Novel aspects of receptor protein RLP30-mediated detection of *Sclerotinia* and *Pseudomonas* patterns

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1 Introduction

1.1 The plant innate immune system

Microbes, like bacteria, fungi or oomycetes are everywhere - in the soil, in the air and in the water. Plants, like all other eukaryotic creatures have to carefully monitor their environment and distinguish between beneficial and potentially harmful microbes. Overshooting immune responses are highly energy consuming processes weakening the plant and can lead to stunted growth (Gómez-Gómez *et al.*, 1999), therefore it is extremely important for the plant to react in an appropriate way to true pathogenic attacks.

1.1.1 Of R-genes, effectors and a zigzag-model - short outline about the development of the concepts of plant innate immunity

In order to protect the plant several defense strategies have evolved. The very first and basal protection of a plant is its thick cell wall providing a mechanical barrier, sometimes covered with a cuticle. Next to the mechanical barrier of the cell wall, the plant apoplast can provide a chemical barrier for potential invaders. Plant cells are able to rapidly shift the extracellular pH to more unfavorable conditions for the pathogen. This alkalinization is one of the earliest defense responses (Wu *et al.*, 2014). Additionally, plants can reinforce their cell walls with the deposition of callose or produce phenolic or other toxic compounds to fight intruders

(Hückelhoven, 2007).

Presently, two different mechanisms are described how plants detect pathogens once they have invaded the plant: Firstly, the pattern- or PRR- (pattern recognition receptor) triggered immunity (PTI), which is induced by microbial structures called PAMPs or MAMPs (pathogen or microbe-associated molecular patterns), and secondly effector-triggered immunity (ETI), both described in the so-called zig-zag model by Jones and Dangl (2006) (Fig. 1.1).

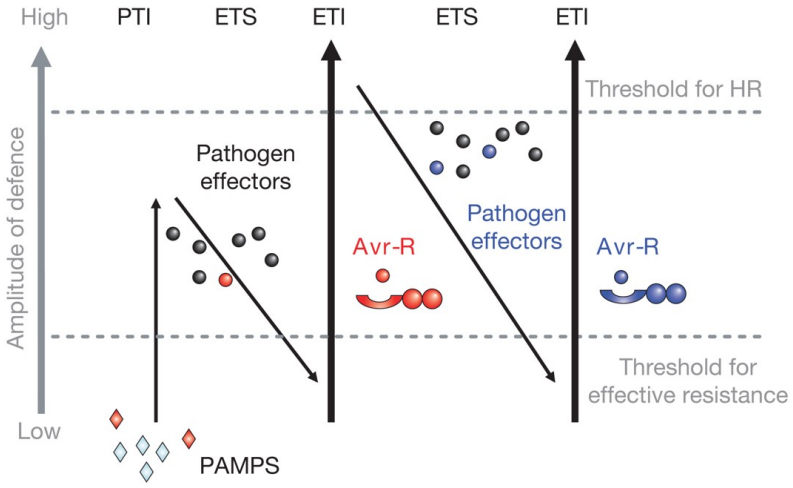

Figure 1.1: The quantitative outputs of plant innate immunity are displayed in the „zigzag-model". Plants detect microbe/pathogen-associated molecular patterns (MAMPs/PAMPs) via plasma-membrane localized pattern-recognition receptors (PRRs) triggering pattern-triggered immunity (PTI), which can be shut-down by successful pathogens through the delivery of effectors, leading to effector triggered susceptibility (ETS). Those effectors can be recognized by plant cytoplasmic nucleotide-binding leucine rich repeat proteins (NB-LRR), activating effector-triggered immunity (ETI), an amplified version of PTI, often passing a threshold for induction of the hypersensitive response (HR), causing cell death. As evolution never stops, pathogens evolve new effectors leading again to ETS, until they get in turn recognized by novel NB-LRR allele (adapted from Jones and Dangl (2006)).

Originally, Flor (1945) described that plant immunity and pathogenicity is inherited and controlled by a corresponding gene pair, a plant resistance (R) gene and an avirulence (Avr) gene carried by the pathogen. The Avr genes seemed to be race- or pathovar specific and not widely distributed. This gene-for-gene resistance was thought to be mediated by a direct interaction (Keen, 1990). However, several studies failed to prove these interactions, leading to the guard hypothesis in which R proteins "guard" the state of a host component which

is targeted by the pathogen (van der Biezen and Jones, 1998). Alterations in a host target are thus sensed by R proteins (Chisholm *et al.*, 2006; Jones and Dangl, 2006), rather than the pathogen's molecule itself. The previously called Avr proteins, are actually promoting and "effecting" the virulence of a pathogen and thus were termed "effectors" (Bent and Mackey, 2007; Boller and Felix, 2009). Effector proteins are molecules determined to suppress immunity and altering host cell structures or processes promoting the lifestyle of the pathogen, rendering the plant susceptible. This is also called effector triggered susceptibility (ETS). But as a result of co-evolution of hosts and "their" pathogens, plants can circumvent the shut-down of their immune signaling pathway through effectors, by sensing either directly or indirectly the respective effectors, leading to effector triggered immunity (ETI).

In the recent years, it became obvious that there is another layer of plant innate immunity, called PAMP-triggered immunity or PTI. Initially, it was observed that not only race- or pathovar specific compounds, but also other more widespread compounds would "elicit" a defense response (Ebel and Cosio, 1994), which were therefore termed "elicitors". Later on, the term "PAMP" was defined for pathogen-associated molecular patterns (Medzhitov and Janeway, 1997; Nürnberger and Brunner, 2002), which were characterized as highly conserved molecules within a class of microbes being crucial for the lifestyle or fitness of its carrier. As they are not only restricted to pathogenic microbes, the term "MAMP" was introduced, for microbe-associated molecular pattern (Boller and Felix, 2009). With science in progress additional terms, such as damage-associated molecular pattern (DAMP) (Rubartelli and Lotze, 2007), herbivory-associated molecular pattern (HAMP) (Mithöfer and Boland, 2008), nematode-associated molecular pattern (NAMP) (Manosalva *et al.*, 2015) or parasite-associated molecular pattern (parAMP) (Kaiser *et al.*, 2015; Hegenauer *et al.*, 2016; Gust *et al.*, 2017) have been added.

But, as more and more insight is gained into plant innate immunity, it is obvious that this is not a black and white story of ETI and PTI, and the existing terminology becomes more

and more blurred (Thomma *et al.*, 2011). For example the fungal extracellular protein 6 (Ecp6) from *Cladosporium fulvum* sequesters Chitin fragments in the apoplast of the plant, thus interfering with chitin-triggered immunity (de Jonge *et al.*, 2010). This would classify Ecp6 as an effector with a rather narrow distribution to certain *C.fulvum* strains. But looking at the occurrence of Ecp6 and its orthologs revealed a wide occurrence throughout the fungal kingdom (Bolton *et al.*, 2008), therefore qualifying it as a typical PAMP. The rather opposite example is Pep-13, a surface-exposed fragment of a calcium-dependent cell wall transglutaminase, which is only conserved within *Phytophthora* species (Nürnberger *et al.*, 1994; Brunner *et al.*, 2002), but being recognized in a PAMP-like manner.

According to the current paradigm, PAMPs are recognized by cell-surface localized pattern recognition receptors (PRRs) and effectors are typically recognized by intracellular nucleotide-binding (NB) leucine-rich repeat (LRR) receptors. But again, there are exeptions from the rule, like Avr2 produced by *C.fulvum* strains. The receptor-like protein (RLP) Cf2 is a classical surface-located PRR, but instead of binding Avr2 it monitors the presence of *C.fulvum* by guarding the Rcr3 protease, which is targeted by Avr2 (Rooney *et al.*, 2005; Shabab *et al.*, 2008; van Esse *et al.*, 2008). The receptor-type would suggest a classification in PTI, whereas the mode of action is typical for ETI.

Another aspect of the differentiation between ETI and PTI is that PTI usually confers broad-spectrum disease resistance, whereas ETI is thought to mediate race- or pathovar-specific resistance. However, recent findings contradict this general view. Zhu *et al.* (2017) found out that the tomato intracellular NLR receptor Sw-5b recognizes a 21 amino acid long PAMP-like region within a viral effector protein, which is enough to confer broad-spectrum disease resistance against American type tospoviruses.

To overcome these and several other issues with the nomenclature, Boutrot and Zipfel (2017) suggest a differentiation between *surface immunity* (a.k.a. PTI) and *intracellular immunity* (a.k.a. ETI).

1.1.2 Surface immunity vs intracellular immunity

After clarifying the terminology in plant innate immunity, the question remains about the differences between PTI and ETI or surface and intracellular immunity. The zigzag-model of Jones and Dangl (2006), already suggests a difference in the strength of the induced immune response, with ETI being the stronger immune response often leading to a local hypersensitive response (HR) in infected cells. By sacrificing the already infected cells, the plant tries to stop further infection.

Surface immunity confers basal immunity towards both adapted and non-adapted pathogens through activation of a local as well as a systemic immune response (Böhm *et al.*, 2014a; Boller and Felix, 2009; Couto and Zipfel, 2016; Win *et al.*, 2012). The detection of the microbes takes place at the plant plasma membrane via pattern-recognition receptors (PRRs) (Fig. 1.2 A). The downstream signaling after detection of the respective pattern is under the tight control of co-receptors like BAK1 (BRI1 activating like kinase 1) (Kemmerling *et al.*, 2007).

Plants lack a circuit signaling and transportation system, like the blood vessels in vertebrates, therefore each single cell is equipped with all the required immune receptors to detect pathogens and defend itself (Litman *et al.*, 2005; Pancer and Cooper, 2006).

In order to protect the whole plant a mechanism called systemic acquired resistance (SAR) has evolved (Ross, 1961). This long-lasting immune response puts the plant in alert after infection on one local site, and pre-activates its immune system via salicylic acid-dependent signaling, leading to the faster accumulation of pathogenesis-related proteins in the rest of the plant upon attacks (Durrant and Dong, 2004).

In contrast, intracellular immunity or ETI is usually associated with more adapted and more specialized pathogens, conferring plant resistance only to specific strains, races or pathovars (Jones and Dangl, 2006).

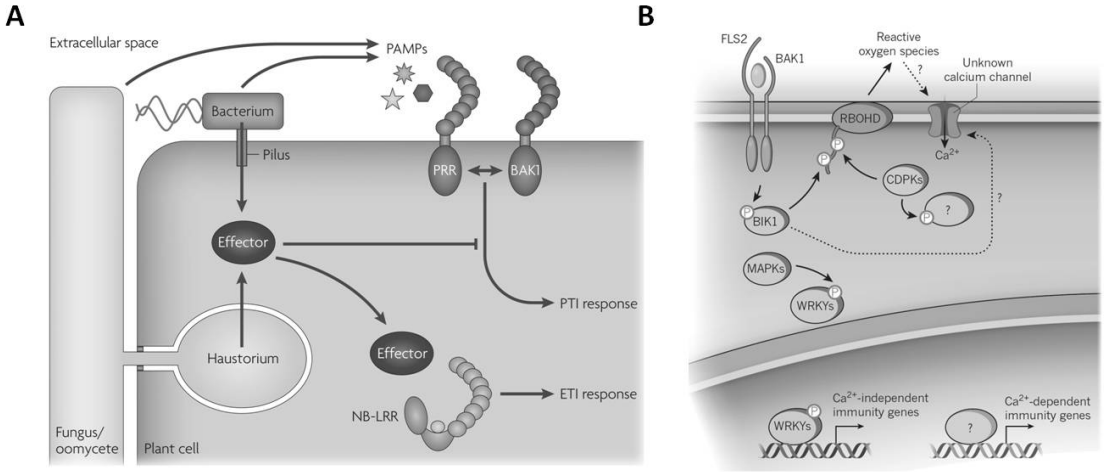

Figure 1.2: Overview of the plant innate immune system and its intracellular signaling pathways. **(A)** Overview of the plant PTI- and ETI-system. Bacterial or fungal pathogens proliferate in the plant apoplast. PAMPs/MAMPs are released from the pathogen and can be recognized by cell surface-located PRRs. After successful recognition, PTI is induced. Pathogens can also release so-called effectors, that are secreted into the plant cell via the bacterial type-III secretion pilus or are delivered via a yet unknown secretion mechanism from fungal haustoria or other structures from oomycetes into the plant cell. These effectors can suppress PTI, however a lot of them can be recognized by intracellular NB-LRR proteins and elicit ETI (adapted from Dodds and Rathjen (2010)). **(B)** Simplified model of plant immune signalling pathways, explained for the perception of flagellin (flg22). Flg22 is recognized via the FLS2-BAK1 receptor complex leading to the phosphorylation of the cytoplasmic kinase BIK1, which phosphorylates the RbohD oxidase on the plasma membrane, resulting in the production of reactive oxygene species (ROS). This may activate a yet unknown calcium channel resulting in the influx of Ca^{2+}-ions, which are recognized by caldium-dependent protein kinases (CDPKs). CDPKs further phosphorylate RbohD and maybe other unknown targets. In parallel, the activation of the mitogen-activated protein kinase (MAPK) cascade leads to the phosphorylation and activation of WRKY transcription factors and their re-localization into the nucleus, where immunity dependent genes are activated. (adapted from Zipfel and Oldroyd (2017)).

Bacteria inject effectors via their type III-secretion system into the plant cell (indicated by the pilus in Fig. 1.2 A) (Galan and Wolf-Watz, 2006). For both, fungi and oomycetes, such a secretion system remains unknown. Fungi and oomycetes produce instead a specialized structure, called Haustorium to establish a very close connection with the host to exchange molecules, but never penetrate the plant cell membrane. Despite the lack of a known secretion system, the identified effectors derived from oomycetes are all carrying an RxLR-motif (Rehmany *et al.*, 2005), which seems to be important for the effector secretion from the pathogen, rather than for direct interaction with the host cells (Wawra *et al.*, 2017). Such a significant motif remains elusive for fungal effector proteins.

An overview of molecular mechanisms and key proteins in the signal transduction pathway of surface immunity are shown in Fig. 1.2 B, exemplified by the MAMP flg22, the 22 amino acid

long immunogenic motif of bacterial flagellin, and its respective PRR, FLS2 (Flagellin-sensing 2). Typical immune responses after binding of MAMPs are initiated through plant membrane depolarisation via an increased influx of Ca^{2+}- and H^{+}- ions and a simultaneous efflux of K^{+}-, Cl^{-}- and NO^{3-}- ions (Jabs *et al.*, 1997; Zimmermann *et al.*, 1997; Wendehenne *et al.*, 2002; Garcia-Brugger *et al.*, 2006). Changes in the intracellular Ca^{2+}-levels are monitored by calcium-dependent protein kinases (CDPKs), which mediate further signaling steps (Blume *et al.*, 2000; Hrabak *et al.*, 2003; Ludwig *et al.*, 2004; Romeis and Herde, 2014).

Activation of the PRR-signaling complex leads to the activation of BIK1 (Botrytis induced kinase 1). BIK1 has two major functions: one is the phosphorylation of RbohD (Respiratory burst oxidase homologue D) (Kadota *et al.*, 2014) and the other one is the activation of MAP (mitogen-activated protein) kinases (Lu *et al.*, 2010a). Phosphorylation of RbohD leads to the production of reactive oxygen species (ROS), further triggering membrane depolarization and Ca^{2+}- influx.

The activation of the MAP kinase cascade induces activation of WRKY (N-terminal "WRKY" motif) transcription factors, which are translocalized into the nucleus and induce immunity genes (Mao *et al.*, 2011). Other typical immune responses are the production of ethylene and the deposition of callose.

Intracellular immunity is usually associated with a hypersensitive response (HR), but several PAMPs or MAMPs, like elicitins from oomycetes or flagellin from bacteria can also induce HR (Ricci *et al.*, 1989; Taguchi *et al.*, 2003). HR is a rapid induction of cell death in the surrounding cells of a local infection site to prevent further spreading of the pathogen. It is induced via the accumulation of salicylic acid in the infected area (Fu *et al.*, 2012).

Intracellular immunity or ETI signaling pathways overlap at least partially with the ones described earlier for extracellular immunity, like the induction of MAPK cascades or the activation of WRKY transcription factors (Muthamilarasan and Prasad, 2013).

1.2 Different classes of receptors recognize different classes of ligands

In recent years, more and more ligand-receptor pairs have been unraveled, not only in *Arabidopsis*, but also in tomato, rice, wheat, lotus, or potato (Boutrot and Zipfel, 2017). In this chapter some selected ligand-receptor pairs, with the main focus on *Arabidopsis*, will be introduced in more detail.

The different plasma-membrane located receptors fall generally in two distinct groups, receptor-like kinases (RLKs) and receptor-like proteins (RLPs) (Fig. 1.3 A and B). RLPs lack an intracellular signaling domain, instead they have only a short intracellular domain like the receptor-like protein 30 (RLP30) in *Arabidopsis* (Zhang *et al.*, 2013) or are attached to the plasma-membrane via a glycosylphosphatidylinositol- (GPI) anchor, like the *Arabidopsis* Lysin-motif (LYM) proteins (Borner *et al.*, 2003).

Besides the differentiation between RLKs and RLPs, the receptors can be categorized according to their extracellular ligand-binding domains. The biggest group are the leucine rich repeat (LRR)-domain containing receptors. In *Arabidopsis* more than 600 LRR-RLKs and more than 50 LRR-RLPs are annotated (Shiu and Bleecker, 2001; Fritz-Laylin *et al.*, 2005). For most of these RLKs and RLPs the function still remains elusive and therefore they were termed receptor-“like“ kinases/proteins, indicating a speculative classification based on structural similarity, rather than proven function (Gust and Felix, 2014). LRR-domains usually bind proteinaceous ligands, like the FLAGELLIN-SENSING 2 (FLS2) receptor recognizing bacterial flagellin (Chinchilla *et al.*, 2006) or the receptor-like protein 23 (RLP23) from *Arabidopsis* binding necrosis and ethylene-inducing protein 1-like proteins (NLP) (Albert *et al.*, 2015).

The Lysin-motif (LysM) carrying receptors recognize N-acetylglucosamine-containing glycan

structures like bacterial peptidoglycan or fungal chitin (Kaku *et al.*, 2006; Miya *et al.*, 2007; Wan *et al.*, 2008; Willmann *et al.*, 2011).

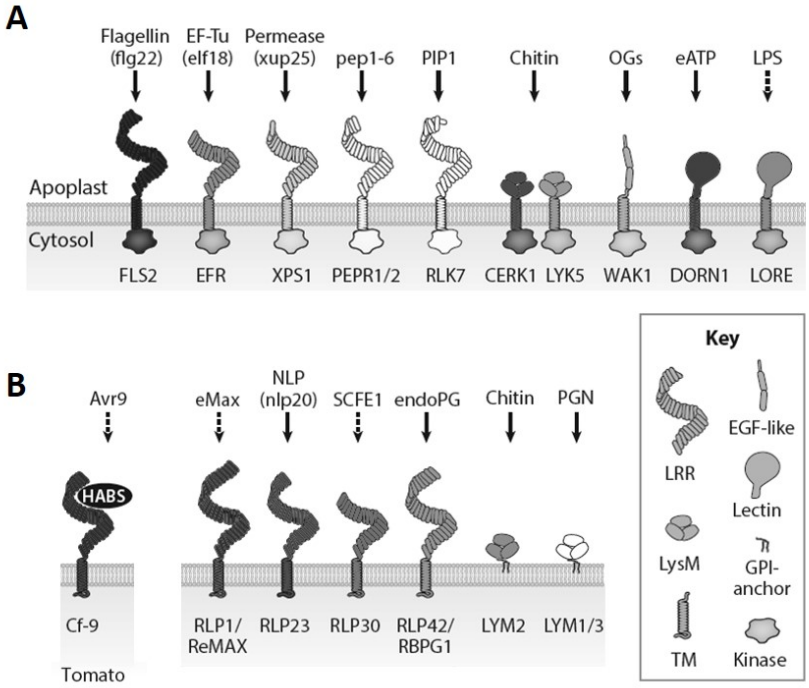

Figure 1.3: Selected examples of proven and potential pattern recognition receptors with known ligands. (A) Selected *Arabidopsis* receptor-like kinases (RLKs) with their respective ligands, or in brackets the recognized immunogenic motif. CERK1 (Chitin Elicitor Receptor Kinase 1); DORN1 (DOes not Respond to Nucleotides 1); eATP (extracellular Adenosine Triphosphate); EFR (Elongation Factor Tu Receptor); EF-Tu (Elognation factor Tu); FLS2 (FLagellin Sensing 2); LORE (Lipo-Oligosaccharide-specific Reduced Elicitation); LPS (Lipopolysaccharide); LYK5 (Lysin Motif Receptor Kinase 5); OGs (Oligogalacturonides); pep1-6 (Peptide 1-6); PEPR1/2 (Pep Receptor 1/2); PIP1 (PAMP-induced secreted peptide 1); WAK1 (Cell Wall-Associated Kinase 1); XPS (1-Deoxy-D-Xylulose 5-Phosphate Synthase 1). **(B)** Selected *Arabidopsis* receptor-like proteins (RLPs) and one example, Cf9, for an RLP in tomato, with their respective ligands, or in brackets the recognized immunogenic motif. Avr9 (Avirulence protein 9); Cf9 (*Cladosporium fulvum* resistance gene 9); eMAX (enigmatic MAMP of *Xanthomonas*); endoPG (Endopolygalacturonases); HABS (high-affinity binding site); LYM1/2/3 (Lysin-motif protein 1/2/3); NLP (Necrosis- and ethylene indcing like protein); PGN (Peptidoglycan); RBPG1 (Responsiveness to *Botrytis* Polygalacturonases 1); ReMAX (Receptor of eMax); SCFE1 (*Sclerotinia* Culture Filtrate Elicitor 1). Solid arrows indicate demonstrated direct binding; dashed arrows indicate a current lack of evidence for direct binding. Key: EGF-like (Epidermal Growth Factor-like); LRR (Leucine-rich repeat); LysM (Lysin motif); GPI (Glycosylphosphatidylinositol); TM (Transmembrane). Adapted from Boutrot and Zipfel (2017).

Other classes of extracellular binding domains are the EGF- (epidermal growth factor) like receptors and the receptors carrying a Lectin domain. Figure 1.3 gives an overview of selected ligand-receptor pairs in *Arabidopsis* and one from tomato (Cf9 with Avr9). Chosen pairs are being discussed, in more detail, in the following sections.

1.2.1 FLS2 and flg22

Most likely the best-studied ligand-receptor pair is FLAGELLIN SENSING 2 (FLS2) and the bacterial flagellin (Gomez-Gomez and Boller, 2000). Bacterial flagella mainly consist of Flagellin (FliC) (Samatey *et al.*, 2001), which is relatively conserved. The flagellum is indispensable for the bacterial lifestyle as it is required for both motility and virulence, in the case of pathogenic bacteria (Tans-Kersten *et al.*, 2001; Taguchi *et al.*, 2010; Josenhans and Suerbaum, 2002). These circumstances make Flagellin a prime target for a PRR. The recognized epitope by the *Arabidopsis* FLS2 receptor is a 22 amino acid long stretch of the most conserved part of the N-terminus of flagellin, called flg22 (Felix *et al.*, 1999). Flg22 is detected by FLS2 (Gomez-Gomez and Boller, 2000) via a direct and physical binding (Chinchilla *et al.*, 2006). Although, the first identified FLS2-receptor was from *Arabidopsis*, functional homologs have been identified for example in *Oryza sativa*, *Nicotiana benthamiana*, *Solanum lycopersicum* or *Vitis vinifera* (Takai *et al.*, 2008; Hann and Rathjen, 2007; Robatzek *et al.*, 2007; Trdá *et al.*, 2014). The ability to perceive bacterial flagellin seems to be conserved in all higher plants, indicating an evolutionary ancient perception system. The moss *Physcomitrella patens* has no FLS2 ortholog and is also not responsive to flg22 (Boller and Felix, 2009). Crystal structure analysis of the FLS2 ectodomain in the presence of flg22 gave further insights into the ligand-receptor interaction, especially into the interaction with the co-receptor, BRASSINOSTEROID-INSENSITIVE 1 (BRI1)-ASSOCIATED KINASE 1 (BAK1) [Sun *et al.* (2013); detailed information about the role of BAK1 in section 1.3].

1.2.2 RLP23 and nlp20

The LRR-domain containing receptor-like protein 23 (RLP23) detects a widespread, but conserved twenty amino acid long epitope in NLPs (Albert *et al.*, 2015). This so-called nlp20 motif is present in NLPs from fungi, oomycetes and bacteria (Böhm *et al.*, 2014b). This is especially interesting, because the recognized pattern is not restricted to one domain of life, but present in both prokaryotic and eukaryotic kingdoms. Nlp20 is another prime example of a PAMP. On the one hand it is widely distributed among different species and on the other hand the motif is located in an important virulence factor for necrotrophic pathogens. NLPs can be cytotoxic by forming pores in the plant plasma membrane and thereby promoting the infection process of the pathogen (Qutob *et al.*, 2007; Ottmann *et al.*, 2009). This example displays the co-evolution of pathogens and their hosts. NLPs are required for the pathogenicity of the microbes and are therefore indispensable, making them a prime target for a PRR. The weapon of the pathogen (NLPs) makes it at the same time more vulnerable, conferring recognition and inducing an immune response.

1.2.3 RLP30 and SCFE1

The receptor-like protein 30 (RLP30) from *Arabidopsis* was described as being required for the detection of the partially purified SCLEROTINIA CULTURE FILTRATE ELICITOR 1 (SCFE1) from the necrotrophic fungus *Sclerotinia sclerotiorum* (Zhang *et al.*, 2013) (for further information about *S.sclerotiorum* see section 1.4). SCFE1 induces typical immune responses in *Arabidopsis*, like the production of ethylene and reactive oxygen species or the activation of MAP kinases and the induction of defense related genes like *FRK1, PR-4, CYP71A13* or *PAD4*. *Rlp30*- mutant plants are fully insensitive to SCFE1 and exhibit a higher susceptibility towards *S.sclerotiorum* and the closely related fungus *Botrytis cinerea*,

indicating the relevance of PAMP-triggered immunity in resistance to necrotrophic fungi (Zhang *et al.*, 2013).

In a study examining the roles of *Arabidopsis* RLPs, Wang *et al.* (2008a) showed that *rlp30*-mutant plants are more susceptible towards the bacterial pathogen *Pseudomonas syringae pv. phaseolicola 1448A*. Interestingly, this phenotype was not seen for infection with any other pathogen tested. The authors concluded at that time that RLP30 confers basal resistance towards bacterial infection.

1.2.4 Cf9 and Avr9

Arabidopsis, belonging to the *Brassicaceae*, is not the only plant species which has evolved a distinct set of PRRs. Tomato, belonging to the *Solanaceae*, has its own distinct set of PRRs, especially of LRR-RLPs. The LRR-RLP family of the Cf proteins confers resistance to the non-obligate biotrophic fungus *Cladosporium fulvum*, carrying the respective Avr-gene. Small cysteine-rich effector proteins are injected into the plant apoplast by the fungus to promote virulence. For some of those injected proteins it was shown that they can be detected by the plant via these Cf receptor-like proteins, localized at the plasma membrane (Rivas and Thomas, 2005). The very first identified plant LRR-RLP was Cf9 being required for the perception of Avr9 (Hammond-Kosack *et al.*, 1994), which was also the very first fungal avirulence gene to be cloned (Van den Ackerveken *et al.*, 1992). It could be proven, that Cf9 does not directly bind Avr9, but that at least one other protein is involved in the binding of Avr9 (Luderer *et al.*, 2001). Surprisingly, binding studies with radioactive-labeled Avr9 revealed that Avr9 can also bind to purified plasma membranes lacking Cf9, indicating the presence of a putative high-affinity binding site (HABS) in tomato plants independent of Cf9 (Kooman-Gersmann *et al.*, 1996).

LRR-RLPs and LRR-RLKs are postulated to bind proteinaceous ligands and very often a

simple peptide motif, rather then the correctly folded full-length protein, is sufficient for recognition and induction of immune responses, exemplified by flg22 or nlp20 (Felix *et al.*, 1999; Böhm *et al.*, 2014b). But in contrast to other known ligands, the folding of Avr9 is important for its function as a ligand. Only when three disulfid bridges between six different cysteine residues are correctly established, Avr9 is able to induce immune responses (Kooman-Gersmann *et al.*, 1997; van den Hooven *et al.*, 2001).

1.2.5 RLKs and RLPs in other plant pathways

RLKs and RLPs play not only essential roles in plant innate immunity, but are also implicated in many other cellular processes such as developmental regulations, maintenance of meristems or floral organ abscission, highlighted with selected examples in this section.

In *Arabidopsis*, the pattern of stomata, micropores to facilitate gas exchange which are located in the epidermis of plant leaves, is regulated by RLP17, also named TOO MANY MOUTH (TMM), the ERECTA (ER) FAMILY RECEPTOR KINASES (ERF) and EPIDERMAL PATTERNING FACTOR (EPF) peptides. TMM and ER or ER-LIKE 1 (ERL1) form constitutive complexes, which can recognize EPF1 and EPF2. The complex formation is not only required for ligand binding, but the LRR-ectodomain of ER or ERL1 facilitated the co-expression of the LRR-ectodomain of TMM in insect cells, suggesting a stabilizing effect of ER and ERL1 (Lin *et al.*, 2017).

For the proper development and maintenance of shoot apical meristems the homedomain protein WUSCHEL is required, of which the expression is regulated in a negative feedback-loop by CLAVATA3 [CLV3, Brand *et al.* (2000); Schoof *et al.* (2000)]. CLV3 is a 13-amino acid long and arabinosylated glycopeptide, being recognized by three different receptors: CLV1, a LRR-receptor kinase, CLV2, a LRR-receptor-like protein and CORYNE (CRN), a receptor-like cytoplasmic kinase (Clark *et al.*, 1997; Jeong *et al.*, 1999; Müller *et al.*, 2008). CLV1 can

homodimerize at the plasma membrane, but also interacts with CLV2 and CRN. Additionally, CLV2 and CRN require each other for the transport from the endoplasmatic reticulum to the plasma membrane (Bleckmann *et al.*, 2009).

Floral organ abscission after pollination is regulated by the perception of the small secreted peptide INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) via the two closely related LRR-RLKs HAESA (HAE)and HAESA-LIKE 2 (HSL2), which function redundantly (Butenko *et al.*, 2014; Cho *et al.*, 2008; Stenvik *et al.*, 2008). After ligand-binding transcription factors like BREVIPEDICELLUS (BP) and AGAMOUS-LIKE 15 (AGL15) (Patharkar and Walker, 2015; Shi *et al.*, 2011) get activated via MAPK induction (Cho *et al.*, 2008).

These examples nicely illustrate the great variety of the different cellular processes being all mediated and regulated via RLPs and RLKs and it is interesting to further dissect those signaling pathways to elucidate similarities and differences in the signaling cascades.

1.3 LRR-RLK and LRR-RLP dependent signaling and complex formation

The major difference between LRR-RLKs and LRR-RLPs is the presence or absence of an intracellular kinase domain, being only present in RLKs. But how do LRR-RLPs transfer their extracellularly received signals into the inside of the cell when an obvious signal domain is absent?

1.3.1 SOBIR1

The LRR-RLK SUPPRESSOR OF BIR1 (BAK1-INTERACTING RECEPTOR-LIKE KINASE 1) SOBIR1/EVR (EVERSHED) was identified as an adaptor kinase for plant

LRR-RLPs (Liebrand *et al.*, 2013, 2014; Gust and Felix, 2014).

It was firstly shown in tomato that SOBIR1 associates with different RLPs, such as Cf4, and is essential in RLP-mediated immunity to fungal pathogens in tomato (Liebrand *et al.*, 2013). In addition, it was shown that RLP-mediated resistance in *Arabidopsis* requires SOBIR1 (Zhang *et al.*, 2013) and recently the ligand-independent complex formation of both RLP30 and RLP23 with SOBIR1, respectively, was confirmed (Albert *et al.*, 2015). To date, all known LRR-RLPs, described as PRRs, require SOBIR1 for full function. The constitutive interaction of those RLPs with SOBIR1 resembles a genuine RLK, with an extracellular ligand-binding domain and an intracellular kinase domain (Gust and Felix, 2014).

Initially, SOBIR1 was identified in a suppressor screen of the *Arabidopsis bir*-mutant, hence the name SUPPRESSOR OF BIR1 (Gao *et al.*, 2009). BIR1 is also a member of the LRR-RLK family; it is interacting with BAK1, and *bir1*-mutant plants exhibit a constitutive defense phenotype, suggesting a role as a negative regulator of defense responses for BIR1. Consequently, overexpression of SOBIR1 in *Arabidopsis* leads to a constitutive activation of immune responses, describing SOBIR1 as a positive regulator of immunity, being normally inhibited by BIR1. A direct interaction of BIR1 and SOBIR1 could not be confirmed, but it was hypothesized that BIR1 functions in a SOBIR1-dependent pathway promoting pathogen resistance and cell death (Gao *et al.*, 2009).

Additionally, SOBIR1 was described playing a role in Systemic Acquired Resistance (SAR) (Pan *et al.*, 2004), an additional form of the plant innate immune system (Ross, 1961).

But SOBIR1 is not only required for LRR-RLP-mediated signaling in plant innate immunity, it also plays a crucial role during floral organ abscission in *Arabidopsis*. Mutants of the ADP-Ribosylation Factor GTPase-Activating Protein (ARF GAP) NEVERSHED (NEV) show defects in floral organ shedding, cellular trafficking and Golgi architecture (Lewis *et al.*, 2010; Liljegren *et al.*, 2009). *Sobir1*-mutants were identified to revert the *nev* phenotype, leading to the second name of SOBIR1, EVERSHED (Leslie *et al.*, 2010). SOBIR1/EVR

was supposed to function downstream of NEV in the floral abscission pathway, interacting with the receptor-like cytoplasmic kinase (RLCK) CAST AWAY (CST) (Burr *et al.*, 2011). In the model of Burr *et al.* (2011) CST sequesters SOBIR1/EVR at the plasma membrane, where otherwise SOBIR1/EVR would form a complexe with HAE or HSL2, which leads to the internalization of the receptor-complexes into the trans-Golgi network. Binding of the secreted IDA peptide to HAE and HSL2 could lead to the stabilization of the HAE/HSL2 receptors at the plasma membrane leading to the activation of MAPKs and subsequently to floral organ abscission (as described in chapter 1.2.5).

RLKs and RLPs, synthesized in the endoplasmatic reticulum, are transported to the plasma membrane via the cellular trafficking machinery and can subsequently be removed from the plasma membrane by endocytosis (Beck *et al.*, 2012). Transient overexpression of SOBIR1-eGFP fusion constructs suggested a role of SOBIR1 in endosomal trafficking (Liebrand *et al.*, 2013). Moreover, co-silencing of *sobir1* and *sobir1-like* in *N.benthamiana* led to reduced Cf4 protein levels in a transient expression assay (Liebrand *et al.*, 2013), suggesting a role for SOBIR1 and its homolog SOBIR1-LIKE in the accumulation of LRR-RLPs at the plasma membrane. Additionally, SOBIR1 might promote the internalization of receptor complexes und regulate Golgi dynamics during cellular stress, like abscission or pathogen attacks (Leslie *et al.*, 2010).

Furthermore, an increase of *sobir1*-expression was induced by high light stress in *Arabidopsis* cell culture (González-Pérez *et al.*, 2011) and *SOBIR1* T-DNA insertion mutants showed altered phenotypes in tolerance to high auxin and high salt concentrations (ten Hove *et al.*, 2011).

1.3.2 BAK1

The receptor-like kinase BAK1/SERK3 (BRASSINOSTEROID-INSENSITIVE 1 (BRI1)-ASSOCIATED KINASE 1) is part of the SERK (SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE)-family, which consists of five members and is highly conserved among plant clades (Schwessinger and Rathjen, 2015). SERK-proteins have been initially identified for their role in embryogenesis (Schmidt *et al.*, 1997), hence the name. SERK3 also regulates brassinosteroid signaling by modulating BRASSINOSTEROID-INSENSITIVE 1 (BRI1) (Li *et al.*, 2002; Vert *et al.*, 2005), leading to the second name BAK1. It has been shown that sequential transphosphorylation between the two kinase domains of BRI1 and BAK1 mediates brassinosteroid signaling (Wang *et al.*, 2008b). Furthermore, SERK1/2/3 (BAK1) and SERK4 are all interacting with HAE and HSL2, regulating floral organ abscission (Meng *et al.*, 2016), in a very similar way like it was shown for BRI1 and BAK1 in brassinosteroid signaling. The first link of BAK1 to immunity was established in 2007 with the involvement of BAK1 in the containment of the cell-death response in a brassinosteroid-independent manner (Kemmerling *et al.*, 2007) and its ligand-dependent complex formation with FLS2 (Chinchilla *et al.*, 2007). Furthermore, it was shown that *bak1*-mutant plants were impaired in FLS2-mediated immunity (Heese *et al.*, 2007). The involvement of BAK1 in MAMP-triggered immunity is not restricted to FLS2 and other LRR-RLKs, like EFR or PEPR1 (Chinchilla *et al.*, 2009; Schulze *et al.*, 2010), but is also required for immune responses mediated by LRR-RLPs. SCFE1 induced immune responses are not only RLP30-dependent, but require also BAK1 in addition to SOBIR1 (Zhang *et al.*, 2013). Furthermore, the tripartite receptor complex formation between RLP30, SOBIR1 and BAK1 in the presence of SCFE1 and RLP23, SOBIR1 and BAK1 in the presence of the ligand nlp20 was shown in pulldown experiments (Albert *et al.*, 2015), emphasizing the hypothesis of RLPs and SOBIR1 resembling a bimolecular RLK.

1.3.3 Positive and negative regulators of PRR signaling

Plant innate immunity needs to be tightly regulated, as it is a highly energy-consuming process and leads to trade-offs in e.g. growth (Lozano-Durán and Zipfel, 2015).
As already described in 1.1.2, BIK1 mediates early immune responses, like the ROS-burst, by direct phosporylation of RbohD (Kadota *et al.*, 2014) and is also involved in transphosphorylation events between the respective PRR and BAK1 (Lu *et al.*, 2010b; Zhang *et al.*, 2010; Liu *et al.*, 2013a). BIK1 is part of the large RLCK family called PBS-LIKE (PBL, avrPphB SUSCEPTIBLE-LIKE) kinases. Several homologs of BIK1, namely PBL1, PBL2 and PBS1 seem to be functionally additive (Zhang *et al.*, 2010; Liu *et al.*, 2013b), working all together as positive regulators of PRR-mediated immunity.
Surface immunity is not only under the control of positive regulators, but even more negative regulators have been identified until now. The "model" RLK, FLS2, was intensively studied and revealed different mechanisms for signal attenuation after flg22 perception. Once flg22 binds to FLS2, the ligand-receptor complex gets internalized and the receptor is no longer available at the plasma membrane (Robatzek *et al.*, 2006). Additionally, it was shown that FLS2 gets ubiquitinated by two PLANT U-BOX E3 UBIQUITIN (PUB) ligases, PUB12 and PUB13 (Lu *et al.*, 2011) and interacts with the KINASE-ASSOCIATED PROTEIN PHOSPHATASE (KAPP), which also negatively regulates signaling (Gómez-Gómez *et al.*, 2001). Several negative regulatory proteins directly target the common co-receptor BAK1, like BIR1 (Gao *et al.*, 2009) or the PROTEIN SER/THR PHOSPHATASE TYPE 2A (PP2A), which constitutively interacts with BAK1 modulating its phosphorylation status (Segonzac *et al.*, 2014). Belonging to the same protein family like BIR1, BIR2 and BIR3 have been identified as additional negative regulators of PRR-signaling complexes in a ligand-dependent manner, by targeting directly both BAK1 and the respective PRR (Halter *et al.*, 2014a,b; Imkampe *et al.*, 2017).

1.4 *Pseudomonas* and *Sclerotinia* - two devastating pathogens

Looking back in history several disastrous outbreaks of pathogen infections happened, like the "Irish Potatoe Famine" in the 1840s, caused by the oomycete *Phytophthora infestans*, killing over one million people and leading to the emigration of another million people (Strange and Scott, 2005) or the "Great Bengal Famine" of 1943 caused by the fungus *Cochliobolus miyabeanus*, affecting rice, leading to starvation of two million people (Padmanabhan, 1973). During the "Southern corn leaf blight epidemic" of 1970-71 in the USA, caused by the fungus *Cochliobolus heterostrophus*, no one died, but the agricultural economy was highly affected (Ullstrup, 1972). These examples and others illustrate the need to protect crop plants by knowing more about the infection strategies of the pathogens on one side, but also by learning more about the defense strategies of plants on the other side. The two different pathogens, *Pseudomonas* and *Sclerotinia sclerotiorum*, used in this study, will thus be introduced in this chapter.

The genus *Pseudomonas* belongs to the family of *Pseudomonadaceae* of Gram-negative bacteria with 191 validly described species (Euzéby, 1997). The best-studied species are the opportunistic human pathogen *P.aeruginosa*, the plant pathogen *P.syringae* and the plant growth-promoting *P.fluorescens*. The species *P.syringae* contains approximately 50 pathovars, which differ in their relatively strict host specificity (Gardan *et al.*, 1999), many of them causing economically important diseases in crop plants. The *P.syringae* lifestyle is maybe best described as that of a locally infecting, hemibiotrophic pathogen (Xin and He, 2013), with an initial epiphytic phase on the plant surface, leading to an endophytic phase in the apoplast, after successfully entering through plant stomata or accidental wounding, ending in extensive necrosis at late stages of pathogenesis. Infection does not spread throughout the whole plant, but is often contained within a few millimeters of the initial infection sites (Xin and He, 2013). The emergence of *P.syringae pv. tomato DC3000* (*Pst*DC3000) as the

prime model pathogen in the lab is due to the landmark work of the lab of Brian Staskawicz, showing that *Pst*DC3000 can infect both *Arabidopsis* and tomato plants under laboratory conditions (Whalen *et al.*, 1991). Approximately 5% of the genome of *Pst*DC3000 encodes virulence-related genes, rendering *Pst*DC3000 a successful plant pathogen (Buell *et al.*, 2003; Xin and He, 2013).

Sclerotinia sclerotiorum and the closely related fungus *Botrytis cinerea* are two devastating plant pathogens, causing white or grey mold, respectively. Both fungi have a very broad host range of more than 400 (*S.sclerotiorum*) or 200 (*B.cinerea*) plant species, they occur worldwide and cause multi-million US dollar losses each year due to pre- and post-harvest infection (Bolton *et al.*, 2006; Williamson *et al.*, 2007). Both fungi are considered as true necrotrophic plant pathogens. The pathogenicity of both fungi appears to be essentially dependent on the production and secretion of oxalic acid (Dutton and Evans, 1996), most likely by suppressing the oxidative burst of the host plant (Cessna *et al.*, 2000). The infection symptoms of both fungi can differ a lot between different plant species and infected tissues. *S.sclerotiorum* usually causes water-soaked lesions in the leaves, which expand rapidly through the petioles into the stem. The lesions usually become necrotic and subsequently develop fluffy white mycelium patches (Bolton *et al.*, 2006). The most typical symptoms for *B.cinerea* are probably soft rots, together with collapse and water-soaking of parenchyma tissue, usually followed by the appearance of a grey mass of conidia. Very often, the infection starts on attached, senescent flowers and spreads as soft rot to affect adjacent developing fruits (Williamson *et al.*, 2007).

Gaining more knowledge about the infection strategies of as many different plant pathogens as possible can help in the future to secure food supply and boosting the intrinsic defense of crop plants can help to reduce the costly and potentially environmental-harmful application of pestizides.

1.5 Aim of this study

The aim of this study was to identify the molecular identity of SCFE1 from the necrotrophic fungus *S.sclerotiorum*, being recognized by the RECEPTOR-LIKE PROTEIN 30 (RLP30) in *Arabidopsis thaliana*.

For the identification of SCFE1 different purification methods should be established, including reversed phase chromatography and a bioinformatical approach. Furthermore, the complex formation of the already described adapter-kinases SOBIR1 and BAK1 should be studied by performing pull-down assays after transient expression of tagged proteins in *N.benthamiana* both in the presence and absence of the ligand SCFE1. Additionally, novel interactors or regulators of RLP-mediated immune signaling should be identified by using the Membrane-based Interactome Database (MIND) (Jones *et al.*, 2014a) and studying other RLP-dependent regulatory pathways, like floral organ abscission. In a proof-of-concept experiment the general idea of LRR-RLPs forming a genuine LRR-RLK by interacting with SOBIR1 should be proven via the generation of chimeric fusion constructs and subsequent testing in a functional assay, like the accumulation of ethylene. Transferring resistance to economically important crop plants is becoming more and more important and by generating stable transformed tomato and *N.tabacum* lines expressing RLP30 it should be proven that the RLP30-dependent SCFE1-perception machinery can be transferred to solanaceous plants enhancing disease resistance against pathogens displaying SCFE1. In addition, further pathogens should be checked for the presence of an SCFE1-like RLP30-dependent activity.

2 Material and methods

2.1 Plants, fungi and bacteria and their cultivation conditions

2.1.1 Plants and their cultivation conditions

The *A.thaliana* accessions used in this thesis are listed in Table 2.1.

Table 2.1: *A.thaliana* accessions used in this study

Col-0	Ler	Ws	Bak-2	Br-0	Gu-0
ice111	Lerik1-3	Lov-1	Lov-5	Mt-0	Sq-1

The following three tables summarize the *Arabidopsis* T-DNA insertion lines used in this work. T-DNA insertion lines were either published before (see references) or obtained from the Nottingham Arabidopsis Stock Centre (NASC). Table 2.2 lists the T-DNA insertion lines of RLP30 and SOBIR1 used in this study.

Table 2.2: *A.thaliana* T-DNA insertion lines of RLP30 and SOBIR1

AGI-ID	**Gene name**	**Mutant name**	**Stock name**	**reference**
At3g05360	RLP30	*rlp30-1*	SALK_122528	(Zhang *et al.*, 2013)
		rlp30-2	SALK_008911	
		rlp30-4	SALK_145342	
At2g31880	SOBIR1	*sobir1-12/ evr-3*	SALK_050715	(Gao *et al.*, 2009)

The *A.thaliana* T-DNA insertion mutant lines of putative interactors of RLP30 and/or SOBIR1 used in this thesis are listed in Table 2.3.

Table 2.3: *A.thaliana* T-DNA insertion lines of putative interactors based on the MIND-database

AGI-ID	**protein name, description**	**Stock name**	**NASC ID**	**MIND-interactor of**
At1g21240	WAK3, EGF-like domain	SALK_071555 SALK_080632 SALK_038813	N686265 N675905 N678376	RLP30, SOBIR1
At1g29060	Target SNARE coiled-coil domain protein	SALK_054695C	N654656	RLP30, SOBIR1
At1g45145	Thioredoxin 5	SALK_144251	N644251	RLP30, SOBIR1
At3g12180	Cornichon family protein	SALK_207275C	N697220	RLP30, SOBIR1
At3g28220	TRAF-like family protein	GK-116E03	N411091	RLP30
At4g20790	LRR-RLK	ET5589 GK-388B07 GK-375F11	N26714 N437171 N435975	RLP30, SOBIR1
At4g30850	heptahelical protein 2	SAIL_822_C02	N863280	RLP30, SOBIR1
At4g37680	heptahelical protein 4	WiscDsLoxHs18710E SAIL_569_F11	N917925 N824222	RLP30, SOBIR1
At4g39890	RAB GTPase homolog H1C	WiscDsLox451_C07	N856457	RLP30
At5g16480	Phosphotyrosine protein phosphatases	SALK_208387C	N698524	SOBIR1
At5g42980	Thioredoxin 3	SALK_133632C SAIL_314_G04	N633632 N862619	RLP30, SOBIR1

AGI-ID	protein name, description	Stock name	NASC ID	MIND-interactor of
At5g49540	Rab5-interacting family protein	GK-684B01	N465581	RLP30, SOBIR1
At5g59650	LRR-RLK	SALK_022711C GK-072A01 WiscDsLox4_F09	N655352 N406817 N849112	RLP30, SOBIR1
At5g63030	Thioredoxin superfamily	GK-509E02	N448818	RLP30, SOBIR1

The used T-DNA insertion lines in this thesis of putative players in innate immunity based on the floral abscission pathway are listed in table 2.4.

Table 2.4: *A.thaliana* T-DNA insertion lines of putative players in innate immunity based on the floral abscission pathway

AGI-ID	Protein name	Donor number	NASC ID/ Reference
At3g17660	ARF-GAP domain containing protein 15, AGD15	WiscDsLoxHs017_11D SALK_150224	N901620 N650224
At4g35600	CAST AWAY	cst1, Gly157Arg SAIL_296_A06	(Burr *et al.*, 2011) N874342
At5g54310	ARF-GAP domain containing protein 5, NEVERSHED	SALK_075680 SALK_079928C	N575680 N666509

The stable transformed *Nicotiana tabacum* lines used in this study are listed in Table 2.5.

Table 2.5: Stable transformed *Nicotiana tabacum* lines used in this study

Line name	Description	Reference
Cf9	stable transformed petite havana tobacco with *Cf9:Cf9*	(Hammond-Kosack *et al.*, 1998)
Avr9	stable transformed petite havana tobacco with *PR1a:Avr9*	(Piedras and Hammond-Kosack, 1998)

Moreover, wild type *N.benthamiana* or *N.tabacum* and *S.lycopersicum* were used for transient (see chapter 2.6.1) and stable transformation (see chapter 2.1.2), respectively.
A.thaliana plants were grown on GS90-soil (Gebr. Patzer GmbH) under standard conditions (8 h light, 150 μmol/cm^{2}s light, 40-60 % humidity, 22 °C) without lid and used for the experiments at an age of 5-6 weeks. For seed production, plants were transferred to long day conditions (16 h light).
N.benthamiana, *N.tabacum* and *S.lycopersicum* were grown in the greenhouse (16 h light, 22 °C).

2.1.2 Stable transformation of plants

Stable transformation of *N.tabacum L. var. Samsun NN* and *S.lycopersicum "Moneymaker"* was performed by Caterina Brancato. Seeds (*S.lycopersicum*, *N.tabacum*) were sterilized by washing them first in 70% ethanol for 3 min (shaking), then in 1.25% hypochlorite containing some drops of 0.001% Triton W-100 for 8-10 min (shaking) and three times washing in sterile distilled water (3x200 ml). Afterwards the seeds were resuspended in 0.1% agarose and distributed on agar plates. The plates were vernalized for 2 days at 4 °C in the dark. 250 ml of *A.tumefaciens*-culture carrying the desired vectors for transformation were grown

in LB-medium as described in 2.1.4. The harvested cells were adjusted to OD_{600} of around 1.0 with 10 mM $MgCl_2$ without antibiotics and leaf pieces of *N.benthamiana* were incubated for 3 min in the cell suspension. Afterwards the leaf pieces were transferred to sterile plates containing solid MS medium with 2% sucrose (MS2%, see table 2.9 for all used media) and were incubated for 48 hrs in the dark. Transgenic calli were selected on MS selection medium containing BASTA and/or the respective antibiotics. After approx. 2 months the shoots were cut and transferred to rooting medium. After developing roots, transgenic plants selected in sterile culture were transferred to soil and grown in the greenhouse under long day conditions. For the transformation of *S.lycopersicum*, 10 days old cotyledons, grown on germination medium, were cut and incubated for 2 days in the dark at 22 °C on conditioning medium. The *A.tumefaciens* solution was prepared as described earlier in this section and two drops of *A.tumefaciens* solution per cotyledon was added and incubated for another two days at 22 °C in the dark. After that, the cotyledons were transferred to selection medium and incubated for 3 days in the light (14 h light and 10 h dark at 23 °C, 50% humidity). After approx. 2 months the shoots were cut and transferred to rooting medium. After developing roots, transgenic plants selected in sterile culture were transferred to soil and grown in the greenhouse under long day conditions.

2.1.3 Fungal strains and their cultivation conditions

The fungal strains used in this study, together with their lifestyle, are listed in table 2.6.

Table 2.6: Fungal strains used in this study

Fungal species	Strain	Life cycle	Division
Botrytis cinerea	*B05.10*	necrotrophic	Ascomycota
Sclerotinia sclerotiorum	*1946* *1980*	necrotrophic	Ascomycota

An agar plug covered with fungal mycelium was cut from a grown fungal plate and transferred to a fresh Malt-peptone plate (10 g malt, 2.5 g peptone, 15 g agar/l). Fungi were incubated at room temperature (RT, 21 °C) until a well growing mycelium was observed. One plug of sub-cultured fungi was used to inoculate liquid medium (200 ml medium in a 1 l flask). The culture was incubated at RT (21 °C) without shaking in the dark for 10 days up to two weeks.

2.1.4 Bacterial strains and their cultivation conditions

The *Escherichia coli* strains used in this study are listed in table 2.7.

Table 2.7: *E.coli* strains used in this study

Strain	**Genotype**	**Reference**
DH5α	*fhu*A2 *lac*ΔU169 *pho*A *gln*V44 ϕ80' *lac*ZΔM15 *gyr*A96 *rec*A1 *rel*A1 *end*A1 *thi*-1 *hsd*R17	(Hanahan, 1983)
One Shot® TOP10	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*Z Δ(M15 Δ(*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Thermo Fisher Scientific
One Shot® ccdB Survival™ 2 T1^R	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*Δ139 Δ*(ara-leu)*7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G *fhuA::IS2*	Thermo Fisher Scientific

For purification of PCFE1, the *Pseudomonas* strains listed in table 2.8 were used.

Table 2.8: Pseudomonas strains used in this study

Species	**Strain**
Pseudomonas syringae pv. tomato	DC3000
Pseudomonas syringae pv. syringae	B278A
Pseudomonas syringae pv. phaseolicola	1448A
Pseudomonas protegens	PF-5
Pseudomoans fluorescens	SBW25
Pseudomonas stutzeri	DSM10701
Pseudomonas monteilii	YK-310

For transient expression of proteins in *Nicotiana benthamiana* the *Agrobacterium tumefaciens* strain GV3101::pmP90 (T-DNA^{-} vir^{+} rif^{r}, pMP90 gen^{r}) (Koncz and Schell, 1986) was used. *E.coli* strains were grown at 37 °C on LB-plates or in liquid LB-medium at 200 rpm with the respective antibiotics for selection.

A.tumefaciens strains were cultivated for 48 h at 28 °C on LB-plates or in liquid LB-medium at 180 rpm with the respective antibiotics for selection.

All *Pseudomonas* strains were grown in liquid PDB medium for purification in baffled flasks (100 ml medium in 500 ml flask; 200 ml medium in 1 l flask) for 12 h at 21 °C with 210 rpm shaking. For growth of *Pseudomonas syringae pv syringae DC3000* Rifampicin was added to the culture medium.

2.2 Chemicals, buffers and solutions

If not otherwise indicated chemicals from the following companies were used in standard purity: Sigma-Aldrich (Taufkirchen), Carl Roth (Karlsruhe), Merck (Darmstadt), Qiagen

(Hilden), Invitrogen (Karlsruhe), Duchefa (Haarlem, Netherlands), Molecular Probes (Leiden, Netherlands), Fluka (Buchs, Switzerland) and BD (Sparks, USA). Restriction enzymes, ligases and DNA modification enzymes were used from Thermo Fisher Scientific (Waltham) and New England Biolabs (Beverly, USA). Oligonucleotides were ordered from Eurofins MWG Operon (Ebersberg). Kits were purchased from SLG (Gauting).

2.2.1 Culture media and antibiotics

The used media for the respective organisms are described in table 2.9. If not otherwise indicated all compounds were weighed out and dissolved in ddH_2O. For the preparation of solid agar plates 15 g/l of Agar-Agar (Roth) were additionally added to the respective medium. All media were subsequently autoclaved at 120 °C for 20 min and stored at RT or after opening the bottle at 4 °C. All Agar-plates were stored at 4 °C.

Table 2.9: Culture media used in this study

Medium	**Ingredients per 1 liter**	**Species**
Gamborg B5	3,2 g Gamborg B5 including vitamins (Duchefa)	*S.sclerotiorum*
King's B	20 g Glycerol, 40 g Proteose Pepton No. 3; addition of sterile 0,1 % (v/v) $MgSO_4$ and KH_2PO_4 after autoclaving	*P.syringae*
LB	10 g Bacto-tryptone, 5 g Yeast extract, 5 g NaCl, pH 7.0	*E.coli*
MP	10 g malt extract (usually used: "standard"; but "microbiological" also works, Roth), 2,5 g peptone ex casein (Roth)	*S.sclerotiorum*
PDB	24 g PDB (Potato Dextrose Broth, Duchefa)	*S.sclerotiorum*, *Pseudomonas*

Medium	**Ingredients per 1 liter**	**Species**
MS2%	Dissolve in 900 ml ddH_2O: 4.31 g MS-salt (Duchefa), 20 g sucrose, 5 ml NPT Vitamins stock-solution (10 mg/l Thiamine-HCl, 1 mg/l Nicotine acid, 1 mg/l Pyridoxine-HCl). Adjust pH to 5.7-5.8 with KOH and fill up to 1 l	*N.benthamiana* transformation
Selection medium	Dissolve in 900 ml ddH_2O: 4.31 g MS-salt (Duchefa), 16 g glucose, 5 ml Vitamin mix. Adjust pH to 5.7-5.8 with KOH and fill up to 1 l. After autoclaving, cool down to 60 °C and add 1 mg 6-Benzyl Amino Purin , 0.2 mg, α-Naphtalenacetic acid, 500 mg Cefotaxime and respective antibiotica	*N.benthamiana* transformation
Rooting medium	MS2% with 500 mg Cefotaxime and respective antibiotica	*N.benthamiana* transformation
Germination Medium	Dissolve in 900 ml ddH_2O: 4.31 g MS-salt (Duchefa), 30 g sucrose, 100 mg myo-Inositol, 1 ml NPT Vitamins stock-solution (10 mg/l Thiamine-HCl, 1 mg/l Nicotine acid, 1 mg/l Pyridoxine-HCl). Adjust to pH 5.8 with KOH	*S.lycopersicum* transformation
Conditioning medium	Same as germination medium. Add after autoclaving and cooling down to 60 °C 0,1 mg 6-Benzyl Amino Purin and 1 mg α-Naphtalenacetic acid	*S.lycopersicum* transformation
Selection medium	Same as germination medium. Add after autoclaving and cooling down to 60 °C 1 mg trans-Zeatin, 250 mg Ticarcillin-clavulanate and antibiotics for selection	*S.lycopersicum* transformation
Rooting medium	Same as germination medium. Add after autoclaving and cooling down to 60 °C 0.1 mg Indole-3-Acetic Acid, 500 mg Vancomycin and antibiotics for selection	*S.lycopersicum* transformation

The used concentrations for the respective antibiotics, as well as the concentrations of the

stored stock solutions and the respective solvents are described in table 2.10. The antibiotics were added to the medium after the autoclaving process, when the respective medium was cooled down to around 60 °C.

Table 2.10: Antibiotics used in this study

Antibiotics	**Final concentration in µg/ml**	**Solvent**
Carbenicillin	100	Water
Cycloheximid	50	Water
Kanamycin	50	Water
Rifampicin	50	Methanol
Spectinomycin	100	Water
Tetracyclin	50	Ethanol

2.2.2 Enzymes and antibodies

In table 2.11, there are the proteinases listed, which were used in this study.

Table 2.11: Proteinases used in this study

Name	**Company**	**catalog number**
Endoproteinase AspN	NEB	P8104S
Endoproteinase GluC	NEB	P8100S
Trypsin-ultra^{TM}, Mass Spectrometry Grade	NEB	P8101S
Proteinase K	Invitrogen	4333793

Table 2.12 lists the antibodies used in this study for Western Blotting.

Table 2.12: Antibodies used in this study for immunoblotting

Name	produced in	working dilution	Company
α-Myc	rabbit	1:5000	Sigma-Aldrich
α-HA	mouse	1:5000	Sigma-Aldrich
α-GFP	goat	1:15 000	Sicgen
α-rabbit lgG, horseradish peroxidase conjugated	goat	1:10 000	Sigma-Aldrich
α-mouse lgG, horseradish peroxidase conjugated	rabbit	1:10 000	Sigma-Aldrich
α-goat lgG, horseradish peroxidase conjugated	rabbit	1:50 000	Sigma-Aldrich

2.2.3 Vectors and Primers

The vectors used in this study are listed in table 2.13.

Table 2.13: Vectors used in this study

Name	Description	Reference
pCR8/GW/TOPO (TA cloning vector)	Entry vector for the Gateway system	Invitrogen
pGWB2	Binary Gateway destination vector for expression of fusion proteins under control of CaMV 35S promoter, without any epitope tag	(Nakagawa *et al.*, 2007)
pGWB5	Binary Gateway destination vector for expression of fusion proteins under control of CaMV 35S promoter with a C-terminal GFP tag	(Nakagawa *et al.*, 2007)

Name	Description	Reference
pGWB8	Binary Gateway destination vector for expression of fusion proteins under control of CaMV 35S promoter with a C-terminal 6xHis tag	(Nakagawa *et al.*, 2007)
pGWB14	Binary Gateway destination vector for expression of fusion proteins under control of CaMV 35S promoter with a C-terminal 3xHA tag	(Nakagawa *et al.*, 2007)
pGWB15	Binary Gateway destination vector for expression of fusion proteins under control of CaMV 35S promoter with a N-terminal 3xHA tag	(Nakagawa *et al.*, 2007)
pGWB17	Binary Gateway destination vector for expression of fusion proteins under control of CaMV 35S promoter with a C-terminal 4xMyc tag	(Nakagawa *et al.*, 2007)

In table 2.14, there are the primers listed, which were used in this study.

Table 2.14: Oligonucleotides for fusion constructs of RLP30 and SOBIR1

Oligonucleotides for fusion constructs of RLP30 and SOBIR1		
Name	**Sequence**	**Description**
B002fw B002rev	TTTTGGGAATATGGCTGTTC GAACAGCCATATTCCCAAAA	$RLP30^{LRR5}$+SOBIR1, chimeric fusion
A001fw A001rev	ACTCTACGGTATGGCTGTTC GAACAGCCATACCGTAGAGT	$RLP30^{allLRR}$+SOBIR1, chimeric fusion
RLP30-F	ATGATTCCAAGCCAATCTAATTCC	RLP30 forward
SOBIR1-R	GTGCTTGATCTGGGACAACATG	SOBIR1 reverse

Oligonucleotides for cloning RLP30^{accession}		
Name	**Sequence**	**Description**
RLP30-F	ATGATTCCAAGCCAATCTAATTCC	RLP30 forward
RLP30-R	ACGAGCACTTGTGGTGACTAC	RLP30 reverse without stop codon
RLP30-S2F	AGCAATCAGTTCACATTGG	RLP30 sequencing, starting after 500 bp

Oligonucleotides for cloning of apoplastic expression vectors		
Name	**Sequence**	**Description**
PR1a_fw_XbaI	AATTTCTAGAATGGGATTTG	PR1a-signal peptide, forward
PR1a_rev_XbaI	TTAATCTAGATTGGCACGGC	PR1a-signal peptide with two bp more, reverse
NIP1_fwXbaIneu	TTAATCTAGAATGAACCTCCGCCCTGCACTC	NIP1-signal peptide, forward
NIP1_revXbaIneu	TTAATCTAGATTGGCGCTCACGTACGCGAATG	NIP1-signal peptide with two bp more, reverse
PR1XbaINoAA_R	TTAATCTAGAGGCACGGCAAG	PR1a-signal peptide, reverse
NIP1XbaINoAA_R	TTAATCTAGAGGCGCTCACGTACGCGAATG	NIP1-signal peptide, reverse

2.3 Detection assay for biosynthesis of ethylene

Arabidopsis leaves were cut into squares (0,3 cm x 0,3 cm) and floated overnight on ddH_2O. Three leaf pieces were put into one glas vial (6 ml volume) with 400 µl 20 mM MES buffer pH 5.4. Only in SOBIR1-overexpressing solanaceous plants 400 µl ddH_2O was used. The desired elicitor was added and the vials were closed with a rubber cap. The samples were shaken for 5 h at 100 rpm on a horizontal shaker. 1 ml air was taken out through the rubber cap with a syringe and injected into a gas chromatograph (GC-14A, Shimadzu, Japan).

2.4 Partial purification of SCFE1 and PCFE1 by chromatography

2.4.1 Cation exchange chromatography using an FPLC

The culture medium of *S.sclerotiorum* or *Pseudomonas* was centrifuged at 6000 rpm for 20 min and then vacuum-filtrated through a filter paper (MN 615, Macherey-Nagel) and subjected to a two-step cation exchange chromatography protocol using an ÄKTA Explorer FPLC system (GE Healthcare) kept at 4 °C. In a first step, the culture filtrate was loaded onto 1-4 x 5 ml HiTrap SP FF (GE Healthcare) column(s) equilibrated in buffer A (50 mM MES, pH 5.4) with the sample pump at a flow rate of up to 15 ml/min. After washing with buffer A, a 100% elution step with buffer B (500 mM KCl, 50 mM MES, pH 5.4) was performed at a flow rate of 1-2 ml/min and the elution peak was monitored with OD_{280nm}, OD_{254nm} and OD_{214nm} and collected manually. The collected eluate was dialysed against 2 l 25 mM MES, pH 5.4, overnight at 4 °C in a dialysis membrane (ZelluTrans, nominal MWCO: 3,5; 46 mm, Roth). The dialysed fraction was again vacuum-filtrated through a cellulose acetate membrane (Ciro Manufacturing Corporation, pore size: 0,2 µm) and loaded with a flow rate

of 1 ml/min onto a Source 15S 4.6/100 PE column (GE Healthcare) equilibrated with buffer A. After washing with buffer A the bound proteins were eluted with a linear gradient of buffer B (0 % to 50 % in 10 column volumes). 500 µl fractions were collected using automated fractionation.

2.4.2 Reversed phase chromatography using an HPLC

FPLC-purified active fractions of SCFE1 were subjected to reversed phase chromatography on a C4 column. The samples were first run over a CHROMABOND® C4 Polypropylen bench-top 1 ml column (Macherey-Nagel) to prevent clogging of the HPLC-column. The bench-top column was run only by gravity. It was first washed with 2 ml Methanol, 2 ml 0,1% (v/v) Trifluoroacetic acid (TFA) in ddH_2O, 2 ml Acetonitrile (ACN) and then 3 ml 0,1% (v/v) TFA in ddH_2O. The elicitor was mixed with two times 0,1% (v/v) TFA in ddH_2O and then loaded on the column. After washing the column two times with 750 µl of 5% (v/v) ACN in 0,1% (v/v) TFA in ddH_2O a stepwise elution was performed with 750 µl of 30% (v/v), 60% (v/v) and 90% (v/v) ACN in 0,1% (v/v) TFA in ddH_2O. The samples were then put in a vacuum-concentrator centrifuge (Concentrator *plus*, Eppendorf) to evaporate the TFA and the ACN. The samples were then resuspended in 50 mM MES, pH 5.4, or ddH_2O, depending on the further use. For further purification, the 30%-fraction was mixed with two times 0,1% (v/v) TFA in ddH_2O and loaded on a EC250/4.6 NUCLEOSIL® 120-5 C4 column (Macherey-Nagel) using an ÄKTA micro HPLC system (GE Healthcare). After washing the column with 5% (v/v) ACN in 0,1% (v/v) TFA in ddH_2O the sample was eluted using an ACN-gradient of 1%/min and a flow rate of 0.5 ml/min. The eluate was collected in 250 µl fractions and then put in a vacuum-concentrator centrifuge (Concentrator *plus*, Eppendorf) to evaporate the TFA and the ACN. Depending on further use, the samples were resuspended in 50 mM MES, pH 5.4, or ddH_2O.

2.5 Molecular biological methods

2.5.1 Isolation of plasmid DNA from *E.coli*

Plasmid was extracted from 4 ml overnight inoculated *E.coli* cell culture using the column-based purification system HiYield® Plasmid Mini Kit (SLG) according to the manufacturer's protocol.

2.5.2 Isolation of genomic DNA from plants

The method is based on the protocol by Edwards *et al.* (1991). One leaf piece was crunched in a 1.5 ml Eppendorf tube with a blue pestle. 200 µl Edwards buffer (200 mM Tris/HCl, pH 7.5, 250 mM NaCl, 25 mM EDTA pH 8.0, 0,5% (w/v) SDS) was added and samples were completely homogenized at room temperature. After centrifugation for 5 min at 13 000 rpm the supernatant was transferred to a fresh tube and 200 µl isopropanol (2-propanol) were added and thoroughly mixed. DNA was precipitated at RT for 5 min (large leaf piece) up to 45 minutes (small leaf piece). After another round of centrifugation for 10 min at 4 °C with 14 000 rpm the supernatant was discarded and the pellet was washed with 200 µl 70% (v/v) EtOH and incubated for 5 min at RT. The pellet was centrifuged for 5 min at RT with 13 000 rpm. After removing the supernatant the pellet was air dried. Finally, the DNA pellet was dissolved in 50 µl (small leaf piece) or 100 µl (large leaf piece) ddH_2O overnight at 4 °C or alternatively heated for 10 min at 65 °C.
Alternatively, the "Phire Plant Direct PCR Kit" (Thermo Fisher Scientific) was used according to the manufacturer's protocol.

2.5.3 Isolation of RNA from plants and fungi

The protocol is based on the method developed by Chomczynski and Sacchi (1987). 50-100 mg frozen leaf material or freeze-dried fungal mycelium was grinded to fine powder in an Eppendorf tube cooled in liquid nitrogen. 1 ml Trizol (400 mM Ammonium-thiocyanat, 800 mM Guanidinium-thiocyanat, 100 mM NaAcetat, 5% (v/v) Glycerin pH 5.0, then add 38% (w/v) Aqua-Phenol or alternatively ready-to-use peqGOLD TriFast™ [peqlab]) was added and the sample vortexed thoroughly and incubated for 10 min at RT. After adding 200 µl Chloroform and incubation for 10 min at RT, the samples were centrifuged for 10 min with 14 000rpm. The upper phase was carefully transferred to a fresh tube and 1 Vol Isopropanol was added and the RNA was precipitated for 15 min at RT or O/N at -20 °C. After centrifugation for 10 min at 4 °C with 14 000 rpm the pellet was washed with 1 ml 70% (v/v) EtOH. The EtOH was removed after another centrifugation step for 5 min at 4 °C with 14 000 rpm. The pellets were completely air-dried and resuspended in 20-40 µl fresh ddH_2O.

2.5.4 DNase treatment of RNA

1 U of DNase I, RNase-free (Thermo Fisher Scientific) was mixed with the included 10x Reaction Buffer with $MgCl_2$ and 0,5 µl RiboLock RNase Inhibitor (40 U/µL; Thermo Fisher Scientific). 2-5 µg of RNA were added in a final volume of 10 µl. The reaction was incubated at 37 °C for 30 min and the reaction was afterwards terminated by adding 2 µl of 50 mM EDTA and incubation for 10 min at 65 °C.

2.5.5 Reverse transcription

1 µl of the recombinant M-MuLV RT "RevertAid reverse transcriptase" (200 U/µl; Thermo Fisher Scientific) was used for 2-5 µg of DNase treated RNA (see chapter 2.5.4), together with 4 µl 10x reaction buffer, 2 µl 10µM Oligo-dT and 2 µl 2.5 mM dNTPs. Additionally, 0,5 µl RiboLock RNase Inhibitor (40 U/µL; Thermo Fisher Scientific) was added and the reaction was filled up to a final volume of 20 µl with ddH_2O. The reaction was incubated for 60 min at 42 °C, followed by enzyme deactivation at 70 °C for 10 min.

2.5.6 Polymerase chain reaction (PCR)

Standard protocols and thermal profiles

Standard PCR reactions were performed using a home-made Taq DNA polymerase. For cloning, recombinant Phusion High-Fidelity DNA Polymerase (Thermo Scientific Fisher) with proofreading function was used.

The standard reaction mix and thermal profile for Taq polymerase is shown in table 2.15 and 2.16, respectively.

Table 2.15: Standard reaction mix for Taq polymerase

Component	Volume
Template DNA	0.1-20 ng
10x Taq-buffer	2 µl
dNTP mix (together 2.5 mM)	2 µl
10 µM of fw-/rev-primer	1 µl + 1 µl
Taq DNA polymerase	0.5 µl (1 U)
ddH_2O	up to 20 µl

Table 2.16: Standard thermal profile for Taq polymerase

Step	Temperature (°C)	Time period	N° of cycles
Initial denaturation	95	5 min	1
Denaturation	95	30 sec	
Annealing	T_M-3 °C	30 sec	35
Extension	72	1 min per kb	
Final extension	72	7 min	1
Cooling	10	∞	

The standard reaction mix and thermal profile for Phusion High-Fidelity polymerase is shown below in table 2.17 and 2.18:

Table 2.17: Standard reaction mix for Phusion High-Fidelity polymerase

Component	Volume
Template DNA	10-50 ng
5x Reaction-buffer	10 µl
dNTP mix (together 2.5 mM)	1 µl
10 µM of fw-/rev-primer	1 µl + 1 µl
Phusion High-Fidelity polymerase	0.5 µl (1 U)
ddH_2O	up to 50 µl

Table 2.18: Standard thermal profile for Phusion High-Fidelity polymerase

Step	Temperature (°C)	Time period	N° of cycles
Initial denaturation	98	30 sec	1
Denaturation	98	10 sec	
Annealing	T_M+3 °C	30 sec	35
Extension	72	30 sec per kb	

Step	Temperature (°C)	Time period	N° of cycles
Final extension	72	7 min	1
Cooling	10	∞	

Cloning of RLP30 from different *Arabidopsis* accessions

The cDNA of the respective *Arabidopsis* accessions was generated as described in chapter 2.5.3, 2.5.4 and 2.5.5. The PCR was set up as described in tables 2.19 and 2.20 using the primers listed in table 2.14.

Table 2.19: Reaction mix for cloning RLP30 from different *Arabidopsis* accessions

Component	Volume
Template cDNA	100 ng
10x HF + $MgCl_2$ Reaction-buffer	2 µl
dNTP mix (together 2.5 mM)	2 µl
10 µM of RLP30-F	1 µl
10 µM of RLP30-R	1 µl
Phusion High-Fidelity polymerase	0.5 µl (1 U)
ddH_2O	up to 20 µl

Table 2.20: Thermal profile for cloning RLP30^{ecotype}

Step	Temperature (°C)	Time period	N° of cycles
Initial denaturation	94	2 min	1
Denaturation	94	30 sec	
Annealing	55	30 sec	35
Extension	72	2.5 min	
Final extension	72	10 min	1
Cooling	8	∞	

Cloning of apoplastic expression vectors

The cloning strategy was to flank the PR1a signal peptide from *N.benthamiana* and the NIP1 signal peptide from the *Phytophtora soiae* NIP1-protein with XbaI-cutting sites on the 3'- and 5'-end. Those fragments were then ligated into the respective, as well XbaI-cut pGWB-vector.

As templates for the signal peptide, respective PR1a- and NIP1-oligonucleotides were ordered from Eurofins MWG Operon. For cloning the signal peptides into pGWB2, pGWB8 and pGWB14 two additional adenines were added to the 3'-end of the signal peptide sequence just before the XbaI cutting site to keep the gateway cassette in frame. See tables 2.21 to 2.23 using the primers listed in table 2.14.

Table 2.21: Reaction mix for cloning signal peptides for apoplastic expression vectors

Component	Volume
Template Oligonucleotide	10 ng PR1a/NIP1
5x GC-Reaction-buffer	10 µl
dNTP mix (together 2.5 mM)	5 µl
10 µM of fw-/rev-primer	2 µl + 2 µl
Phusion High-Fidelity polymerase	0.5 µl (1 U)
ddH_2O	up to 50 µl

Table 2.22: 3-Step thermal profile for cloning the signal peptide PR1a for apoplastic expression vectors

Step	Temperature (°C)	Time period	N° of cycles
Initial denaturation	98	30 sec	1
Denaturation	98	5 sec	
Annealing	55	10 sec	35

Step	Temperature (°C)	Time period	N° of cycles
Extension	72	15 sec	
Final extension	72	5 min	1
Cooling	10	∞	

Table 2.23: 2-Step thermal profile for cloning the signal peptide NIP1 for apoplastic expression vectors

Step	Temperature (°C)	Time period	N° of cycles
Initial denaturation	98	30 sec	1
Denaturation	98	10 sec	35
Extension	72	15 sec	
Final extension	72	5 min	1
Cooling	10	∞	

2.5.7 Restriction endonuclease digestion of DNA

The restriction digests were performed according to the manufacturer's protocols in 20 µl reaction volume with 1 U enzyme/µg DNA.

2.5.8 Isolation of DNA fragments from agarose gels

PCR products were separated by 1 % (w/v) agarose gel electrophoresis or for very small products by a 3 % (w/v) agarose gel electrophoresis (peqGOLD MS1000 Agarose, peqlab). After cutting out the bands with a clean razor blade the DNA fragments were purified using

the "HiYield® PCR Clean-up/ Gel Extraction Kit" from SLG according to the manufacturer's recommended protocol.

2.5.9 Dephosphorylation of vectors

Before the ligation of the linearized vector with the insert the vector ends were dephosphorylated with FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific) according to the recommended protocol of the manufacturer.

2.5.10 DNA ligation

DNA ligation was performed for 8 h or O/N at 20 °C in a total volume of 25 µl. 10 µl of the dephosphorylated vector was mixed with 10 µl of insert DNA (the concentrations were priorly adjusted to the manufacturer's recommendations). 2.5 µl 10x T4 DNA ligase buffer and 1 µl T4 DNA Ligase (Thermo Fisher Scientific) were added and the sample was filled up with 1.5 µl ddH_2O.

2.5.11 Site-specific recombination of DNA in Gateway compatible vectors

The pCR8/GW/TOPO Cloning kit (Invitrogen) was used for cloning of PCR products into the pCR8-vector with a polyA-overhang to generate an entry clone for the Gateway system according to the manufacturer's instructions. For adding the polyA-overhang to the PCR product, 2.4 µl of 10x Taq buffer (100 mM Tris-HCl, 500 mM KCl, and 15 mM $MgCl_2$, pH 8.3), 1 µl 10 mM dATP, 1 µl homemade Taq-Polymerase (Inoue *et al.*, 1990) and 20 µl of the purified PCR product was incubated for 30 min at 72 °C. 4 µl of this reaction was afterwards used, together with 1 µl provided salt solution (1.2 M NaCl, 0.06 M $MgCl_2$) from

the pCR8/GW/TOPO Cloning kit (Invitrogen) and 1 µl of the pCR™8 /GW/TOPO® vector (TOPO®-adapted). For transfering the fragment of interest into the gene expression vector, an LR reaction between the entry clone and a Gateway destination vector was performed using the Gateway® LR Clonase® II Enzyme Mix (Invitrogen) according to the manufacturer's recommended protocol.

2.5.12 Transformation of chemically competent *E.coli* cells

200 µl of chemically competent *E.coli* DH5alpha (homemade according Inoue *et al.* (1990)) or *E.coli* TOP10 (commercial, Invitrogen) cells were thawn on ice. 5-20 µl of plasmid DNA were added to the cells and incubated for 30 min on ice. After a 30 sec heat-incubation step at 42 °C the cells were immediately transferred on ice again for 2 min. 600 µl of LB-medium was added and the *E.coli* cells were incubated for 1 h at 37 °C with 220 rpm shaking. Depending on the transformed construct, 5 µl (retransformation of plasmid) or up to all cells (ligation) were plated on selective LB-agar-plates and incubated at 37 °C O/N until colonies were visible.

2.5.13 Transformation of electro-competent *A.tumefaciens* cells

For the transformation of electro-competent GV3101::pmP90 (T-DNA^{-} vir^{+} rif^{r}, pMP90 gen^{r}) *A.tumefaciens* cells, prepared as described in chapter 2.5.14, 40 µl of cells were thawn on ice, mixed with 100 ng of plasmid DNA and stored on ice in a pre-cooled electroporation cuvette (1 mm electrode distance) for 30 min. The cells were pulsed one time with 1800 V for 5 ms using an Electroporator2510 (Eppendorf) and 600 µl LB-medium was directly added to the cuvette. The cells were carefully transferred to an Eppendorf-tube and incubated for

4 h at 28 °C with 180 rpm shaking, before they were plated on selective LB-agar-plates and incubated for 48 h at 28 °C until colonies were visible.

2.5.14 Preparation of electro-competent *A.tumefaciens* cells

5 ml LB medium with the required antibiotics was inoculated with the desired *A.tumefaciens* strain and incubated overnight at 28 °C. 500 ml LB medium with the required antibiotics was inoculated with the overnight culture and again incubated at 28 °C until the culture reached an OD_{600} of 0.5-1.0. From now on, the bacterial cells were maintained at 4 °C or on ice. The bacterial culture was spun down at 4500 g for 15 min at 4 °C. The bacterial cells were two times washed with ddH_2O. Therefore, the pellet was first resuspended in 200 ml of ice-cold sterile ddH_2O. The resuspended cells were again centrifuged at 4500 g for 15 min at 4 °C and the pellet was a second time resuspended in 100 ml of ice-cold sterile ddH_2O and centrifuged as described above. Afterwards the bacterial pellet was resuspended in 4 ml of ice-cold 10% glycerol and centrifuged as described above. The bacterial cells were resuspended in 1-1.5 ml of ice-cold 10% glycerol and 40 µl aliquots were frozen in liquid nitrogen and stored at -80°C.

2.5.15 Sequencing

All sequencing reactions were performed by GATC Biotech AG. Samples for sequencing were prepared according to the provider's recommendations. Usually, the "Light run" method was used. 5 µl DNA (80-100 ng/µl of Plasmid-DNA, 20-80 ng/µl of PCR products) was pre-mixed with 5 µl 10 µM Primer and sent for sequencing. Sequence analysis was conducted using the CLC Main Workbench programme (Qiagen).

2.6 Protein biochemistry

2.6.1 Transient protein expression in *N.benthamiana*

N.benthamiana plants were transformed with *A.tumefaciens* for transient protein expression. The bacterial strains (5 ml) carrying the appropriate expression constructs were cultured as described in 2.1.4. The cells were harvested in a 15 ml Falcon tube by centrifugation for 10 minutes at 4500 g and then re-suspended in 5 ml 10 mM $MgCl_2$. The density of the culture was adjusted to an OD_{600} of 1 with 10 mM $MgCl_2$ and a final concentration of 150 µM Acetosyringone was added. The bacterial suspension was incubated at RT for 2 hours in the dark. Afterwards, the bacteria were mixed 1:1 with a suspension of bacteria carrying an p19 expression construct (Voinnet *et al.*, 2003) and adjusted to an OD_{600} of 0,2. The mixture was infiltrated with a 1 ml syringe into leaves of 3-week-old *N.benthamiana* plants (two young leaves per plant) and the leaf tissue was analyzed 2-3 days post infection for the presence of the protein.

2.6.2 Protein extraction from plant tissue

Frozen leaf material was ground in liquid nitrogen. 500 mg of ground powder was re-suspended in 1 ml solubilization buffer (25 mM TRIS-HCl pH 8.0, 150 mM NaCl, 1% (v/v) NP40, 0,5% (w/v) DOC, 2 mM DTT and 1 tablet of "cOmplete ULTRA Tablets, Mini, *EASY*pack" (Roche) per 10 ml). The samples were solubilized for 1 h at 4 °C in an overhead rotation shaker (8 rpm). Centrifugation for 20 min at 4 °C and 20 000g separated the soluble proteins from the cell debris and the supernatant was transferred to a new Eppendorf-tube and centrifuged for another 10 min at 4 °C and 20 000g. The supernatant was then used for further analysis.

2.6.3 Immunoprecipitation on GFP-trap beads

Protein extraction was performed as described in section 2.6.2. The solubilized proteins were incubated with the pre-washed and in solubilization buffer equilibrated GFP-trap beads (ChromoTek). After 1 h of incubation at 4 °C with 8 rpm in an overhead rotation shaker the GFP-trap beads were then two times carefully washed with solubilization buffer and two times with washing buffer (25 mM TRIS-HCl pH 8.0, 150 mM NaCl, 2 mM DTT). The precipitated beads were resuspended in SDS-PAGE loading dye and were boiled at 95 °C for 10 min and the supernatant was then subjected to SDS-PAGE and Western Blot analysis. For the pull-down experiment with transiently overexpressed BAK-1, SOBIR-1 and RLP30 in *N.benthamiana*, the NaCl concentration was reduced to 20 mM and only 0,3 % (v/v) NP40 and 0,15 % DOC was used.

2.6.4 SDS-PAGE

For SDS polyacrylamide gel electrophoresis the protocol of Laemmli *et al.* (1970) by the method of Sambrook and Russell (2001) was used. The acrylamid-bisacrylamid mixture (37,5 : 1) was used from Carl Roth (Rotiphorese Gel 30). Separating gels of 8 % with 5 % stacking gels were used in a Mini PROTEAN 3 system (Biorad). The protein separation was conducted at a constant current of 30 mA per gel. As protein marker the prestained PageRuler™ protein ladder mix (Thermo Scientific Fisher) was used.

2.6.5 Tricine SDS-PAGE

Tricine SDS PAGE was used to separate small proteins below 20 kDa according to the protocol established by Schägger and Jagow (1987). For this study a 16 % separating and a 4 % stacking gel was used. A constant current of 30 mA per gel was used to separate the

proteins. 0.2 µl of the prestained PageRuler™ protein ladder mix (Thermo Fisher Scientific) was used as a protein marker.

2.6.6 Silver staining

Tricine SDS PA gels were incubated in fixing solution (50 % (v/v) methanol, 12 % (v/v) acetic acid and 0.5 % (v/v) 37 % formaldehyde) for at least one hour or O/N at RT. After three times washing for 20 min with 50 % (v/v) ethanol the gels were pretreated with fresh 0.02 % (w/v) $Na_2S_2O_3$*5H_2O for 1 min. The gels were then rinsed three times for 20 sec with ddH_2O and then impregnated with 0.2 % (w/v) $AgNO_3$ and 0.75 % (v/v) 37 % formaldehyde for one hour. After quickly rinsing the gels two times in ddH_2O, the gels were developed with 6 % (w/v) Na_2CO_3, 0.5 % (v/v) 37 % formaldehyde and 4 mg/l $Na_2S_2O_3$. The developing process was stopped after 5 – 10 min (or when the signal was strong enough), first with rinsing the gels two times with ddH_2O and then with a solution of 50 % (v/v) methanol and 12 % (v/v) acetic acid. The gels were scanned for later documentation.

2.6.7 Protein elution from a Tricine SDS PA gel

The Tricine SDS PA gel was cut into 1 mm thick segments and each segment was incubated in 500 µl ddH_2O overnight at 4 °C. 300 µl of the supernatant were taken and the water was evaporated using a vacuum-concentrator centrifuge (Concentrator *plus*, Eppendorf). The pellet was resuspended in 30 µl 20 mM MES buffer pH 5.4 or ddH_2O.

2.6.8 Western Blot

The proteins were transferred from the Laemmli SDS PA gel onto a Amersham™ Protran™ 0.2 µm NC membrane (GE Healthcare) in Transfer-buffer (25 mM TRIS, 192 mM Glycine, 1 %

(w/v) SDS, 20 % (v/v) methanol) using a Mini PROTEAN 3 system (Biorad) for 1 h at 100 V with prefrozen cooling packs. After transfer, the membrane was stained with Ponceau S-Red (0.1 % (w/v) Ponceau S Red in 5 % (v/v) acetic acid) and scanned for later documentation. The membrane was then incubated with TBS-T (10 mM TRIS pH 7.5, 150 mM NaCl , 0.1 % Tween-20) containing 5 % (w/v) BSA for 1 h at RT to block unspecific binding sites. The membrane was washed 3 times with 15 ml TBS-T for 10 min at RT. The membrane was incubated with the desired primary antibody in 10 ml TBS-T containing 5 % (w/v) BSA over night at 4°C or at RT for 1 h. After washing 3 times with 15 ml TBS-T for 10 min at RT, the membrane was incubated with the respective secondary antibody for 1 h in 10 ml TBS-T containing 5 % (w/v) BSA at RT. Then the membrane was 3 times washed with 15 ml TBS-T for 10 min at RT. Chemiluminescent substrate (ECL; GE Healthcare) was applied and incubated for 5 min before exposure to an x-ray film (CL-XPosure, Thermo Scientific) or using an Amersham Imager600 detection system from GE Healthcare.

2.7 Bioinformatical analysis

The following bioinformatics script was written using Bioperl. In order to run the script, different files need to be prepared in advance. This script will then put all the collected information together into one excel-file. The required information contain the the predicted proteome used for the analyses as a fasta-file, a list of the predicted proteins having a signal peptide using the SignalP-prediction algorithm (Petersen *et al.*, 2011), a list of proteins being classified as effectors using the EffectorP algorithm (Sperschneider *et al.*, 2016), one file containing the best hit of a chosen BLAST-search (here *Botrytis cinerea* against *Sclerotinia sclerotiorum* or *vice versa*), RNA-seq data and one file containing only the headers of the proteins. The hashtags # are not part of the code, but will guide you through the

program and help you to understand the executed operations. The original code is in chapter 8.

2.8 Statistical analysis

Statistical analysis was performed using Microsoft Office Excel. The data represent the average of replicates with +/- standard deviation (SD) of the mean.

3 Results

3.1 RLP30 forms a heterotrimeric complex with SOBIR1 and BAK1

RLP30, which lacks any intracellular signaling domain, is the receptor required for the recognition of the MAMP SCFE1 derived from *Sclerotinia sclerotiorum*. Therefore, RLP30, like other RLPs, has to interact with other proteins in order to transduce signals from the extracellular space into the cell. RLP30-dependent signaling of SCFE1 requires both BAK1 and SOBIR1, as functional assays like ethylene measurements (Zhang *et al.*, 2013) have shown. To investigate whether RLP30 forms stable complexes with these two kinases, the differently C-terminal tagged proteins (BAK1-myc, SOBIR1-HA and RLP30-GFP) were together overexpressed in *N.benthamiana* and pulled down using GFP-trap beads both in the absence and presence of the ligand SCFE1.

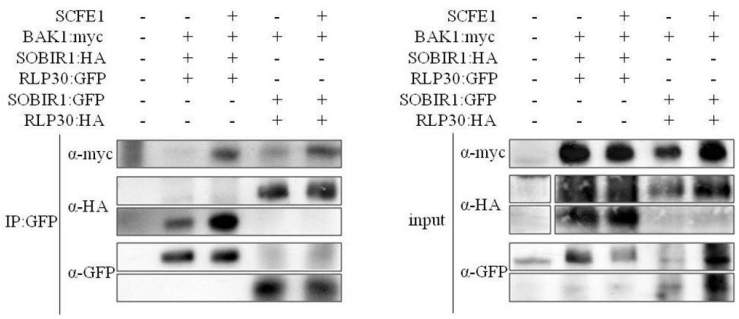

Figure 3.1: Heterotrimeric complex formation of RLP30, SOBIR1 and BAK1. Western Blot analysis with tag-specific antisera of co-immunoprecipitated RLP30-GFP, SOBIR1-HA and BAK1-myc. Proteins were transiently overexpressed in *N.benthamiana* for 3 days and after protein extraction pulled down with GFP-trap beads. BAK1-myc was expressed together with RLP30-GFP and SOBIR1-HA or RLP30-HA and SOBIR1-GFP. Leaf material was collected 5 minutes after infiltration with water (-) or SCFE1 (+) with a final dilution of 1:10.

Analysis of the Co-IP experiments (Fig. 3.1) revealed that SOBIR1 and RLP30 are always interacting, independently of the ligand SCFE1. However, BAK1-myc, together with the RLP30-SOBIR1 complex, was only pulled down in the presence of SCFE1. Switching the target of the pull-down, either SOBIR1-GFP or RLP30-GFP, did not change the SCFE1-dependent pulldown of BAK1-myc. A weak band representing BAK1-myc was visible in the absence of SCFE1, when SOBIR1-GFP was pulled down, but it was much more pronounced in the presence of SCFE1, indicating that similar to LRR-RLKs such as FLS2 (Chinchilla *et al.*, 2007) and EFR (Schulze *et al.*, 2010), also the RLP30-SOBIR1 complex recruits BAK1 as a co-receptor upon ligand-detection.

3.2 RLP30 can be transformed into an RLK

RLPs form bimolecular receptor complexes with the adaptor kinase SOBIR1. By comparing RLKs like FLS2 or EFR with RLPs like RLP30, one can see immediately the striking similarity of the LRR-repeats. The only difference is the absence or presence of a kinase domain. To test whether it is possible to transform an RLP into an RLK by fusing a kinase domain on its C-terminal end, different chimeric fusion constructs of RLP30 and SOBIR1 were generated (Fig. 3.2 A-F) and transiently overexpressed in *N.benthamiana*. Their functionality was tested in an ethylene assay, because RLP30 expression alone did not trigger any ethylene response after SCFE1 treatment in *N.benthamiana* (Fig. 3.2 G).

All fusion constructs were labeled with a C-terminal GFP-tag. Transient expression of RLP30-GFP, SOBIR1-HA or both proteins together were used as controls. After three days the accumulation of ethylene upon SCFE1-treatment was measured (Fig. 3.2 G) and the presence of the proteins was confirmed using Western Blot detection of the GFP- or HA-tag of the proteins (Fig. 3.2 H and I).

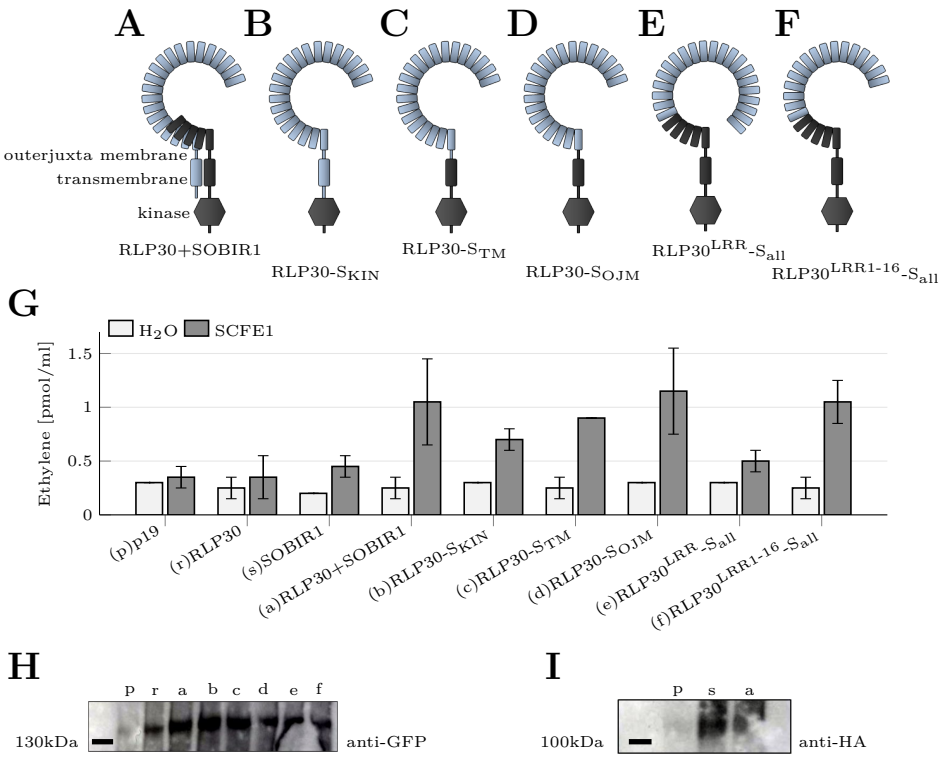

Figure 3.2: Chimeric fusion constructs of RLP30 and SOBIR1 are funtional in an ethylene assay. The different chimeric fusion constructs are shown in a schematic overview. In **(A)** the interacting full-length RLP30 and SOBIR1 proteins are shown. **(B)** The SOBIR1-kinase domain was fused to the full-length RLP30 protein (RLP30-S$_{KIN}$). **(C)** The transmembrane region together with the kinase domain of SOBIR1 was fused to the LRR-domain and outer juxta-membrane region of RLP30 (RLP30-S$_{TM}$). **(D)** The RLP30 LRR-domain was fused to SOBIR1 starting with the outer juxta-membrane region (RLP30-S$_{OJM}$). **(E)** The complete LRR-domain of RLP30 was fused to the full-length SOBIR1 protein (RLP30^{LRR}-S$_{all}$). **(F)** On top of the full-length SOBIR1 protein the LRR-repeats 1-16 of RLP30 were cloned (RLP30$^{LRR1-16}$-S$_{all}$). **(G)** Ethylene assay of transiently expressed proteins in *N.benthamiana*, three days after infiltration. Plants were treated with ddH_2O or with a final dilution of 1:100 SCFE1, and accumulation of ethylene was measured after 8 hours. The assay was repeated three times with similar results. Bars represent average values ± S.D. (n=2) of one representative experiment. **(H, I)** Western Blot analysis of transiently overexpressed proteins in *N.benthamiana* three days after infiltration. Blots were probed with **(H)** GFP- or **(I)** HA-specific antisera. Small letters represent the proteins as described in **G**.

Expression of all fusion constructs did not result in ethylene production towards the negative control H_2O. The infiltration control with p19, as well as the expression of RLP30-GFP or SOBIR1-HA alone did not lead to an ethylene response upon SCFE1-treatment. Only the co-expression of RLP30-GFP and SOBIR1-HA led to an SCFE1-dependent ethylene response. All of the fusion proteins (Fig. 3.2 B-D, F), except the fusion of the full-length LRR-domain of RLP30 on top of the full-length SOBIR1-protein (Fig. 3.2 E) mediated an ethylene response towards SCFE1 similar to the control with RLP30-GFP and SOBIR1-HA co-expression. The

fusion construct with the LRR^{RLP30} domain fused to the full-length SOBIR1-protein (Fig. 3.2 E) showed always a lower or almost no ethylene production towards SCFE1.
The expression analysis using Western Blot confirmed the expression of RLP30-GFP with a signal at the expected size of around 140 kDa. Signals with a slightly increased molecular weight were detected for all of the fusion constructs tagged as well with GFP. SOBIR1-HA gave the characteristic ladder-like pattern centered around 100 kDa in a Western Blot probed with HA-specific antisera.
Taken together, fusion proteins of different RLP30-ectodomain parts with different parts of SOBIR1 could be expressed in *N.benthamiana* leaves and could be detected in Western Blot analyses against the respective tags. Furthermore, the engineered receptors seem to recognize and response in an ethylene assay to SCFE1-treatment after transient expression in otherwise non-responsive *N.benthamiana* plants.

3.3 RLP30-dependent disease resistance can be transferred to solanaceous plants

Enhancing disease resistance of agricultural important plants is one of the major goals of plant breeding. One attempt is the transfer of PRRs to plants which initially lack the respective receptor, resulting in plants that are more resistant towards a certain pathogen displaying the respective MAMP or PAMP. Transgenic expression of the rice Xa21 pattern recognition receptor, for example, conferred resistance against different *Xanthomonas* pathovars in susceptible rice varieties (Wang *et al.*, 1996), sweet orange cultivars (Mendes *et al.*, 2010) or bananas (Tripathi *et al.*, 2014). The transfer of the *Arabidopsis* EFR LRR-RLK made *N.benthamiana* and *S.lycopersicum* more resistant against bacterial infection with *Ralstonia solanacearum* (Lacombe *et al.*, 2010) and stable, ectopic expression of the *Arabidopsis* RLP23

LRR-RLP in potato enhances resistance against the devastating pathogens *Phytophthora infestans* and *Sclerotinia sclerotiorum* (Albert *et al.*, 2015).

3.3.1 Tomato plants expressing RLP30 and SOBIR1 gain resistance towards *S.sclerotiorum*

Previous data indicated that the two solanaceous plants, tomato and tobacco, were unable to respond to SCFE1 (Fig. 3.3). However, expression of RLP30 together with SOBIR1 restored

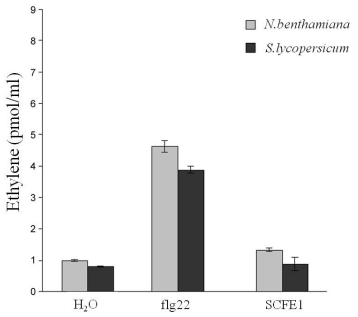

Figure 3.3: *N.benthamiana* and *S.lycopersicum* plants are insensitive to SCFE1. Leaf pieces of *N.benthamiana* or *S.lycopersicum* leaf pieces were treated with 0.25 μg/ml SCFE1 or 500 nM flg22 and the accumulation was measured. Experiment was performed by Dr. Weiguo Zhang and is published in (Zhang, 2013).

SCFE1 responsiveness (see Fig. 3.2 G). Notably, former experiments of transient expression of RLP30 in *N.benthamiana* showed that RLP30 seems to require the SOBIR1-protein from *A.thaliana* to produce ethylene upon stimulation with SCFE1, as RLP30 expression alone was not sufficient to confer SCFE1-responsiveness.

Based on those results, tomato plants of the cultivar *Moneymaker* were stable transformed with both *35S:RLP30-RFP* and *35S:SOBIR1-GFP* (Transformation was performed by Caterina Brancato, Transformation Unit ZMBP). The regenerated tomato plants were initially screened with an ethylene assay for their ability to respond upon SCFE1 stimulation. The three plants with the most robust phenotype are shown in Fig.3.4 A.

A picture of these three tomato lines with the highest ethylene responses upon SCFE1 stimulation are shown in Fig. 3.4 B. Plant #5.3 exhibited a stunted growth phenotype

compared to WT, which was also visible to a lesser extent in plant #13.1. Only plant #12.6 displayed a growth phenotype similar to WT. Unfortunately, so far we were not able to perform a successful Western Blot, to determine the expression levels of RLP30-RFP and SOBIR1-GFP in those transgenics.

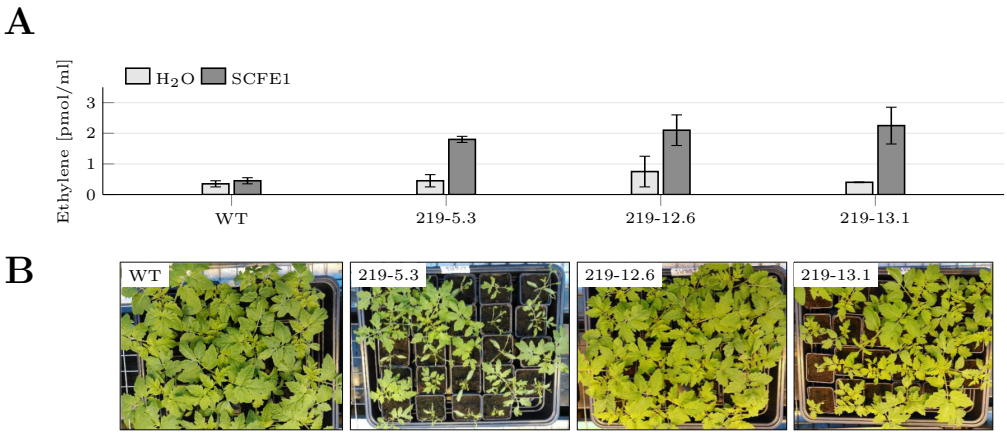

Figure 3.4: RLP30-RFP and SOBIR1-GFP stable transformed tomato lines are able to sense SCFE1 in an ethylene assay (A) Ethylene assay of stable transformed tomato lines with RLP30-RFP and SOBIR1-GFP (Transformation no. 219). Plants were treated with ddH_2O or a final concentration of 1:20 SCFE1, and accumulation of ethylene was measured after 5 hours. Bars represent average values ± S.D. (n=2). **(B)** Pictures of WT, or transgenic tomato lines no. 219-5.3/ 12.6/ 13.1 (pictures were taken by Dr. Frederic Brunner, Plant Response Biotech, Madrid).

In collaboration with Plant Response Biotech (Madrid), these transgenic tomato lines were infected with *S.sclerotiorum, isolate CH109*. Due to still on-going patent applications I'm however not able to show any original data of the performed infection assays with *S.sclerotiorum*, but can report that all of the transgenic lines expressing RLP30-RFP and SOBIR1-GFP had a lower disease symptome index (DSI) compared to WT tomato plants, with line #5.3 showing a significant reduction of the DSI after 5, 7 and 10 day post infection (dpi) compared to WT.

Stable overexpression of RLP30 and SOBIR1 in tomato led to a response after SCFE1-treatment in an ethylene assay and to decreased symptome development after infection with *S.sclerotiorum*. The results also indicated that an overexpression of RLP30 and/ or SOBIR1 can lead to a stunted growth phenotype.

3.3.2 Tobacco plants expressing RLP30 and SOBIR1 gain resistance towards *B.cinerea* but not *S.sclerotiorum*

Similar to tomato, *Nicotiana tabacum* was transformed with both *35S:RLP30-RFP* and *35S:SOBIR1-GFP* (Transformation was performed by Caterina Brancato, Transformation Unit ZMBP) in order to transfer the RLP30-dependent recognition system of *A.thaliana* to a plant species lacking the respective receptor (Fig. 3.3).

The transformed plants were tested in an ethylene assay for their ability to respond to SCFE1 (Fig. 3.5 A). Plant #49 and #55 showed the highest ethylene accumulation in response towards SCFE1, whereas plant #50 showed no response.

The expression of RLP30-RFP and SOBIR1-GFP was afterwards confirmed using Western blotting and immunostaining with antibodies raised against the GFP- or RFP-tag. RLP30-RFP was only detected in plant #49 and #55 corresponding to the results observed in the etyhlene assay. SOBIR1-GFP could be detected in all of the lines shown in this Western Blot, but the strongest signal was again seen for plant #49 (Fig. 3.5 B).

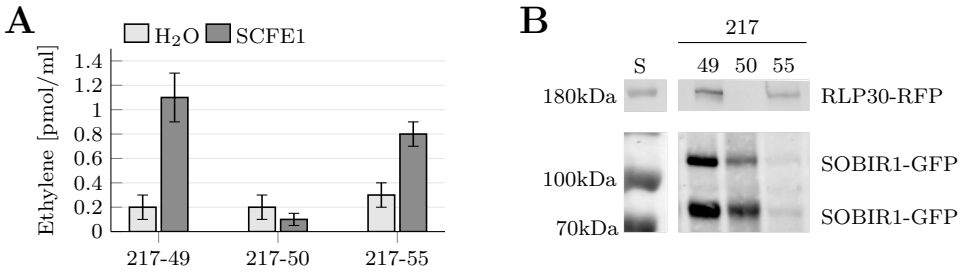

Figure 3.5: Stable transformed tobacco lines with RLP30 and SOBIR1 can sense SCFE1 in an ethylene assay. (A) Ethylene assay of stable transformed tobacco lines with RLP30-RFP and SOBIR1-GFP (Transformation no. 217). Plants were treated with ddH_2O or a final concentration of 1:20 SCFE1, and accumulation of ethylene was measured after 5 hours. Bars represent average values ± S.D. (n=2). **(B)** Western Blot analysis of RLP30-RFP and SOBIR1-GFP with tag-specific antisera raised against RFP or GFP, respectively. S=protein standard.

The two most sensitive lines towards SCFE1 (#49 and #55) were chosen for infection assays with *S.sclerotiorum, isolate CH109* and *B.cinerea*, performed by our collaborators at Plant

Response Biotech. But again, due for issues with the ongoing patent applications I'm not able to show any original data here. For the infection with *S.sclerotiorum* no difference could be observed between the WT tobacco lines and the transgenic lines. But the infection assay with *B.cinerea* revealed a significant lower disease symptom index (DSI) of the transgenic line #55 towards the fungi compared to WT. Transgenic line #49 showed no difference compared to WT.

The obtained results showed a correlation between the presence of RLP30 and SOBIR1, detected in Western Blot analyses, and the ability to accumulate ethylene after SCFE1-treatment and increased resistance to at least *B.cinerea*.

3.4 Putative interactors of RLP30 and SOBIR1

For identifying putative players in the RLP-SOBIR1-dependent immunity signaling pathway we screened the Membrane-based Interactome Database (MIND) (Jones *et al.*, 2014a) for reported interactors of RLP30 and/or SOBIR1. For the MIND-database, more than 3000 *Arabidopsis* membrane and signaling proteins were cloned to study more than 3 million binary protein-protein interactions in a split-ubiquitin based yeast two-hybrid assay (www.associomics.org). The chosen candidates are shown in table 3.1. The respective T-DNA insertion lines were ordered from the NASC-stock center and plants homozygous for the respective T-DNA insertion were tested in an ethylene assay for their response to SCFE1, nlp20 and flg22. All of the tested plants from the different T-DNA insertion lines showed the same response like WT Col-0 *Arabidopsis* plants (data not shown), indicating that the targeted proteins do not play major roles in innate immunity.

SOBIR1 was not only found to be a crucial co-receptor in immune signaling pathways, but was also described to play an important role during flower abscission (Leslie *et al.*, 2010). Based on

that, we were interested if proteins, initially described in flower abscission, are also involved in innate immunity. NEVERSHED (NEV) (Lewis *et al.*, 2010; Liljegren *et al.*, 2009) and CAST AWAY (CST) (Burr *et al.*, 2011) were chosen to be tested first.

Table 3.1: Putative interactors of RLP30 and/or SOBIR1 based on the MIND-database were tested for their role in plant immunity

AGI-ID	protein name, functional description	MIND-interactor of
At1g21240	WAK3, EGF-like domain	RLP30, SOBIR1
At1g29060	Target SNARE coiled-coil domain protein	RLP30, SOBIR1
At1g45145	Thioredoxin 5	RLP30, SOBIR1
At3G12180	Cornichon family protein	RLP30, SOBIR1
At3g28220	TRAF-like family protein	RLP30
At4g20790	LRR-RLK	RLP30, SOBIR1
At4g30850	HHP2, heptahelical protein 2	RLP30, SOBIR1
At4g37680	HHP4, heptahelical protein 4	RLP30, SOBIR1
At4g39890	RAB GTPase homolog H1C	RLP30
At5g16480	Phosphotyrosine protein phosphatases	SOBIR1
At5g42980	Thioredoxin 3	RLP30, SOBIR1
At5g49540	Rab5-interacting family protein	RLP30, SOBIR1
At5g59650	LRR-RLK	RLP30, SOBIR1
At5g63030	Thioredoxin superfamily	RLP30, SOBIR1

NEV (also called AGD5) belongs to the family of ARF-GAP domain containing proteins, with the ARF-GAP domain containing protein 15 (AGD15) being the closest NEV-homologue. Interestingly, NEV was already described to be a conserved target of powdery and downy mildew effectors (Schmidt *et al.*, 2014). All of the tested genes are listed in table 3.2. Homozygous T-DNA insertion lines of the respective genes, or in the case of *cst1* an EMS-mutagenesis-derived point mutation mutant line (Burr *et al.*, 2011), were tested for their ability to sense SCFE1 and nlp20 in an ethylene assay.

Table 3.2: Known genes involved in abscission or development tested for their role in the PAMP-triggered immunity pathway

AGI-ID	protein name, functional description
At3g17660	AGD15, ARF-GAP domain containing protein 15
At4g35600	CAST AWAY, cytoplasmic RLK
At5g54310	NEVERSHED, ARF-GAP domain containing protein 5

For AGD15, experiments with the obtained lines could not be repeatedly performed, therefore it is not possible to drew any conclusion at this time point. However, the preliminary data would hint towards an increased ethylene production in response to SCFE1 in the *agd15*-mutant plants compared to the wild type control (data not shown).

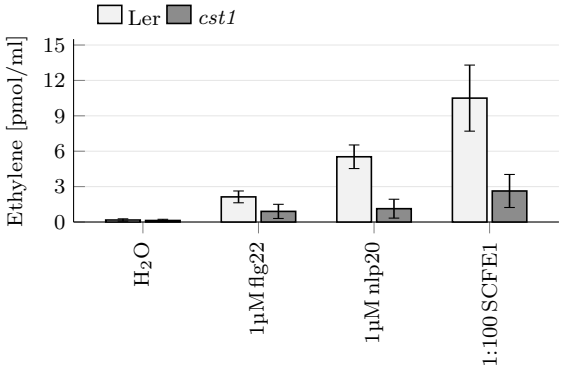

Figure 3.6: *cst1* plants showed lower ethylene production compared to *Landsberg erecta* wildtype plants. Ethylene assay of *Landsberg erecta* WT *Arabidopsis* plants or *cst1* mutant plants. Plants were treated with ddH_2O, 1 µM flg22, 1 µM nlp20 or a final concentration of 1:100 SCFE1 and ethylene accumulation was measured after 5 hours. Bars represent average values ± S.D. (n=2).

Only for *cst1* and *nev7* multiple experiments were performed. The *cst1*-mutant allele in the *Landsberg erecta* background showed a lower ethylene response towards treatment with flg22, nlp20 or SCFE1 compared to *Ler* control plants (Fig. 3.6). *Nev7* plants showed an inconsistent phenotype with sometimes a higher and sometimes a lower response in the etyhlene assay after treatment with nlp20 or SCFE1 compared to Col-0 plants and further replicates are required to determine the phenotype (data not shown).

The identification of novel interactors or regulators of RLP-dependent signaling was not successful using the MIND-database, as none of the targeted proteins seem to have an impact

on the SCFE1- or nlp20-induced accumulation of ethylene. Looking into others pathways, like flower abscission, might provide new insights into the regulation of immune answers, as *cst1*-mutant plants showed a reduced ethylene response upon treatment with SCFE1 or nlp20.

3.5 SCFE1-insensitive *Arabidopsis* accessions display unique SNPs in the *RLP30*-gene

The natural genetic variation of different *Arabidopsis* accessions was used to identify RLP30 as the receptor for SCFE1 (Zhang *et al.*, 2013). In that study, the accessions Br-0, Lov-1, Lov-5, Mt-0 and Sq-1 were identified to be insensitive to SCFE1, but able to sense flg22 in an ethylene assay.

Based on the data of Dr. Li Fan the *Arabidopsis* accessions Bak-2, ice111 and Lerik1-3 were also identified to be insensitive to SCFE1 in an ethylene assay. But, all of these accessions reacted like Col-0 plants upon the stimulation with H_2O, flg22 or nlp20 (Fig. 3.7 A). To unravel why those accessions are not able to sense SCFE1 any longer, the RLP30 gene was cloned from cDNA of the respective *Arabidopsis* accessions, subcloned into the pCR8-vector and sent for sequencing analysis and compared to all other available sequences of RLP30 in different accessions annotated in the *1001 Genomes Project* (www.1001genomes.org).

Sequencing analyses of the accession Bak-2 showed no SNP that was unique to SCFE1-insensitive accessions. All identified SNPs were also present in accessions that are still sensitive to SCFE1, based on comparison of the available sequencing information of the *1001 Genomes Project* (www.1001genomes.org). In order to better understand the impacts of the SNPs leading to amino acid exchanges a model of the extracellular LRR-Loop of RLP30 was generated, using the Phyre^2 server and PyMol. The RLP30 LRR-loop was

modeled based on all available LRR-loop crystal structures (modeling was performed by Christoph Käsbauer, ZMBP University of Tübingen).

The accession Mt-0 has a unique SNP changing the leucine at position 307 to an arginine. According to the model, this leucine/arginine is surface exposed (Fig. 3.7 B) and therefore could alter putative ligand-binding or -interaction sites.

The accessions Lov-1 and Lov-5 have a SNP leading to an amino acid exchange at position 433 from an arginine to a glycine that is unique to SCFE1-insensitive accessions. Arginine 433 is also exchanged in the accession ice111 to a leucine. This amino acid seems to be buried deep within the backbone of the LRR-loop (Fig. 3.7 B) and mutations might lead to the destruction of the folding of the extracellular domain.

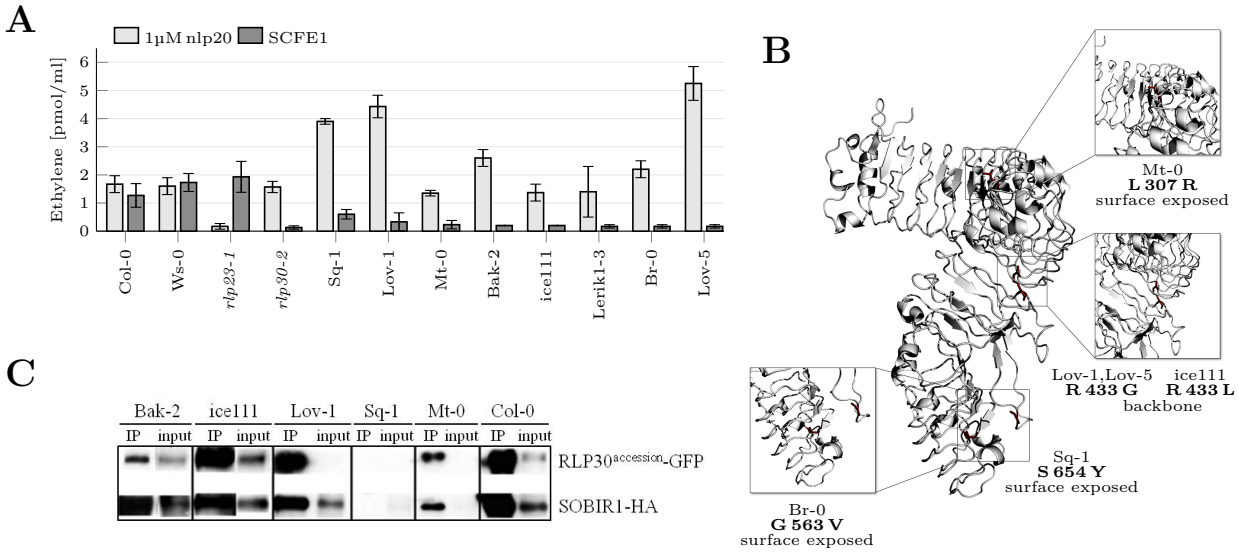

Figure 3.7: Unique SNPs in the *RLP30*-gene abolish SCFE1 perception. (A) Ethylene assay of different *Arabidopsis* accessions and *rlp23-1* and *rlp30-2* mutants with 1 µM nlp20 as positive control and a final concentration of 1:100 SCFE1. Accumulation of ethylene was measured after 5 hours. Bars represent average values ± S.D. (n=2). **(B)** Model of the extracellular LRR-Loop of RLP30. Modeling was performed by Christoph Käsbauer, using the Phyre[2] server and PyMol. The LRR-loop was modeled based on all available crystal structures of LRR-loops. Highlighted in a box are the regions where the specific SNPs are located for each accession. The number of the respective amino acid is given and the substitution, as well as the relative localization of the amino acid within the predicted structure, surface exposed or buried in the backbone. **(C)** Western Blot analysis of pull down experiments of transiently expressed RLP30^{accession}-GFP with SOBIR1-HA in *N.benthamiana* probed with the respective antisera against GFP or HA.

In the accession Br-0 a surface exposed glycine residue at position 563 is changed to a valine and in close proximity in the accession Sq-1 a serine at position 654 is changed to a tyrosine, which both seem to be surface exposed (Fig. 3.7 B). This could lead to possible alterations in binding or interaction sites for proteins like BAK1 or SOBIR1.

The Lerik1-3 accession is the only identified one that has a premature stopcodon. Serine 12 was changed to an early stop codon resulting in the termination of the protein sequence.

The SCFE1-insensitive RLP30^{accession}-proteins (corresponding to the Bak-2, ice111, Lov-1, Sq-1 and Mt-0 accession) were tested for their ability to interact with SOBIR1, a prerequisite for successful signal transduction. The C-terminal GFP-tagged RLP30^{accession}-proteins were transiently co-expressed with C-terminal HA-tagged SOBIR1 in *N.benthamiana* and after protein extraction subjected to a pull-down experiment using a GFP-trap (Fig. 3.7 C). All of the RLP30^{accession}-GFP-proteins, except RLP30$^{\text{Sq-1}}$ were expressed and SOBIR1-HA was always pulled down together with the respective RLP30$^{\text{accession}}$-protein (Fig. 3.7 C). None of the attempts to express RLP30$^{\text{Sq-1}}$ were successful and also changing the tag to HA didn't lead to the expression of RLP30$^{Sq\text{-}1}$ in detectable amounts, indicating that protein stability or the protein transport to the plasma membrane is affected.

Previously, it was shown that transcript levels of RLP30 were not altered in the accessions Lov-1, Lov-5 and Mt-0 compared to Col-0 (Zhang *et al.*, 2013). The expression levels of the remaining insensitive *Arabidopsis* accessions were not tested, so far.

Taken together, several important single amino acid residues could be identified that seem to be either required for putative ligand binding or interaction with other proteins like SOBIR1 or BAK1, or that might destroy the architecture of the protein.

3.6 SCFE1 has expected and unexpected molecular features

3.6.1 SCFE1 occurs in different species and under different growth conditions

Zhang *et al.* (2013) showed that SCFE1 is present in the *Sclerotinia sclerotiorum 1946* strain, and that *rlp30*-mutant lines are more susceptible towards the *Sclerotinia sclerotiorum 1980* strain. To test whether *S.sclerotiorum 1980* also contains SCFE1, the fungus was grown and the culture medium was purified according to the established protocol. The cation-exchange (CEX)-purified fractions were tested in an ethylene assay. A clear RLP30-dependent ethylene response of SCFE1^{Ss1980} could be observed (Fig. 3.8 A), which showed that SCFE1 is not only specific to the *S.sclerotiorum 1946* strain, but at least also present in the *S.sclerotiorum 1980* strain.

To check whether SCFE1 is also present in other fungal species the closest relative to *S.sclerotiorum, Botrytis cinerea B05.10*, was exactly grown and processed like *S.sclerotiorum*. The purified fractions were then subjected to an ethylene assay and could again induce an RLP30-dependent ethylene response (Fig. 3.8 B), indicating that SCFE1 is even not only restricted to one specific *Sclerotinia* strain, but also occurring in the closely related fungus *B.cinerea*.

To rule out the possibility that SCFE1 is an arbitrary product that is only produced during artificial growth of the fungus in the lab, tomato plants were infected with *S.sclerotiorum 1980* and the infected tomato plant pieces were used as starting material to purify SCFE1 (purification was performed by Birgit Löffelhardt). The CEX-purified fractions were then tested in an ethylene assay and showed an ethylene response only when RLP30 was present (Fig. 3.8 C). This result indicated that SCFE1 is not only produced under arbitrary conditions of *in vitro* cultivation, but also through the natural occurring infection process of a host plant.

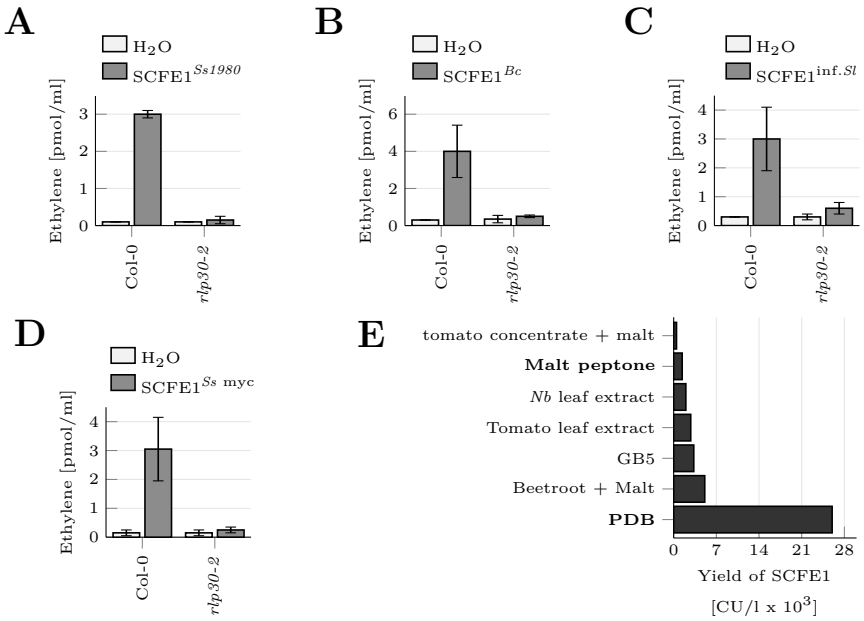

Figure 3.8: SCFE1 occurs in different fungal species and is produced under different growth conditions. (A) SCFE1 can be purified from culture medium of *S.sclerotiorum 1980* and **(B)** *B.cinerea*. Both fungi were grown in malt peptone medium for two weeks in the dark and the culture medium was harvested and subjected to CEX-chromatography. Purified fractions were diluted to a final concentration of 1:40 and were then tested in an ethylene assay in Col-0 and *rlp30-2* mutant plants. **(C)** SCFE1 can be purified from tomato plants infected with *S.sclerotiorum*. Tomato plants infected with *S.sclerotiorum* were mashed and the filtrate was subjected to CEX-chromatography. Purified fractions were diluted to a final concentration of 1:80 and were then tested in an ethylene assay in Col-0 and *rlp30-2* mutant plants (Purification and ethylene measurement was done by Birgit Löffelhardt). **(D)** SCFE1 can also be purified from the mycelium of *S.sclerotiorum*. The mycelium of *S.sclerotiorum* was harvested after 2 weeks of fungal growth in liquid malt peptone medium. The mycelium was homogenized in a mixer and subjected to CEX-chromatography. Purified fractions were diluted to a final concentration of 1:1000 and were then tested in an ethylene assay in Col-0 and *rlp30-2* mutant plants. Bars represent average values ± S.D. (n=2). All of the shown graphs are only giving qualitative and not quantitative hints about the RLP30-dependent ethylene inducing activity. Accumulation of ethylene was always measured after 5 hours. **(E)** SCFE1 can be purified from *S.sclerotiorum* grown in different culture media. *S.sclerotiorum* was grown in different growth media indicated in the figure. Bars represent estimated purified activity based on the eliciting activity of SCFE1, measured in arbitrary CU/l (Christina Units per liter) in an ethylene assay.

Usually, SCFE1 was purified from the culture medium of the fungus. Additionally, the mycelium of the fungus was mashed and subjected to CEX-chromatography. The purified fractions were tested in an ethylene assay and an RLP30-dependent activity could be observed (Fig. 3.8 D). Quantification of the ethylene-inducing activity of SCFE1 purified from the mycelium in comparison to that from the culture medium revealed the similar potential to induce the accumulation of ethylene, indicating that SCFE1 could be a secreted protein or targeted to be in the extracellular space.

Different culture media were tested in order to increase the yield of SCFE1 produced by

the fungus. The standard medium initially used, contained malt extract and peptone. The peptone was substituted by different leaf extracts (Tomato and *N.benthamiana*) or just leaf extracts alone were used. Additionally, concentrated tomato paste or beet root juice from the supermarket have been tested, as well as the commercially available minimal medium Gamborg's B5 (GB5) and the nutrient rich potato dextrose broth (PDB). All of the tested media produced an SCFE1-yield in more or less the same range, measured in CU/l ("Christina Units/liter"), an arbitrary number corresponding to the dilution factor of SCFE1 that would still induce an ethylene response of 1 pmol/ml Ethylene. The only exception was the PDB medium which yielded up to five times more SCFE1 than all of the other tested media (Fig. 3.8 E). The occurrence of SCFE1 after cultivation of *S.sclerotiorum* in this huge variety of different culture media strengthens the hypothesis of SCFE1 being truly fungal-derived.

3.6.2 SCFE1 is a proteinaceous ligand with a peptide motif sufficient to trigger ethylene

For further characterization of the elicitor activity of SCFE1, different biochemical properties were determined. First the heat-stability of the eliciting activity was tested. Unboiled and boiled SCFE1 was used in an ethylene assay. In addition, it was tested whether the elicitor is still active after boiling with 0,1% SDS. Neither boiling nor treatment with SDS could destroy the eliciting activity of SCFE1 when applied to *Arabidopsis* leaf pieces (Fig. 3.9 A), indicating that a motif or a non-proteinaceous structure rather than an active or functional protein is required for ethylene accumulation.

The next question to answer was about the nature of the elicitor. The SCFE1 elicitor was treated with different proteinases (AspN, GluC, Trypsin, Proteinase K) or Cyanogenbromid and the eliciting activity was determined afterwards in an ethylene assay. All of the tested

proteinases destroyed the activity of SCFE1, whereas SCFE1 incubated in the respective buffers or H_2O was still active (Fig. 3.9 B), leading to the assumption that SCFE1 is mainly proteinaceous.

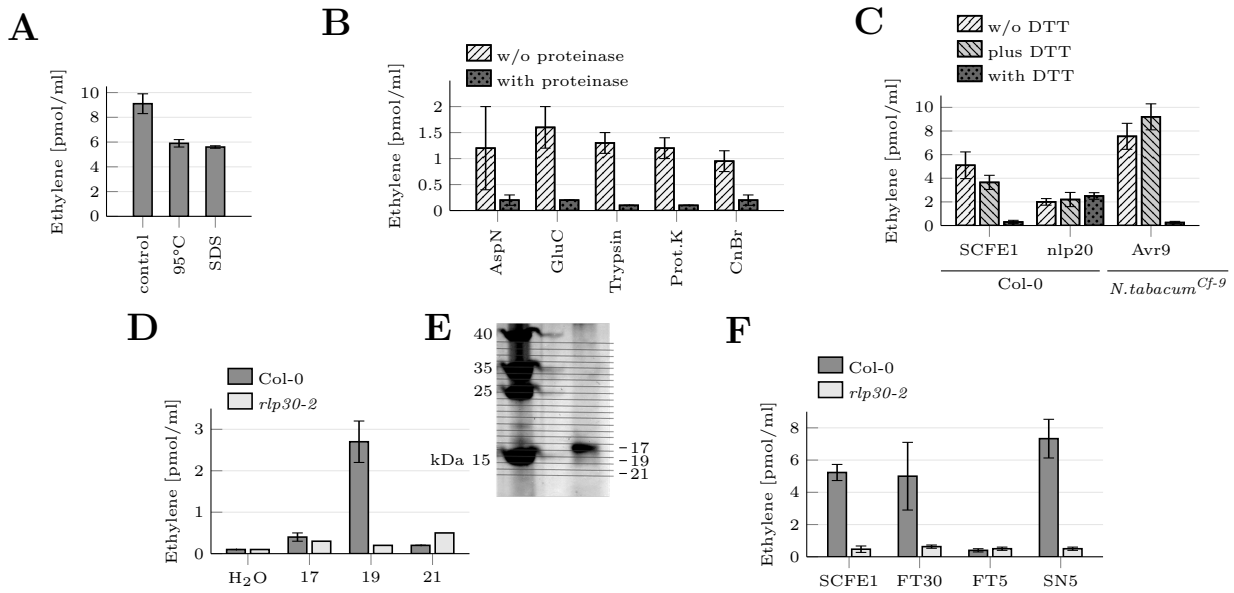

Figure 3.9: SCFE1 is a proteinaceous ligand with a peptide motif sufficient to trigger an ethylene response. (A) SCFE1 is heat-stable and stable to SDS-treatment. SCFE1 was boiled for 20 min with or without 0,1 % SDS and then tested in an ethylene assay with a final concentration of 1:100 using Col-0 leaf pieces. Unboiled SCFE1 in a final dilution of 1:100 served as control. **(B)** SCFE1 activity can be destroyed with different proteinases. Boiled SCFE1 was incubated O/N at 37 °C with or without different proteinases in the respective buffers. After heat inactivation of the enzymes the samples were tested in an ethylene assay with a final concentration of 1:100 using Col-0 leaf pieces. **(C)** SCFE1-dependent activity can be destroyed with reducing agents. Boiled SCFE1, nlp20 and Avr9 were incubated O/N at 37 °C with 5 mM DTT and then subjected for an ethylene assay using *Arabidopsis* Col-0 or Cf-9-tobacco leaf pieces, respectively ("with DTT"). Ethylene accumulation was also measured of a mixture of the respective elicitor without any pre-incubation ("plus DTT"). **(D, E)** SCFE1 can be eluted from SDS-PAG slices at around 15 kDa. An active fraction of SCFE1 was separated on a 14% Tricine-SDS-PAG and the gel was afterwards cut into thin slices, indicated by the horizontal lines **(E)**. The proteins were eluted with 200 µl of ddH_20 from the gel slices and 50 µl of the supernatants were tested in an ethylene assay on *Arabidopsis* Col-0 and *rlp30-2* leaf pieces for their eliciting activity **(D)**. Shown are only the results obtained for gel slices 17, 19 and 21. **(F)** VivaSpin Columns with different pore sizes were used to fractionate SCFE1. The flow-trough, diluted to a final concentration of 1:100, of a spin column with a cut off of 30 kDa ("FT30") was still active in Col-0 leaf pieces, whereas only the supernatant of a spin column with a cut off of 5 kDa ("SN5"), diluted to a final concentration of 1:100, remained active. The corresponding flow through ("FT5") showed no ethylene-inducing activity. Accumulation of ethylene was measured after 5 hours. Bars represent average values ± S.D. (n=2).

It is known that the elicitor Avr9 forms three disulfid bridges between different cysteines which are all required for its immunogenic activity (Kooman-Gersmann *et al.*, 1997; van den Hooven *et al.*, 2001). Reducing agents like Dithiothreitol (DTT) reduce the sulfur and the cysteine bridges are released. SCFE1, nlp20 as a negativ control, and Avr9 as a positive control, were incubated overnight at 37 °C or boiled for 40 min at 95 °C with or without DTT

and then tested in an ethylene assay on *Arabidopsis* wild type or *N.tabacum* Cf9 leaf pieces (Fig. 3.9 C, "w/o DTT" and "with DTT"). Additionally, 5 mM DTT was mixed with the respective elicitor and tested in the ethylene assay without any pre-incubation (Fig. 3.9 C, "plus DTT"). Nlp20 did not loose its immunogenic activity after treatment with DTT as reducing agent, whereas Avr9 and surprisingly also SCFE1 completely lost their ability to induce any ethylene accumulation. The experiment was repeated, using ß-mercaptoethanol as reducing agent, leading to the same result (data not shown). These experiments could indicate that disulfide bridges between cysteines are crucial for the ethylene-inducing activity of SCFE1.

To determine the molecular size of SCFE1 an active fraction was loaded onto a Tricine-SDS-PAG. After running the gel, it was cut into 1 mm thin slices and incubated O/N in ddH_2O to elute the proteins out of the gel. The supernatants of the incubated gels were then subjected to an ethylene assay and an ethylene-inducing activity could be recovered from a gel slice of around 15 kDa, representing gel slice #19 (Fig. 3.9 D and E).

Additionally, an active fraction of SCFE1 was further fractionated using VivaSpin columns (Sartorius, Göttingen) with different cut offs. The first used spin column had a cut off of 30 kDa and all of the activity was found in the flow-through. Reducing the pore size to a cut off of 5 kDa retained the activity in the supernatant and the flow-through didn't show any ethylene-inducing activity (Fig. 3.9 F), indicating that SCFE1 is between 5 and 30 kDa, which is also confirmed by the SDS-PAGE analysis. Here, SCFE1 could be eluted from gel slices that would indicate a molecular weight of SCFE1 of around 15 kDa.

Taken together, SCFE1 seems to be a proteinaceous ligand with a molecular weight of around 15 kDa and important, reduceable disulfide bridges.

3.7 Different approaches to identify SCFE1

After characterizing SCFE1 as a proteinaceous, heat-stable and DTT-sensitive elicitor of approximately 15 kDa in size, the next experiments were aiming at the molecular identification of this novel *Sclerotinia* MAMP. The following paragraphs will explain the different approaches to identify SCFE1.

3.7.1 Fishing the ligand with its receptor RLP30

The first approach to identify SCFE1 was fishing the ligand with its receptor. The receptor of SCFE1, RLP30, was C-terminal GFP-tagged and transiently overexpressed in *N.benthamiana* leaves. The receptor-like protein RLP23 was as well C-terminal GFP-tagged and served as a negative control. After protein extraction the receptor-GFP proteins were bound to a GFP-trap. An active fraction of SCFE1 was added to the GFP-trap-coupled receptors and after incubation the GFP-trap was carefully washed to remove unbound protein. The bound proteins were eluted with SDS-loading buffer and sent for LC-MS/MS analysis on a Proxeon Easy-nLC coupled to an LTQ Orbitrap Elite mass spectrometer (method: 130 min, Top15, HCD; done by Dr. Mirita Franz-Wachtel, Proteome Center, University of Tübingen).
In the first experiment 71 *S.sclerotiorum* proteins in total were identified. 52 of these proteins were present in both samples with the coupled RLP30 but also RLP23 (see Table 8.6 for full results). Table 3.3, shows in the upper section the three most abundant proteins according to the number of found peptides in the LC-MS/MS analysis and the 7 proteins which were found to be only present in the RLP30-sample. 12 proteins were found only in the RLP23-sample (see Table 8.6).

Table 3.3: Most abundant proteins and proteins exclusively bound to RLP30 found in the LC-MS/MS-analysis by fishing the ligand with its receptor RLP30. The protein descriptions are often based on already identified homologs in other fungi. The numbers given in the "RLP30" and "RLP23" column indicate the number of identified peptides in the respective sample. The upper part of the table shows the results of the first analysis, whereas in the lower part, the identified proteins of the second repetition are listed.

Protein-ID	**protein description**	**RLP30**	**RLP23**	**Mol.Weight [kDa]**
A7F878	Glycosyl hydrolase family	9	12	68
A7ETS6	Actin	7	6	42
A7E4E9, A7F0B8, A7E993	Polyubiquitin, Ubiquitin-ribosomal fusion protein	4	4	34,18,15
A7ET57	Rho-GDI	3	0	23
A7EPL9	Tripeptidyl peptidase	3	0	73
A7E4P1	Predicted protein	1	0	16
A7EWA0	Histone H4	1	0	11
A7EQQ8	Carbohydrate esterase family/Cutinase	1	0	20
A7F1G5	U1 small nuclear ribonucleoprotein	1	0	49
A7F0Y2	60S ribosomal protein	1	0	16
A7E677	40S ribosomal protein	4	5	34
A7E4E9, A7F0B8, A7E993	Polyubiquitin, Ubiquitin-ribosomal fusion protein	4	4	34,18,15
A7ETS6	Actin	4	4	42
A7ECT9	Rab family GTPase	2	0	23
A7F3F7	26S protease regulatory subunit	2	0	47
A7E3Y4	Alpha-tubulin suppressor protein	1	0	34
A7EN34	Hypothetical mitochondrial ribosomal protein	1	0	37
A7EPE0	Hypothetical mitochondrial ribosomal protein	1	0	31
A7EQ69	Ribosome biogenesis protein	1	0	38
A7F091	Dihydrolipoyl dehydrogenase	1	0	54
A7EP73	40S ribosomal protein	1	0	28
A7EET7	Glutamate oxaloacetate transaminase	1	0	43
A7ERR3	Predicted protein	1	0	49
A7E431	Translation elongation factor EF-Tu	1	0	49

To validate the identified proteins the experiment was repeated exactly as described earlier in this section. In the second LC-MS/MS analysis 60 *S.sclerotiorum* proteins could be identified,

with 35 proteins present in both samples (see Table 8.7 for full results). The 3 most abundant *S.sclerotiorum* proteins, identified in the LC-MS/MS analysis, are listed in table 3.3, lower section. 11 proteins were exclusively present in the sample pulled down with RLP30-GFP (table 3.3, lower section) and 14 proteins were pulled down with RLP23-GFP (Table 8.6). None of the proteins that were found in the first experiment to be exclusively present in the RLP30-sample could be confirmed in the second experiment, but Actin as well as Polyubiquitin/Ubiquitin-ribosomal fusion proteins were both times among the most abundant proteins.

Taken together, this approach did not result in reproducible data and therefore further purification and enrichment of SCFE1 seem to be required to unravel the molecular nature of SCFE1.

3.7.2 Improved purification of SCFE1 using reversed phase chromatography and SDS-PAGE

The approaches tried so far, not only for this thesis, but also by Dr. Frederic Brunner and Dr. Weiguo Zhang, were most likely not successful, due to low protein abundances and too many contaminating proteins. In order to increase the purity and the concentration of SCFE1, reversed phase chromatography was used. This technique introduces a new dimension of protein separation, namely by hydrophobicity. C2-, C4-, C8- and C18-columns with Acetonitril, Isopropanol or Methanol as elution buffer were tested and C4-column with Acetonitril as elution buffer was chosen for further purification, because this combination led to a recovery of around 80 % RLP30-specific SCFE1-activity in an ethylene assay. Purification of the proteins on the C4-column was monitored with UV_{280nm} and afterwards with a silver stained 14% Tricine-SDS-PAG (Fig. 3.10 B). The active fractions have been identified using an ethylene assay (Fig. 3.10 A). Fraction E3, E4 and E5 were

pooled and concentrated in a vacuum-concentrator centrifuge and afterwards loaded onto a 14% Tricine-SDS-PAG. After running the gel, it was cut into thin slices (Fig. 3.10 C) and incubated in 2-(N-morpholino)ethanesulfonic acid (MES)-Buffer to elute the proteins out of the gel slices. This enabled a third dimension of protein purification - the separation by size in an SDS-PAG. The different supernatants with the eluted proteins were tested in an ethylene assay (Fig. 3.10 D) and gel slice "k" was chosen for sending for LC-MS/MS analysis on a Proxeon Easy-nLC coupled to an LTQ Orbitrap Elite mass spectrometer (method: 130 min, Top15, HCD; done by Dr. Mirita Franz-Wachtel, Proteome Center, University of Tübingen), together with the whole fraction E6 of the C4-purification run.

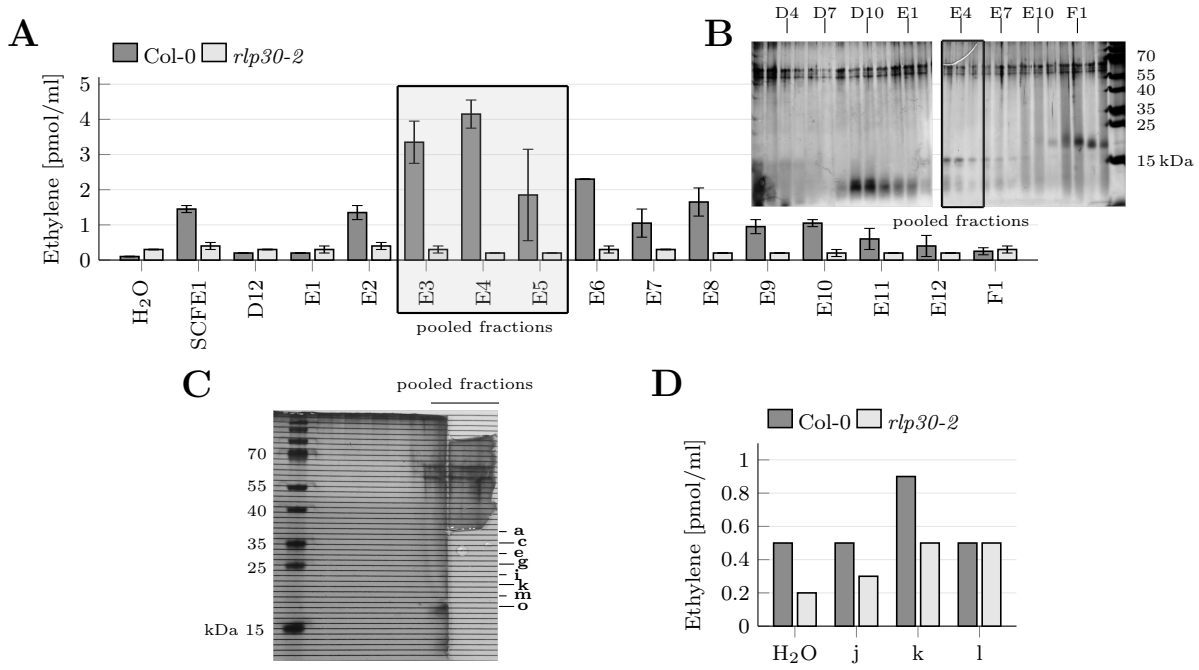

Figure 3.10: Improved purification of SCFE1 by reversed phase chromatography and Tricine-SDS-PAGE - Part I. (A) Ethylene assay of collected fractions after the C4-reversed phase chromatography. Fractions were diluted to a final concentration of 1:100 in the ethylene assay and Col-0 and *rlp30-2* plants were tested. Accumulation of ethylene was measured after 5 hours. Bars represent average values ± S.D. (n=2). Highlighted with a box are the fractions giving the highest RLP30-dependent ethylene response, which were pooled for further analysis. **(B)** Silver stained 14% Tricine-SDS-PAG of 5 µl of collected fractions after the C4-reversed phase chromatography. Highlighted with a box are the fractions corresponding to the highest RLP30-dependent ethylene response shown in A. **(C)** Silver stained 14% Tricine-SDS-PAG loaded with the whole pooled and concentrated fractions E3, E4 and E5. Horizontal lines indicate the cut slices. **(D)** The supernatants of the cut Tricine-SDS-PAG gel slices were tested in an ethylene assay with Col-0 and *rlp30-2* plants in a final concentration of 1:5. Bars represent single values due to constraints in material. Accumulation of ethylene was measured after 5 hours.

Additionally, a second round of SCFE1 purification with a new batch of starting material using C4-reversed phase chromatography was performed and after identification of the most active fractions in an ethylene assay (Fig. 3.11 A), Fraction D9 to E4 were pooled, concentrated in a vacuum-concentrator centrifuge and loaded onto a 14% Tricine-SDS-PAG (Fig. 3.11 B). The gel was cut into thin slices, indicated in Fig. 3.11 B with horizontal lines, and the gel pieces were incubated in ddH_2O to elute the proteins. The supernatants with the eluted proteins were again tested for their RLP30-dependent etyhlene-inducing activity (Fig. 3.11 C). The supernatant of gel slice "S" as well as the corresponding gel slice were sent for LC-MS/MS analysis.

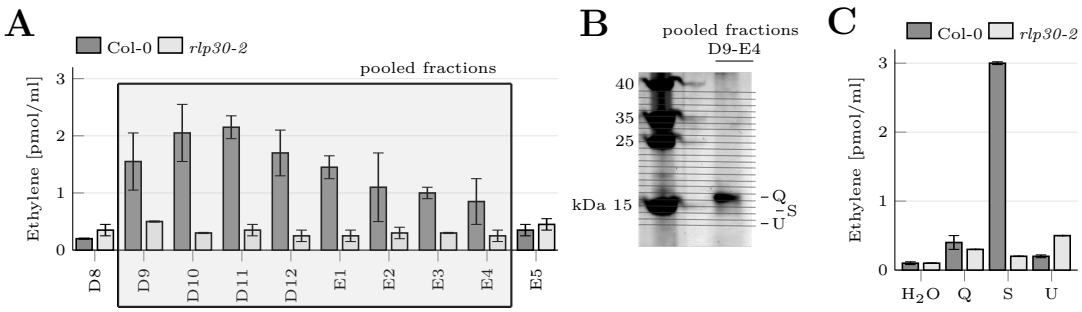

Figure 3.11: Improved purification of SCFE1 by reversed phase chromatography and Tricine-SDS-PAGE - Part II. (A) Ethylene assay of selected fractions collected after the C4-reversed phase chromatography. Fractions were diluted to a final concentration of 1:200 in the ethylene assay and Col-0 and *rlp30-2* plants were tested. Accumulation of ethylene was measured after 5 hours. Bars represent average values ± S.D. (n=2). Highlighted with a box are the fractions giving the highest RLP30-dependent response, which were pooled for further analysis. **(B)** Silver stained 14% Tricine-SDS-PAG of pooled fractions (D9-E4) after the C4-reversed phase chromatography. Horizontal lines indicate the cut gel slices. **(C)** The supernatants of the cut Tricine-SDS-PAG gel slices were tested in an ethylene assay with Col-0 and *rlp30-2* plants. The supernatants were diluted to a final concentration of 1:80. Accumulation of ethylene was measured after 5 hours. Bars represent average values ± S.D. (n=2).

The *S.sclerotiorum* proteins with the highest number of identified peptides are shown in table 3.4. Polyubiquitin, Rho-GDI and Actin were again among the identified proteins, like before in the fishing experiment (table 3.3). An acyl-CoA dehydrogenase was the protein with the highest number of identified proteins in the analyzed fraction E6 and a predicted protein of 16 kDa was present in the gel slice "k", in fraction E6 and in the supernatant of gel slice "S".

Table 3.4: Identified proteins in the LC-MS/MS-analysis after improved purification via reversed phase chromatography and SDS-PAGE. E6= whole active fraction after C4 reversed phase chromatography; slice k= gel slice harbouring an RLP30-dependent ethylene inducing activity (Fig. 3.10); supernatant S/slice S= supernatant and gel slice harbouring an RLP30-dependent ethylene inducing activity (Fig. 3.11); Numbers indicate the number of identified peptides in the respective sample.

Protein-ID	**protein description**	**E6**	**slice k**	**supernatant S**	**slice S**	**Mol.Weight [kDa]**
A7F3Y0	Acyl-CoA dehydrogenase	6	1	0	0	90
A7E610	Predicted protein	2	1	1	0	16
A7E993; A7F0B8; A7E4E9	Polyubiquitin, Ubiquitin-ribosomal fusion protein	2	0	1	0	34,18,15
A7ET57	Rho-GDI	2	0	1	0	23
A7ETS6	Actin	2	0	1	1	42

Based on the results shown in table 3.4 synthetic nested peptides spanning the whole region of the Rho-GDI, Actin, Ubiquitin-ribosomal fusion protein (A7E993) and the predicted protein sequence were ordered and tested for their immunogenic activity in an ethylene assay with different concentrations on *Arabidopsis* Col-0 and *rlp30-2* mutant plants (see the amino acid sequences in Table 8.2, 8.3, 8.4, 8.5). None of the tested peptides showed an RLP30-dependent activity in an etyhlene assay (data not shown). Additionally, all of those peptides were tested for ethylene-induction in leaf pieces of *Solanum pennellii*, *Solanum lycopersicum* and *N.benthamiana* plants, in case one of those peptides showed an immunogenic activity dependent on a solanaceous PRR, but no specific activity was observed (data not shown).

Taken together, the aimed purification using reversed phase chromatography was successful, as the number of identified proteins in the MS/MS-analyses decreased, but testing of nested peptides of the most promising candidate proteins was not successful and the molecular identity of SCFE1 still remains elusive.

3.7.3 Use of the new sequenced and annotated *Sclerotinia sclerotiorum* genome together with six-frame translation

Another critical part for a successful identification of SCFE1 is the quality of the sequenced genome and therefore of the predicted proteome as this is the base for the identification of the found masses in the LC-MS/MS analyses. Derbyshire *et al.* (2017) released a new annotated genome of *Sclerotinia sclerotiorum 1980* with a higher overall sequence coverage as the previous one.

Table 3.5: The most abundant identified *S.sclerotiorum* proteins in the LC-MS/MS analysis of various gel slices using the newly released *S.sclerotiorum* genome, together with six-frame translation after CEX-purification and separation via SDS-PAGE ranked according their abundance.

Protein-ID	protein description	Mol.Weight [kDa]
Sscle10g077860	Rho-GDI	22
Sscle03g029230	Polyubiquitin	34
Sscle08g068580	Glucosamine 6-phosphate N-acetyltransferase	19
Sscle15g104590	PAF acetylhydrolase family protein	42
Sscle16g108160	Pectinesterase	34
Sscle03g022920	Cytochrome C	12
Sscle03g024790	Predicted protein	18
Sscle02g018350	Putative c2 domain-containing protein	166
Sscle10g080270	Glucoamylase	67
Sscle12g090030	Acyl-CoA dehydrogenase	62
Sscle03g030530	Extracellular aldonolactonase	42
Sscle02g015410	Thioredoxin-like/ Co-Chaperone YnnB	22
Sscle02g017490	Glucosidase-like protein	63

Dr. Frederic Brunner purified via CEX-chromatography 10 x 10 l of *S.sclerotiorum* culture medium and loaded purified samples onto an SDS-PAG and cut the gels into thin slices. The slices were then tested for their ethylene inducing activity by Birgit Löffelhardt. Out of those gel slices I chose the most active ones and sent them for LC-MS/MS analysis to

"The Sainsbury Laboratories" (Dr. Frank Menke). The obtained LC-MS/MS data was analyzed using the lately released new version of the proteome of *Sclerotinia sclerotiorum 1980* (Derbyshire *et al.*, 2017) and additionally, a six-frame translation of the *S.sclerotiorum* genome to make sure that SCFE1 is not overseen by being a non-annotated protein.
The most abundant *S.sclerotiorum* proteins identified in the LC-MS/MS analysis are listed in table 3.5. Applying the six-frame translation did not reveal any further proteins, that were not already predicted in the annotated genome. Again, the Rho-GDI, Polyubiquitin and an acyl-CoA dehydrogenase were found, like in the previous analyses.
Taken together, the results of the LC-MS/MS analyses didn't reveal any new candidate-proteins that would immediately strike you, but confirmed the already obtained data. Conformation and then expression of interesting candidate genes is now required to narrow down the list and subsequently identify SCFE1, for example by using the later on introduced Gateway-compatibel expression system for transient apoplastic expression in *N.benthamiana* (see chapter 3.7.5).

3.7.4 Bioinformatical approach for SCFE1 identification

With the use of bioinformatical tools a completely different approach was taken to identify SCFE1. This part of the thesis was performed at Wageningen University in the Phytopathology department of Prof. Bart Thomma, with the help of Dr. Michael Seidl.
The aim of this approach was the generation of a list of all *S.sclerotiorum* and *B.cinerea* proteins that would fulfill the characteristics of SCFE1 identified so far. The criteria that putative candidate proteins should fulfill are the following (indicated in brackets are the reasons for the criteria):

- secretion signal (presence in the culture medium)

- molecular weight between 5-30 kDa, most likely around 15 kDa (determined by the cut off ranges of VivaSpin columns; molecular size of proteins in gel slices showing an ethylene response)
- contain all of the following amino acids: methionines, aspartates and glutamates (proteinase digestion)
- contain at least two cysteines (Loss of activity after treatment with reducing agents)
- homologue in *B.cinerea*
- no overall conservation (initial screen revealed fungi that seem to not produce any measurable eliciting proteins under the used conditions)
- expressed during infection and growth in medium

The analysis was started using the complete annotated proteome of *S.sclerotiorum* and *B.cinerea*. Based on the prediction of a signal peptide using the SignalP algorithm (Petersen *et al.*, 2011) the list of annotated proteins was significantly shortened. For each protein its molecular weight with and without the signal peptide was determined. After that, the number of all cysteines, methionines, aspartates and glutamates of each protein were counted. The EffectorP algorithm (Sperschneider *et al.*, 2016) was used to predict putative effector proteins. This information was just collected as additional information and was not used as an exclusion argument. A BLAST search against the *B.cinerea* or *S.sclerotiorum* proteome, respectively, was performed and the best hit for each protein was identified. Additionally, the expression level of each corresponding gene was placed in the table, based on RNAseq data (RNAseq data were kindly provided by Dr. Michael Seidl and Prof. Jan van Kan, Wageningen University). Finally, a search for putative domains was performed using Interpro Scan version 5.17-56.0 (Jones *et al.*, 2014b). After identifying the most promising candidate proteins a BLAST search against all sequenced fungal proteomes was performed and the proteins

were ranked according to their homology throughout all the available fungal proteomes, excluding the *B.cinerea* and *S.sclerotiorum* proteome. Proteins which showed no or only little conservation in other fungi were of particular interest.

After applying all above mentioned criteria a total of 32 *B.cinerea* proteins and 42 *S.sclerotiorum* proteins were compared with each other to identify proteins that would match each other. Out of those, 15 proteins could be identified, that were present both in the final list of *B.cinerea* and *S.sclerotiorum* proteins, respectively (Table 3.6).

Table 3.6: Identified proteins using a bioinformatical approach applying all known characteristics of SCFE1 and comparing the *S.sclerotiorum* proteome with that of *B.cinerea*.

Protein-ID	Protein-ID	Protein-ID	Protein-ID	Protein-ID
Sscle01g008950	Sscle01g011350	Sscle02g016170	Sscle03g022550	Sscle03g024510
Sscle03g028510	Sscle03g029740	Sscle06g051210	Sscle06g052360	Sscle06g055280
Sscle13g095230	Sscle13g097000	Sscle14g097630	Sscle15g102390	Sscle15g106560

All of the here identified proteins are so-called "predicted proteins" having no sequence homology to an already described protein. Having a closer look on those proteins, three of them were of particular interest. The protein Sscle03g024510 was differently annotated in the new genome compared to the previous one. The protein Sscle13g095230 belongs to a class of proteins which is called Hydrophobins, that all have the same cysteine pattern: "-C–$(x)_n$-CC–$(x)_n$-C–$(x)_n$-C–$(x)_n$-CC–$(x)_n$-C-" (C=Cysteines, x=any amino acid, n=any number). And the protein Sscle03g028510 was already identified in a previous LC-MS/MS analysis (see Table 3.3, protein code A7E4P1; additionally this protein was identified in two LC-MS/MS analyses performed by Dr. Frederic Brunner and Dr. Weiguo Zhang [data not shown]). Primers were ordered for a subset of apparently interesting genes, including Sscle03g024510, Sscle06g055280 and Sscle13g095230. Unfortunately, none of the attempts to get a PCR product were successful, thus this approach was stopped at that point.

Taking a bioinformatical approach can offer new insights and provide important information, but can also have its drawbacks and always needs confirmation in the wet lab.

3.7.5 Establishing an easy cloning and purification system to test putative candidate proteins

The elicitor Avr9 was successfully expressed in the apoplast of *N.tabacum* by fusing a secretion peptide on the N-terminal part of the protein sequence (Piedras and Hammond-Kosack, 1998). For testing the putative candidates a straight forward cloning system was required which allows an easy expression and which didn't comprise a time-consuming purification afterwards. Therefore, the apoplastic expression of putative candidate proteins based on a Gateway-compatible cloning system was the method of choice.

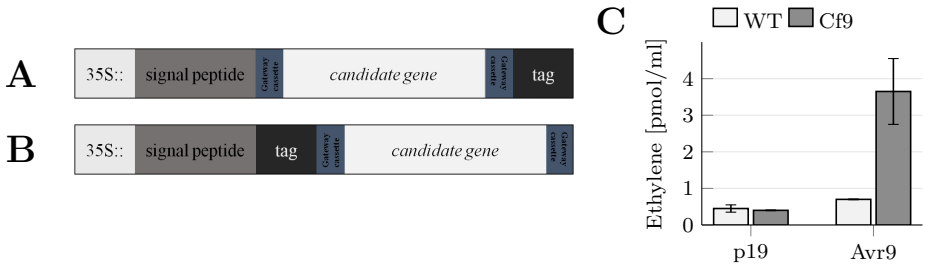

Figure 3.12: Gateway-compatible expression system for apoplastic expression. **(A)** Schematic drawing of the 35S-promoter, signal peptide, Gateway-cassette and C-terminal tag. **(B)** Schematic drawing of the 35S-promoter, signal peptide, N-terminal tag and Gateway-cassette. **(C)** Proof-of-principle experiment with Avr9 cloned into pGWB8 containing a 35S-promoter, the PR1a-signal peptide and a C-6xHis tag (*35S:PR1a-AVR9-6xHis*). The vector was transiently expressed in *N.benthamiana* using *Agrobacteria*-mediated transformation and the apoplastic fluid was harvested after 64 hours. The ethylene assay was performed using leaf pieces of wild type *N.tabacum* and *N.tabacum* expressing Cf9. The apoplastic fluid of plants expressing p19, as negativ control or the Avr9-contruct were diluted to a final concentration of 1:8 and accumulation of ethylene was measured after 5 hours. Bars represent average values ± S.D. (n=2).

For this system binary expression vectors of the pGWB-series (Nakagawa *et al.*, 2007) were used. The chosen vectors should already contain a 35S-promoter and should be with or without small C-/N-terminal tags (Fig. 3.12 A and B). Two different signal peptides were chosen to be classically cloned into the desired vector backbones. One was the well

characterized and described signal peptide of PR1a from *N.benthamiana* and the other one was a signal peptide of *Phytophthora sojae* NIP protein. In table 3.7 the successfully cloned expression vectors are shown.

Avr9 was cloned into all of the vectors containing a tag and transiently expressed in *N.benthamiana*. The harvested apoplastic fluid was then tested in an ethylene assay without any further purification. In a proof-of-principle experiment the transient expression of *35S:PR1a-AVR9-6xHis* was successful, leading to a Cf9-specific accumulation of ethylene in *N.tabacum*, whereas expression of p19 as a negativ control did not induce any ethylene accumulation (Fig. 3.12 C).

Table 3.7: Gateway-compatible expression vectors for apoplastic expression.

Vector backbone	**Signalpeptide**	**Tag**
pGWB2	PR1a-Signalpeptide	No tag
pGWB2	Signalpeptide from *Phytophthora sojae*	No tag
pGWB8	PR1a-Signalpeptide	C-6xHis
pGWB8	Signalpeptide from *Phytophthora sojae*	C-6xHis
pGWB14	PR1a-Signalpeptide	C-3xHA
pGWB14	Signalpeptide from *Phytophthora sojae*	C-3xHA
pGWB15	PR1a-Signalpeptide	N-3xHA
pGWB15	Signalpeptide from *Phytophthora sojae*	N-3xHA

Further testing of the different expression vectors is required to determine the constructs that are suited best for a rapid, but very simple expression of identified candidate genes in *N.benthamiana*. The preliminary experiments already indicate that the expression-system is working in general but further optimization is required, to get a reliable system.

3.8 Identification of a novel SCFE1-like bacterial elicitor

Wang *et al.* (2008a) showed that *rlp30*-mutant plants are more susceptible to the bean pathogen *Pseudomonas phaseolicola 1448A* which is not able to colonize WT *A.thaliana*. This indicated that RLP30 is required for full resistance against *Pseudomonas phaseolicola 1448A*. *Rlp30-2* mutant plants lacking the RLP30-receptor are also more susceptible to *S.sclerotiorum* and *B.cinerea* (Zhang *et al.*, 2013), which both produce SCFE1. Consequently, the higher susceptibility of *rlp30*-mutant plants could be due to the inability of sensing a novel bacterial elicitor via the RLP30-receptor.

3.8.1 RLP30 is the receptor for PCFE1 detection

To prove the hypothesis that *Pseudomonas* bacteria contain a molecule that can be perceived by RLP30, the culture medium of different *Pseudomonas* strains was subjected to exactly the same purification protocol established for SCFE1 from *S.sclerotiorum*. The *Pseudomonas* strains were chosen based on the availability of a sequenced genome, covering a big range of the phylogenetic tree of the genus of *Pseudomonas*. The following *Pseudomonas* strain were tested, representing five out of nineteen subgroups spread all over the phylogenetic tree of the genus *Pseudomonas* based on four concatenated genes (Gomila *et al.*, 2015): *P.phaseolicola 1448A*, *P.syringae pv.tomato DC3000*, *P.syringae pv.syringae B278A*, *P.fluorescens SBW25*, *P.protegens PF-5* and *P.stutzeri DSM10701*. The CEX-purified fractions were afterwards tested in an ethylene assay and surprisingly all three tested *rlp30*-mutant lines were not able to respond to the fractions purified from the *Pseudomonas* culture medium, whereas Col-0 WT plants and *rlp30-2* plants complemented with *35S:RLP30-YFP* were able to respond to the tested fractions (Fig. 3.13 A). These findings indicated the presence of a novel bacterial elicitor now named PCFE1 (Pseudomonas Culture Filtrate Elicitor 1).

To further support the hypothesis that RLP30 together with its adaptor kinase SOBIR1 might be required for PCFE1 perception, *sobir1-12* mutant plants were tested in an ethylene assay and, as expected, did not react to the novel elicitor PCFE1 (Fig. 3.13 B).

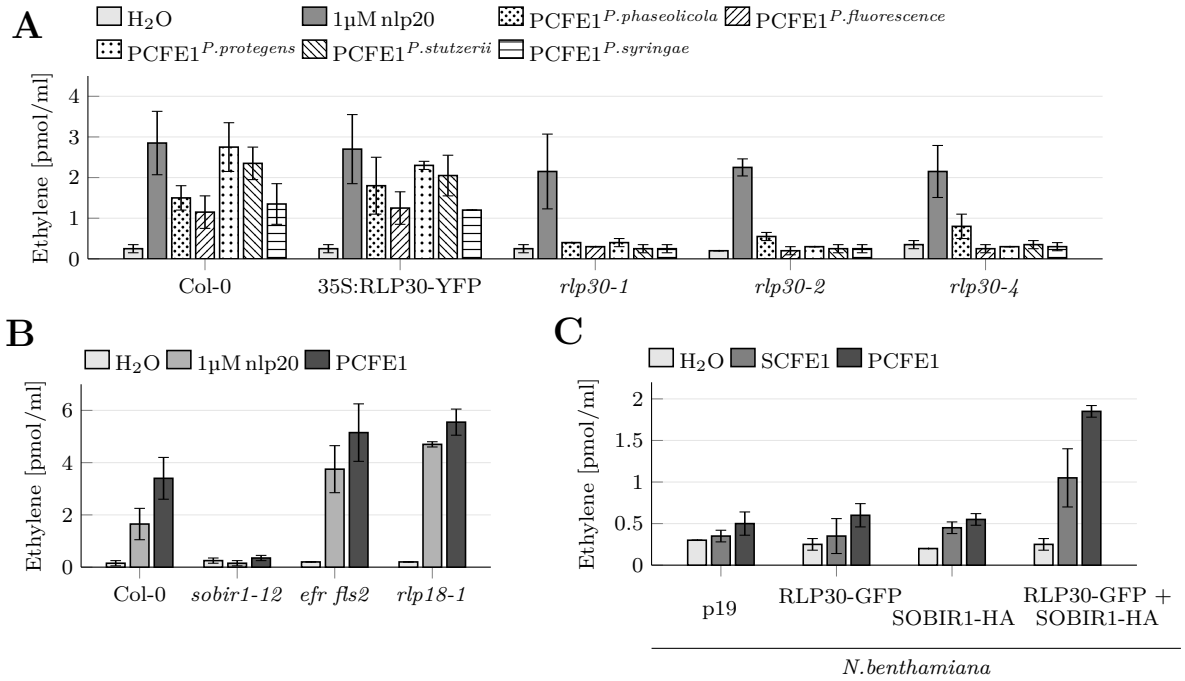

Figure 3.13: RLP30 is required for PCFE1 detection. Ethylene assays testing PCFE1 purified from different *Pseudomonas* strains with CEX-chromatography. 1 µM nlp20 served as positive control and ddH_2O as negative control. **(A)** PCFE1 is only detected in the presence of RLP30. Complemented *rlp30-2* mutant plants with *35S:RLP30-YFP* react like WT Col-0 towards PCFE1, whereas all tested *rlp30*-mutant alleles showed no response. A final dilution of 1:25 of PCFE1 was tested. **(B)** PCFE1 activity is also SOBIR1-dependent, as the *sobir1-12* mutant line shows no response, but not dependent on EFR, FLS2 or RLP18, as all of the respective mutant lines showed a WT-like response. A 1:100 dilution of PCFE1$^{P.syringaeDC3000}$ was tested. **(C)** Transient overexpression in *N.benthamiana* of RLP30-GFP together with SOBIR1-HA confers the detection of PCFE1. Single-expression or the expression of p19 does not lead to an ethylene response upon PCFE1 treatment. 1:100 SCFE1 and 1:50 PCFE1$^{P.syringaeDC3000}$ was tested. Accumulation of ethylene was measured after 5 hours. Bars represent average values ± S.D. (n=2).

It was also reported that *rlp18-1* mutant plants showed an increased susceptibility towards *Pseudomonas phaseolicola 1448A* (Wang *et al.*, 2008a). Because of that, the response of *rlp18-1* mutant plants towards PCFE1 was tested in an ethylene assay, however, no difference compared to Col-0 plants could be observed (Fig. 3.13 B). Additionally, the double mutant line *efr fls2* was tested as well. The response to PCFE1 was again comparable to Col-0 and

no major difference was detectable (Fig. 3.13 B), indicating that PCFE1 is not contaminated with flagellin or EF-T.

Also heterologous co-expression of RLP30-GFP together with SOBIR1-HA in *N.benthamiana* leaves led to a response to SCFE1 and PCFE1 in an ethylene assay, whereas only p19-infiltrated *N.benthamiana* plants or plants expressing only RLP30-GFP or SOBIR1-HA alone did not show any response towards SCFE1 or PCFE1 (Fig. 3.13 C).

Taken together, PCFE1 seems to be widely distributed within the genus of *Pseudomonas* and is recognized in a RLP30-dependent specific manner, likewise to SCFE1.

3.8.2 PCFE1 exhibits the same molecular properties like SCFE1

The biochemical properties of PCFE1 were determined exactly like it was described for SCFE1 in chapter 3.6.2. Firstly, the heat-stability of PCFE1 was tested by boiling the elicitor for 5 min at 95 °C before applying it in an ethylene assay. Neither boiling nor adding 0.1 % SDS to the elicitor could destroy its ethylene inducing activity (Fig. 3.14 A).

PCFE1 showed also the same behaviour towards all the tested proteinases and cyanogenbromide. Like it was observed for SCFE1, also PCFE1 lost its immunogenic activity after incubation with AspN, GluC, Trypsin, Proteinase K or cyanogenbromide, whereas the elicitor remained fully active after incubation with the respective buffers alone (Fig. 3.14 B).

Moreover, also PCFE1 showed the same property like SCFE1 with respect to the treatment with reducing agents. PCFE1 lost, like SCFE1 or Avr9, after extensive boiling or overnight incubation with DTT its ability to induce ethylene in wild type *Arabidopsis* Col-0 leaf pieces, whereas nlp20 showed no difference in its immunogenic activity after DTT treatment (Fig. 3.14 C, "with DTT"). Adding DTT without any pre-incubation to the respective elicitors

did not alter the accumulation of ethylene compared to the untreated samples (Fig. 3.14 C, "plus DTT").

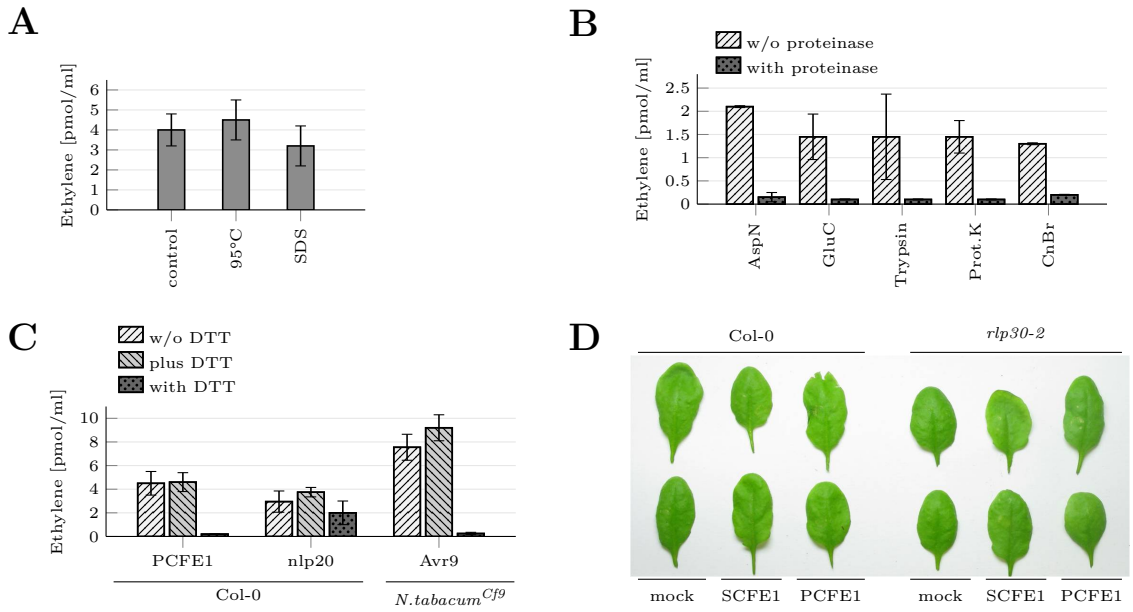

Figure 3.14: PCFE1 is a proteinaceous ligand with a peptide motif sufficient to trigger ethylene accumulation in *Arabidopsis* Col-0 plants. **(A)** PCFE1 is heat-stable and stable to SDS-treatment. PCFE1$^{P.syringaeDC3000}$ was boiled for 20 min with or without 0,1 % SDS and then tested in an ethylene assay on Col-0 leaf pieces with a final PCFE1$^{P.syringaeDC3000}$ dilution of 1:100. Unboiled PCFE1$^{P.syringaeDC3000}$ served as control. **(B)** PCFE1 activity can be destroyed with different proteinases. Boiled PCFE1$^{P.syringaeDC3000}$ was incubated O/N at 37 °C with or without different proteinases and their respective buffers. After heat inactivation of the enzymes the samples were tested in an ethylene assay with a final dilution of 1:160. **(C)** PCFE1-dependent activity can be destroyed with reducing agents. PCFE1$^{P.syringaeDC3000}$, nlp20 and Avr9 were boiled for 40 min at 95 °C with or without 5 mM DTT ("w/o DTT", "with DTT") and then subjected for an ethylene assay on Col-0, *rlp30-2* or *N.tabacum* Cf9 leaf pieces. Additionally, 5 mM DTT was added to the different elicitors without any pre-incubation ("plus DTT"). A final concentration of 1:100 PCFE1$^{P.syringaeDC3000}$, 1 µM nlp or 1:1000 Avr9 was used. Accumulation of ethylene was measured after 5 hours. Bars represent average values ± S.D. (n=2). **(D)** *Arabidopsis* Col-0 and *rlp30-2* mutant plants were infiltrated with either 50 mM MES-buffer, pH 5.4 (mock) or 1:20 SCFE1 or 1:20 PCFE1$^{P.syringaeDC3000}$. Pictures of two treated leaves were taken 2 days after infiltration.

Another feature of SCFE1 is its inability to induce an HR response or necrosis (Zhang *et al.*, 2013). *A.thaliana* Col-0 and *rlp30-2* mutant plants were infiltrated with either MES-buffer, as control, SCFE1 or PCFE1. PCFE1 is also inducing no HR-response or necrosis up to two days after infiltration into *Arabidopsis* Col-0 leaves as it is shown in Fig. 3.14 D.

Taken together, PCFE1 exhibited exactly the same molecular features like SCFE1. It is as well heat-stable and insensitive to SDS, can be destroyed via all tested

proteinases and looses its immunogenic potential upon the treatment with reducing agents.

3.8.3 SCFE1-insensitive *Arabidopsis* accessions are also insensitive to PCFE1

To learn more about putative ligand binding sites of PCFE1, the SCFE1-insensitive *Arabidopsis* accessions, described in chapter 3.5 were also tested in an ethylene assay for their ability to respond to PCFE1.

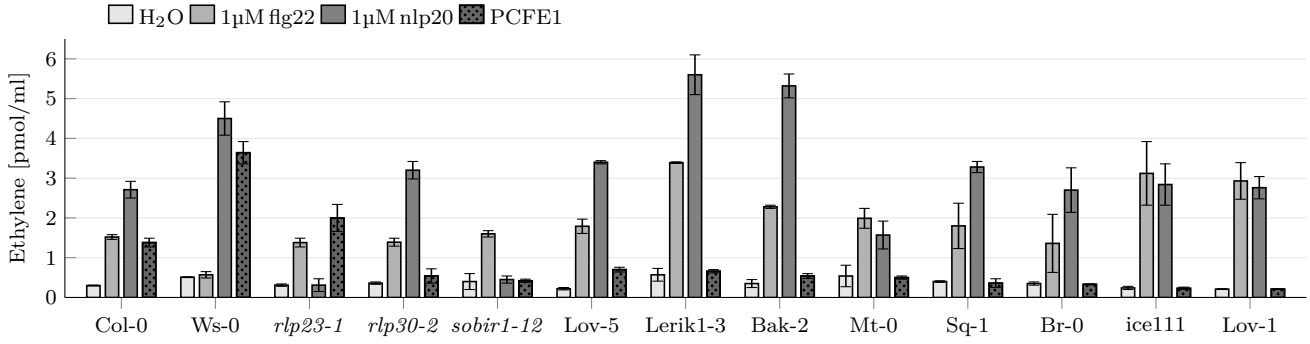

Figure 3.15: SCFE1-insensitive *Arabidopsis* accessions are also insensitive to PCFE1. Ethylene assay on leaf pieces of different *Arabidopsis* accessions and *rlp23-1*, *rlp30-2* and *sobir1-12* mutants with ddH_2O, or a final concentration of 1 µM flg22, 1 µM nlp20 or 1:50 PCFE1$^{P.syringaeDC3000}$. Accumulation of ethylene was measured after 5 hours. Bars represent average values ± S.D. (n=2). Experiment was performed by Marcel Conrady.

All of the tested accessions responded like WT Col-0 plants with respect to flg22 and nlp20 treatment, and no ethylene production could be induced by water treatment. Similarly to SCFE1, PCFE1 was also not detected by plants of the Lov-5, Lerik1-3, Bak-2, Mt-0, Sq-1, Br-0, ice111 and Lov-1 accession and the plants did not respond with the production of ethylene (Fig. 3.15, done by Marcel Conrady), indicating that SCFE1 and PCFE1 signaling is mediated in a similar way and moreover that SCFE1 and PCFE1 harbor the same immunogenic minimal motif, which is recognized by the same ligand binding site of RLP30.

3.8.4 SCFE1 is a fungal-derived MAMP

The occurrence of a MAMP across different kingdoms is a very rare event, and so far only described for NLPs (Böhm *et al.*, 2014b) and Ave1 (de Jonge *et al.*, 2012). To test whether SCFE1 is truly a fungal-derived protein, three different *S.sclerotiorum* cultures were prepared containing either mock, 100 µg/ml Carbenicillin, 100 µg/ml Streptomycin and 50 µg/ml Tetracycline or 50 µg/ml Cycloheximid. With these different set-ups a bacterial contamination of the *S.sclerotiorum* culture should be excluded.

As expected no fungal growth was observed in the culture containing the Cycloheximid, as it is a eukaryote protein synthesis inhibitor and subsequently no ethylene accumulation could be observed in the fractions purified out of this culture (Fig. 3.16).

In the mock treated culture and the culture containing the different antibiotics a more and less fungal growth could be observed, measured by the fresh weight of the *S.sclerotiorum* mycelium (18,5 g in the mock control vs 15,9 g in the antibiotic-treated culture). Additionally, no difference could be observed in accumulation of ethylene after treatment of *Arabidopsis* Col-0 leaf pieces with the different purified fractions (Fig. 3.16).

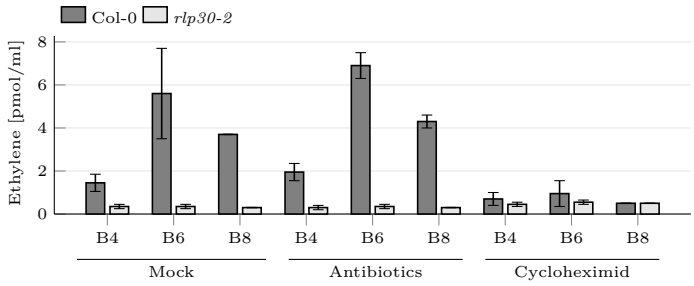

Figure 3.16: SCFE1 is a fungal-derived MAMP produced by *S.sclerotiorum*. Ethylene assay of Col-0 WT *Arabidopsis* plants or *rlp30-2* mutant plants. Plants were treated with corresponding fractions of differently treated *S.sclerotiorum* cultures with a final concentration of 1:66 and ethylene accumulation was measured after 5 hours. Bars represent average values ± S.D. (n=2).

Taken together, these results indicate that SCFE1 is truly a fungal-derived MAMP, as the addition of antibiotics did not alter the production of SCFE1 and therefore the ethylene-inducing activity of the antibiotica-treated culture. This experiment excluded the contamination of the *S.sclerotiorum* stock with bacteria.

4 Discussion

4.1 RLP30-dependent signaling and complex formation

4.1.1 RLP30, SOBIR1 and BAK1 form a ligand-dependent tripartite complex

In the last years an increasing number of ligand-receptor pairs have been identified. Several LRR-RLKs and LRR-RLPs have been shown to be required for the perception of proteinaceous ligands (Monaghan and Zipfel, 2012; Böhm *et al.*, 2014b). One of those pairs is the ligand SCFE1 (Sclerotinia culture filtrate elicitor 1) from *Sclerotinia sclerotiorum* and its receptor RLP30 from *Arabidopsis thaliana* (Zhang *et al.* (2013) and this thesis). Additionally, a novel bacterial elicitor derived from *Pseudomonas* is also recognized in a RLP30-dependent manner and therefore termed PCFE1 (Pseudomonas culture filtrate elicitor1) (chapter 4.3).
RLP30 is indispensably required for the recognition of both SCFE1 (Zhang *et al.*, 2013) and PCFE1 (Fig. 3.13). In addition, it was shown that both, SOBIR1 and BAK1 are required to establish an immune response in an infection assay with *S.sclerotiorum* or *B.cinerea* or in an immune assay, like the SCFE1-/PCFE1-dependent production of ethylene (Zhang *et al.* (2013), and this study). But SOBIR1 and BAK1 are not only required for RLP30-mediated signaling, but rather are associated with most, if not all, RLPs involved in at least plant immunity (Gust and Felix, 2014). Furthermore, complex formation or the requirement of more than one receptor-like protein or kinase is not restricted to LRR-type receptors, but

occurs also with receptors carrying LysM-domains. The RLPs LYM1 and LYM3 are together with the LysM-RLK CERK1 required to mediate peptidoglycan-recognition in *Arabidopsis* (Willmann *et al.*, 2011). And in rice the RLP CEBIP (CHITIN ELICITOR BINDING PROTEIN) dimerizes upon chitin recognition and recruits CERK1 (Hayafune *et al.*, 2014). Complex formation of receptors is also not only restricted to signaling during PTI, but it was also shown that perception of the CLAVATA3-peptide requires the RLK CLAVATA1, the RLP CLAVATA2 and the kinase CORYNE (Bleckmann *et al.*, 2009), similarly the IDA-peptide is recognized by the RLKs HAESA and HAESA-LIKE2, which together with SOBIR1 and the RLCK CAST AWAY mediate in a so-far not completely understood way floral organ abscission (Gubert and Liljegren, 2014).

Despite complex formation seemed to be a common mechanism during signal recognition, it remained elusive how the three signaling components, RLP30, SOBIR1 and BAK1 work and interact with each other. The results discussed in this chapter are partially published in Albert *et al.* (2015). Different scenarios would be possible, how RLP30, BAK1 and SOBIR1 interact with each other, both in the absence and presence of the ligand SCFE1. One possibility would be that all three proteins are already interacting within a preformed complex and get only activated upon ligand binding. Other options would involve the recruitment of one or both RLKs to RLP30 upon ligand recognition, like it was shown for FLS2 and BAK1 upon flg22-binding (Chinchilla *et al.*, 2007; Schulze *et al.*, 2010). A third scenario would be the dissociation of an already existing complex, to allow new interactions or activation of one or all of the partners, similar to a mechanism introduced for BIR2, which is released from BAK1 upon flg22-perception allowing only then interaction with FLS2 (Halter *et al.*, 2014a). Transient overexpression in *N.benthamiana* of the tagged proteins (RLP30, SOBIR1, BAK1) followed by pull-down experiments and Western Blotting allowed us to study the temporal complex formation of RLP30, SOBIR1 and BAK1 both in the absence and presence of SCFE1. RLP30 and SOBIR1 were found to constitutively interact with each other independent of

SCFE1 and independent of the pulldown target, SOBIR1 or RLP30 (Fig. 3.1). This finding is in line with a couple of previous results, all indicating a constitutive interaction of SOBIR1 with different RLPs. Two groups independently found in their pulldown-experiments RLP23 and SOBIR1 interacting with each other in a ligand-independent manner (Bi *et al.*, 2014; Albert *et al.*, 2015). And also co-immunoprecipitations assays analyzed by Western Blot analyses showed an interaction between SOBIR1 and RLP42 (Zhang *et al.*, 2014) and also in *solanaceous* plants *Sl*SOBIR1 is interacting with RLPs, as it was shown for Cf4 and Ve1 (Liebrand *et al.*, 2013). Ratiometric bimolecular fluorescence complementation assays (BiFC) performed in transgenic *N.benthamiana* plants and transiently transformed *Arabidopsis* protoplasts revealed a physical interaction between RLP23 and SOBIR1 in the absence of the ligand nlp20 (Albert *et al.*, 2015). The interaction between SOBIR1 and the different RLPs is in general thought to be mediated via the GxxxG-motif, a so-called glycine zipper, located within the transmembrane region of SOBIR1 and the RLP (Cymer *et al.*, 2012; Fink *et al.*, 2012). Bi *et al.* (2016) showed that the glycine motif is indispensable for the interaction of SOBIR1 with Cf4, Ave1, *Sl*CLV2 and *Le*EIX2. Additionally, the interaction between SOBIR1 and RLPs could be supported via the oppositely charged outer juxtamembrane regions of SOBIR1 and RLPs. In SOBIR1 the outer juxtamembrane region is highly enriched with lysines, whereas in RLPs a lot of acidic amino acids like aspartic and glutamic acid are located (Gust and Felix, 2014). Interestingly, there are differences in the binding preferences of different RLPs with the *N.benthamiana* SOBIR1. RLP23 is fully functional, when expressed in *N.benthamiana* (Albert *et al.*, 2015), whereas RLP30 or RLP1/ReMAX seem to need the *Arabidopsis* version of SOBIR1 to be responsive towards their respective ligands, SCFE1/PCFE1 and eMAX (Zhang *et al.* (2013); Jehle *et al.* (2013), Fig. 3.2 G).

In contrast to the ligand-independent interaction of SOBIR1 and RLPs, BAK1 is only recruited to the SOBIR1/RLP30 complex in the presence of SCFE1 (Fig. 3.1). The

co-immunoprecipitation experiment with SOBIR1 being precipitated led to a faint band on the Western Blot representing BAK1 in the absence of the ligand SCFE1, suggesting that BAK1 is already in close proximity to the SOBIR1/RLP30 complex, in a kind of pre-assembled complex, that gets glued together in the presence of the respective ligand. The idea of clustered signaling components within certain areas is not new and for BAK1 and FLS2 it was suggested that they are localizing in lipid rafts (Ali *et al.*, 2007) or are clustering around plasmodesmata (Faulkner, 2013). Furthermore it was shown that the interaction between BAK1 and FLS2 after ligand binding occurs almost instantaneously (Schulze *et al.*, 2010), suggesting that BAK1 and FLS2 are already in very close proximity. The idea of a ligand working as a molecular glue between a PRR and the adaptor-kinase BAK1 was proven by Sun *et al.* (2013) resolving the crystal structure of the FLS2- and BAK1-LRR domain both binding flg22. Additionally, it was shown in a gel filtration experiment that FLS2 and BAK1 ectodomains are sufficient to form a monomeric heterodimer induced by flg22, which was also shown with the heterologously expressed ectodomains of BAK1 and RLP23, which co-migrate and co-elute in the presence of the nlp20 ligand in a gel-filtration experiment (Albert *et al.*, 2015). This indicates a similar mechanism both for RLKs and RLPs with regard to the interaction with BAK1.

Combining the available evidence so far, I would suggest that RLP30, SOBIR1 and BAK1 form a ligand-dependent heterotrimeric complex with a protein ratio of 1:1:1, with a direct constitutive interaction of RLP30 and SOBIR1 as well as a direct ligand-mediated interaction of RLP30 and BAK1. The remaining question is the interaction between SOBIR1 and BAK1. In the BiFC assays mentioned before no interaction between the LRR-RLK EFR and SOBIR1 was detected (Albert *et al.*, 2015), indicating a binding specificity of SOBIR1 for RLPs. Additionally, SOBIR1 is not required in RLK-mediated signaling, indicating that a SOBIR1-BAK1 interaction is not required for further downstream signaling.

Taken together, it could be shown in a heterologous overexpression experiment that RLP30

and SOBIR1 constitutively interact and that BAK1 is recruited in a ligand-dependent manner into the complex.

4.1.2 The bimolecular receptor complex of RLP30 and SOBIR1 can be fused to a genuine RLK-protein

RLP30 and SOBIR1 are interacting in a constitutive manner (chapter. 4.1.1). This is not only true for RLP30, but also for other so-far identified RLPs, like RLP23 and RLP42 in *Arabidopsis* or Cf4 in tomato (Albert *et al.*, 2015; Zhang *et al.*, 2014; Liebrand *et al.*, 2013). Gust and Felix (2014) compare the RLP/SOBIR1 complexes with genuine RLKs, like FLS2. The idea is that the RLP provides the extracellular domain for ligand-binding specificity, whereas the kinase-domain of SOBIR1 is required for signaling. Additional evidence for this hypothesis is provided by the observation that RLP- and RLK-mediated immunity seem to share at least most of the downstream signaling cascade (Tang *et al.*, 2017), indicating that RLPs are only split-up RLKs. Following the idea of this model, fusing the SOBIR1-kinase domain with the LRR-domain of RLP30 should lead to an RLK that can perceive SCFE1. It was already shown that receptor fusion constructs are still working in domain swapping experiments (Albert *et al.*, 2010). Therefore, different chimeric fusion constructs between SOBIR1 and RLP30 were generated and transiently overexpressed in *N.benthamiana* and their functionality was tested in an ethylene assay (Fig. 3.2). Controls with RLP30 or SOBIR1 solely expressed did not result in an ethylene response towards SCFE1. Only when both proteins were co-expressed an SCFE1-dependent ethylene-response was measurable. This indicated that RLP30 can only interact with the *Arabidopsis* version of SOBIR1-protein and not with the *N.benthamiana* homologues *Nb*SOBIR1 and *Nb*SOBIR1-like.

The generated chimeric constructs differ in the localization of their respective fusion site (Fig. 3.2 A-F). All of the generated fusion constructs gave an SCFE1-dependent ethylene-response,

except RLP30^{LRR}-S$_{all}$. In this construct on top of the full-length SOBIR1-protein all 21 LRR-repeats of RLP30 were fused. As already shown, not only SOBIR1 is required for RLP-mediated signaling, but also BAK1, both in RLP- and RLK-mediated signaling (Chinchilla *et al.*, 2007; Zhang *et al.*, 2013; Albert *et al.*, 2015). As discussed in chapter 4.1.1, RLP30, SOBIR1 and BAK1 form a tripartite complex with most likely a direct interaction between the LRR-domains of RLP30 and BAK1, glued together by the ligand SCFE1, therefore it is possible that in the RLP30^{LRR}-S$_{all}$ fusion construct steric hindrance of the now very long extracellular domain or other spatial constrictions prevent a sensing of SCFE1.
The fusion construct with 5 RLP30-LRR-repeats less seemed to be functional (RLP30$^{LRR1-16}$-S$_{all}$). All of the other fusion constructs are functional, leading to an SCFE1-dependent increase in the production of ethylene (Fig. 3.2 G).
Of course, in this set-up it can never be excluded, that the chimeric constructs are not really functional, but that the signal transduction after perception of SCFE1 occurs via an interaction of the fusion constructs and the endogenous *Nb*SOBIR1 or *Nb*SOBIR1-like, rather than be transmitted via the chimeric fusion construct. To test this hypothesis *Arabidopsis rlp30 sobir1* double mutants have been generated and stably transformed with the described chimeric fusion constructs. Unfortunately, due to time constrains the transformed plants could not yet be tested to prove the hypothesis that an RLK is nothing more than an RLP with a kinase domain.

4.1.3 SCFE1/PCFE1-insensitive *Arabidopsis* accessions reveal putative interaction sites

The usage of different *Arabidopsis* accessions, together with the available sequence information, provided on www.1001genomes.org, is a powerful tool to identify natural occuring variations and mutations within the model plant *Arabidopsis thaliana*. The receptor RLP30 was

identified by screening available accessions and identifying insensitive ones (Zhang *et al.*, 2013).

All of the identified SCFE1/PCFE1-insensitive accessions were still able to respond like WT Col-0 upon the stimulation with nlp20 (perceived by RLP23) or flg22, excluding the possibility of any defects within the ethylene signaling or production cascade, especially of the RLP-dependent cascade. Having a closer look on the *RLP30*-gene locus and comparing the gene sequences of SCFE1/PCFE1-sensitive with insensitive accessions, revealed unique single nucleotide polymorphisms (SNPs) within the *RLP30*-gene, leading to amino acid substitutions or premature stop codons (Zhang *et al.* (2013), and Fig. 3.7). There were several additional SNPs present, also leading to amino acid exchanges, but those were not unique to the insensitive accessions but were also found in SCFE1-sensitive ones, leading to the conclusion that they are not impairing the proper function of RLP30.

Other genes/proteins were not checked, as the SCFE1/PCFE1-insensitive accessions behaved like Col-0 with respect to the ethylene response upon treatment with nlp20 or flg22, excluding mutations e.g. in the *SOBIR1* or *BAK1* gene.

For seven out of the eight identified SCFE1/PCFE1-insensitive accessions, unique SNPs could be identified, which most likely led to their insensitivity. The Lerik1-3 accession has a SNP, changing a serine on position 12 to a premature stopcodon, resulting in the lack of a functional full-length RLP30-protein.

The Bak-2 accession has no unique SNPs, which would lead to distinct amino acid exchanges. One possibility is that the combination of identified SNPs would diminish ligand binding. On position 83 a glutamic acid residue is changed to a lysine and the phenylalanine residue on position 599 is changed to serine. But these amino acid exchanges are also present in accessions which are sensitive to SCFE1 or PCFE1. The co-immunoprecipitation experiment showed that $RLP30^{Bak\text{-}2}$ is still able to interact with SOBIR1 (Fig. 3.7 C). Another possibility is that additional SNPs are present in the promoter region of RLP30, altering the expression

levels of RLP30$^{\text{Bak-2}}$. Analysis of expression levels of RLP30 in the Bak-2 accession or transient overexpression in *N.benthamiana* followed by an ethylene assay would help to clarify whether Bak-2 is insensitive to SCFE1 and PCFE1 because of altered protein expression or an altered protein structure.

The other SNPs that lead to amino acid substitutions can be divided into three different groups, based on their localization within the RLP30-protein: firstly, destruction of the putative ligand binding site, secondly, putative messed-up overall structure and thirdly, putative loss-of-interaction with co-receptors like BAK1 or SOBIR1. The first two possibilities are discussed in chapter 4.3.1.

Notably, all tested protein variants of RLP30 (Bak-2, ice111, Lov-1 and Mt-0), when transiently expressed in *N.benthamiana*, could still interact with SOBIR1, except of Sq-1 (Fig. 3.7 C). The mutation in the Sq-1 accession changes a serine at position 654 to a tyrosine residue and in the Br-0 accession a glycine at position 563 is changed to a valine. These two positions are located close to the plasma membrane and might make contact to the much shorter LRR-domains of SOBIR1 and BAK1, which have only 5 LRR-repeats. RLP30$^{\text{Sq-1}}$ could never be detected on a Western Blot. Neither changing the tag of the expression construct, nor re-cloning of the sequence led to a successful expression of RLP30$^{\text{Sq-1}}$ in *N.benthamiana*. Our hypothesis is that the point mutation S 654 Y in the Sq-1 accession prevents an interaction of RLP30 with its co-receptor SOBIR1. SOBIR1 was shown to be required for the localization and stabilization of PRRs at the plasma membrane. Liebrand *et al.* (2013) showed that co-silencing of SOBIR1-homologues in *N.benthamiana* reduces the protein levels, but not the expression levels of transiently overexpressed Cf4 and Ve1 constructs. Additional proof of this theory was provided by Dr. Weiguo Zhang in his dissertation. He described, besides a membrane localization of transiently overexpressed RLP30-GFP in *N.benthamiana*, also a localization of RLP30-GFP around the cell nucleus, which is drastically decreased in the presence of overexpressed SOBIR1-HA (Zhang, 2013).

Alternatively, the S 654 Y mutation might lead to an overall instability of RLP30, possibly by introducing an additional, artificial phosphorylation site. In addition to the already discussed possibilities, it is also possible that a ligand-induced interaction with BAK1 is diminished in the insensitive accessions which would also explain the lack of an SCFE1/PCFE1-induced ethylene response. Further experiments are necessary to prove this hypothesis, for example using pull down assays of transiently expressed RLP30^{accession}, SOBIR1 and BAK1 after incubation with SCFE1 or PCFE1.

So far only the expression levels for Lov-1, Lov-5 and Mt-0 have been shown to be like wild type level (Zhang *et al.*, 2013), for the other accessions the expression levels of RLP30 still remain elusive and need to be determined.

Taken together, the identified insensitive SNPs are a powerful tool to study interacting proteins like SOBIR1 and BAK1, as there are already hints towards important regions for putative interactions present and do not need to be identified by tedious deletion experiments. Additionally, it could be shown that SOBIR1 interaction of RLP30 seems to be required for the correct localization of RLP30 at the plasma membrane and that a diminished interaction of RLP30 and SOBIR1 could subsequently lead to a possible degradation or miss-localization of RLP30.

4.1.4 Known and unknown players in RLP signaling pathways

For a better understanding of plant innate immunity it is important to know more about the signaling mechanisms leading e.g. to RLP-mediated immunity. Two different approaches were taken to identify putative interactors or regulators of, especially RLP-mediated, PTI. The first approach was based on the publicly available Membrane-based Interactome Database (MIND) (Jones *et al.*, 2014a). We focused on predicted interacting proteins of RLP30 and/or SOBIR1. The second approach was to transfer already acquainted knowledge from other

signaling pathways to the PTI signaling pathway and examine respective proteins, for a putative role in PTI.

Eleven proteins were predicted to interact with both RLP30 and SOBIR1 and two proteins were additionally chosen that only interacted with RLP30 and one with SOBIR1 (see Table 3.1). As a positive control, the interaction of RLP30 and SOBIR1 was used, which was confirmed in a Western Blot in this study (see Fig. 3.1) and which is a reported interaction in the MIND-database. Among the identified and selected proteins several of them, or at least close homologs, were already described to play roles in PTI. One of the identified putative interactors of both RLP30 and SOBIR1 is WAK3, a cell wall-associated kinase (WAK) with an extracellular epidermal growth factor (EGF)-like domain (He *et al.*, 1999). WAKs are involved in the response to pathogens, like infection with *Pseudomonas syringae* or *Alternaria brassicicola* (He *et al.*, 1998; Schenk *et al.*, 2000) and are additionally upregulated during systemic acquired resistance (Maleck *et al.*, 2000).

Three proteins belonging to the superfamily of Thioredoxins were identified. In tomato the Cf9 interacting thioredoxin (CITRX) was found to be required for Cf9-mediated immune responses and to interact with Cf9 in a yeast two-hybrid screen (Rivas *et al.*, 2004). Moreover, CITRX acts as an adaptor protein for the Avr9/Cf9 induced kinase 1 (ACIK1) (Nekrasov *et al.*, 2006), which thereby interacts with Cf9, indicating a role of CITRX in the regulation of plant disease resistance in tomato. ACIK1 belongs to the large family of plant receptor-like cytoplasmic kinases (RLCKs), belonging to the family of AVRPPHB Susceptibel (PBS1)-like (PBL) proteins, to which also the BOTRYTIS INDUCED KINASE 1 (BIK1) belongs, which links PRR-complex activation to downstream signaling via phosphorylation of Respiratory burst oxidase homologue D (RbohD) (Kadota *et al.*, 2014) and activation of mitogen-activated protein kinases (MAPK) (Lu *et al.*, 2010a). The corresponding homologue of ACIK1 in *Arabidopsis* is PBL13 which was also shown to interact with RbohD and the interaction is disrupted upon flagellin-induced immune signaling (Lin *et al.*, 2015).

The MIND-database is an unbiased database reporting only observed interactions of membrane-bound or signaling proteins and can contain false positive interactions. To minimize the likelihood of such false positive results the identified proteins were double checked, if other LRR-RLPs are also predicted to be interacting partners, indicating a broader, general role of the identified protein in RLP-dependent signaling. Interestingly, always the same RLPs appeared in the lists of putative interactors, namely RLP16, 34, 53 and 55, besides RLP30. A lot of LRR-RLPs could not be found at all in the MIND-database, maybe indicating that they have not been tested yet and explaining why already published examples like RLP23 or RLP42 are not listed in the MIND-database.

For all the putative RLP30 and/or SOBIR1 interacting proteins identified in the MIND database, T-DNA insertion lines were characterized in immune assays (table 3.1). However, none of the studied homozygous T-DNA insertion lines showed a phenotype different from wild type *Arabidopsis* Col-0 plants in an ethylene assay or a ROS burst assay after treatment with flg22, nlp20 or SCFE1. On one hand these results suggest that the studied proteins are not involved in the signaling cascade of PTI and the production of ROS or ethylene, but on the other hand it can not be excluded, that those proteins belong to larger families of proteins with redundant functions. In the latter case multiple knockouts or amiRNA-lines, targeting the whole gene family or at least close homologues, would be necessary to study the role of these proteins in PTI.

Taken together, the MIND-database is a powerful resource, but the reported interactions should be handled with the necessary respect and carefulness. Every heterologous expression system has the potential to deliver false-positive results. And even a proven interaction does not have to have an impact on the studied biological process.

The second approach was looking at other RLP-mediated signaling pathways, in this case floral organ abscission and checking selected proteins involved in this process for their role in PTI. This idea is based on the finding that SOBIR1 does not only have a role as an adaptor

kinase in PTI for RLPs like RLP23, RLP30 or Cf9, but was also described as negative regulator in floral organ abscission restoring the *NEVERSHED (NEV)*-mutant phenotype, hence its second name EVERSHED (Leslie *et al.*, 2010).

NEV/ AGD5 belongs to the family of ADP-ribosylation factor-GTPase-activating protein (ARF-GAP) domain containing proteins (AGD), containing 15 members in *Arabidopsis* (Liljegren *et al.*, 2009). Key regulators of membrane trafficking are the small ADP-ribosylation factor (ARF) G-proteins, which are regulated via ARF-guanine exchange factors (ARF-GEFs) that activate ARFs, whereas ARF-GAPs promote the inactivation of ARFs by stimulating GTP hydrolysis (D'Souza-Schorey and Chavrier, 2006). *Nev*-mutants are not able to shed their outer floral organs after fertilization (Liljegren *et al.*, 2009). It was shown that NEV localizes to the trans-Golgi network and endosomes in *Arabidopsis*, suggesting that NEV is involved in the recycling and trafficking of cargo molecules (Liljegren *et al.*, 2009). For the *agd5/nev*-mutants no consistent phenotype in the ethylene assay upon stimulation with SCFE1, PCFE1 or nlp20 could be determined so far and further experiments with additional T-DNA insertion lines are required to determine a putative role of NEV in RLP-mediated signaling.

The closest homologue of NEV is AGD15. The *agd15*-mutants showed in a preliminary first experiment an increased ethylene production in response to treatment with SCFE1 compared to wild type Col-0 *Arabidopsis* plants. It is possible that NEV and/or AGD15 are required for localization of RLPs at the plasma membrane or these two proteins might have an important function during internalization or recycling of RLPs. Interestingly, Schmidt *et al.* (2014) showed that AGD15 and its closest homologue AGD5/NEV are conserved targets of powdery and downy mildew effectors and that mutants of *AGD15* and *AGD5/NEV* are more susceptible towards the obligate biotrophic, non-adapted powdery mildew pathogen *Erysiphe pisi* and a virulent isolate of the adapted oomycete *Hyaloperonospora arabidopsidis* (Schmidt *et al.*, 2014). Taken together, our previous observations of an altered phenotype of *agd15* and

agd5/nev mutants in an ethylene assay and the report from Schmidt *et al.* (2014) of those two proteins being conserved effector targets could hint towards a role of AGD5/NEV and AGD15 in RLP-mediated PTI. Further experiments are required to determine the function of those two proteins in RLP-mediated immune responses.

Another protein important during floral organ abscission is CAST AWAY (CST), a cytoplasmic receptor-like kinase (Burr *et al.*, 2011). CST interacts at the plasma membrane with SOBIR1/EVERSHED and may be involved in the regulation or localization of a ligand-binding receptor (Burr *et al.*, 2011). To test CST in an ethylene assay only one mutant line containing a point-mutation in the *cst*-gene in the *Landsberg erecta* background was available and this line showed a reduced response after treatment with flg22, nlp20 or SCFE1 compared to the Ler control plants. To rule out the possibility of side effects of unknown mutations in the *cst1*-mutants it is necessary to test further mutant lines, which were not homozygous to date. Like it was shown for floral organ abscission CST may play a role in sequestering receptors at the plasma membrane, a similar function for CST may be conceivable during PTI responses and the absence of CST could lead to an increased internalization of plasma-membrane located receptors, resulting in decreased responsiveness towards extracellular triggers like the tested MAMPs. For stem cell niche maintenance it was shown that the cytoplasmic kinase CRN is required for stabilization and recruitment of CLV2 to the plasma membrane (Bleckmann *et al.*, 2009). A similar role could be also possible for CST. Additionally, it was shown in BiFC assays that CST interacts with both SOBIR1 and the receptor-like kinase HAESA (HAE) (Burr *et al.*, 2011). The redundant RLKs HAE and HAESA-LIKE2 (HSL2) are activating organ abscission in *Arabidopsis* flowers after binding of the peptide INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) by switching on a MAP kinase cascade (Jinn *et al.*, 2000; Cho *et al.*, 2008). It is very surprising that SOBIR1 is found in this complex, as HAE and HSL2 are both RLKs usually not requiring the adaptor kinase SOBIR1. This could indicate that a yet unknown RLP is additionally required in the process

of floral organ abscission, further strengthening an involvement of CST in RLP-mediated PTI.

In summary, the two proteins NEV (or its homolog AGD15) and CST, which were implicated in floral organ abscission, also involving SOBIR1, might indeed also have a function in plant immunity. However, further experiments are required to establish a proposed role of these proteins during PTI. Nevertheless, having a closer look on other signaling pathways may help to identify new regulators or at least add additional roles to already described proteins.

4.2 Making plants "see" - Transferring disease resistance to crop plants

Plant diseases cause tremendous losses in agriculture worldwide. Until now, the mostly used way to combat pests is by applying agrochemicals, which are not only poisoning the environment but cause even today up to 300 000 deaths worldwide (Ronald, 2011). This is one of the reasons why more sustainable methods are required (Baulcombe *et al.*, 2009). One already successfully applied approach in the lab is strengthening the plant's immune system by transferring resistance genes or pattern recognition receptors. This was already shown for the transfer of *Arabidopsis* EFR into *solanaceous* species, like *N. benthamiana* or tomatoes, conferring enhanced resistance towards a huge range of bacterial pathogens like *Pseudomonas, Agrobacterium, Xanthomonas* or *Ralstonia* (Lacombe *et al.*, 2010) or for the *Arabidopsis* RLP23 into potato enhancing resistance to *Phythophthora* or *Sclerotinia* (Albert *et al.*, 2015). The advantage of PRRs compared to classical resistance genes lies within the definition of their ligands: PAMPs or MAMPs are usually very conserved and essential for the lifestyle of the pathogen, thus reducing the likelihood of a fast evolution of the pathogen

to evade host immunity through mutations, compared to classical effector proteins. Stam and McDonald (2017) describe how even resistance gene pyramids can easily be overcome by pathogens and that R-genes are unlikely to provide a durable control for many pathogens. Another advantage of this transgenic approach compared to classical breeding strategies is the ability to quickly introduce identified genes in elite varieties or apply it to crops, which are not amenable to classical breeding approaches, like banana (Lacombe *et al.*, 2010).

This thesis describes another example of a successful transfer of an *Arabidopsis* PRR into *solanaceous* species, namely *N.tabacum* and tomato. However, a single transfer of the *Arabidopsis* RLP30 would not have been effective, as RLP30 is not able to interact with the *solanaceous* SOBIR1 or SOBIR1-like protein. Therefore, it was necessary to transfer additionally the *Arabidopsis SOBIR1* gene. A functional immune assay, in this case the production of ethylene as well as an infection experiment with *Sclerotinia sclerotiorum* or *Botrytis cinerea* revealed an RLP30-dependent recognition of SCFE1 and decreased infection symptoms compared to untransformed plants (Fig. 3.4, 3.5). These results broadens the spectrum of available PRRs that can confer resistance to crops.

It was already shown that functional stacking of broad spectrum resistance genes could be an effective strategy for more durable resistance, for example, to potato late blight caused by *Phytophthora infestans* (Zhu *et al.*, 2012). The use of one single vector containing all three resistance genes, leads presumably to the insertion at only one locus. This is very useful for breeders, as these plants can be used as parental lines to transfer all resistance genes simultaneously into offspring plants (Zhu *et al.*, 2012).

The same could be now also achieved by not only transferring one PRR to crop plants with the required co-receptors, but by also stacking different PRRs. The devastating plant pathogens *S.sclerotiorum* and *B.cinerea*, for example, harbor not only SCFE1, but also the nlp20-motif (this thesis and Böhm *et al.* (2014b)), thus the transfer of both RLP30 and RLP23 could significantly increase the resistance to those pathogens.

But overexpression of proteins can also lead to unwanted side-effects. Some of the transgenic tomato plants expressing SOBIR1 and RLP30 under the control of the *35S*-Promoter are really dwarfed and fruit and seed setting is heavily impaired (chapter 3.3.1), characteristics which are of course unwanted by breeders for crop plants. The tobacco plants on the other side, do not show any dwarf phenotypes. These differences might be due to different expression levels of SOBIR1 and/or RLP30. One way to circumvent the problem could be by using the endogenous promoters of SOBIR1 and RLP30, in order to reduce protein levels. SOBIR1 is involved in so many different regulatory cell processes, like innate immunity or floral organ abscission that overexpression might easily disturb important regulatory pathways.

These results can be seen as a proof-of-concept, that not only the PRRs should be transferred to a new host plant, but that it might be required to provide additional co-receptors or adaptor proteins. The increasing knowledge of involved proteins in PRR signaling opens the field for the transfer of resistance also to very distantly related plant families.

Another interesting lesson learned from those experiments is the fact that it is possible to transfer more than one gene into a target plant at the same time, opening the field for multiple PRR-gene-stacking. This raises the chances to prevent a fast evolution of resistance in the pathogens by spreading the evolutionary pressure on more than one gene resulting in more durable resistance.

4.3 SCFE1 and PCFE1 - two cross kingdom MAMPs being recognized by the same receptor RLP30

4.3.1 PCFE1 is a novel bacterial MAMP perceived in an RLP30-dependent manner

Susceptibility or resistance of a plant towards a certain pathogen is dependent on the ability of the plant to recognize the invading pathogen. Therefore, the plant needs to be equipped with the right set of PRRs recognizing the corresponding PAMPs or MAMPs displayed by the pathogen. It was reported that *rlp30*-mutant plants not only show a higher susceptibility towards *S.sclerotiorum* and *B.cinerea* (Zhang *et al.*, 2013), but also towards the non-adapted bacterial pathogen *Pseudomonas phaseolicola 1448A* (Wang *et al.*, 2008a). Based on that knowledge, culture filtrates of different *Pseudomonas* strains were subjected to cation-exchange chromatography (CEX), similar to SCFE1-purification. The purification was performed using new CEX-columns, which were exclusively used for the purification of the bacterial culture filtrate.

All of the tested, purified fractions were able to stimulate the production of ethylene in Col-0 *Arabidopsis* plants (Fig. 3.13 A), as well as in *efr fls2* double mutant plants, excluding a dominating contamination with the well-known bacterial elicitors flagellin and elongation factor TU (Fig. 3.13 B).

Furthermore, *sobir1-12* mutant plants showed no response in an ethylene assay upon the stimulation with the partially purified bacterial fractions (Fig. 3.13 B), indicating that a SOBIR1-dependent RLP is required for the perception of this novel bacterial elicitor.

Wang *et al.* (2008a) described two *rlp*-mutant lines, *rlp18* and *rlp30* that showed a higher susceptibility towards the infection with *P.phaseolicola*. In an ethylene assay with the partially purified bacterial fractions, *rlp18-1* mutant plants exhibited a WT-like response

(Fig. 3.13 B), whereas all three tested *rlp30*-mutant alleles (*rlp30-1*, *rlp30-2* and *rlp30-4*) showed no ethylene production after elicitation with the novel bacterial elicitor (Fig. 3.13 A). Complementation of an *rlp30*-mutant line with an overexpressing *35S:RLP30-YFP*-construct restored the responsiveness towards the partially purified bacterial culture filtrate in an ethylene assay, as did the transient co-expression of RLP30-GFP together with SOBIR1-HA in *N.benthamiana*.

Taken together, these results indicate a clear RLP30-dependency of the perception of the novel partially purified *Pseudomonas* culture filtrate elicitor. Due to the same purification protocol applied for both, the fungal SCFE1 and this novel bacterial elicitor, in addition to the RLP30-dependent recognition of both ligands, this partially purified activity was named Pseudomonas Culture Filtrate Elicitor 1 (PCFE1).

SCFE1 induces typical immune responses in *A.thaliana* such as ethylene production, ROS burst, MAP kinase activation and induction of defense-related genes, but no induction of a hypersensitive response (HR) (Zhang *et al.*, 2013). Also for PCFE1 the induction of ethylene was shown and the inability to induce an HR-response in *Arabidopsis* (Fig. 3.14 D). Further experiments are required to investigate whether PCFE1 is also able to induce all the other known immune responses showed for SCFE1.

Until now, the proof of a direct physical interaction between RLP30 and SCFE1 or PCFE1 is still missing. To date a physical interaction of an LRR-RLP and its ligand was only shown for the tomato *Sl*EIX2/ EIX (ethylene inducing xylanase) (Ron and Avni, 2004), the RLP23/ nlp20 (Albert *et al.*, 2015) and the RLP42/ endopolygalacturonase PG3 (Zhang *et al.*, 2014) receptor-ligand pairs. However, for the tomato Cf9 receptor no direct binding of its ligand Avr9 could be observed (Luderer *et al.*, 2001). But still, Cf9 confers resistance to *C.fulvum* strains that carry the avirulence gene *Avr9* (Jones *et al.*, 1994). These findings lead to the hypothesis of at least one additional player involved in the binding of Avr9, which is only present in *solanaceous* plants (Kooman-Gersmann *et al.*, 1996). These so called HABS

(high affinity binding sites) are present in plasma membranes purified from tomato leaves of near-isogenic lines (NILs), independent of the presence of the Cf9-resistance gene as binding studies with radio-labeled Avr9 indicated (Luderer *et al.*, 2001). However, the nature of these HABS still remain elusive.

At the moment, for RLP30 no conclusion is possible whether it physically interacts with SCFE1 or PCFE1. It is tempting to hypothesize that SCFE1 binds directly to RLP30, because firstly the heterologous overexpression of the *brassicaceous At*RLP30 together with *At*SOBIR1 in *solanaceous N.benthamiana* plants confers recognition of SCFE1. This was also successfully tested for RLP23 and RLP42 (Albert *et al.*, 2015; Zhang *et al.*, 2014), whereas so far a recognition of Avr9 in *A.thaliana* expressing the *solanaceous* Cf9 was never achieved, further strengthening the hypothesis of HABS-involvement in the Cf9-Avr9-perception system. Such HABS are either not required for the perception of nlp20, PG3, SCFE1 or PCFE1 by RLP23, RLP42 or RLP30, respectively, or they are conserved between *solanaceous* and *brassicaceous* plants.

Secondly, different SNPs in the extracellular domain of RLP30 from different *Arabidopsis* accessions have been identified (chapter 3.5 and 3.8.3), which render the respective accessions insensitive towards both SCFE1 and PCFE1. All of these accessions were still able to sense flg22 or nlp20 in a Col-0-like manner, indicating that these accessions are still able to respond to external stimuli with the accumulation of ethylene. By comparing the coding sequences of the *RLP30*-gene of the different insensitive accessions with Col-0 and with the available sequence information provided on the *1001 Genomes Project*-webpage (www.1001genomes.org), several unique SNPs could have been identified leading to amino acid exchanges in the LRR-domain of RLP30. Some of them may present mutations in the ligand-binding sites of RLP30, because co-immunoprecipitation assays showed that for RLP30^{ice111}, RLP30$^{Lov\text{-}1}$ and RLP30$^{Mt\text{-}0}$ an interaction with SOBIR1 was still possible, after transient expression in *N.benthamiana*, indicating that the insensitivity is not due to impaired complex formation with SOBIR1 (also

see chapter 4.1.3).

The Mt-0 accession has on position 307 a leucine changed to an arginine compared to Col-0. This amino acid residue lies within LRR9 of RLP30 (www.uniprot.org). Based on a model of the RLP30 extracellular LRR-loop (Fig. 3.7 B), this amino acid residue is located on the surface of the protein. Furthermore, this upper part of the LRR-loop is thought to form a putative ligand binding site, like it was shown for flg22-binding to FLS2. Here, the N-terminal part of flg22 binds to the third LRR-loop of FLS2 and the C-terminal part has contact with LRR-loop 16 (Sun *et al.*, 2013). The amino acid exchange L 307 R in $RLP30^{Mt\text{-}0}$ may prevent the binding of the ligand. The unpolar hydrophobic leucine is replaced by a positively charged arginine that could alter the binding affinities of SCFE1 and PCFE1, preventing an induction of the signaling cascade.

The other unique SNPs affect all the same arginine on position 433. In the Lov-1 and Lov-5 accessions this arginine is replaced by a glycine and in the ice111 accession it is substituted by a leucine. This amino acid residue 433 seems to be buried deep within the structure of the LRR-loop according to the predicted model structure and an amino acid exchange may lead to altered folding or even mis-folding of the protein and this may affect its ligand-binding ability and/ or signal transduction capability.

These insensitive *Arabidopsis* accessions further strengthens the hypothesis of direct ligand binding to RLP30 as a single amino acid exchange in the receptor is enough to abolish recognition of both SCFE1 and PCFE1. Of course it can not be excluded that RLP30 is recognizing instead of SCFE1 and PCFE1 another protein, supporting the guard-hypothesis, and that a single amino acid exchange is also enough to abolish recognition. To finally proof the exact reasons why the $RLP30^{accession}$-variants fail to perceive SCFE1 or PCFE1, binding experiments with the purified receptor and the purified ligand would be necessary. For heterologous expression of the RLP30-LRR domain, the best-choice would be an insect-cell based expression system. Heterologous overexpression of LRR-loops in bacteria turned out to

be rather difficult, most likely due to the high degree of glycosylation. With binding studies using Micro Scale Thermophoresis (MST) or surface plasmon resonance (SPR) measurements, it would be possible to determine the affinity and binding kinetics of SCFE1 and PCFE1 to RLP30. These parameters together with the already identified insensitive RLP30-mutants would offer a powerful tool to learn more about the binding and signaling mechanisms of RLP30 with respect to SCFE1 and PCFE1 perception.

Taken together, the performed experiments showed that PCFE1, like SCFE1, is perceived in an RLP30-dependent manner, most likely via a direct interaction of the ligand with its receptor as the single amino acid exchanges in the insensitive *Arabidopsis* accessions would suggest, together with the possibility of the transfer of the RLP30-receptor from *brassicaceous* plants to *solanaceous* plants conferring recognition of both SCFE1 and PCFE1.

4.3.2 SCFE1 and PCFE1 are cross-kingdom MAMPs sharing similar properties

SCFE1 and PCFE1 are both recognized in *Arabidopsis* in an RLP30-dependent manner. But do they have other features in common supporting the idea of one and the same MAMP being present both in fungi and bacteria?

The very first and basal observation is the fact that both, SCFE1 and PCFE1, can be purified from the respective culture's supernatant. This indicates that even in the absence of a living host, SCFE1 and PCFE1 are released into the culture medium. To date it is not known, whether this is a directed process, meaning that SCFE1 and PCFE1 contain secretion signals targeting them extracellularly, or if this is just a by-product of dying and bursting cells, releasing their otherwise intracellular content into the culture medium. Prominent examples do exist for both scenarios. For instance, the necrosis and ethylene-inducing peptide

1 (Nep1)-like proteins (NLPs) are actively secreted by different bacteria, fungi and oomycetes (Dong *et al.*, 2012; Qutob *et al.*, 2007; Oome and Van den Ackerveken, 2014) and its conserved 20 amino acid long pattern, called nlp20, is perceived as a MAMP by the RLP23-receptor (Böhm *et al.*, 2014b; Albert *et al.*, 2015). The bacterial elongation factor TU (EF-TU), harboring the elf18 immunogenic motif, is an example of a MAMP, usually localized within the cell, but being present outside of bacteria in high enough amounts to be recognized by the plant elongation factor-receptor (EFR) (Zipfel *et al.*, 2006).

SCFE1 and PCFE1 were both tested for their biochemical properties. Both elicitors lost their immunogenic activity after treatment with different proteinases or cyanogenbromid, a chemical, cleaving methionines (Villa *et al.*, 1989) (Fig. 3.9 B, Fig. 3.14 B). A first conclusion is that SCFE1 and PCFE1 are both proteinaceous ligands that require at least an intact protein-backbone for their immunogenic activity. Based on the used proteinases and their specific cleavage sites, further insights can be obtained about the amino acid sequence of the immunogenic motif, most likely containing aspartate, glutamate, arginine, lysine and methionine. All of those amino acids are either important in the immunogenic motif of SCFE1 or PCFE1, because they are directly located within a putative binding motif for RLP30 or they are required to obtain the correct folding of the protein structure leading to the recognition by RLP30.

Both elicitors showed no difference in their immunogenic activity before and after boiling with or without SDS (Fig. 3.9 A, Fig. 3.14 A), indicating that a peptide motif is enough for recognition.

The most distinct similarity SCFE1 and PCFE1 are sharing is their sensitivity towards reducing agents. Both elicitors loose their immunogenic activity after treatment with reducing agents like DTT or ß-mercaptoethanol (Fig. 3.9 C, Fig. 3.14 C). Adding the reducing agent and the elicitor separately to the ethylene assay, without previous incubation, did not result in a loss of activity. This indicates that not the reducing agent itself disturbs

the ethylene assay, but rather that the reducing agents modify SCFE1 and PCFE1 in a way which prevents recognition by RLP30. The most likely effect is the reduction of one or more disulfide bridges between the thiol groups of cysteine residues. These disulfide bridges might be important for the correct folding of the protein and its recognition via its corresponding receptor. A prominent example is Avr9, which looses its immunogenic activity after destroying its three disulfide bridges, formed between Cys2-Cys16, Cys6-Cys19 and Cys12-Cys26 (Kooman-Gersmann *et al.*, 1997; van den Hooven *et al.*, 2001). As a secreted peptide into the apoplastic space the Avr9 elicitor is likely to require additional stability, ideally provided by disulfide bridges. Reducing the disulfide bond Cys2-Cys16 already strongly decreased the necrosis-inducing activity of Avr9 and further reduction completely abolished necrosis (van den Hooven *et al.*, 2001). The folding of Avr9 resembles the typical structure of cystine-knotted peptides, typical for small cysteine-rich proteins like serine proteinase inhibitors (Pallaghy *et al.*, 1994), ion channel blockers (Olivera *et al.*, 1994) and growth factors (McDonald and Hendrickson, 1993).

SCFE1 was initially described being present in the culture filtrate of the necrotrophic fungus *Sclerotinia sclerotiorum 1946*, indicated by its name. Additionally, SCFE1 could also be purified from a second *S.sclerotiorum* strain (*S.sclerotiorum 1980*) (Fig. 3.8 A). SCFE1 is recognized by the receptor-protein RLP30; however, it was described that RLP30 not only confers resistance towards *S.sclerotiorum*, but also to the closely related necrotrophic fungus *Botrytis cinerea* in *Arabidopsis* (Zhang *et al.*, 2013). As shown in Fig. 3.8 B, SCFE1 is not only produced by *S.sclerotiorum*, but can also be purified from the culture medium of *B.cinerea*. This finding strengthens the hypothesis that SCFE1 is a genuine MAMP, being present in two closely related, but different fungal species. PCFE1 is also not restricted to one specific *Pseudomonas* strain, in contrary PCFE1 could be identified so far in six different *Pseudomonas* species distributed all over the phylogenetic tree of the genus *Pseudomonas*, indicating that PCFE1 qualifies for a MAMP, rather than a strain-specific

effector or avirulence protein. SCFE1 and PCFE1 are also not qualifying as typical effector proteins, as they firstly seem not to be race-specific, rather the opposite with a distribution in the eukaryotic and prokaryotic kingdom and secondly are most likely recognized at the plasma-membrane by RLP30. Furthermore, both SCFE1 and PCFE1 do not induce any hypersensitive response in plants carrying RLP30 and SOBIR1, which is one of the hallmarks of ETI-triggered immunity.

To rule out the possibility that SCFE1 is a phenomenon of the artificial growth of *S.sclerotiorum* in the lab on liquid media, infected tomato plants with *S.sclerotiorum* were subjected to the established protocol for the purification of SCFE1 and indeed an RLP30-dependent activity could be purified (Fig. 3.8 C). SCFE1 is produced by *S.sclerotiorum* independently of the used culture medium. The RLP30-dependent activity could be found in all tested media (Fig. 3.8 E), no matter if they contained plant material or only artificial nutrients, like in the Gamborg's B5 medium. Interestingly, the potato dextrose broth (PDB) showed a more than five times higher yield of SCFE1 compared to the other tested media. The reasons for that remain elusive, but according to Prof. Bart Thomma, WUR, the same observation was made for the hemibiotrophic fungus *Verticillium dahliae*, that produces a greater yield of effectors when it is grown in PDB medium (oral communication).

Taken together, it could be shown that SCFE1 is a fungal derived MAMP that is produced in at least two different *S.sclerotiorum* strains, as well as in *B.cinerea* under different growth conditions, both artificially in the lab in very different culture media and during infection of tomato plants.

One remaining question is, whether SCFE1 is truly fungal derived and PCFE1 has really a bacterial origin. To rule out any cross-contamination, two separate sets of CEX-columns were used dedicated either for the purification of SCFE1 or PCFE1. Additionally, to rule out a bacterial contamination of the *S.sclerotiorum* culture, the fungus was grown in the presence of three different antibiotics, targeting both gram-negative as well as gram-positive

bacteria. SCFE1 could be purified in comparable amounts to the mock-treated control culture (Fig. 3.16), indicating that SCFE1 is truly a fungal-derived MAMP. In the presence of Cycloheximid no fungal growth was observed, as expected, but also no SCFE1 could be purified in measurable amounts. This experiment does not rule out the possibility, that certain bacteria are associated within the fungus, which need a living fungal host, but are also protected by the fungus and are actually producing SCFE1. But these bacteria are then associated with at least two *Sclerotinia* strains as well as with *B.cinerea*.

The corresponding experiment excluding an eukaryotic origin of PCFE1 was not yet performed, as the *Pseudomonas* culture was only grown overnight, a time-span that is usually too short for *Sclerotinia* or *Botrytis* to grow. Several MAMPs, like flagellin, lipopolysaccharides or peptidoglycans are widely distributed among microbes and likewise chitin is present in almost all fungi, but not many examples of MAMPs are known, which are occurring in both bacteria and fungi or oomycetes. One very well studied example are the necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs), produced by bacteria, fungi and oomycetes (Dong *et al.*, 2012; Qutob *et al.*, 2007; Oome and Van den Ackerveken, 2014). More than one thousand NLP sequences from bacteria, fungi and oomycetes can be identified in databases, with more than half of it harboring the so-called "nlp20" PAMP-motif, consisting of 20 more or less conserved amino acids triggering typical immune responses after recognition through RLP23 (Böhm *et al.*, 2014b; Oome and Van den Ackerveken, 2014; Albert *et al.*, 2015).

It is also possible that SCFE1 and PCFE1 were independently obtained via horizontal gene transfer, like it was shown for Ave1 (Avirulence on Ve1 tomato) being recognized by the LRR-RLP Ve1 from tomato (de Jonge *et al.*, 2012). Ave1 was identified being a race-specific effector of the fungus *Verticillium dahliae* using comparative population genomics. Further analyses of the identified locus revealed that it is only present in the fungi *Fusarium oxysporum f. sp. lycopersici*, *Colletotrichum higginsianum* and *Cercospora beticola* (de Jonge *et al.*, 2012). Additionally, an Ave1 homologue was previously identified in the plant-pathogenic bacterium

Xanthomonas axonopodis pv. citri, which is the causal agent of citrus canker (Nembaware *et al.*, 2004). Surprisingly, more than 200 Ave1 plant homologs have been identified using BLASTp analyses (de Jonge *et al.*, 2012). Further analysis established horizontal gene transfer from plants to the respective pathogens as the most likely reason for the observed distribution of Ave1, following no species phylogeny (de Jonge *et al.*, 2012).

The recently characterized MAMP SCFE1 produced by the fungus *S.sclerotiorum* (Zhang *et al.*, 2013) and the novel MAMP PCFE1 derived from the bacterial genus *Pseudomonas*, described in this study, are another example of the seldom occurrence of a cross kingdom MAMP. Until SCFE1 and PCFE1 are identified, it is only possible to speculate about the intrinsic function of most likely a protein, harboring the immunogenic motif of SCFE1 and PCFE1 and the occurence in both the prokaryotic and eukaryotic kingdom. Further analyses are required to determine if SCFE1 and PCFE1 are derived from the same protein or share just the same minimal immunogenic motif. It is possible that the motif being recognized by RLP30 is also present in other bacteria, fungi or maybe also extending to oomycetes. With that knowledge it would be also possible to see whether SCFE1 and PCFE1 are evolutionary conserved proteins widely distributed, acquired only by some species via horizontal gene transfer or have independently evolved.

Taken together, SCFE1 and PCFE1 are typical MAMPs being recognized by the PRR RLP30 and induce typical PTI responses. To my knowledge SCFE1 and PCFE1 would be the third example of a MAMP being present in both the prokaryotic and eukaryotic kingdom.

4.3.3 Different attempts to identify SCFE1 and PCFE1

The identification of the nature of SCFE1 and later also of PCFE1 was an desirable aim, but until now the identity of both SCFE1 and PCFE1 still remains elusive.

Different approaches were taken to identify SCFE1. No time was left for any identification approaches of PCFE1. The first approach to identify SCFE1 was by fishing the ligand with its receptor RLP30, which was coupled via GFP-tag to GFP-trap beads. RLP23-GFP served as a negative control to analyze arbitrary binding of proteins to the GFP-tag or the GFP-trap beads. Unfortunately, a confirmation of the identified proteins that would bind exclusively to RLP30 was not possible in an independent experiment, raising the question about the reliability of the used approach. Testing of the fractions that were analyzed by LC-MS/MS for their ethylene-inducing activity failed, already indicating a very low amount of potentially bound SCFE1 (data not shown). Another issue was the relatively high number of identified proteins (around 60-70), also suggesting an unspecific protein binding which made it impossible to test all of them in more detailed experiments.

In order to increase the abundance of SCFE1 and decrease the number of contaminating proteins, reversed phase chromatography was applied after CEX-chromatography and active fractions were afterwards separated with SDS-PAGE. This added two new parameters to the purification of SCFE1, namely by hydrophobicity (reversed phase) and by size (SDS-PAGE). One sample, collected after the reversed phase chromatography step, was sent to LC-MS/MS in order to get a feeling about the efficiency as a purification step. Additionally, active gel slices together with the corresponding eluted proteins were also sent for LC-MS/MS analyses. The overall number of identified proteins was significantly reduced to only 15 identified proteins, with only 4 being present in all analyzed samples. From these 4 proteins (A7ET57 Rho-GDI; A7E993, A7F0B8, A7E4E9 Polyubiquitin and Ubiquitin ribosomal fusion proteins; A7ETS6 Actin; A7E610 predicted protein), nested 30 amino acid long synthetic peptides, with an overlap of 15 amino acids, spanning the whole protein sequences were ordered and tested in an ethylene assay. None of the tested peptides showed an RLP30-dependent activity and any activities that could be observed were not RLP30-dependent and only present after addition of high peptide concentrations (10 µM), suggesting contamination from the peptide

production process. Additionally, commercially available Actin from rabbit and Ubiquitin from *Bovine* erythrocytes (Sigma Aldrich) were tested, also giving no RLP30-dependent ethylene-response. For an ultimate exclusion of those proteins as possible candidates for SCFE1, heterologous expression in the eukaryotic expression system *Pichia pastoris* was performed. Pre-experiments were conducted, but no protein expression of the desired proteins was detectable on an SDS-PAG or in a functional ethylene assay. Due to the time and labor intensive optimization process required for every single protein, no further testing was performed. With the knowledge now that PCFE1 is also able to induce an RLP30-dependent ethylene response and the assumption that SCFE1 and PCFE1 contain the same immunity-inducing epitope, a bacterial expression system should be considered in the future, because eukaryotic post-translational modifications do not seem to be required for an ethylene-inducing RLP30-dependent response.

Derbyshire *et al.* (2017) released a new sequenced and annotated genome of *S.sclerotiorum 1980* with a higher accuracy and sequence coverage than the previous version. This better annotated genome, together with a six frame translation of the genome, was used as basis for a new sequencing approach in collaboration with Dr. Frank Menke from the Sainsbury Laboratories, Norwich, UK, using "active" gel slices, which were produced by Dr. Frederic Brunner. After CEX-chromatography, Dr. Frederic Brunner subjected purified samples to SDS-PAGE and cut out thin slices, which were afterwards tested in an ethylene assay for their RLP30-dependent activity. By analyzing multiple gel slices, the chance to exclude impurities being present in only one or few samples and to find the SCFE1-candidate being present in all of the samples, should be increased. In total around 120 proteins could be identified, with around 30 proteins being present with different abundances in all of the analyzed samples. The six-frame translation did not reveal any non-annotated gene products.

Additionally, a BLAST search was performed to identify homologous proteins in *Pseudomonas* based on the identified proteins from the LC-MS/MS analyses done by Dr. Frank Menke.

Only two out of 42 analyzed proteins (see table 8.10 for the BLASTed protein list) gave the highest key alignment score of over 200 in a BLAST search. The ATP-dependent chaperone ClpB (Sscle06g053820, NP_790675) is highly conserved in fungi, bacteria, plants and to a smaller degree also in humans. The Enoyl-CoA hydratase (Sscle03g028710, NP_792953) is very well conserved within fungi and bacteria and a high conservation is only seen for mosses like *Physcomitrella*, *Marchantia* and *Selaginella*. Interestingly, the predicted molecular weight of those two proteins does not fit within in the range of the excised gel slices, indicating that maybe only parts or further modified versions of the proteins were present in the sample.

Simple BLASTing of candidate proteins has a very big drawback, as it is based on the assumption that SCFE1 and PCFE1 are two homologous proteins. So far, there is no proof that this is the case and the same immunogenic motif could be present in two completely different proteins, which would not be identified using a simple BLAST search, but would require more elaborated search algorithms. It is also possible that the amino acid sequence between SCFE1 and PCFE1 is not highly conserved, but that the folding and the assumed cysteine disulfide bridges are more important for the immunogenic potential of SCFE1 and PCFE1.

For the future two collaborations are already established to further narrow down the list of candidate genes. The first one is again with Dr. Frank Menke. The idea is to repeat the experiment, using this time gel slices containing active PCFE1 and compare the list of identified peptides, not whole proteins, with the previously identified peptides from *S.sclerotiorum*.

The other approach is in collaboration with Dr. Christina Ludwig, BayBioMS, TU München. Together with her, different fractions of SCFE1, each showing a different ethylene-inducing capability thus containing different amounts of the SCFE1 protein should be analyzed by LC-MS/MS analyses. Afterwards, quantification of the abundance of proteins within each sample should be performed using the MaxQuant software (Cox and Mann, 2008)

by determining the Label-free quantification (LFQ) value and the intensity based absolute quantification (iBAQ) value, excluding candidates that would not fit the previously observed ethylene-inducing activity pattern of SCFE1. The LFQ values reflect the relative amounts of the proteins, but work best for homogeneous samples, which in the case of SCFE1 is not necessarily given. The iBAQ algorithm can roughly estimate the relative abundance of the proteins within each sample, by dividing the raw intensities by the number of theoretical peptides. With these two values a comparison of different samples showing a different ethylene-inducing capacity thus having different amounts of SCFE1 should be possible.

A completely different approach was taken with the bioinformatical analyses and comparison of the available *S.sclerotiorum* and *B.cinerea* proteome. By applying all the so far known characteristics that SCFE1 should most likely fulfill, the list of potential genes was narrowed down and in a final step the received list of *Sclerotinia* proteins was compared with the list of *Botrytis* proteins, resulting in 15 candidate proteins. Unfortunately, all of these proteins were "predicted proteins" and it was not possible to get a gene product via PCR for further analysis. One drawback of the bioinformatical approach could be the fact that the analysis was made on the assumption that SCFE1 should contain a signal peptide, as it is present in the culture medium. Having a closer look on the LC-MS/MS data obtained by Dr. Frank Menke from the "active" gel slices, most of the here identified proteins do not contain a predicted signal peptide. In the used bioinformatics approach, all of the proteins having no signal peptide were dismissed from the beginning. Bioinformatics can be a very powerful tool, but for this approach, which is based on assumptions, it can only be a as powerful as the assumptions on which it is based. Investigation and verification in a wet lab approach is still needed.

Until now, all of the proteins that were chosen to be good candidates to harbor the SCFE1 immunogenic motif never fulfilled all the criteria at the same time. Good candidate genes like the Rho-GDI (A7ET57, Sscle10g077860), which was identified in almost every analysis, the very abundant Polyubiquitin and Ubiquitin-ribosomal fusion proteins (A7E4E9, A7F0B8,

A7E993, Sscle03g029230) or a predicted protein (A7E610, Sscle03g024790) which was identified in every sample after the improved purification using reversed phase chromatography, do not contain a predicted signal peptide. Furthermore the last mentioned predicted protein also has no annotated homologue in *B.cinerea*. Other proteins like a DUF985-containing protein (A7ELN3, Sscl05g043810) or the Rho-GDI protein (A7ET57, Sscle10g077860) contain only one cysteine residue, which make them more unlikely to be a good candidate protein, as disulfide bridges seem to be required for the immunogenic function of SCFE1. Only very few proteins are highly conserved between *S.sclerotiorum* and *Pseudomonas*. Only two out of 42 analyzed proteins (see table 8.10 being the most abundant identified proteins in the gel slices analyzed by Dr. Frank Menke) are highly conserved. The ATP-dependent chaperone ClpB (Sscle06g053820, NP_790675) and an Enoyl-CoA hydratase (Sscle03g028710, NP_792953) could be identified, but especially the ClpB with a predicted molecular weight of 104 kDa is not really matching the size profile of SCFE1. With the very recently obtained data from Dr. Christina Ludwig matching SCFE1-activity to the peptide abundance in the respective samples, this has changed. There is now one very good candidate protein identified that perfectly matches all of the so far known criteria of SCFE1. The predicted protein Sscle03g028510/ A7E4P1 was found to interact exclusively with RLP30 in one of the fishing experiments, it fulfills all of the criteria and was therefore among interesting candidate proteins identified by applying the bioinformatics approach. It is highly upregulated during the infection process, but also decently expressed during axenic *in vitro* culture in the lab, indicated by the RNA-seq data provided by Dr. Michael Seidl. It was additionally found in all of the 13 gel slices analyzed by Dr. Frank Menke and it matches perfectly the SCFE1-activity profile with the peptide abundance analyzed by Dr. Christina Ludwig. The only drawback is that the protein as a whole sequence is not conserved in *Pseudomonas*.

Once SCFE1 and PCFE1 are identified, it would be interesting to further determine the function of the protein. It is possible, that both SCFE1 and PCFE1 are implicated in the

infection process, but both proteins could also be indispensable for the general lifestyle of the microbes not related to infection. With the generation of *SCFE1*- or *PCFE1*- knockout mutants in *S.sclerotiorum* and *B.cinerea* or *Pseudomonas*, respectively, this question can easily be addressed and the pathogenicity as well as the general fitness of the mutants would be tested.

The most important part, after the identification of interesting candidate proteins is the conformation of the hypothesis by heterologous expression of the respective candidate proteins and testing their RLP30-dependent immunogenic potential. Every heterologous expression system has its advantages but also drawbacks. For the expression of SCFE1 and PCFE1 candidate proteins a rather unusual expression system was chosen: A transient, gateway-based expression system in *N.benthamiana* mediated by *A.tumefaciens*. The beauty of this system lies within its easy protein purification after expression. The expressed proteins are targeted to the apoplast via a signal peptide (PR1a from *N.benthamiana* or a signal peptide from *P.sojae*, NIP) and after vacuum-infiltration of the respective leaves the apoplastic wash-fluid is harvested by centrifugation and can be directly applied in an ethylene assay as it was nicely shown for Avr9 (Piedras and Hammond-Kosack (1998) and Fig. 3.12 C). In order to simplify the cloning process the Invitrogen™Gateway™recombination cloning technology was chosen. The binary expression vectors of the pGWB-series (Nakagawa *et al.*, 2007) were used as backbones and by using conventional cloning strategies the respective signal peptides were cloned in-frame into the expression vectors (see table 3.7), both for tagged and untagged expression. Using Avr9 as a positive control for testing the newly designed expression vectors revealed soon problems. The expression efficiency was very low, indicated by a low ethylene-inducing potential in *N.tabacum* plants expressing the Cf9-receptor (see Fig. 3.12 C), compared to the conventionally cloned Avr9-expression vector (Honée *et al.*, 1998) which was kindly provided by Dr. Matthieu Joosten, Wageningen University and which was also tested (data not shown). One problem of the Gateway-technology is the presence of

additional amino acids due to the attB-binding sites, but this should not alter the expression efficiency as there are more than enough examples of successful *Agrobacterium*-mediated protein expression in *N.benthamiana* using the pGWB-vectors, including this thesis (see Fig. 3.1, Fig. 3.2 H, I, Fig. 3.7 C, Fig. 3.13 C). Another problem could be that the used pGWB-vector serie (Nakagawa *et al.*, 2007) has all necessary information encoded on one single vector, resulting in a very large vector, which could also reduce transformation efficiency. Changing the vector backbone to the pGREEN-serie (Hellens *et al.*, 2000) would reduce the size of the plasmid dramatically, thereby increasing the transformation efficiency, as it is inversely proportional to the size of the plasmid DNA (Sambrook and Russell, 2001). Another option would be the use of the Golden Gate Assembly technique, using restriction enzymes cutting outside of their binding site, creating non-palindromic overhangs and by a careful design of the overhangs a seamless cloning is possible (Engler *et al.*, 2008).

Further optimization of this heterologous expression is still required, but it would offer then an easy and fast cloning system, followed by a cheap expression, as no expensive media are required, like for the maintenance of insect cells, and most importantly would allow a very easy protein purification.

Taken together, there are very good candidate proteins already in the pipeline waiting for the experimental testing and with a strong team supporting the analyses it will be possible to unravel the molecular identity of both SCFE1 and PCFE1 very soon.

5 Summary

Arabidopsis thaliana has evolved a large number of transmembrane cell surface receptors that play major roles during development, organogenesis and responses to external stimuli. One *A.thaliana* leucine-rich repeat (LRR) receptor-like protein, RLP30, was already associated with a specific function as pattern recognition receptor (PRR) for the novel microbe-associated molecular pattern (MAMP) SCFE1 (Sclerotinia culture filtrate elicitor 1) from the necrotrophic fungus *Sclerotinia sclerotiorum*, but the molecular identity of SCFE1 remained elusive. Different approaches were used to try to identify the identity of SCFE1 using different chromatography methods, followed by LC-MS/MS analyses or by using a bioinformatical approach. Additionally, an easy Gateway-based cloning and expression system for transient expression in the apoplast of *N.benthamiana* plants was established, in order to test putative candidate proteins. The two LRR-receptor like kinases (RLK), BRI1-ASSOCIATED KINASE 1 (BAK1) and SUPPRESSOR OF BIR1 (SOBIR1), were already shown to be involved in RLP-dependent immune responses. Using co-immunoprecipitation assays a constitutive ligand-independent interaction of RLP30 and SOBIR1 could be shown, additionally, to the ligand-dependent recruitment of BAK1 to the RLP30-SOBIR1 complex. RLP30 was identified by mapping naturally occurring SCFE1-insensitive *Arabidopsis* accessions and important amino acid residues for proper function of RLP30 could be identified, targeting most likely putative ligand binding sites or interaction sites for SOBIR1 and/or BAK1. Furthermore, RLP30 was transformed into a

functional receptor-like kinase, by fusing the kinase domain of SOBIR1 to the C-terminal end of RLP30, thus transforming the RLP into a genuine RLK. The brassicaceous RLP30 was so far not functional in solanaceous plants, but by co-transforming the *Arabidopsis* SOBIR1 together with RLP30 in tomato and tobacco plants, an increased resistance in those two plant species against *S.sclerotiorum* and *Botrytis cinerea* could be achieved. Furthermore, a novel RLP30-dependent MAMP was purified from different strains of the bacterial genus *Pseudomonas*, which showed exactly the same biochemical properties like SCFE1, indicating a putative shared immunogenic minimal motif. This is one of very few examples of cross-kingdom occurence of MAMPs.

6 Zusammenfassung

Im Genom von *Arabidopsis thaliana*, wie auch in allen anderen höheren Landpflanzen, findet sich eine beträchtliche Anzahl an Genen die für Rezeptoren kodieren die in der Zellmembran lokalisiert sind. Diese Rezeptoren spielen eine wichtige Rolle während der Embryonalentwicklung, der Differenzierung der Organe, sowie in der Perzeption von externen Stimuli. Für ein LRR rezeptorähnliches Protein, RLP30, konnte gezeigt werden, dass es den neu entdeckten MAMP SCFE1 (Sclerotinia culture filtrate elicitor 1) aus dem necrotrophen Pilz *Sclerotinia sclerotiorum* erkennen kann, jedoch konnte bis jetzt die molekulare Identität von SCFE1 nicht aufgeklärt werden. Durch den Einsatz verschiedener Chromatographie-Techniken und anschließender LC-MS/MS-Analyse, sowie einem bioinformatischen Ansatz sollte versucht werden die molekulare Identität von SCFE1 zu entschlüsseln. Um mögliche Kandidaten testen zu können, wurde ein einfaches Gateway-basiertes Klonierungssystem entwickelt, mit dessen Hilfe Kandidatengene transient im Apoplasten von *Nicotiana benthamiana* exprimiert werden sollen. Die LRR rezeptor-ähnlichen Kinasen BAK1 und SOBIR1 werden beide in der RLP-abhängigen Signaltransduktion benötigt und in Co-immunoprezipitationsversuchen konnte gezeigt werden, dass SOBIR1 und RLP30 konstitutiv und ligandenunabhängig miteinander interagieren. Außerdem konnte gezeigt werden, dass BAK1 ligandenabhängig zum RLP30-SOBIR1-Komplex rekrutiert wird. RLP30 konnte mit Hilfe von natürlich vorkommenden *Arabidopsis* Ökotypen identifiziert werden, die insensitiv gegenüber SCFE1 sind und mit dessen

Hilfe nun auch wichtige Aminosäure identifiziert werden konnten, die für die Funktion von RLP30 unabdingbar sind und höchstwahrscheinlich mögliche Ligandenbindestellen oder Interaktionsstelle mit SOBIR1 und/oder BAK1 betreffen. Zusätzlich konnte das rezeptorähnliche Protein RLP30 durch die C-terminale Fusion der SOBIR1-Kinasedomäne in eine funktionelle rezeptorähnliche Kinase umgewandelt werden. RlP30 aus *Brassicaceae* war bislang in Pflanzen die zu den *Solanaceae* gehören nicht funktionell, aber durch das gleichzeitige Transformieren von SOBIR1 aus *Arabidopsis* und RLP30 in Tomaten- und Tabakpflanzen konnte eine höhere Resistenz in diesen Pflanzen gegenüber *S.sclerotiorum* und *Botrytis cinerea* erreicht werden. Abschließend konnte noch ein neuer, ebenfalls RLP30-abhängiger MAMP aus verschiedenen bakteriellen Stämmen, die alle der Gattung *Pseudomonas* angehören, partiell aufgereinigt werden. Dieser neu-gefundene MAMP zeigte die gleichen biochemischen Eigenschaften wie SCFE1, was darauf hindeuten könnte, dass beide das gleiche immunogene Minimalmotiv teilen. Das ist eines von sehr wenigen Beispielen für ein MAMP, das sowohl in Prokaryoten als auch in Eukaryoten vorkommt.

7 Bibliography

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8 Appendix

Abbreviations

AGD	ARF-GAP containing domain
AGL15	AGAMOUS-LIKE 15
ARF-GAP	ADP-Ribosylation Factor GTPase-Activating Protein
Avr	Avirulence
BAK1	BRI1-associated receptor kinase 1
BIK1	BOTRYTIS INDUCED KINASE 1
BIR	BAK1-INTERACTING RECEPTOR-LIKE KINASE
BP	BREVIPEDICELLUS
BRI1	BRASSINOSTEROID INSENSITIVE 1
CDPK	Calcium-dependent protein kinase
CEBIP	CHITIN ELICITOR BINDING PROTEIN
CERK1	Chitin Elicitor Receptor Kinase 1
CEX	cation exchange
Cf	Cladosporium fulvum resistance protein
CLV	CLAVATA
Co-IP	Coimmunoprecipitation
Col-0	Columbia-0

CST	CAST AWAY
CU/l	"Christina units per liter" (arbitrary unit for ethylene inducing activity)
CRN	CORYNE
DAMP	damage-associated molecular pattern
DORN1	DOes not Respond to Nucleotides 1
dpi	days post-infection
DSI	Disease symptome index
DTT	Dithiothreitol
eATP	extracellular Adenosine Triphosphate
Ecp6	extracellular protein 6
EFR	Elongation Factor Tu Receptor
EF-Tu	Elognation factor Tu
EGF	epidermal growth factor
eGFP	extracellular green fluorescent protein
eMAX	enigmatic MAMP of Xanthomonas
endoPG	Endopolygalacturonases
EPF	EPIDERMAL PATTERNING FACTOR
ER	ERECTA
ERF	ERECTA FAMILY RECEPTOR KINASES
ERL1	ER-LIKE 1
ETI	effector-triggered immunity
flg22	flagellin epitope of 22 amino acids
FLS2	FLAGELLIN-SENSITIVE 2
FRK1	FLG22-induced receptor-like kinase 1
GFP	green fluorescent protein

GPI	Glycosylphosphatidylinositol
HA	hemagglutinin
HABS	HIGH-AFFINITY BINDING SITE
HAE	HAESA
HHP	heptahelical protein
HR	hypersensitive response
HSL2	HAESA-LIKE 2
IDA	INFLORESCENCE DEFICIENT IN ABSCISSION
KAPP	KINASE-ASSOCIATED PROTEIN PHOSPHATASE
LC-MS/MS	Liquid chromatography–mass spectrometry/mass spectrometry
Ler	Landsberg erecta
LORE	Lipo-Oligosaccharide-specific Reduced Elicitation
LPS	Lipopolysaccharide
LRR	leucine rich repeat
LYK5	Lysin Motif Receptor Kinase 5
LYM	lysin-motif
MAMP	microbe-associated molecular pattern
MAPK	mitogen-activated protein kinase
MES	2-(N-morpholino)ethanesulfonic acid
MIND	Membrane-based Interactome Database
NAMP	nematode-associated molecular pattern
NASC	Nottingham Arabidopsis Stock Centre
N.benthamiana	Nicotiana benthamiana
NB-LRR	nucleotide-binding leucine rich repeat
NEV	NEVERSHED

NLP	NIP like protein
nlp20	NIP like protein epitope of 20 amino acids
NLR	nucleotide-binding domain, leucine rich containing
OGs	Oligogalacturonides
PAD4	Protein arginine deiminase 4
PAMP	pathogen-associated molecular pattern
parAMP	parasite-associated molecular pattern
PBL	PBS-like
PBS-like	avrPphB SUSCEPTIBLE-LIKE
PCFE1	Pseudomonas culture filtrate elicitor 1
pep1-6	Peptide 1-6
PEPR1/2	Pep Receptor 1/2
PGN	Peptidoglycan
PIP1	PAMP-induced secreted peptide 1
PP2A	PROTEIN SER/THR PHOSPHATASE TYPE 2A
PR-4	pathogenesis-related 4
PRR	Pattern recognition receptor
PUB	PLANT U-BOX E3 UBIQUITIN
pv	pathovar
R-gene/-protein	resistance-gene/-protein
Rab	Ras-related in brain
RbohD	Respiratory burst oxidase homologue D
RBPG1	Responsiveness to Botrytis Polygalacturonases 1
Rcr3	required for C. fulvum resistance 3
ReMAX	Receptor of eMax

RFP	red fluorescent protein
Rho-GDI	Rho GDP-dissociation inhibitor
RLCK	receptor-like cytoplasmic kinase
RLK	receptor like kinase
RLP	receptor like protein
ROS	reactive oxygen species
SAR	systemic acquired resistance
SCFE1	Sclerotinia culture filtrate elicitor 1
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS-Polyacrylamid gel electrophoresis
SERK	SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE
SNP	single nucleotide polymorphism
SOBIR1	suppressor of BIR1 1
S.sclerotiorum	Sclerotinia sclerotiorum
TM	Transmembrane
TMM	TOO MANY MOUTH
TRAF	TNF receptor associated factor
WAK	CellWall-Associated Kinase
XPS	1-Deoxy-D-Xylulose 5-Phosphate Synthase 1

Perl-Code for bioinformatical analysis of *S.sclerotiorum* and *B.cinerea* proteome

```
#!/usr/bin/perl -w
# How to run this program (command line): PERL program ARGV0:signalp-file
  ARGV1:effectorP-file ARGV2:Blast-file ARGV3:fasta-file ARGV4:RNAseq
  ARGV5:header

use strict;
use warnings;
use Bio::SeqIO;
use Bio::Tools::SeqStats;

#get all proteins with a signal peptide from the SignalP-prediction file
  and ignore the two first lines starting with "#"

open(IN,"<$ARGV[0]");
my %ids;
while(<IN>){
  chomp;
  next if (/^#/);
  my @list = split(/\s+/,$_);
  next if ($list[9] eq "N");
  $ids{$list[0]} = $list[2];
}
close(IN);

#get all proteins from the EffectorP-prediction file
  and ignore the two first lines starting with "#"
open(IN,"<$ARGV[1]");
my %effectorP;
while(<IN>){
  chomp;
  next if ($_ !~ /^>/);
  my ($id) = $_ =~ m/>(.+) \|/g;
  $effectorP{$id} = 1;
}
close(IN);

#get the best hit from the BLAST against Botrytis cinerea/
  Sclerotinia sclerotiorum, respectively
open(IN,"<$ARGV[2]");
my %blast;
while(<IN>){
  chomp;
  my @list = split(/\s+/,$_);
  next if (exists $blast{$list[0]});
  $blast{$list[0]} = $list[1] . "\t" .  $list[10] . "\t" . $list [12] . "\t" .
  $list [13] . "\t" . $list [14];
}
```

```
48  close(IN);
49
50  #get the data from the Sclerotinia/Botrytis RNAseq data
51  open(IN,"<$ARGV[4]");
52  my %RNAseq;
53  while(<IN>){
54    chomp;
55    next if ($_ !~ /^mRNA/);
56    my @list = split(/\s+/,$_);
57    $RNAseq{$list[0]} = $list[1] . "\t" .  $list[2] . "\t" .  $list[3] . "\t" .
58    $list [4] . "\t" . $list [5] . "\t" . $list [6] . "\t" . $list [7]
59  }
60  close(IN);
61
62  #find the cutting site for the signal peptid and write the protein sequence
63    without the signal peptide
64  my $inseq = Bio::SeqIO->new(-file   => $ARGV[3],
65                              -format => 'fasta', );
66
67  # write the headers for the table in the excel-sheet
68  print "SequenzID" . "\t";
69  print "Sequenz" . "\t";
70  print "Sequenz without SP" . "\t";
71  print "# of AA" . "\t";
72  print "# of AA without SP" . "\t";
73  print "MW in Da" . "\t";
74  print "# of Cys" . "\t";
75  print "# of Asp" . "\t";
76  print "# of Glu" . "\t";
77  print "# of Met" . "\t";
78  print "EffectorP prediction" . "\t";
79  print "Best BLAST hit sequenzID" . "\t";
80  print "e-value" . "\t";
81  print "query length" . "\t";
82  print "hit length" . "\t";
83  print "sequence coverage" . "\t";
84  print "RNAseq 0h" . "\t";
85  print "RNAseq 1h" . "\t";
86  print "RNAseq 3h" . "\t";
87  print "RNAseq 6h" . "\t";
88  print "RNAseq 12h" . "\t";
89  print "RNAseq 24h" . "\t";
90  print "RNAseq 48h" . "\n";
91
92  while (my $seq = $inseq->next_seq) {
93    next if (!exists $ids{$seq->id});
94    my $cut_site = $ids{$seq->id};
95    my $test = $seq->seq;
96    $test =~ s/\.//;
97    $seq->seq($test);
98    my $len = length($seq->seq());
99    my $start = $cut_site;
```

```
100
101 #find all sequences with D and E and M and <200AA; and count all the Cs, Ds,
102   Es, Ms in the sequence
103   #if ($seq->subseq($start,$len) =~m/D/ && $seq->subseq($start,$len) =~ m/E/
104     && $seq->subseq($start,$len) =~ m/M/ &&length($seq->subseq($start,$len))
105     < 50500){
106     my $sub_seq = $seq->subseq($start,$len);
107     my @countC = $seq->subseq($start,$len) =~ m/C/g;
108     my @countD = $seq->subseq($start,$len) =~ m/D/g;
109     my @countE = $seq->subseq($start,$len) =~ m/E/g;
110     my @countM = $seq->subseq($start,$len) =~ m/M/g;
111
112 #the following part is only important for the data output
113     #if (scalar@countC > 1){
114       print $seq->id . "\t";
115     print $seq->seq . "\t";
116     print $seq->subseq($start,$len) . "\t";
117     #print "Number of AA\t";
118     print $seq->length . "\t";
119     #print "Number of AA without SP\t";
120     print length($seq->subseq($start,$len)) . "\t";
121     #print "Cysteins\t";
122
123 my $mass = calc_mass($seq->subseq($start,$len));
124 print $mass . "\t";
125     print scalar(@countC) . "\t";
126     print scalar(@countD) . "\t";
127     print scalar(@countE) . "\t";
128     print scalar(@countM) . "\t";
129   if (exists $effectorP{$seq->id}){
130     print "Effector" . "\t";
131   }
132   else{
133     print "No Effector" . "\t";
134   }
135
136 if (exists $blast{$seq->id}){
137     print $blast{$seq->id} . "\t";
138   }
139   else{
140     print "No hit" . "\t";
141     print "-" . "\t";
142     print "-" . "\t";
143     print "-" . "\t";
144     print "-" . "\t";
145 }
146
147 if (exists $RNAseq{$seq->id}){
148     print $RNAseq{$seq->id} . "\n";
149 }
150 if (!exists $RNAseq{$seq->id}){
151     print "No data" . "\t";
```

```
152       print "No data" . "\t";
153       print "No data" . "\t";
154       print "No data" . "\t";
155       print "No data" . "\t";
156       print "No data" . "\t";
157       print "No data" . "\n";}
158   }
159   #calculation for the determination of the molecular mass of the proteins
160   sub calc_mass {
161       my $a = shift;
162       my @a = ();
163       my $x = length $a;
164       @a = split q{}, $a;
165       my $b = 0;
166       my %data = (
167           A=>88,  R=>173,  D=>132,  N=>131,
168           C=>120,  E=>146,  Q=>145,  G=>74,
169           H=>154,  I=>130,  L=>130,  K=>145,
170           M=>198,  F=>164,  P=>114,  S=>104,
171           T=>118,  W=>203,  Y=>180,  V=>116,
172           X=>0,U=>0,Z=>0,"."=>0
173       );
174       for my $i( @a ) {
175           $b += $data{$i};
176       }
177       my $c = sprintf("%0.2f",$b - (18.01528 * ($x - 1)));
178       return $c;
179   }
```

Supplemental tables

Table 8.2: Amino acid sequences of synthetic peptides of Actin.

Peptide name	AA sequence
Actin1	MEEEVAALVIDNGSGMCKAGFAGDDAPRAV
Actin2	GMCKAGFAGDDAPRAVFPSIVGRPRHHGIM
Actin3	FPSIVGRPRHHGIMIGMGQKDSYVGDEAQS
Actin4	IGMGQKDSYVGDEAQSKRGILTLRYPIEHG
Actin5	KRGILTLRYPIEHGVVTNWDDMEKIWHHTF
Actin6	VVTNWDDMEKIWHHTFYNELRVAPEEHPVL
Actin7	YNELRVAPEEHPVLLTEAPINPKSNREKMT
Actin8	LTEAPINPKSNREKMTQIVFETFNAPAFYV
Actin9	QIVFETFNAPAFYVSIQAVLSLYASGRTTG
Actin10	SIQAVLSLYASGRTTGIVLDSGDGVTHVVP
Actin11	IVLDSGDGVTHVVPIYEGFSLPHAIARVDM
Actin12	IYEGFSLPHAIARVDMAGRDLTDYLMKILA
Actin13	AGRDLTDYLMKILAERGYTFSTTAEREIVR
Actin14	ERGYTFSTTAEREIVRDIKEKLCYVALDFE
Actin15	DIKEKLCYVALDFEQEIQTASQSSSLEKSY
Actin16	QEIQTASQSSSLEKSYELPDGQVITIGNER
Actin17	ELPDGQVITIGNERFRAPEALFQPSVLGLE
Actin18	FRAPEALFQPSVLGLESGGIHVTTFNSIMK
Actin19	SGGIHVTTFNSIMKCDVDVRKDLYGNIVMS
Actin20	CDVDVRKDLYGNIVMSGGTTMYPGISDRMQ
Actin21	GGTTMYPGISDRMQKEITALAPSSMKVKII
Actin22	KEITALAPSSMKVKIIAPPERKYSVWIGGS
Actin23	APPERKYSVWIGGSILASLSTFQQMWISKQ
Actin24	ILASLSTFQQMWISKQEYDESGPSIVHRKCF

Table 8.3: Amino acid sequences of synthetic peptides of Ubiquitin-ribosomal fusion protein (A7E993).

Peptide name	AA sequence
Ubi1	MFTSMQIFVKTLTGKTITLEVESSDTIDNV
Ubi2	KTITLEVESSDTIDNVKAKIQDKEGIPPDQ
Ubi3	KAKIQDKEGIPPDQQRLIFAGKQLEDGRTL
Ubi4	QRLIFAGKQLEDGRTLSDYNIQKESTLHLV
Ubi5	SDYNIQKESTLHLVLRLRGGIIEPSLKALA
Ubi6	LRLRGGIIEPSLKALASKFNCEKMICRKCY
Ubi7	SKFNCEKMICRKCYARLPPRATNCRKKKCG
Ubi8	ARLPPRATNCRKKKCGHTNQLRPKKKLK
Ubi9	EPSLKALASKFNCEKMICRKCYARLPPRAT

Table 8.4: Amino acid sequences of synthetic peptides of Rho-GDI.

Peptide name	AA sequence
Rho1	MADQQDNDLLPETTDGFKVGEKKTLDEY
Rho2	GFKVGEKKTLDEYSKMDAEDESLQRYKESL
Rho3	SKMDAEDESLQRYKESLGLGGGGKDLSDP
Rho4	GLGGGGKDLSDPNDPRDCIILTLEMNSEGR
Rho5	NDPRDCIILTLEMNSEGRPPVKLELSTPDA
Rho6	PPVKLELSTPDALNTLKDHPFKIKEGSKFN
Rho7	LNTLKDHPFKIKEGSKFNLTATFKVQHNVL
Rho8	LTATFKVQHNVLSGLQYVQVIKRKGIRIDK
Rho9	SGLQYVQVIKRKGIRIDKLQEMIGSYA
Rho10	LQEMIGSYAPNTDKNPVHTKRFADEDAP
Rho11	PNTDKNPVHTKRFADEDAPTGMMARGHYTA
Rho12	TGMMARGHYTAISTFIDDDKKKHLEFEWSF
Rho13	ISTFIDDDKKKHLEFEWSFDITKDW

Table 8.5: Amino acid sequence of synthetic peptide of a predicted protein of 16 kDa.

Peptide name	AA sequence
Pred1	GGIKKDGLLEQKAGGTIPDDQVQVVEDGMKTWSHGKYT

Table 8.6: Identified proteins in the LC-MS/MS-analysis by fishing the ligand I.

Protein IDs	Peptide Counts (all)	Peptides RLP23-GFP I	Peptides RLP30-GFP I	Peptides RLP30-GFP II	Peptides RLP23-GFP II	Mol. Weight [kDa]	Sequence Length	Experiment RLP23-GFP I	Experiment RLP30-GFP I	Experiment RLP30-GFP II	Experiment RLP23-GFP II	PEP	Sequence Coverage RLP23-GFP I [%]	Sequence Coverage RLP30-GFP I [%]	Sequence Coverage RLP30-GFP II [%]	Sequence Coverage RLP23-GFP II [%]	Intensity	Intensity RLP23-GFP I	Intensity RLP30-GFP I	Intensity RLP30-GFP II	Intensity RLP23-GFP II
RLP23	45	42	5	0	32	98,481	890	108	5		66	0	56,5	7,6	0	51,3	1,62E+10	8526700000	1782600	0	7697300000
RLP30	24	1	20	18	0	87,526	786	1	32	29		1	28,6	29,9	0	34179000000		790550	97638000	24407000000	0
A7EJM6	3	2	3	2	1	49,993	460	3	3	2	1	2,12E-09	4,6	6,3	4,6	2,4	7212260000	174130000	82912000	2772000000	187020000
A7E4E9;A7F0B8; A7E993	5;5;5	4	5	4	4	34,263	305	9	8	13	8	2,12E-09	49,8	58,7	49,8	49,8	3778600000	1002800000	53355000	7032300000	153900000
A7E4B2	3	2	3	3	3	68,531	629	4	4	5	3	7,42E-35	2,7	5,1	5,1	5,1	3286800000	83917000	45354000	1039500000	95453000
A7E677	7	7	4	4	5	33,999	313	9	6	4	9	8,08E-43	21,7	12,5	12,5	15,7	2880200000	100550000	27631000	3908900	113550000
A7EQ69	1	1	1	1	0	38,047	344	1	1	1		0,012414	3,5	3,5	3,5	0	232880000	113900000	6629700	52679000	0
A7EVV6	2	2	2	0	0	43,745	399	5	6			2,91E-30	4,3	4,3	0	0	191790000	11793000	7386500	0	0
A7F878	13	12	8	0	0	67,638	622	15	12			5,08E-130	25,1	16,1	0	0	1436900000	105960000	3773100	0	0
A7EWA0	1	0	1	1	1	11,37	103	2	2	3	2	1,70E-21	11,7	11,7	11,7	11,7	143590000	0	1373200	61002000	69215000
A7EGA2	6	5	5	0	0	62,929	605	6	7		2	6,12E-78	13,2	13,2	0	0	13201000	65957000	6605600	0	0
A7ET56	7	6	7	4	4	41,639	375	2	2	2	2	1,06E-39	15,7	20	8,5	8,5	1263200000	40884000	21674000	20168000	43598000
A7E468	1	0	0	1	1	15,983	150		1	1	1	0,0034261	0	0	7,3	7,3	110740000	0	0	57251000	53493000
A7ERD2	2	1	1	1	1	31,017	284	2	2	1	3	9,21E-26	4,6	4,6	4,6	8,5	1104200000	49842000	10630000	14645000	35305000
A7EW99	2	1	1	1	2	15,319	136	1	1	2	2	6,33E-10	5,1	5,1	10,3	10,3	994220800	18008000	16564000	33815000	47162000
A7EN31	7	6	4	3	4	20,984	183	7	4	3	6	1,06E-85	41	28,4	25,7	29,5	94732000	32402000	8261800	11728000	42341000
A7ER40	4	1	2	3	2	55,676	518	1	2	3	2	5,32E-19	2,3	4,4	7,7	3	633010000	0	2839400	40392000	22625000
A7F2S3	1	1	1	0	1	70,228	625	1	1		1	0,010263	1,9	1,9	0	1,9	39807000	24167000	2154300	0	13486000
A7EGT2	2	0	1	2	2	24,626	220		1	2	2	9,96E-05	0	5	8,6	8,6	36468000	0	1458400	13700000	21309000
A7F3F7	5	3	3	2	0	47,188	421	3	3	2	2	3,06E-13	7,1	8,1	4,8	0	31068000	16504000	9764900	4798900	0
A7EPZ2	2	1	2	1	1	79,463	699	1	2	2	1	8,36E-19	1,7	3,1	1,4	1,4	304110000	56197700	8224200	12647000	3920300
A7E3Y3	2	1	0	2	2	28,659	249	1	1	2	2	5,92E-06	6	0	8,8	8,8	28599000	3769600	0	12060000	16162000
A7EFC1	1	1	1	1	1	22,171	191	1	1	1	1	2,08E-14	4,2	4,2	4,2	4,2	25238000	15912000	1113300	11065000	11469000
A7EM88	2	1	1	2	1	45,498	399	1	1	2	1	1,82E-15	2,5	2,5	2,5	2,5	23788000	13217000	730200	10902000	10833000
A7EH60	3	3	3	1	1	49,792	447	4	3	2	2	5,03E-23	5,6	5,6	3,1	3,1	23706000	12451000	2969300	3917700	4367700
A7ECL2	2	0	0	2	1	11,143	97			2	1	0,001047	0	0	34	12,4	23448000	0	0	11241000	12208000
A7F5P3	1	1	0	1	1	30,095	269	2		2	2	0,0002193	3,7	0	3,7	3,7	23347000	6578800	0	9317000	13372000
A7F7J3	1	0	0	0	1	54,745	521				1	0,0079835	0	0	0	2,1	23035000	0	0	0	23035000
A7EUG8;A7F8B4	2;1	1	1	1	2	34,521	323	1	1	1	2	9,17E-08	3,3	3,3	3,6	6,9	20054000	6801800	2943800	3608600	15471000
A7F631;A7E9K5	2;1	2	1	1	1	22,52	203	2	1	1	1	8,29E-05	9,4	5,4	5,4	5,4	19373000	9944820	1437900	3502400	4484800
A7E193	1	1	0	1	1	83,141	774	1		1	1	0,0008587	1,6	1,6	1,6	1,6	18564000	1200800	0	4727200	12608000
A7EAD4	2	2	1	0	1	73,058	668	3	1		2	1,04E-06	1,6	1,5	0	1,6	17042000	13240000	742760	0	3059600
A7EN60	1	0	1	0	1	50,796	475		1		1	0,01304	0	1,5	0	1,5	13504000	0	878370	0	12625000
A7EVG6	1	0	0	1	1	98,29	871			1	1	0,0059617	0	0	0,8	0,8	13249000	0	0	6645500	6603200
A7EHY2	2	0	0	1	1	50,371	450			1	1	4,03E-05	0	2,2	2,2	1,8	11280000	0	0	3898000	7381500
A7E3Y4	1	1	1	1	0	34,198	321	1	1	1		3,84E-06	5,9	5,9	5,9	0	10551000	4416100	2364400	3770100	0
A7EYV5	1	1	1	0	0	30,677	278	1	1			0,0083856	4	4	0	0	10441000	8364100	2077300	0	0
A7EH24	3	3	2	0	0	14,282	136	3	2			2,29E-14	18,4	18,4	0	0	8729900	4497600	4232300	0	0
A7EP81	1	1	1	1	1	21,505	189	1	1	1	1	3,54E-06	5,8	5,8	5,8	5,8	8416700	947480	318250	1904600	5246400
A7ECT9	2	0	0	2	0	22,998	205			2		1,79E-09	0	0	10,2	0	7300300	0	0	7300300	0
A7F847	3	2	3	0	0	38,506	343	2	3			8,09E-43	7	9,3	0	0	6835700	2428400	4407300	0	0
A7E431	1	0	0	1	0	49,196	447			1		0,0072653	0	0	1,6	0	6709000	0	0	6709000	0
A7E7R3	1	1	1	1	1	87,431	790	1	1	1	1	0,005631	1,5	1,5	1,5	1,5	6640800	627440	183710	1135600	4694100
A7EP20	1	0	0	1	0	30,687	280				1	0,0005389	0	0	3,6	0	5920800	0	0	5920800	0
A7F2H8	1	1	0	1	1	56,468	488	1			1	0,0095802	1,8	0	1,8	1,8	5702300	1101400	0	2578000	2023000
A7EXV0	3	3	1	0	0	34,201	327	3	1			1,84E-33	14,4	4	0	0	5687600	4638400	1049100	0	0
A7F6H0	4	3	3	0	0	44,099	412	3	3			1,83E-25	8,5	10	0	0	5556800	2924900	2632000	0	0
A7EJY3;A7E449	3;1	3	2	2	1	71,219	678	3	2	2	1	1,10E-57	4,4	2,7	2,7	1	5510800	1413700	1092600	1586700	1417700
A7E4I2	2	2	1	0	0	31,375	280	2	1			2,92E-18	4,3	3,9	0	0	5110200	4214600	895540	0	0
A7EK15	2	2	2	0	0	50,012	450	1	1		1	1,07E-07	2,1	6	0	3,9	5103800	660500	1827100	0	2616200
A7E8C0	2	2	2	0	0	50,012	450	1	1		1	5,04E-30	7,3	7,3	0	0	5096500	4431700	664800	0	0
A7F4H7	1	0	0	1	1	17,89	156			1	1	0,0082926	0	0	4,5	4,5	5055000	0	0	1683100	3371900
A7E8L3	2	2	2	0	0	43,571	391	1	1			4,00E-07	5,4	5,4	0	0	4654800	2906900	1748000	0	0
A7E116	6	4	5	0	0	64,6	585	4	5			1,38E-17	8,5	10,9	0	0	4600500	2615800	1984700	0	0
A7E947	1	1	1	0	0	49,04	439	1	1			0,0085872	2,5	2,5	0	0	4511100	2708200	1802900	0	0
A7F5G5	1	0	0	1	0	56,162	518			1	1	0,0067106	0	0	1,7	1,7	4508600	0	0	2373300	2136300
A7F074	1	1	1	0	0	170,53	1494	1	1			0,000581	0,8	0,8	0	0	4391300	1913600	2477600	0	0
A7F4P8	1	1	1	1	1	33,747	323	1	1		1	2,28E-19	3,4	3,4	3,4	3,4	4068300	1812600	796970	1458800	0

Table 8.7: Identified proteins in the LC-MS/MS-analysis by fishing the ligand II.

Protein IDs	Peptide Counts (all)	Peptides RLP23-GFP I	Peptides RLP30-GFP I	Peptides RLP30-GFP II	Peptides RLP23-GFP II	Mol. Weight [kDa]	Sequence Length	Experiment RLP23-GFP I	Experiment RLP30-GFP I	Experiment RLP30-GFP II	Experiment RLP23-GFP II	PEP	Sequence Coverage RLP23-GFP I [%]	Sequence Coverage RLP30-GFP I [%]	Sequence Coverage RLP30-GFP II [%]	Sequence Coverage RLP23-GFP II [%]	Intensity	Intensity RLP23-GFP I	Intensity RLP30-GFP I	Intensity RLP30-GFP II	Intensity RLP23-GFP II
A7ERR3	1	1	1	0	1	0	48,723	432	1		1	0,00298687	1,6	0	1,6	0	4062100	1268600	0	2793600	0
A7EP73	2	1	0	1	0	0	28,498	263	1		1	0,00034562	3	0	2,7	0	4049200	633480	0	3415700	0
A7EY67	1	1	1	1	1	1	33,17	292	1	1	1	2,54E-07	4,5	4,5	4,5	4,5	3804300	600120	308390	1705900	1189900
A7E9W7	2	2	1	0	0	2	23,431	213	2	1		1,07E-10	9,9	4,7	0	0	3801700	3357800	443870	0	0
A7E157	1	0	0	1	1	1	66,997	763			1	0,0038241	0	0	0,9	0,9	3444000	0	0	1104600	2339400
A7EH00	3	3	3	0	0	0	37,224	341	3	3		2,09E-09	11,4	11,4	0	0	3180700	1751900	1428900	0	0
A7EZB5	2	0	0	1	1	1	57,409	517			1	0,00028176	0	0	3,1	2,9	2945800	0	0	2471600	474270
A7E5Q2	1	1	0	0	0	0	52,103	476	1			0,003207	2,3	0	0	0	2939100	2939100	0	0	0
A7F2R1	1	1	0	0	0	0	67,213	626	2			0,00127753	2,9	0	0	0	2885100	2885100	0	0	0
A7F3Q4	1	0	0	0	0	1	26,039	229				0,0094536	0	0	0	3,5	2691800	0	0	0	2691800
A7FBJ1	2	2	1	0	0	0	66,699	611	2	1		0,00015804	3,1	1,3	0	0	2686000	2094800	591220	0	0
A7EP00	1	1	1	0	0	0	51,387	466	1	1		2,02E-05	2,8	2,8	0	0	2549400	1923800	625640	0	0
A7E952;A7F042	1;1	0	0	0	1	1	15,136	142			1	2,42E-06	0	0	0	4,9	2539100	0	0	0	2539100
A7F4N3	1	0	0	0	1	0	104,44	943			1	0,014019	0	0	0	0,8	2317200	0	0	0	2317200
A7F3A9	1	0	0	0	1	1	57,463	510			1	0,016217	0	0	0	2,4	2273200	0	0	0	2273200
A7F8D9	1	1	1	0	0	0	140,86	1306	1			0,0038313	1,3	0	0	0	2205400	2205400	0	0	0
A7EN34	1	0	0	0	1	0	37,116	345			1	3,45E-05	0	0	3,8	0	2009500	0	0	2009500	0
A7EV80	1	1	1	0	0	0	68,529	632	1	1		0,0036832	1,6	1,6	0	0	1969500	1969500	0	0	0
A7EQR9;A7EL54;	1;1;1;1	1	0	0	0	0	121,78	1071	1			4,30E-10	0,7	0	0	0	1882000	1882000	0	0	0
A7ESH0	1	0	0	0	1	0	16,895	147			1	0,016074	0	0	0	5,4	1852300	0	0	0	1852300
A7EC20	1	1	1	0	0	0	75,119	680	2	1		3,07E-56	2,9	2,9	0	0	1809900	1626600	183230	0	0
A7EU18	1	0	0	0	1	1	53,83	483			1	0,0075588	0	0	1,7	1,7	1717300	0	0	649410	1067900
A7ECE6	1	0	0	0	1	0	16,832	151			1	0,0095802	0	0	0	6	1602300	0	0	0	1602300
A7EXD8	2	2	0	0	0	0	63,596	578	2			9,40E-16	4,3	0	0	0	1498300	1498300	0	0	0
A7E157	3	0	3	0	0	0	22,539	199		3		3,39E-06	0	17,6	0	0	1261000	0	1261000	0	0
A7EU48	1	1	1	0	0	0	66,408	588	2	2		2,15E-09	2,7	2,7	0	0	1251700	671460	580260	0	0
A7EFI0	2	1	2	0	0	0	43,932	393		1		1,27E-13	3,1	6,4	0	0	1216000	0	1216000	0	0
A7EXL0	1	0	0	0	1	0	90,251	821			1	0,0006948	0	0	0	0,9	1183100	0	0	0	1183100
A7F091	1	0	0	0	1	0	54,351	510			1	0,012636	0	0	3,1	0	1142400	0	0	1142400	0
A7ECQ8	1	0	1	0	0	0	20,432	202		1		0,0002974	0	6,9	0	0	1138100	0	1138100	0	0
A7EVV5	1	1	1	0	0	0	64,988	596	1	1		0,0046797	1,8	1,8	0	0	1076100	680420	395650	0	0
A7E7W1	2	1	2	0	0	0	28,531	273	1	2		1,16E-18	6,6	11,7	0	0	1065400	586960	478450	0	0
A7F043	1	0	0	0	1	1	14,783	139			1	6,95E-05	0	0	0	5,8	1032200	0	0	0	1032200
A7EET7	1	0	0	1	0	0	43,02	394		1		0,0072141	0	0	2,3	0	977880	0	0	977880	0
A7F0Y2	1	0	1	0	0	0	15,611	135		1		0,011996	0	8,1	0	0	894750	0	894750	0	0
A7E774	1	1	1	0	0	0	42,976	413	1	1		3,31E-21	6,3	6,3	0	0	834300	551090	283210	0	0
A7F6J2	1	0	0	0	1	0	53,858	471			1	0,0038241	0	0	0	1,5	787810	0	0	0	787810
A7E4P1	1	0	1	0	0	0	16,484	155		1		1,96E-05	0	8,4	0	0	748920	0	748920	0	0
A7E759	2	1	1	0	0	0	51,668	463	1	1		8,80E-05	3,5	2,6	0	0	548070	357190	190880	0	0
A7EPL9	3	0	3	0	0	0	72,965	669		3		3,37E-06	0	5,4	0	0	502680	0	502680	0	0
A7ESF8	1	0	1	0	0	0	146,82	1532		1		0,015346	0	1,1	0	0	465180	0	465180	0	0
A7EQS0	1	1	1	0	0	0	58,768	531	1	1		0,0065144	1,9	1,9	0	0	0	0	0	0	0
A7ESR3	1	1	0	0	0	0	127,55	1153	1			0,0011492	1,6	0	0	0	0	0	0	0	0

Table 8.8: Identified proteins in the LC-MS/MS-analysis after reversed phase chromatography and SDS-PAGE.

Protein IDs	Peptide counts (all)	Peptides E6	Peptides slice k	Peptides SN S	Peptides slice S	Mol. weight [kDa]	Sequence lengths	Score	Sequence coverageE5 [%]	Sequence coverage slice k [%]	Sequence coverage SN S [%]	Sequence coverage slice S [%]	Intensity	Intensity E6	Intensity slice k	Intensity SN S	Intensity slice S
A7E993;A7F0B8;A7E4E9	2;2;2	2	0	1	0	14,566	128;155;305	15,625	14,1	0	7	0	30162000	26239000	0	3923100	0
A7E610	3	2	1	0	0	15,959	143	24,694	18,2	4,9	12,6	0	53712000	38669000	7377400	7665400	0
A7E7B4	1	1	0	0	0	48,904	449	7,7899	2	0	0	0	3530900	3530900	0	0	0
A7ECT9	1	1	0	0	0	22,998	205	9,3411	4,9	0	0	0	1071200	1071200	0	0	0
A7EE85	1	0	0	0	1	91,721	833	5,7771	0	0	0	2,5	0	0	0	0	0
A7EJY8	1	1	0	0	0	99,971	900	6,1383	0,8	0	0	0	165680	165680	0	0	0
A7ELW6	1	1	0	0	0	99,313	884	6,028	1,2	0	0	0	164630	164630	0	0	0
A7EQ30	2	1	0	0	1	133,4	1179	11,265	0,7	0	0	0,6	8380000	0	0	0	8380000
A7ER74	1	1	0	0	0	52,351	464	5,8941	1,7	0	0	0	1225200	1225200	0	0	0
A7ET57	2	2	0	1	0	22,539	199	55,704	13,1	0	6,5	0	26796000	25912000	0	884500	0
A7ETS6	3	2	0	1	1	41,639	375	39,461	9,1	0	2,9	2,9	4895300	2884600	0	1385900	624840
A7EUW2	1	1	0	0	0	48,954	450	5,8155	2,7	0	0	0	942400	942400	0	0	0
A7F3K1	1	0	0	0	1	59,23	523	5,873	0	0	0	4	0	0	0	0	0
A7F3Y0	6	6	1	0	0	89,529	809	52,717	2,6	1,1	0	0	416930000	411730000	5194700	0	0
A7F4W9	1	0	0	0	1	6,8204	58	5,7947	0	0	0	13,8	6634700	0	0	0	6634700

Table 8.9: Identified proteins in the LC-MS/MS-analysis from SDS-PAGE gel slices analysed by Dr. Frank Menke.

Identified Proteins (96)	Molecular Weight	gel slice 1.4	gel slice 2.3	gel slice 2.4	gel slice 2.5	gel slice 3.4	gel slice 4.4	gel slice 4.5	gel slice 5.5	gel slice 6.4	gel slice 7.4	gel slice 8.4	gel slice 9.4	gel slice 10.4
Sscle10g077860	22 kDa	75	341	203	49	220	342	79	100	182	477	272	374	260
Sscle03g029230	34 kDa	47	30	50	44	32	55	51	62	67	60	68	43	54
Sscle08g068580	19 kDa	33	58	56	16	48	57	32	41	45	50	57	67	39
Sscle15g104590	42 kDa	20	73	55	14	46	42	15	11	29	30	58	55	31
Sscle16g108160	34 kDa	28	29	26	24	23	26	28	27	36	62	27	29	29
Sscle03g022920	12 kDa	42	19	28	24	20	33	30	26	31	33	32	26	23
Sscle03g024790	18 kDa	28	15	17	17	16	18	28	23	20	23	19	14	12
Sscle02g018350	166 kDa	25	13	19	13	16	13	18	18	20	17	21	23	19
Sscle10g080270	67 kDa	21	20	16	21	21	24	23	19	17	33	18	22	18
Sscle12g090030	62 kDa	12	13	21	23	13	20	14	19	19	26	22	19	19
Sscle03g030530	42 kDa	13	24	20	10	17	16	14	19	14	15	18	18	21
Sscle02g015410	22 kDa	25	16	21	21	16	22	21	17	21	19	21	18	11
Sscle02g017490	63 kDa	18	24	19	11	16	17	17	10	15	21	14	18	16
Sscle16g108170	38 kDa	4	12	7	5	8	7	4	7	7	11	6	9	9
Sscle01g008170	43 kDa	13	11	12	14	11	10	15	12	13	9	13	15	6
Sscle07g057170	99 kDa	18	4	7	22	14	5	16	6	12	3	7	6	9
Sscle08g063080	67 kDa	5	15	9	6	12	13	10	8	10	10	8	11	9
Sscle07g056420	18 kDa	0	3	2	0	0	2	0	0	1	3	1	2	2
Sscle07g059880	28 kDa	10	9	8	11	10	8	9	8	8	9	8	8	12
Sscle06g048920	15 kDa	1	0	0	1	0	0	2	1	1	1	1	0	0
Sscle09g073610	56 kDa	0	0	1	0	0	0	0	0	0	0	0	0	0
Sscle09g074570	110 kDa	7	7	10	6	8	6	10	5	10	6	9	9	5
Sscle03g028510	16 kDa	3	10	6	4	5	6	6	5	5	11	6	14	5
Sscle03g028710	31 kDa	9	4	4	4	7	9	6	8	7	13	6	4	7
Sscle16g107660	24 kDa	2	12	5	2	5	9	4	0	4	4	3	6	4
Sscle06g049550	80 kDa	4	2	2	5	4	4	5	4	4	2	5	2	5
Sscle08g064170	27 kDa	4	6	8	3	7	4	4	4	6	8	6	8	8
Sscle01g011530	16 kDa	6	6	6	6	4	6	6	6	6	4	6	6	6
Sscle05g043810	20 kDa	4	7	5	2	5	6	6	5	6	4	5	4	4
Sscle06g049350-DECO	?	0	0	0	0	0	0	0	0	0	1	0	0	0
Sscle03g031000-DECO	?	0	0	1	0	0	0	0	0	0	0	0	0	0
Sscle02g019600	31 kDa	1	12	4	1	6	3	2	2	4	4	4	5	8
Sscle04g037130-DECO	?	0	0	0	0	0	0	0	0	0	0	0	1	0
Sscle09g070540-DECO	?	0	0	0	1	0	0	0	0	0	0	0	0	0
Sscle07g061530-DECO	?	0	0	1	0	0	0	0	0	0	0	0	0	0
Sscle08g063580	105 kDa	1	1	3	0	0	2	2	0	4	0	4	2	2
Sscle10g076250	29 kDa	2	8	0	2	2	2	3	2	5	2	2	2	2
Sscle06g053820	104 kDa	2	2	2	2	0	2	0	2	0	0	2	2	0
Sscle02g014540	115 kDa	0	0	0	0	0	0	1	0	0	1	0	0	0
Sscle02g011880	206 kDa	0	0	0	0	0	0	0	0	0	1	0	0	0
Sscle15g103750	47 kDa	1	0	1	1	0	2	0	1	1	3	3	1	1
Sscle05g046610-DECO	?	0	0	2	0	1	0	0	0	0	1	0	1	1
Sscle01g006430-DECO	?	0	0	0	0	0	0	0	0	0	0	0	1	0
Sscle10g077120	160 kDa	0	0	0	0	0	0	0	1	1	0	0	0	0
Sscle13g094220	246 kDa	0	0	1	0	0	0	0	0	0	0	0	0	0
Sscle03g025450	95 kDa	0	0	0	5	2	1	5	4	4	0	1	0	0
Sscle14g099090	42 kDa	0	1	0	0	0	1	0	0	9	0	0	0	0
Sscle15g104020	113 kDa	0	0	0	0	0	0	0	0	0	0	0	0	1
Sscle10g080460-DECO	?	0	0	0	0	0	0	0	0	0	1	0	0	0
Sscle03g031360	56 kDa	0	2	2	0	2	2	2	2	2	2	2	2	2
Sscle09g069810	77 kDa	0	0	0	1	0	1	0	0	0	0	0	0	1
Sscle11g085550	74 kDa	2	2	2	0	2	2	2	2	2	3	2	2	2
Sscle01g008820-DECO	?	0	1	1	0	0	2	0	0	0	1	0	1	0
Sscle04g034310	58 kDa	1	0	1	0	0	0	2	0	1	0	0	0	0
Sscle12g090390	70 kDa	3	2	0	0	2	2	2	2	2	3	3	2	0
Sscle11g083470	25 kDa	0	0	2	1	0	2	4	0	0	5	1	0	0
Sscle03g022530	73 kDa	0	0	0	0	0	0	1	0	0	0	1	0	0
Sscle01g009480	25 kDa	2	2	2	1	0	0	2	2	2	0	1	2	2
Sscle11g084380	20 kDa	2	0	0	2	0	1	2	2	2	1	1	2	0
Sscle10g075210	68 kDa	1	0	0	2	2	0	2	0	4	2	2	0	2
Sscle05g043410	54 kDa	2	0	0	2	2	0	1	0	0	0	0	0	0
Sscle15g104230-DECO	?	0	0	0	0	0	0	0	0	0	0	0	0	1
Sscle03g027870	64 kDa	1	0	0	0	2	0	1	2	2	0	2	1	0
Sscle15g106280	69 kDa	1	2	2	0	2	0	3	1	2	0	2	2	2
Sscle05g044050	14 kDa	3	0	0	2	2	0	1	1	3	2	0	0	0
Sscle05g048580	139 kDa	0	0	0	0	0	0	0	0	0	0	0	0	1
Sscle10g080050	40 kDa	2	2	1	1	0	2	0	1	3	0	2	2	0
Sscle10g079010	15 kDa	2	1	2	1	0	2	3	0	2	0	2	0	0
Sscle02g016510	82 kDa	0	0	0	0	2	2	2	2	2	0	2	2	2
Sscle04g033200	68 kDa	0	2	0	0	0	0	0	0	0	0	0	0	0
Sscle13g092110-DECO	?	0	0	0	0	0	0	0	0	1	0	0	0	0
Sscle01g002430	62 kDa	0	0	1	0	0	0	0	0	1	0	2	0	0
Sscle11g084720	19 kDa	0	0	0	0	2	2	2	3	2	0	2	2	0
Sscle08g062240	43 kDa	0	2	2	2	1	0	2	0	0	2	0	0	0
Sscle08g067290	50 kDa	0	3	1	0	0	0	0	0	0	0	2	2	0
Sscle01g007550	14 kDa	2	0	2	3	0	0	0	2	1	0	0	2	0
Sscle11g085570-DECO	?	0	0	0	0	0	0	0	0	0	1	0	0	0
Sscle13g093110	60 kDa	0	0	0	0	0	0	0	0	0	3	0	0	0
Sscle12g090430	34 kDa	0	0	0	0	0	0	1	2	0	2	0	0	3
Sscle02g013770	32 kDa	0	1	0	0	0	1	0	0	1	1	1	1	1
Sscle02g020140	20 kDa	0	0	0	0	0	2	0	0	0	0	1	0	0
Sscle08g066140	35 kDa	0	0	0	0	0	0	0	0	0	0	0	0	1
Sscle10g077770	13 kDa	2	0	0	2	0	0	0	0	1	0	0	0	0
Sscle14g100060	70 kDa	0	0	0	0	0	2	1	0	0	0	0	0	0
Sscle08g065770	12 kDa	0	0	0	1	0	0	1	1	0	0	0	0	0
Sscle11g083230	38 kDa	0	0	1	0	0	2	0	0	0	0	1	0	0
Sscle05g041810	69 kDa	0	0	0	0	0	2	0	2	0	0	0	0	0
Sscle14g099300	28 kDa	0	0	0	0	0	0	2	1	0	0	0	0	0
Sscle09g074550	48 kDa	0	0	0	0	0	0	0	0	0	1	0	0	0
Sscle07g057820	55 kDa	0	0	0	0	0	0	0	0	0	2	0	0	0
Sscle01g004620	13 kDa	0	0	0	0	0	0	0	0	0	2	0	0	0
Sscle08g062290	15 kDa	1	0	0	0	0	0	1	0	0	0	2	0	0
Sscle09g074770	28 kDa	1	0	0	2	0	0	0	0	0	0	0	0	0
Sscle06g051210	21 kDa	0	1	0	0	0	0	2	0	0	0	0	0	0
Sscle01g009200	29 kDa	0	1	0	0	0	0	0	0	0	0	0	0	0
Sscle03g026590	26 kDa	0	1	0	0	0	0	0	0	0	0	0	0	0

Table 8.10: Most abundant proteins in the LC-MS/MS-analysis from SDS-PAGE gel slices analysed by Dr. Frank Menke, which were compared to *Pseudomonas*.

Most abundant proteins (42)	Molecular Weight	gel slice 1.4	gel slice 2.3	gel slice 2.4	gel slice 2.5	gel slice 3.4	gel slice 4.4	gel slice 4.5	gel slice 5.5	gel slice 6.4	gel slice 7.4	gel slice 8.4	gel slice 9.4	gel slice 10.4
Sscle01g008170	43 kDa	13	11	12	14	11	10	15	12	13	9	13	15	6
Sscle01g009480	25 kDa	2	2	2	1	0	0	2	2	2	0	1	2	2
Sscle01g011530	16 kDa	6	6	6	6	4	6	6	6	6	4	6	6	6
Sscle02g015410	22 kDa	25	16	21	21	16	22	21	17	21	19	21	18	11
Sscle02g017490	63 kDa	18	24	19	11	16	17	17	10	15	21	14	18	16
Sscle02g018350	166 kDa	25	13	19	13	16	13	18	18	20	17	21	23	19
Sscle02g019600	31 kDa	1	12	4	1	6	3	2	2	4	4	4	5	8
Sscle03g022920	12 kDa	42	19	28	24	20	33	30	26	31	33	32	26	23
Sscle03g024790	18 kDa	28	15	17	17	16	18	28	23	20	23	19	14	12
Sscle03g025450	95 kDa	0	0	0	5	2	1	5	4	4	0	1	0	0
Sscle03g028510	16 kDa	3	10	6	4	5	6	6	5	5	11	6	14	5
Sscle03g028710	31 kDa	9	4	4	4	7	9	6	8	7	13	6	4	7
Sscle03g029230	34 kDa	47	30	50	44	32	55	51	62	67	60	68	43	54
Sscle03g030530	42 kDa	13	24	20	10	17	16	14	19	14	15	18	18	21
Sscle03g031360	56 kDa	0	2	2	0	2	2	2	2	2	2	2	2	2
Sscle05g043810	20 kDa	4	7	5	2	5	6	6	5	6	4	5	4	4
Sscle05g044050	14 kDa	3	0	0	2	2	0	1	1	3	2	0	0	0
Sscle06g048920	15 kDa	1	0	0	1	0	0	2	1	1	1	1	0	0
Sscle06g049550	80 kDa	4	2	2	5	4	4	5	4	4	2	5	2	5
Sscle06g051210	21 kDa	0	1	0	0	0	0	2	0	0	0	0	0	0
Sscle06g053820	104 kDa	2	2	2	2	0	2	0	2	0	0	2	2	0
Sscle07g056420	18 kDa	0	3	2	0	0	2	0	0	1	3	1	2	2
Sscle07g057170	99 kDa	18	4	7	22	14	5	16	6	12	3	7	6	9
Sscle07g059880	28 kDa	10	9	8	11	10	8	9	8	8	9	8	8	12
Sscle08g063080	67 kDa	5	15	9	6	12	13	10	8	10	10	8	11	9
Sscle08g063580	105 kDa	1	1	3	0	0	2	2	0	4	0	4	2	2
Sscle08g064170	27 kDa	4	6	8	3	7	4	4	4	6	8	6	8	8
Sscle08g068580	19 kDa	33	58	56	16	48	57	32	41	45	50	57	67	39
Sscle09g074570	110 kDa	7	7	10	6	8	6	10	5	10	6	9	9	5
Sscle10g075210	68 kDa	1	0	0	2	2	0	2	0	4	2	2	0	2
Sscle10g076250	29 kDa	2	8	0	2	2	2	3	2	5	2	2	2	2
Sscle10g077860	22 kDa	75	341	203	49	220	342	79	100	182	477	272	374	260
Sscle10g080270	67 kDa	21	20	16	21	21	24	23	19	17	33	18	22	18
Sscle11g084380	20 kDa	2	0	0	2	0	1	2	2	2	1	1	2	0
Sscle11g085550	74 kDa	2	2	2	0	2	2	2	2	2	3	2	2	2
Sscle12g090030	62 kDa	12	13	21	23	13	20	14	19	19	26	22	19	19
Sscle15g103750	47 kDa	1	0	1	1	0	2	0	1	1	3	3	1	1
Sscle15g104590	42 kDa	20	73	55	14	46	42	15	11	29	30	58	55	31
Sscle15g106280	69 kDa	1	2	2	0	2	0	3	1	2	0	2	2	2
Sscle16g107660	24 kDa	2	12	5	2	5	9	4	0	4	4	3	6	4
Sscle16g108160	34 kDa	28	29	26	24	23	26	28	27	36	62	27	29	29
Sscle16g108170	38 kDa	4	12	7	5	8	7	4	7	7	11	6	9	9

Danksagung

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