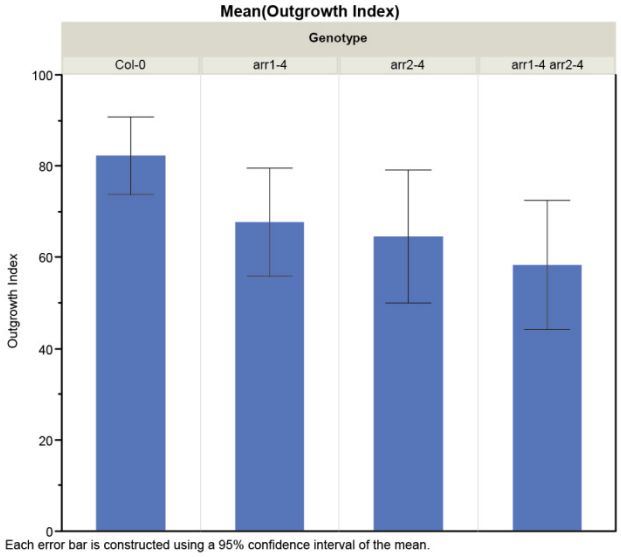
(A)LSD results for plants treated with *Alternaria* only after 7 days (top) and after 10 days (bottom). (B) LSD results for plants treated with *Alternaria* in the presence and absence of cytokinin (1μM t-Zeatin). WT stands for Col-0. Control represents MOCK treatment. LSD tests were conducted at the 0.05 α level.



Supplemental 6 Supplement to Figure 4.16B. Plants treated with cytokinin for pathogen assays with *Alternaria brassicicola*

Cytokinin application was monitored by detecting expression of *ARR4*, which is a known cytokinin responsive gene. Plants treated with 1 µM t-Zeatin showed increased expression of *ARR4* indirectly proving that cytokinin treatment was successful.

A



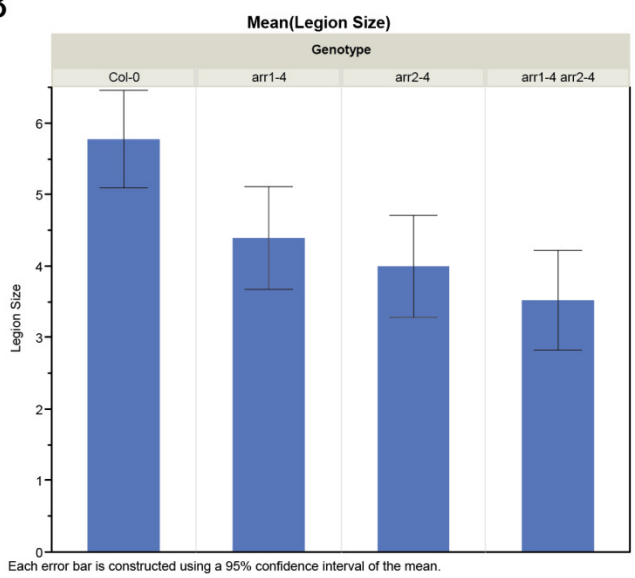
Connecting Letters Report

Level		Mean
Col-0	A	82,291667
arr1-4	B	67,708333
arr2-4	B	64,583333
arr1-4 arr2-4	B	58,333333

Levels not connected by same letter are significantly different.

Level	- Level	p-Value
Col-0	arr1-4 arr2-4	0,0062*
Col-0	arr2-4	0,0411*
Col-0	arr1-4	0,0914*
arr1-4	arr1-4 arr2-4	0,2756
arr2-4	arr1-4 arr2-4	0,4666
arr1-4	arr2-4	0,7155

B



Connecting Letters Report

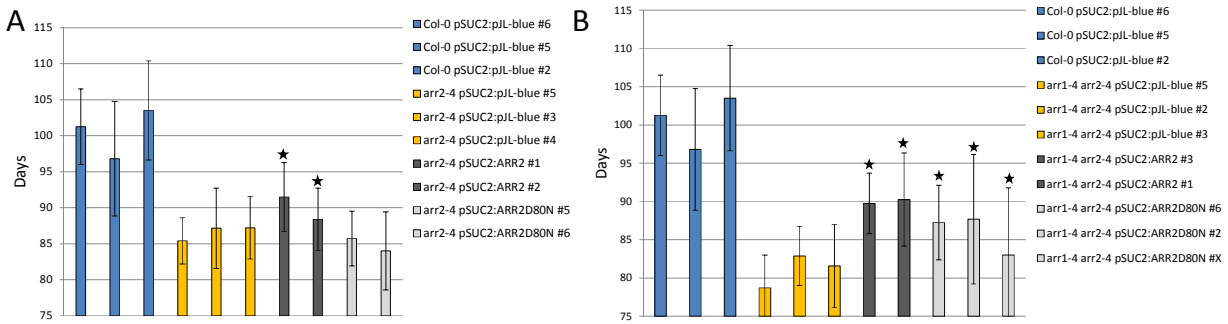
Level		Mean
Col-0	A	5,7726042
arr1-4	B	4,3903125
arr2-4	B	3,9953125
arr1-4 arr2-4	B	3,5207292

Levels not connected by same letter are significantly different.

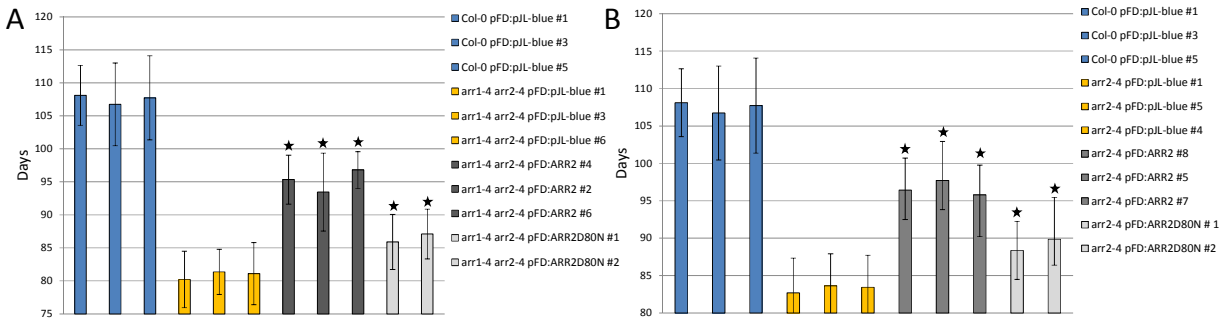
Level	- Level	p-Value
Col-0	arr1-4 arr2-4	<,0001*
Col-0	arr2-4	0,0004*
Col-0	arr1-4	0,0060*
arr1-4	arr1-4 arr2-4	0,0830
arr2-4	arr1-4 arr2-4	0,3434
arr1-4	arr2-4	0,4303

Supplemental 7 Supplement to Figure 4.17. Plants treated with cytokinin for pathogen assays with *Botrytis cinerea*.

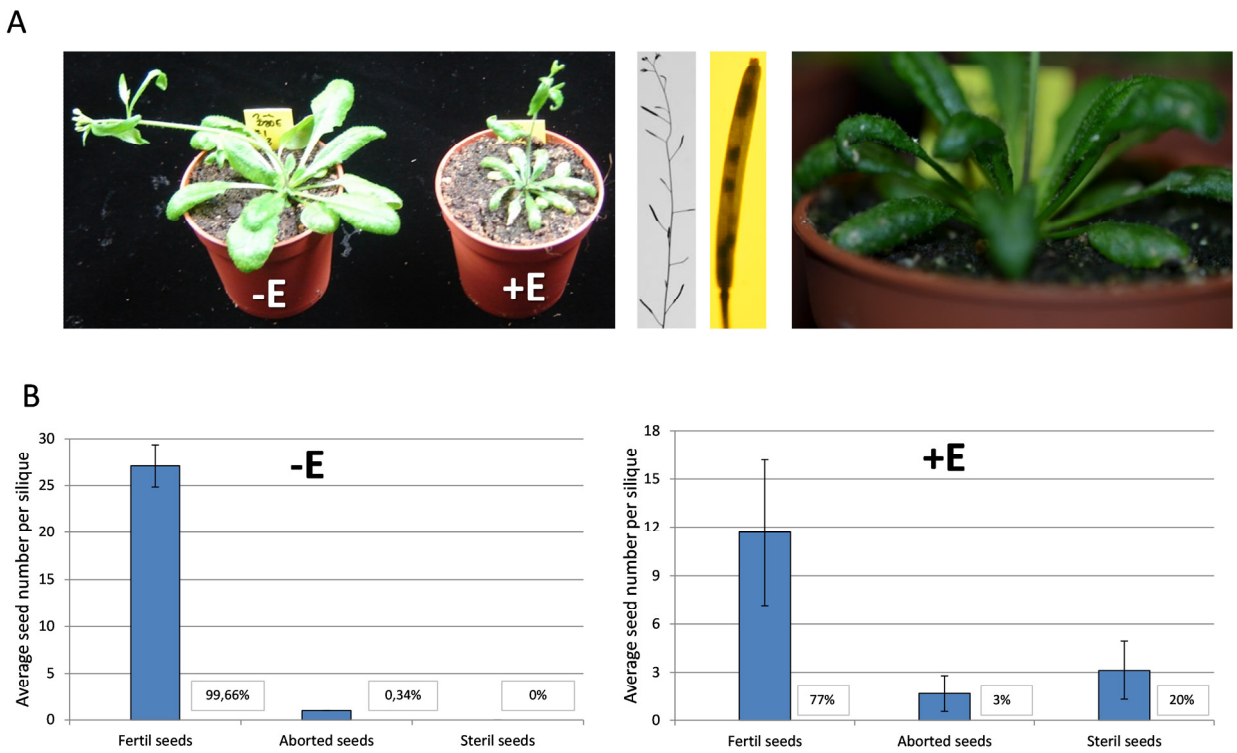
(A) Left: Outgrowth Index data shown as mean and the 95% confidence interval. Right: LSD results for the Outgrowth Index data about the mean at the 0.1 α level. All of the mutants are significantly smaller than the wild-type at the 0.1 alpha level and those with arr2-4 at the 0.05 α level. (B) Left: Lesion Size data shown as mean and the 95% confidence interval. Right: LSD results for the Lesion Size about the mean at the 0.06 α level. All of the mutants are significantly smaller than the wild-type at the 0.06 α level.



Supplemental 8 to table 5.3. Flowering time of transgenic lines driven under tissue specific *pSUC2* promoter under short day non-inductive conditions (alternative representation).
(A) Experiments where *pSUC2::ARR2* was complemented in *arr2-4* mutant background. **(B)** Experiments where *pSUC2::ARR2* was complemented in *arr1-4 arr2-4* mutant background. All independent lines show the same tendency. Lines used for this experiment were in T2 generation and preselected with BASTA. Mean values and standard deviations are shown.

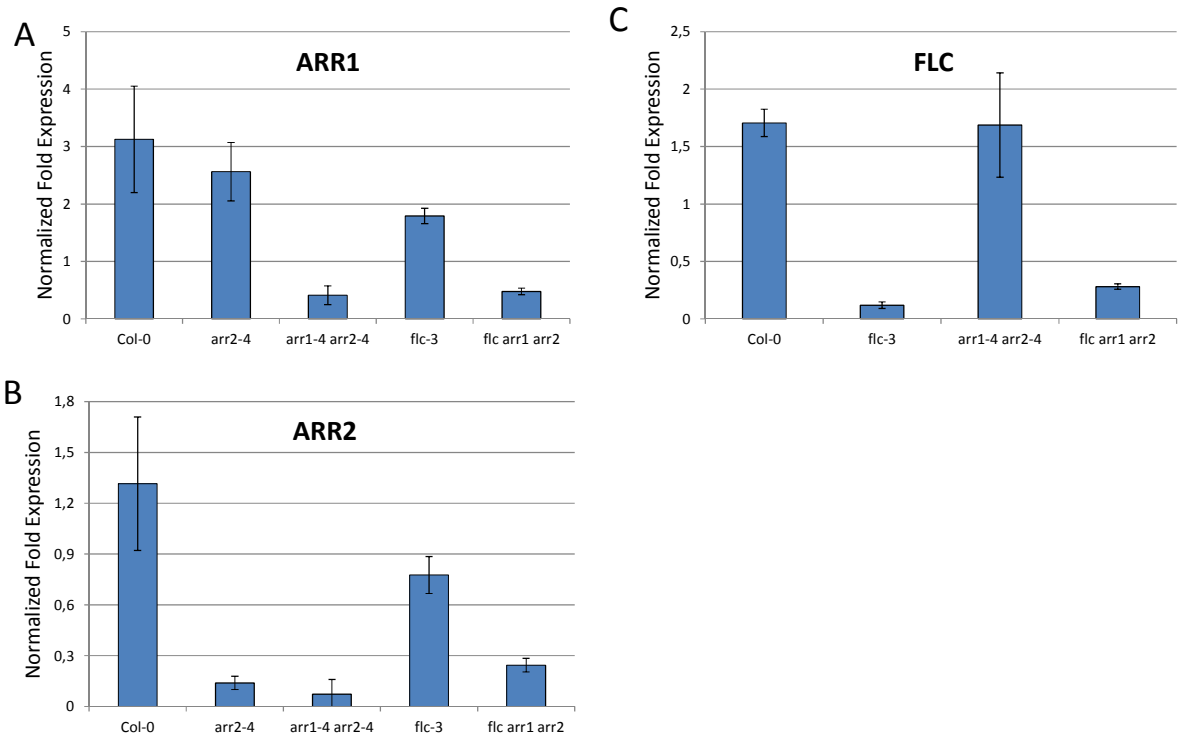


Supplemental 9 to table 5.4. Flowering time of transgenic lines driven under tissue specific *FD* promoter under short day non-inductive conditions (alternative representation).
(A) Experiments where *pFD::ARR2* was complemented in *arr2-4* mutant background. **(B)** Experiments where *pFD::ARR2* was complemented in *arr1-4 arr2-4* mutant background. All independent lines show the same tendency and stronger complementation than under *SUC2* promoter. Lines used for this experiment were in T2 generation and preselected with BASTA. Mean values and standard deviations are shown.



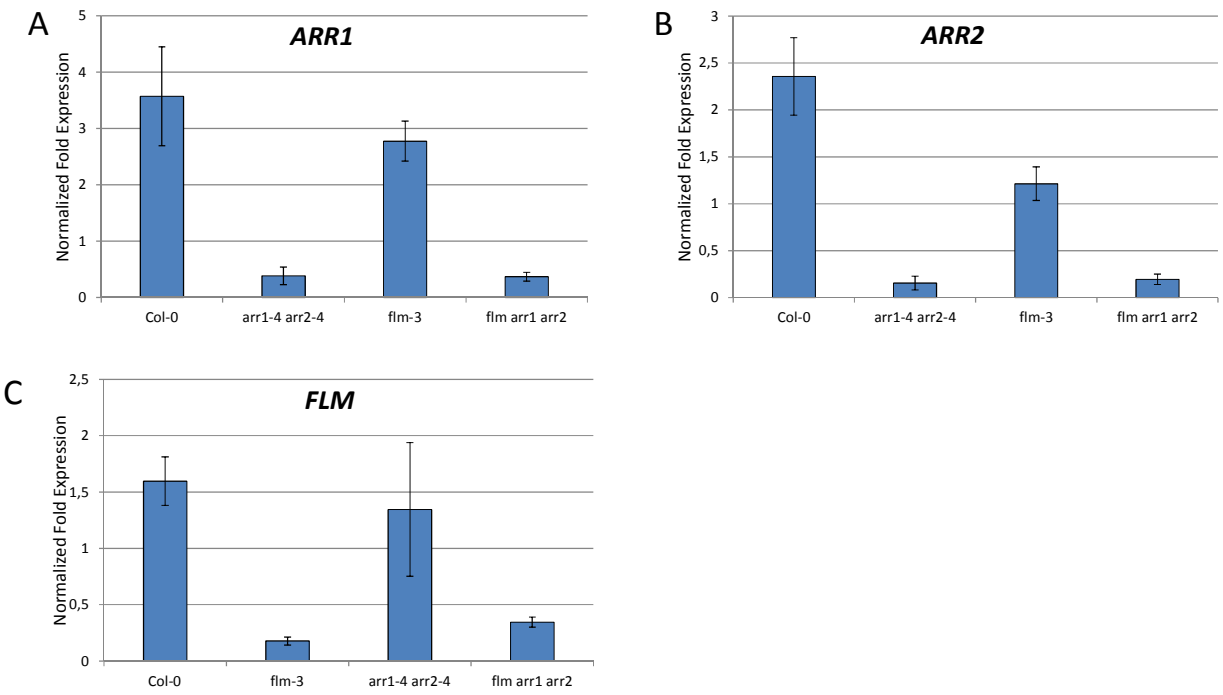
Supplemental 10 Phenotypic differences of *arr1-4 arr2-4* transgenic lines transformed with *pABind::ARR2D80E::GFP* estradiol inducible vector when treated with estradiol or Mock control

(A) *arr1-4 arr2-4* transformed with *pABind::ARR2D80E::GFP* estradiol inducible vector in T2 generation. Left photograph shows phenotypic differences of transgenic plant not induced (-E) and induced (+E) with β-estradiol. In addition photographs of disproportional silique size, reduced seed number and leaf changes, respectively, of transgenic line treated with β-estradiol are shown. (B) Difference in seed number and types of seeds between non-induced (left) and induced (right) transgenic line. For this experiment at least 10 plants per transgenic line were taken, total 3 independent lines were used, and 5 siliques per plant were used for statistical evaluation.



Supplemental 11 Transcriptional levels of *ARR1, ARR2* and *FLC* in the *triple flc-3 arr1-4 arr2-4* mutant knock out.

(A) Expression of *ARR1* in *flc-3, arr2-4, arr1-4 arr2-4* and *flc-3 arr1-4 arr2-4* mutants. **(B)** Expression of *ARR2* in *flc-3, arr2-4, arr1-4 arr2-4* and *flc-3 arr1-4 arr2-4* mutants. **(C)** Expression of *FLC* in *flc-3, arr2-4, arr1-4 arr2-4* and *flc-3 arr1-4 arr2-4* mutants. For RNA isolation leaves were taken from 3-week-old plants grown under SD. RT-qPCR was done using one biological replicate with three technical repetitions each and normalized to *ELF-1-α*. Error bars indicate SD of the mean.



Supplemental 12 Transcriptional levels of *ARR1, ARR2* and *FLM* in the *triple flm-3 arr1-4 arr2-4* mutant knock out.

(A) Expression of ARR1 in Col-0, *flm-3, arr1-4 arr2-4* and *flm-3 arr1-4 arr2-4* mutants. **(B)** Expression of ARR2 in Col-0, *flm-3, arr2-4, arr1-4 arr2-4* and *flm-3 arr1-4 arr2-4* mutants. **(C)** Expression of *FLM* in Col-0, *flm-3, arr2-4, arr1-4 arr2-4* and *flm-3 arr1-4 arr2-4* mutants. For RNA isolation leaves were taken from 3-week-old plants grown under SD. RT-qPCR was done using one biological replicate was used with three technical repetitions and normalized to *ELF-1-α*. Error bars indicate SD of the mean.

6.2 List of Figures

6.3 List of Tables

6.4 List of Supplements

7 Curriculum Vitae

Name:	Marko Vesić
Date of Birth:	April 13th 1986
Place of Birth:	Kruševac, Socialist Federal Republic of Yugoslavia *now* Republic of Serbia
Marital Status:	Unmarried

Since 2009	Doctoral studies at the Center for Plant Molecular Biology (ZMBP) University of Tübingen, Germany Supervisor: Prof. Dr. Klaus Harter Bench Supervisor: Dr. Kenneth Berendzen
2009	Diploma thesis (M.S.) at the Institute of Field and Vegetable Crops, Laboratory for Molecular Markers in Novi Sad, Serbia. Title: "Inheritance of Sunflower *Downy Mildew* resistance gene analogs"
2005-2009	Studies of Molecular Biology at the University of Novi Sad in Novi Sad, Autonomous Province Vojvodina, Serbia
2001-2005	Secondary education at the mathematical Gymnasium in Kruševac, Serbia
1993-2001	Basic education at the primary school in Kruševac, Serbia

8 Acknowledgements

Oh, I thought I will never come to this point of my PhD and therefore I did not even think about what I would write in here...

First person coming on my mind is **Klaus** (and that not because of the fact that he is one who makes important decisions about this dissertation). When I go 6 years back in the past I remember myself, young and ambitious student of molecular biology just finished 3rd year of his studies, sitting in front of computer (exactly like now) and sending an Email to some Professor from Germany whose group is working on very interesting topic and me willing to spend whole my summer holiday in his lab. The "shock "occurred only one hour after that when Klaus wrote me back (using his direct, kind and polite style) and said that he would be very happy having me in his lab for a practical course. No one was happier than me in that moment! Ever since then Klaus has always been there to give me help, support and his never-ending optimism. Klaus you were/are a real DoktorVATER to me and thank you for that!

Kenneth! Thank you for everything! Billion thanks you for correcting my thesis and critically reviewing it. Thank you for all your attempts to make me an independent scientist, I believe you made it☺.

Furthermore, I would like to thank a lot to **PD Dr. Markus Schmid** for the successful collaboration and finding time to read and correct my thesis and constantly reminding me to submit it☺. I am also very grateful to **Prof. Claudia Oecking** for finding time and accepting to be a committee member for my thesis defence making me a great honour. Many thanks to **Dr.** Gabriel Schaaf for a great time spent together at the ZMBP, constructive discussions about various topics and for accepting to read my thesis and be a member of the committee for my defence.

To **Rebecca Schwab** and **Sascha Laubinger**, extraordinary people, I thank you for all your advices, great help and support I was very lucky that I met you guys.

Of course my sincere gratitude to **Prof. Silvana Andrić** and **Prof. Snežana Radulović** (University of Novi Sad, Serbia) and to **Prof. Dejana Panković-Saftić** (EDUCONS University, Serbia) for their constant support and encourigment on the way to accomplishment of my dreams and wishes.

I would also like to thank to the DAAD, DGF, Ministry of Youth and Sport of Republic of Serbia and to Reinhold-und-Maria-Teufel-Stiftung for the financial support making my studies and stay in Germany possible.

Jochen Eisele (Mann mit einem goldenem Herzen), **Christina Chaban**, **Tante Christel** (meine zweite Mutter), **Caterina**, **Mani**, **Steven**, **Babs**, **Franzi-Schw...i** (meine größte Unterstützung), **Bea** (mein bester Kumpel), **Simon der Förschter**, **Tanja**, **Christian**, **Patty**, **Niklas**... Thank you

guys for making my time in Germany beautiful and helping me to solve all the problems I was facing with.

Meli tebi posebno hvala na SVEMU! Special thanks to my new **Meli & D´Boys** Immuno Dream Team (**Niko**, **Dominik**, **Hasan**, **Davide**, **Aki**, **Anu**) for accepting me and making me feel great in your company and integrating me within your team.

Svenito-Kurito Punišić DANKE for wonderfull vacations, sports, mutual support and teaching me how to get my car repaired all by myself. Du biSch omeglich!

Thierry mon ami merci pour tout! I am going to miss our long philosophical discussions about science, art and life early in the morning on the way home after having "successful" evenings down town! These are the things which enriched my life a lot!

To my Serbian mates and the roommates **Zorica**, **Jelena** *alias* **Baba Stamena** and **Verica** thank you for all the priceless moments we spent together in good and bad times and thank you for being entire time with me even when I left and went to Germany. Волим Вас!

Special passage samo za tebe **Honey**! Muchas gracias por todo! Por tu apoyo, comprensión y locuras! Cuando sólo pienso en nosotros en: Madrid, Barsa, Roma, Novi Sad, Cerova, Toledo... ... sólo espero que esta lista de „pichvajza" no se termina aquí!

Непроцењиву заслугу у целокупном мом животном развоју има, наравно, и мој комшилук из Церове (комшије **Снежа** и **Горан**) и касније Новог Сада (моји **Снежа** и **Иван**, **Срнче-Прасе**, **Романа**, дивни **Мира** и **Влада**). Хвала Вам на искреној и безусловној подршци, ручковима и вечерама, трпљења мојих провокација и разноразних испада, силним покушајима да ме „уразумите" и „доведете у ред" (мада ми је драго да се само делимично у томе успели!).

За крај, велико ХВАЛА мојој породици - **родитељима** и **брату**, посебно мојој **мами**, за сав труд и пожртвовање које су уложили да мог брата и мене изведу на „прави" пут.

ХВАЛА Вам **Наталија**, **Милице**, **Милане**, **Дивна**, **Светиславе** где год да сте!

Овај рад посвећујем мојој баки **Нади** и мојим малим *venturi*-јима: **Наталији**, **Иви** и **Сергеју**!

Ваш Марко