Identification and Characterization of the Novel Fungal MAMP SsE1 and its Receptor-Like Protein (RLP30)-based Perception System in *Arabidopsis*

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Summary

Effective plant defense strategies rely on the perception of non-self determinants, so-called microbe-associated molecular patterns (MAMPs), by transmembrane pattern recognition receptors (PRRs) leading to MAMP-triggered immunity (MTI). Plant resistance against necrotrophic pathogens with a broad host-range is complex and yet not well understood. Particularly, it is unclear if resistance to necrotrophs involves MTI. In this thesis, a novel proteinaceous elicitor called SsE1 was partially purified from the necrotrophic fungal pathogen *Sclerotinia sclerotiorum* that induces typical MTI responses in the model plant *Arabidopsis thaliana*. Analysis of natural genetic variation between different *Arabidopsis* accessios revealed five ecotypes (Mt-0, Lov-1, Lov-5, Br-0 and Sq-1) that are fully insensitive to SsE1. Using a F2 segregating population from crosses between Col-0 and the SsE1-insensitive ecotype Lov-1 the locus determining SsE1 sensitivity was mapped to RECEPTOR-LIKE PROTEIN 30 (RLP30). Further reverse genetic screens revealed that SsE1-triggered immune responses depend on two receptor-like kinases, BRASSINOSTEROID INSENSITIVE1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) and SUPPRESSOR OF BIR1 1/EVERSHED (SOBIR1/EVR). Knock-out mutant lines for these three genes are more susceptible to *S. sclerotiorum* and the taxonomically-related fungus *Botrytis cinerea*. Whereas SOBIR1 physically interacts with RLP30 in a ligand-independent manner and is required for RLP30 localization to the plasmamembrane, complex formation of BAK1 and RLP30 could not yet be demonstrated. However, the cytoplasmic tail of RLP30 can be phosphorylated by the kinase domain of BAK1 in an *in vitro* kinase assay. Furthermore, transient co-expression of RLP30 and SOBIR1 confers SsE1 sensitivity to naturally SsE1-insensitive *N. benthaminana* plants. As SOBIR1 is also involved in the MAMP signaling pathway triggered by the novel elicitor EMAX

from *Xanthomonas* species and which is mediated by another RLP-PRR, REMAX/RLP1, it can be assumed that SOBIR1 is a novel co-receptor for PRRs of the RLP-type.

The identification of SsE1 and RLP30 as a potential MAMP-PRR pair demonstrates the relevance of plant MTI in the resistance to necrotrophic fungi. Hence, the application of co-delivered RLP receptor complex components, such as RLP30 and SOBIR1, could serve as novel genetic tool for the generation of fungus-resistant crops.

Zusammenfassung

Effektive Pflanzenabwehrstrategien beruhen auf der Erkennung von "Nicht-Selbst"-Determinanten, den sogenannten Mikroben-assoziierten Molekularen Mustern (*microbe-associated molecular patterns*, MAMPs), durch transmembrane Mustererkennungsrezeptoren (*pattern recognition receptors*, PRRs) und entsprechende MAMP-ausgelöste Immunität (*MAMP-triggered immunity*, MTI). Die pflanzliche Resistenz gegenüber nekrotrophen Pathogenen mit einem breiten Wirtsspektrum ist komplex und bisher noch nicht gut verstanden. Im Besonderen ist unklar, ob Mechanismen der MIT für die Resistenz gegenüber Nekrotrophen benötigt wird.

In dieser Arbeit wurde eine neue proteinöse Elizitoraktivität aus dem nekrotrophen pilzlichen Pathogen *Sclerotinia sclerotiorum* partiell aufgereinigt, die SsE1 (*Sclerotinia sclerotiorum* Elicitor 1) genannt wurde und die typische pflanzliche Abwehrreaktionen in der Modelpflanze *Arabidopsis thaliana* auslöst. Analysen der genetischen Variation zwischen verschiedenen *Arabidopsis* Ökotypen ergaben fünf Ökotypen (Mt-0, Lov-1, Lov-5, Br-0 und Sq-1) mit einer vollständigen Insensitivität gegenüber SsE1. Die segregierende F2-Population der Kreuzung zwischen Col-0 und dem SsE1-insensitiven Ökotypen Lov-1 wurde für die klassische MAP-basierte Klonierung des für die SsE1-Erkennung nötigen Lokus verwendet und ergab das Rezeptor-ähnliche Protein RLP30. Zusätzlich wurden in einem reversen genetischen Ansatz die beiden Rezeptorkinasen BRASSINOSTEROID INSENSITIVE1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) und SUPPRESSOR OF BIR1 1/EVERSHED (SOBIR1/EVR) als weitere Komponenten der SsE1-Erkennungsmachinerie identifiziert. Knock-out Mutanten aller drei Gene sind anfälliger gegenüber einer Infektion mit *S. sclerotiorum* sowie des taxonomisch verwandten Pilzes *Botrytis cinerea*. Während SOBIR1 mit RLP30 liganden-unabhängig interagieren kann und für die Plasmamembran-Lokalisation von RLP30 nötig

ist, konnte eine RLP30/BAK1-Interaktion bisher nicht nachgewiesen werden. Allerdings kann der cytoplasmatische Teil von RLP30 von der BAK1-Kinasedomäne *in vitro* phosphoryliert werden. Transiente Co-Expression von RLP30 und SOBIR1 führte in natürlicherweise SsE1-insensitiven *N. benthamiana*-Pflanzen zur SsE1-Erkennung. Da SOBIR1 auch für die Signalweiterleitung nach Erkennung eines weiteren neuen MAMPs, EMAX (*enigmatic MAMP from Xanthomonas*), durch das Rezeptorprotein RLP1/REMAX nötig ist (Jehle et al., 2013), kann man SOBIR1 als einen neuen Ko-Rezeptor für RLP-PRRs betrachten.

Die Identifikation von SsE1 und RLP30 als neuem MAMP-PRR-Paar veranschaulicht die Wichtigkeit von pflanzlicher MTI in der Resistenzantwort gegenüber nekrotrophen Pilzen. Deshalb könnte eine gleichzeitige Applikation von mehreren Rezeptorkomplex-Komponenten, wie z.B. RLP30 und SOBIR1, ein neues genetisches Mittel zur Herstellung pilzresistenter Kulturpflanzen darstellen.

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Abbreviations

aa	Amino acid
At	*Arabidopsis thaliana*
Ax21	the activator of Xa21-mediated immunity
Avr	Avirulence factor
bp	Base pair
CC	coiled-coil
cDNA	complementary DNA
cfu	colony forming unit
DAMP	Danger-associated molecular pattern
DNA	Deoxyribonucleid acid
EF-Tu	Elongation factor Tu
Elf18	Peptide from EF-Tu
EMAX	Enigmatic MAMP of *Xanthomonas*
ETI	Effector-triggered immunity
Flg22	Peptide from flagellin
FLS2	Flagellin sensing 2
GFP	Green fluorescent protein
HR	Hypersensitive reaction
kb	Kilobase
kDa	KiloDalton
KO	Knock-out
LRR	Leucine-rich repeat
LPS	Lipopolysaccharide
LysM	Lysin motif
MAMP	Microbe-associated molecular pattern
MAPK/MPK	Mitogen-activated protein kinase
NB	nucleotide-binding
MBP	maltose binding protein / myelin basic protein
PAMP	Pathogen-associated molecular pattern
PCD	Programmed cell-death
PCR	Polymerase chain reaction
PEN	Extracts of *Penicillium chrysogenum*
PGN	Peptidoglycan
PR	Pathogenesis-related
PRR	Pattern recognition receptor
PTI	PAMP-triggered immunity
Pto	*Pseudomonas syringae* pv. *tomato*
pv.	Pathovar
REMAX	Receptor of emax
R-Gene	Resistance gene

RNA	Ribonucleic acid
R-Protein	Resistance protein
RLK	Receptor-like kinase
RLP	Receptor-like protein
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic acquired resistance
T-DNA	Transfer-DNA
TIR	Toll/Interleukin
TLR	Toll-like receptor
WT	Wild type
Xa21	*Xanthomonas* resistant protein 21

1. Introduction

Plants are sessile living organisms that have to face various biotic and abiotic challenges. In their whole life time, plants have to be effective to defend attacks by fungi, oomycetes, bacteria, viruses, nematodes and invertebrates for survival (Dangl and Jones, 2001; Jones and Dangl, 2006; Boller and Felix, 2009). Discrimination of self and non-self is a primary challenge for all living organisms to detect microbial invasion and to protect and defend against the invader. Animals have specialized cells and a circulatory immune system that is able to react with somatically generated adaptive immune responses at the infection site (Nürnberger et al., 2004; Ausubel, 2005). Unlike this animal immune system, plants have evolved a special innate immune system that efficiently detects and protects plants against the majority of plant pathogens (Boller and He, 2009; Thomma et al., 2011)

1.1 Plant Immunity: MTI versus ETI

The plant innate immune system is comprised of two layers of defense. The first layer depends on detecting an array of microbial-derived molecules termed pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs) by pattern recognition receptors (PRRs) at the plant cell surface (Chisholm et al., 2006; Jones and Dangl, 2006; Zipfel, 2008; Boller and He, 2009). This so-called MAMP-triggered immunity (MTI) is associated with rapid ion fluxes across the plasma membrane, mitogen-activated protein kinase (MAPK) activation, production of reactive-oxygen species, increase of ethylene biosynthesis, rapid changes in defense gene expression, and cell wall reinforcement (Felix et al., 1999; Asai et al., 2002; Kunze et al., 2004; Zipfel et al., 2004; Chinchilla et al., 2006; Miya et al., 2007; Kwon et al., 2008; Boudsocq et al., 2010; Jeworutzki et al., 2010). However, in order to survive

and grow, successful pathogens have acquired the ability to deliver effector proteins into the plant cell to suppress MTI, leading to plant disease. As a co-evolutionary event, plants have evolved specified proteins, so-called resistance (R) proteins, that can interact directly or indirectly with effector proteins to monitor the presence of pathogens, leading to a rapid activation of defence responses, the so-called effector triggered immunity (ETI) (Chisholm et al., 2006; Ingle et al., 2006; Jones and Dangl, 2006; Pieterse et al., 2009). One prominent reaction during ETI is the hypersensitive response, a form of programmed cell death at the infection site that prevents further spreading of the pathogen (Jones and Dangl, 2006). ETI as a second layer of defense is also referred to as gene-for-gene resistance (Axtell and Staskawicz, 2003; Mackey et al., 2003; Coll et al., 2011). These events reflect the evolutionary arms race between host and pathogen, and suggest that PTI is a major initial driving force in the process of shaping of the dynamic interplay between plants and their pathogens (Ingle et al., 2006; Monaghan and Zipfel, 2012).

1.2 PAMPs/MAMPs: Pathogen or microbe-associated molecular patterns

PAMPs, or microbe-associated molecular patterns (MAMPs), as they are not restricted to pathogenic microbes (Jones and Dangl, 2006; He et al., 2007; Boller and Felix, 2009), are highly conserved characteristics of a whole class of micro-organisms and play pivotal functions for the micro-organism, and therefore are difficult to mutate or delete (Zipfel, 2008; Zipfel, 2009).
Although many MAMPs have been characterized (Table 1), so far only a limited number of corresponding PRRs has been identified (Nürnberger and Kemmerling, 2009).

Table 1. 1 Known PAMPs (Nürnberger and Kemmerling, 2009)

PAMP	Origin	Minimal structural motif required for defense activation	Sensitive plants
Lipopolysaccharide	Gram-negative bacteria (*Xanthomonas*, *Pseudomonas*)	Lipid A, lipooligosaccharides	Pepper, tobacco
Peptidoglycan	Gram-positive and Gram-negative bacteria	Muropeptides	*Arabidopsis*, tomato
Flagellin	Gram-negative bacteria	flg22 (amino-terminal fragment of flagellin)	*Arabidopsis*, tomato
Elongation factor (EF-Tu)	Gram-negative bacteria	elf18 (*N*-acetylated amino-terminal fragment of EF-Tu)	*Arabidopsis* and other Brassicaceae
Harpin (HrpZ)	Gram-negative bacteria (*Pseudomonas, Erwinia*)	Undefined	*Arabidopsis*, cucumber, tobacco, tomato
Cold-shock protein	Gram-negative bacteria Gram-positive bacteria	RNP-1 motif (amino-terminal fragment of the cold-shock protein)	Solanaceae
Necrosis-inducing proteins (NLP)	Bacteria (*Bacillus* spp.), fungi (*Fusarium* spp.), oomycetes (*Phytophthora* spp., *Pythium* spp.)	Undefined	Dicotyledonous plants
Transglutaminase	Oomycetes (*Phytophthora* spp.)	Pep-13 motif (surface-exposed epitope of the transglutaminase)	Grapevine, *Nicotiana benthamiana*, parsley, potato, tobacco
Cellulose-binding elicitor lectin (CBEL)	Oomycetes (*Phytophthora* spp.)	Conserved Cellulose-binding domain	Tobacco, *Arabidopsis*
Lipid-transfer proteins (elicitins)	Oomycetes (*Phytophthora* spp., *Pythium* spp.)	Undefined	Tobacco, turnip
Xylanase	Fungi (*Trichoderma* spp.)	TKLGE pentapeptide (surface-exposed epitope of the xylanase)	Tobacco, tomato
Invertase	Yeast	*N*-mannosylated peptide (fragment of the invertase)	Tomato
β-glucans	Fungi (*Pyricularia oryzae*)	Tetraglucosyl glucitol	Rice, tobacco, Fabaceae
	Oomycetes (*Phytophthora* spp.)	Branched hepta-β-glucoside	
	Brown algae	Linear oligo-β-glucosides	
Sulfated fucans	Brown algae	Fucan oligosaccharide	Tobacco
Chitin	All fungi	Chitin oligosaccharides (degree of polymerisation > 3)	*Arabidopsis*, barley, rice, tomato, wheat
Ergosterol	All fungi		Tomato
Cerebrosides A, C	Fungi (*Magnaporthe* spp.)	Sphingoid base	Rice
Oligouronides	Plant cell wall pectins	Oligomers	*Arabidopsis*, tobacco
Cellodextrins	Plant cell wall cellulose	Oligomers	Grapevine
Cutin monomers	Plant cuticle	Dodecan-1-ol	Apple, cucumber, tomato
Siderophores	*Pseudomonas fluorescens*	Undefined	Tobacco

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1.3 PRRs: Receptor Like Kinases and Receptor Like Proteins

Plants perceive and integrate external and internal stimuli via surface receptors (Boller and Felix, 2009; De Smet et al., 2009) of which the largest family with more than 600 genes in *Arabidopsis* is formed by receptor-like kinase proteins (RLKs). RLKs are modular transmembrane proteins with a cytoplasmic kinase domain participating in intracellular signal transduction and an extracellular domain potentially responsible for ligand perception. A few of these RLKs have been demonstrated to sense MAMPs and to participate in plant defense (Nürnberger et al., 2004; Boller and Felix, 2009). The first PRR identified in plants or animals was the XA21 protein, conferring resistance to the gram-negative bacterial pathogen *Xanthomonas oryzae pv. oryzae* in rice (Song et al., 1995). The best studied PRRs of *Arabidopsis* are FLS2 (FLAGELLIN SENSING 2) and EFR (EF-TU RECEPTOR), two receptors with an extracellular domain harbouring tandemly arranged leucine-rich-repeats (eLRR) that bind a 22-amino acid fragment of bacterial flagellin, flg22, and a 18-amino acid fragment of bacterial elongation factor thermo-unstable (EF-Tu), elf18, respectively (Gomez-Gomez and Boller, 2000; Zipfel et al., 2006). Xa21 (23 LRRs), FLS2 (28 LRRs) and EFR (21 LRRs) belong to the same subfamily LRR-RK XII of receptor-like kinases (Shiu et al., 2004).

In addition to RLKs, receptor-like proteins (RLPs) have been demonstrated to be crucial players in plant immunity (Kruijt et al., 2005; Monaghan and Zipfel, 2012; Yang et al., 2012). The structure of RLPs resembles that of RLKs, except that they lack a cytoplasmic kinase domain (Wang et al., 2008a). The first identified eLRR-RLP was the tomato (*Solanum lycopersicum*) Cf-9, which recognizes the effector protein Avr9 from the fungal leaf mold pathogen *Cladosporium fulvum* (Jones et al., 1994). Other Cf proteins have subsequently been identified and found to specifically sense corresponding *Cladosporium* Avr proteins (Thomas et al., 1998). Two tomato eLRR-RLPs, LeEIX1 and LeEix2, mediate the perception of the cell wall-derived ethylene

inducing xylanase (EIX) from *Trichoderma* species (Ron and Avni, 2004). Recently, it was shown that tomato race-specific resistance against vascular wilt caused by *Verticillium* species is also controlled by two eLRR-RLPs, Ve1 and Ve2 (Kawchuk et al., 2001; Fradin et al., 2009).

RLPs carrying lysin motif (LysM) ectodomains are implicated in the perception of N-acetylglucosamine-containing glycan structures such as peptidoglycan or chitin, both being major structural component of bacterial or fungal cell walls, respectively (Kaku et al., 2006; Miya et al., 2007; Wan et al., 2008; Willmann et al., 2011; Gust et al., 2012; Liu et al., 2012).

In *Arabidopsis*, a reverse genetic-based study of the role of the whole repertoire of eLRR-RLPs in plant development and in the response to abiotic and biotic stresses revealed only few RLPs with an apparent biological function (Wang et al., 2008a). Loss-of-function experiments have shown that *RLP30*, *RLP51* and *RLP52* are involved in basal resistance against a bacterium (*Pseudomonas syringae* pv. phaseolicola), an oomycete (*Hyaloperonospora arabidopsidis*) and a fungus (*Erysiphe cichoracearum*), respectively (Ramonell et al., 2005; Wang et al., 2008a; Zhang et al., 2012). Additionally, the implication of eLRR-RLPs in the developmental program of *Arabidopsis* was demonstrated with TOO MANY MOUTHS (TMM; RLP17), regulating stomata distribution and initiation of stomatal precursor cells (Yang and Sack, 1995; Nadeau and Sack, 2002), and CLAVATA 2 (CLV2; RLP10), being required for proper meristem and organ development (Kayes and Clark, 1998; Jeong et al., 1999).

The absence of the intracellular signaling domain suggests that RLPs must interact with additional components for subsequent activation of immune and developmental responses. RLKs are primary candidates to fulfill such functions. Recent studies have shown that BAK1 (BRI1-ASSOCIATED RECEPTOR KINASE 1), an eLRR-RLK structurally similar to FLS2 and EFR, is involved in Ve1-mediated *Verticillum* resistance in tomato (Fradin et al., 2009). BAK1 belongs to the *Arabidopsis* somatic embryogenesis receptor

kinase (SERK) family that includes five LRR-RLKs (Hecht et al., 2001; Albrecht et al., 2008). BAK1 was first identified as a co-receptor for recognizing brassinosteroids by direct interaction with the brassinosteroid receptor BRI1 (BRASSINOSTEROID INSENSITIVE 1) (Li et al., 2002; Nam and Li, 2002). In addition, BAK1 and its close homolog BKK1 form a complex with FLS2 and EFR in a ligand-dependent manner (Chinchilla et al., 2007; Heese et al., 2007; Roux et al., 2011) underlining their capacity to interact with different types of receptors and their roles as key regulators of both immune and developmental responses.

Interestingly, LysM-RLP-mediated signaling is independent of BAK1 but instead requires the LysM-RLK CERK1 (CHITIN ELICITOR RECEPTOR KINASE 1) (Shimizu et al., 2010; Willmann et al., 2011). Similar to the RLPs involved in disease resistance, both TMM and CLV2 have been demonstrated to recruit RLKs (TMM/ERECTA and CLV2/CRN/CLV1, respectively) to form functional receptor complexes (Guo et al., 2010; Lee et al., 2012).

A few examples for known MAMPs and their recognition systems are described in the following (Figure 1.1) (Monaghan and Zipfel, 2012).

Figure 1. 1 Pattern recognition receptors with known ligands (Monaghan and Zipfel, 2012)
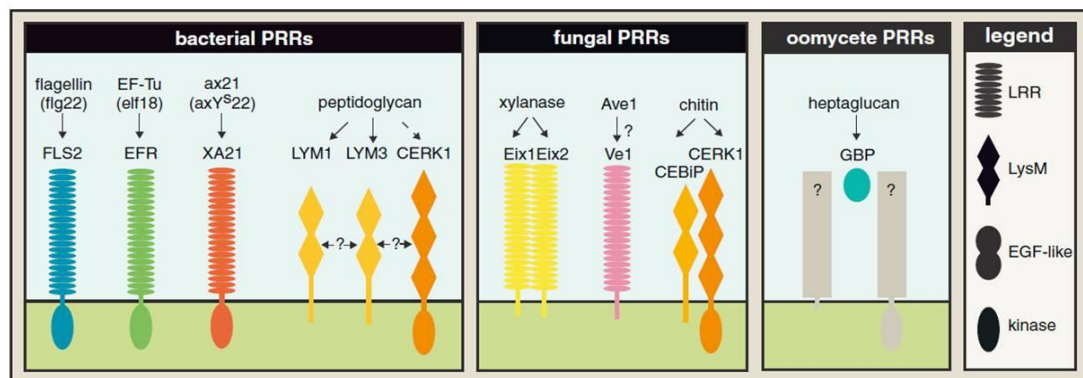
1.4 Bacterial MAMPs and their cognate PRRs

1.4.1 Flagellin / FLS2

A well-documented PAMP is flagellin, as the main building block of bacterial flagella. Flagella are required for bacterial motility and flagellin acts as a general elicitor of plant defense responses (Felix et al., 1999). Flagellin contains a highly conserved N- and C-terminus, whereas the middle part is variable between known species. In *Arabidopsis*, flagellin perception can induce multiple defence responses that include the production of reactive oxygen species (ROS), activation of mitogen-activated protein kinases (MAPKs), callose deposition, expression of defense-related genes and also strong inhibition of seedling root growth (Gomez-Gomez and Boller, 2000; Asai et al., 2002; Zipfel et al., 2004; Gust et al., 2007; Zhang et al., 2007; Boutrot et al., 2010). Perception of flg22 also initiates the closure of stomata that serve as entry sites of pathogen infection, and this may constitute a form of pre-invasive immunity (Melotto et al., 2006).

Most plant species recognize a conserved 22-amino acid epitope, flg22, present at the N-terminus of flagellin (Boller and Felix, 2009). Functional AtFLS2 homologues have been recently identified in rice (*Oryza sativa*), *Nicotiana benthamina* and tomato (Hann and Rathjen, 2007; Robatzek et al., 2007; Takai et al., 2008), indicating that the PRR for flagellin is evolutionary conserved. *Arabidopsis* plants mutated in FLS2 are more susceptible to infections by the pathogenic bacterium *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto* DC3000) when surface-inoculated (Zipfel et al., 2004), but also allow more growth of the non-adapted bacterium *P. syringae* pv. *phaseolicola* (*Pph*, a bean pathogen) (de Torres et al., 2006) or of a *P. syringae* pv. *tabaci* (*Pta*, a tobacco pathogen) strain devoid of flagellin (FliC- mutant) (Li et al., 2005). Finally, *N. benthamiana* plants silenced for *NbFLS2* are more susceptible to a range of adapted and non-adapted bacteria (Hann & Rathjen,

2007).

Mutational, phylogenetic, and structural modeling approaches were employed to investigate exact binding sites of flg22 in the leucine-rich repeat (LRR) domain of AtFLS2, and LRR9 to LRR15 seem to be important for flagellin responsiveness (Dunning et al., 2007). The other experiment was performed with a comparison of AtFLS2 and the orthologous tomato (*Solanum lycopersicum*) receptor SlFLS2 to map the species-specific sites in the recognition of shortened or sequence-modified flg22 (Mueller et al., 2012). LRRs 7 to 10 of SlFLS2 were revealed to confer high affinity binding of SlFLS2 to the core peptide RINSAKDD of flg22. In addition, the LRRs 19 to 24 also play an important role for the responsiveness to C-terminally modified flagellin peptides (Mueller et al., 2012). However, the precise FLS2 binding site for flg22 is still elusive.

Upon ligand-binding, the interaction between FLS2 and BAK1 (chapter 1.3) occurs almost instantaneously after flg22 binding (<15s) (Schulze et al., 2010). The interaction between FLS2 and BAK1 is crucial for FLS2 signal transduction, because the innate immune response is much weakened in the *bak1* mutant (Chinchilla et al., 2007; Heese et al., 2007).

Activation of PRR triggers immune responses, however tight regulation of PRR signaling is also essential for the host, since exaggerated and prolonged immune response would be harmful to the host (Lang and Mansell, 2007). In an approach with a functional fusion of FLS2 to the green fluorescent protein (GFP), which localizes at cell membranes in un-stimulated cells, it was demonstrated that FLS2-GFP was rapidly and specifically internalized from cell surfaces upon flg22 stimulation (Robatzek et al., 2006). The signal intensity of FLS2-GFP decreased with prolonged time of flg22 incubation, indicating a FLS2 degradation following the internalization (Robatzek et al., 2006). Receptor internalization and subsequent degradation is a major mechanism to control receptor abundance and the intensity and duration of receptor signaling (Irani and Russinova, 2009). Recently, it was demonstrated

that flg22-FLS2 signaling can be attenuated by ubiquitination-dependent degradation (Lu et al., 2011). The procedure is dependent on two U-box E3 ubiquitin ligases, PUB12 and PUB13, which associate with FLS2 upon perception of flg22, a process that also requires kinase activity of BAK1 (Lu et al., 2011). Moreover, reticulon-like proteins RTNLB1 and RTNLB2 were also identified as FLS2 interactors and regulate FLS2 immune activity by controlling transport of newly synthesized FLS2 to the plasma membrane (Lee et al., 2011).

1.4.2 EF-Tu / EFR

Another well-known bacterial PAMP is the elongation factor Tu (EF-Tu), one of the most abundant and most conserved proteins of bacteria, which can be recognized by *Arabidopsis* and other members of the *brassicaceae* family (Kunze et al., 2004; Boller and Felix, 2009).

EFR (EF-Tu receptor) was identified as receptor for EF-Tu by screening T-DNA insertion lines of various receptor-like kinases related to FLS2 in *Arabidopsis* (Zipfel et al., 2006). In addition to lacking EF-Tu-triggered immune responses, *Arabidopsis efr* mutants are more susceptible to infection with *Agrobacterium tumefaciens*, demonstrating the *in vivo* importance of EF-Tu perception in immunity against bacteria (Zipfel et al., 2006). Moreover, *Nicotiana benthamiana* which naturally is unable to perceive EF-Tu acquires EF-Tu binding sites and PAMP responsiveness upon transient expression of *EFR* (Zipfel et al., 2006). Microarray analysis reveals that the whole transcriptome changes 30 min after flg22 and 60 min after elf26 treatments were highly correlated, indicating a strong overlap of FLS2 and EFR signaling pathways (Zipfel et al., 2006).

Studies with chimeric receptors were used to map subdomains of EFR ligand binding and receptor activation (Albert et al., 2010). Replacement of different parts of the LRR-domain of EFR with the corresponding LRR-domain parts of

FLS2 displayed that the first six LRRs and/or the last two LRRs play a critical role in elf18 binding and EFR activation (Albert and Felix, 2010). The results also indicated that modular assembly of chimeras from different receptors can be used to form functional receptors (Albert et al., 2010). Unfortunately, the exact binding sites for elf18 still need to be further narrowed down.

Forward genetic screens for elf18 insensitive mutants revealed that components of the EQ quality control machinery are required for EFR function (Li et al., 2009; Nekrasov et al., 2009; Ottmann et al., 2009; Saijo et al., 2009). Interestingly, although these mutants were affected in elf18 triggered immune responses, they still responded normally to flg22 (Li et al., 2009; Nekrasov et al., 2009; Ottmann et al., 2009; Saijo et al., 2009) .

1.4.3 Peptidoglycan & LYM3 / LYM1 / CERK1

Peptidoglycans (PGNs) are essential and unique components of the bacterial envelope. Virtually all bacteria contain a layer of PGN, but the amount, location and specific composition vary (Schleifer and Kandler, 1972; Meroueh et al., 2006). PGNs from Gram-negative and Gram-positive bacteria are effective elicitors of defense responses in the model plant *Arabidopsis thaliana* (Gust et al., 2007; Erbs et al., 2008).

Lysin-motif proteins LYM1, LYM3 and CERK1 proved to have a critical role in the perception of bacterial peptidoglycan and in innate immunity to bacterial infection in *Arabidopsis* (Willmann et al., 2011). LYM1 and LYM3 are plasma membrane proteins that directly interact with structurally different PGNs from Gram-negative and Gram-positive bacteria (Willmann et al., 2011). CERK1, previously identified as chitin receptor (see chapter 1.5.2), is a LysM receptor kinase that cannot bind PGN, but is indispensable for PGN sensitivity and immunity to bacterial infection -(Gimenez-Ibanez et al., 2009; Willmann et al., 2011). The authors' hypothesis is that the three proteins can form a heterotrimeric receptor complex for recognizing PGNs and transferring the

extracellular signal across the plasma membrane to initiate immune responses (Willmann et al., 2011). The direct interaction between LYM1/LYM3 and CERK1 however still needs to be demonstrated.

1.5 Oomycete and fungal MAMPs and their PRRs

1.5.1 Heptaglucan & GBP

Soybean (*Glycine max* L.) detects a β-1,6-linked and β-1,3-branched heptaglucoside (HG), present in cell walls of the oomycetal pathogen *Phytophthora sojae*, and launches defense responses (Cosio et al., 1990a; Cosio et al., 1990b). A soluble β-glucan-binding protein (GBP) was identified that has a specific binding site for the HG (Umemoto et al., 1997; Fliegmann et al., 2004). This glucan binding protein was shown to be an extracellular glucanase anchored to the plasma membrane (Umemoto et al., 1997; Fliegmann et al., 2004). Biochemical methods identified the GBP as a binding protein for the soluble glucan heptamer, but a genetic evidence for the role of GBP in innate immunity of soybean is still missing (Schwessinger and Ronald, 2012). Notably, the GBP lakes an obvious signaling domain, raising the possibility that this protein associates with a yet unknown transmembrane protein to initiate signal transduction (Boller and Felix, 2009; Zipfel, 2009).

1.5.2 Chitin & CEBIP / CERK1

Chitin is a potent MAMP in several plant species, including rice, wheat, tomato and *Arabidopsis* (Felix et al., 1993; Shibuya and Minami, 2001; Okada et al., 2002; Kaku et al., 2006; Miya et al., 2007; Wan et al., 2008). The first protein shown to be involved in chitin perception was the rice chitin elicitor-binding protein (OsCEBiP) which contains two extracellular LysMs for chitin-binding, a transmembrane domain and a short cytoplasmic tail (Kaku et al., 2006). Knockdown of OsCEBIP expression reduced the chitin-induced oxidative burst

and suppresses chitin-induced changes in gene expression (Kaku et al., 2006). The lack of a functional intracellular domain of OsCEBiP suggested that additional component(s) should be involved in the chitin signal transduction pathway. Based on this consideration, a second LysM domain containing protein, OsCERK1 (*O. sativa* chitin elicitor receptor kinase), was revealed as an important component in chitin perception of rice (Shimizu et al., 2010). Interestingly, OsCEBIP and OsCERK1 cooperatively regulate chitin signaling by forming a chitin-induced heterodimeric receptor complex in rice (Shimizu et al., 2010).

Before the demonstration of the importance of OsCERK1 in chitin perception in rice, the role of CERK1 was first identified in *Arabidopsis* (Miya et al., 2007; Wan et al., 2008). AtCERK1 (also named Lys-RLK1) is a membrane protein with three extracellular LysMs, a transmembrane domain and an intracellular kinase domain and is required for chitin responses in *Arabidopsis* (Miya et al., 2007; Wan et al., 2008). Genetic inactivation of *AtCERK1* increased susceptibility to the biotrophic and necrotrophic fungal pathogens *Erysiphe cichoracearum* and *Alternaria brassicicola*, respectively (Miya et al., 2007; Wan et al., 2008). Moreover, AtCERK1 is able to directly bind chitin *in vitro* and *in vivo* (Lizasa et al., 2010; Petutschnig et al., 2010; Shinya et al., 2012). The crystal structure of the ectodomain (ECD) of AtCERK1 has been solved (Liu et al., 2012). The three LysM domains of AtCERK1 are tightly packed in a globular structure. LysM domain 2 was found to bind N-acetylglucosamine pentamers, although ligand binding did not alter the conformation of the protein. A chitin octamer acts as a bivalent ligand to induce AtCERK1-ECD dimerization that is inhibited by shorter chitin fragments. Ligand-induced AtCERK1 homodimerization is vital for receptor activation and immune signal transduction (Liu et al., 2012; Willmann and Nurnberger, 2012).

Although AtCERK1 seems to be sufficient for ligand binding and signal transduction, recent studies investigated the role of the three homologs of OsCEBiP in *Arabidopsis*, LYM1, LYM2 and LYM3, in chitin perception (Shinya

et al., 2012). It was demonstrated that only one member of the AtLYM family, AtLYM2/AtCEBiP, displayed a high-affinity binding for chitin similar to rice CEBiP (Shinya et al., 2012). However, the single/triple knockout mutants of AtLYM1, AtLYM2 and AtLYM3 and the overexpression line of AtLYM2/AtCEBiP showed the same chitin-induced defense responses as the wild type, indicating that AtLYM2/AtCEBiP does not contribute to chitin signaling (Shinya et al., 2012). Using a domain swap approach, the authors confirmed that AtCERK1 is sufficient for chitin perception by itself (Shinya et al., 2012).

1.5.3 Xylanase & LeEIX1 / 2

The β-1-4-endoxylanase EIX originates from the fungus *Trichoderma viride*, (Fuchs et al., 1989; Hanania and Avni, 1997). EIX has been proven to be a potent MAMP in tomato and tobacco, also inducing the hypersensitive cell death (HR) (Ron et al., 2000; Ron and Avni, 2004; Bar et al., 2010).

Experiments with EIX mutants lacking enzymatic xylanase activity but still retaining elicitor activity indicated that not the enzyme activity is necessary for the HR elicitation process (Enkerli et al., 1999; Furman-Matarasso et al., 1999; Rotblat et al., 2002). Indeed, the immunogenic portion of the EIX was identified as comprising the pentapeptide TKLGE mapping to an exposed β-strand of the EIX protein (Rotblat et al., 2002).

The two LRR-RLPs LeEix1 and LeEix2 can bind EIX independently, but only LeEIX2 confers signalling when expressed heterologously in tobacco. Upon application of EIX, LeEIX2 can form heterodimers with LeEIX1 (Bar et al., 2010). Recently, LeEix1 was suggested as a decoy receptor for LeEix2 and BAK1 is required for this LeEix1 function (Bar et al., 2010).

1.5.4 Ave1 & Ve1

The tomato gene locus Ve has been shown to provide resistance against

specific strains of *V. dahliae* and *V. albo-atrum* that are soil-borne vascular wilt pathogens (Kawchuk et al., 1994; Kawchuk et al., 2001). This locus comprises the two closely linked inversely oriented genes *Ve1* and *Ve2*. The corresponding proteins of Ve1 and Ve2 share an amino acid identity of 84% (Kawchuk et al., 2001). Importantly, transfer of the *Ve1* or *Ve2* gene into susceptible potato plants conferred resistance against *V. albo-atrum* (Kawchuk et al., 2001).

Recent studies suggested that only *Ve1* plays a role in defence response of tomato against race 1 strains of *V. dahliae* and *V. albo-atrum.* Furthermore, genetic inactivation of *SERK1* or *BAK1* in tomato compromises the Ve1-mediated resistance to *V. dahliae* and *V.albo-atrum* race 1 (Fradin et al., 2009; Fradin et al., 2011).

By using advanced whole genome and RNA sequencing technology, Ave1 (for Avirulence on Ve1 tomato) was revealed as a protein from *Verticillium* that activates Ve1-mediated immunity in tomato and tobacco and is required for fungal virulence (de Jonge et al., 2012). Interestingly, Ave1 is conserved in fungal pathogens such as *Fusarium oxysporum* f. sp. *lycopersici*, *Colletotrichum higginsianum* and *Cercospora beticola* and bacterial pathogens such as *Xanthomonas axonopodis* pv. *citri*. Considering its similarity to plant natriuretic peptides, Ave1 may have been acquired through horizontal gene transfer from plants (de Jonge et al., 2012). However, a direct interaction between Ave1 and Ve1 still needs to be demonstrated.

1.6 Fungal diseases

The most serious plant diseases are caused by fungi. Fungal diseases of plants have had a major impact on society and humans, even had an impact on human history. For instance, late blight of potato was responsible for the loss of 25 % of the population of Ireland; during the 1840s, more than 1 million people died from starvation or famine-related diseases, and more than 1.5

million emigrated from Ireland (Levetin. and McMahon., 2003).

Fungal pathogens can be broadly divided into two groups. One is dependent on living host tissue to finish its life cycle (biotrophs), the other kills their hosts quickly after infection and feeds on the cell content (necrotrophs) (Dangl and Jones, 2001; Voegele and Mendgen, 2003). Still, there are some fungal species called hemibiotrophic fungi. They behave as biotrophs at early stages of their life cycle, and then switch to necrotrophic growth and kill their hosts at the end of their life cycle (Koeck et al., 2011).

Although many measures have been taken to protect plants against fungal diseases, such as powdery mildew, downy mildew, blast, blight and rust infections they are still a serious problem in agriculture. Fungal pathogens can produce huge numbers of spores, which are dispersed from one susceptible host to another by wind or rain (Brown and Hovmoller, 2002). To develop measures to control these fungal diseases becomes more and more important in basic research.

Recently, Cyril Zipfel's group observed that stable transgenic *N. benthamiana* and tomato plants expressing AtEFR are more resistant to bacterial pathogens (Lacombe et al., 2010). Furthermore, tomato Ve1 remains fully functional after interfamily transfer to *Arabidopsis thaliana*, and Ve1-transgenic *Arabidopsis* plants were resistant to race 1 but not to race 2 strains of *V. dahliae* and *V. albo-atrum* (Fradin et al., 2011). These results indicate that signalling components downstream of PRRs are conserved across plant families and open a gate of trans-species transfer of PRRs to improve disease resistance. The identification of novel MAMPs and their cognate PRRs could therefore be important tools for disease control.

1.7 Thesis aims

Disease control in agriculture is important to ensure a sustainable food supply for an increasing human population. Hence, a better understanding of the

interaction of fungal pathogens and plants is an urgent issue for us.

With the exception of the chitin receptor CERK1 (Miya et al., 2007; Wan et al., 2008), no surface pattern recognition receptor involved in the recognition of fungal-derived MAMPs or effectors has been identified in *Arabidopsis.*

The research aims of this thesis were the identification of novel proteinceous fungal MAMP and their cognate receptors in *Arabidopsis.* The first step was to identify fungal-derived MAMPs from fungal pathogens that are important pests and for which the whole genome sequences are available, facilitating the subsequent identification of MAMPs.

The second aim of this thesis was to identify new PRRs and associated signaling components in *Arabidopsis* by virtue of reverse and forward genetic approaches. The forward genetic screening was based on natural genetic variation in *Arabidopsis.* Confronting diversity environment, these variations have resulted in different genotypes. For instance, two accessions of *Arabidopsis, Ws-0* and *Cvi-0*, have been shown to be flg22 insensitive and to be natural FLS2-mutants (Gomez-Gomez et al., 1999; Dunning et al., 2007). Therefore, the response of different ecotypes to the novel MAMPs that was identified will be tested, followed by map based cloning of responsible PRR genes in MAMP-insensitive accessions.

Additionally, a reverse-genetics approach will be conducted based on the previously observed up-regulation of PRRs by MAMP treatments (Zipfel et al., 2006). Microarray analyses in the AtGenExpress Initiative allowed for the identification of 49 MAMP-induced LRR-RLK genes (Kemmerling et al., 2007). Homozygous T-DNA insertion lines for 40 of these are available within our department and are complemented by further lines developed in Cyril Zipfel's lab. Also, a collection of homozygous T-DNA insertion lines for the 5 LysM-RLK genes and 6 out of the 9 LysM-P genes is avaible. All these mutants will be tested for loss of MAMP responsiveness.

2. Materials and Methods

2.1 Materials

2.1.1 *Arabidopsis thaliana*

Arabidopsis wild type, mutant and transgenic lines used in this study are listed in Tables 2.1 and 2. 2. If not stated otherwise, all seeds were obtained from the Nottingham *Arabidopsis* Stock Centre (NASC).

Table 2. 1 *Arabidopsis* wild type accessions used in this study

An-1	Bay-0	Bil-5	Bil-7	Bor-1	Bor-4	Br-0	Bur-0
C24	CIBC-5	Col-0	Ct-1	Cvi-0	Edi-0	Edn-1	Est-1
Fei-0	Fab-2	Got-7	Got-22	Gy-0	Gv-0	HR-10	Kas-1
Kn-0	Kondara	Kz-1	Kz-9	Ler	Lov-1	Lov-5	Lp2-2
Lz-0	Mr-0	Mrk-8	Ms-0	Mt-0	Mz-0	Na-1	Nde-3
NFA-8	NFA-10	Omo2-3	Pna-10	Pro-0	Pu2-23	Ra-0	Rmxa180
Ren-1	Ren-11	Se-0	Sha	Sorbo	Spr1-6	Sq-1	Tamm-27
Ts-1	Ts-5	Tsu-1	Ull2-3	Ull2-5	Uod-1	Uod-7	Van-0
Var2-6	Vod-7	Wa-1	Ws-0	Wt-5	Zdr-6		

Arabidopsis thaliana ecotype Col-0 was the background for all mutants and transgenic lines used in this study. The T-DNA insertional lines used in this work are listed in Table 2-2 and were either purchased (NASC) or received from other labs. The transgenic *pPR-1:GUS* line has been described previously (Shapiro and Zhang, 2001; Gust et al., 2007).

Table 2. 2 *Arabidopsis* mutant and transgenic lines used in this study

AGI	Gene name	Mutant name	Stock name	Reference
At3g05360	*RLP30*	*rlp30-1*	SALK_122528	(Wang et al., 2008a)
		rlp30-2	SALK_008911	
		rlp30-3	SALK_122536	
		rlp30-4	SALK_145342	
At3g05370	*RLP31*	*rlp31-1*	SALK_058586	(Wang et al., 2008a)
		rlp31-2	SALK_094160	
At3g05660	*RLP33*	*rlp33_2*	SALK_087631	(Wang et al., 2008a)
		rlp33_3	SALK_085252	
At5g20480	*EFR*	*efr-1*	SALK_044334	(Zipfel et al., 2006)
At5g46330	*FLS2*	*fls2c*	SAIL_691C4	(Zipfel et al., 2004)
At4g33430	*BAK1*	*bak1-3*	SALK_034532	(Schwessinger et al., 2011)
		bak1-4	SALK_116202	
		bak1-5	EMS-mutant	
At2g13790	*BKK1*	*bkk1-1*	SALK_057955	(Roux et al., 2011)
At1g73080	*PEPR1*	*pepr1*	SALK_059281	(Krol et al., 2010)
At1g17750	*PEPR2*	*pepr2*	SALK_098161	(Krol et al., 2010)
At3g52430	*PAD4*	*pad4-1*	EMS-mutant	(Jirage et al., 1999)
At3g20600	*NDR1*	*ndr1-1*	Fast-neutron-Mutant	(Century et al., 1995)
At3g48090	*EDS1*	*eds1-1*	EMS-mutant	(Aarts et al., 1998)
At1g35710	*XII1*	*xii1-2*	*GK-031G02*	Cyril Zipfel's lab unpublished
At2g24130	*XII2*	*xii2-1*	*SAIL_373_E04*	
At3g47090	*XII3*	*xii3-1*	*SALK_101474*	
At3g47110	*XII4*	*xii4-1*	*SALK_101668*	
At3g47570	*XII5*	*xii5-1*	*GK-415H04*	
At3g47580	*XII6*	*xii6-1*	*SAIL_31_F02*	
At4g08850	*XII7*	*xii7-1*	*SALK_061769*	
At2g31880	*SOBIR1 (EVR)*	*sobir1-12 (evr-3)*	SALK_050715	(Gao et al., 2009)
		sobir1-3 (evr-4)	SALK_009453	(Leslie et al., 2010)
		evr-2	EMS-mutant	(Leslie et al., 2010)

The double mutants *bak1-5 bkk1-1, fls2c efr-1 and pepr1 pepr2* have been characterized previously (Krol et al., 2010; Roux et al., 2011; Schwessinger et al., 2011). To generate *fls2 efr cerk1 xii* quadruple mutants (kindly provided by Freddy Boutrot and Cyril Zipfel, The Sainsbury Laboratory, Norwich, UK), *Arabidopsis xii* single mutants in the Col-0 background were crossed with the *fls2c efr-1 cerk1-2* triple mutant (Gimenez-Ibanez et al., 2009). T-DNA insertion in *fls2c* confers resistance to BASTA and T-DNA insertion in *cerk1-2* confers resistance to sulfadiazine. F2 seeds were surface sterilized and germinated on MS medium supplemented with 10 mg/L phosphinotricine (Duchefa) and 5.25 mg/L sulfadiazine sodium salt (Sigma). 50 mg/L kanamycin was added as a third antibiotic for the selection of seedlings carrying *xii(xx)*. Seedlings were assayed for their responses to elf18 (growth inhibition assay,(Zipfel et al., 2006)), flg22 and chitin (Albrecht et al., 2012). Insensitive plants were subsequently genotyped for the T-DNA insertions in *fls2c*, *efr-1*, *cerk1-2* and the mutation of the family *XII* gene of interest using the primers given in Table 6.4.

2.1.2 Fungal strains

Fugal strains for MAMP purification were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). *Sclerotinia sclerotiorum strain 1980* (kindly provided by H. Stotz, University of Würzburg, Germany) and *Botrytis cinerea* strain B05-10 (Kemmerling et al., 2007) were used for fungal infections (see chapter 2.2.8.1).

Table 2. 3 Fungal strains used in this study

Fungal species	Life cycle	Disease caused
Botrytis cinerea	Necrotrophic	grey mould
Cercospora beticola	Biotrophic	beet leaf spot
Fusarium graminearum	Necrotrophic	head blight on wheat and barley

Magnaporthe oryzae	Hemibiotrophic	rice blast
Mycosphaerella graminicola	Hemibiotrophic	leaf blotch of wheat
Rhizoctonia solani	Necrotrophic	"damping off" of a range of crops as diverse as cereals, canola, and legumes
Rhizopus oryzae	Necrotrophic	post harvest decay
Sclerotinia sclerotiorum	Necrotrophic	stem rot on soybean and *brassica*, host range of greater than 400 plant species
Ustilago maydis	Biotrophic	maize corn smut

2.1.3 Bacterial strains

2.1.3.1 *Escherichia coli* strains

Table 2. 4 Bacterial strains used in this study

Strains	Genotype	Reference
DH5α	fhuA2 lac(del)U169 phoA glnV44 Φ 80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17	Invitrogen
BL21(DE3)	F$^-$ ompT hsdS$_B$ (r$_B^-$m$_B^-$) gal dcm (DE3)	Novagen

2.1.3.2 *Agrobacterium tumefaciens* strains

Agrobacterium tumefaciens strain C58C1 (T-DNA$^-$ vir$^+$ rif^r) was used.

2.1.4 Vectors

Table 2. 5 Vectors used in this study

Vector	Description	Reference
PCR8/GW/TOPO (TA cloning vector)	Entry vector for the Gateway system	Invitrogen
pDEST15	*E. Coli* expression vector with a C-terminal	Invitrogen

	GST tag (Gateway destination vector)	
pET-22b(+)	*E. Coli* expression vector with a C-terminal His tag	Novagen
pMAL-p5x	*E. Coli* expression vector with a N-terminal MBP tag	NEB
pGWB2	Binary Gateway destination vector for expression of fusion proteins under control of CaMV 35S promoter	(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	
pGWB14	Binary Gateway destination vector for expression of fusion proteins under control of CaMV 35S promoter with a C-terminal 3XHA tag	
pGWB17	Binary Gateway destination vector for expression of fusion proteins under control of CaMV 35S promoter with a C-terminal 4Xmyc tag	
pK7FWG2.0 (EFR)	Binary Gateway destination vector for expression of EFR under control of CaMV 35S promoter with a C-terminal GFP tag	(Karimi et al., 2005; Albert et al., 2010)

2.1.5 Primers

The primers used in this study that were synthesized by Eurofins MWG Operon (Ebersberg). Lyophilized primers were resuspended in nuclease-free water to a final concentration of 100 µM. The working concentration was 10 µM. The sequence of primers used in this work for cloning, genotyping, transcript analysis and sequencing are listed in the Table 6.1 to Table 6.4..

2.1.6 Enzymes

2.1.6.1 DNA modification enzymes

Restriction endonucleases were purchased from Fermentas. Enzymes were supplied with 10x reaction buffer that were used for restriction digests.

Standard PCR reactions were performed using home-made *Taq* DNA polymerase. For cloning, *Pfu* DNA polymerase was used. All enzymes used and their providers are listed in Table 2.6.

Table 2. 6 Used enzymes in this study

Restriction endonucleases	Fermentas
Taq DNA polymerase	Home made
Pfu hot start II DNA polymerase	Fermentas
T4 DNA ligase	Fermentas
LR Clonase enzyme mix	Invitrogen

2.1.6.2 Protein modification enzymes

The proteases Proteinase K, AspN, trypsin and GluC and the commercial deglycosylation mixture were purchased from NEB. All enzyme reactions were carried out according to the manufacturer's recommendations (NEB).

2.1.7 Chemicals, buffers and solutions

Laboratory grade chemicals and reagents were purchased from Sigma-Aldrich (Taufkirchen), Carl Roth (Karlsruhe), Merck (Darmstadt), Qiagen (Hilden), Invitrogen (Karlsruhe), Duchefa (Haarlem, Niederlande), Molecular Probes (Leiden, Niederlande), Fluka (Buchs, Schweiz) und BD (Sparks, USA), unless otherwise stated.

All buffers and solutions were prepared with Milli-Q water. Buffers and solutions for molecular biological experiments were autoclaved or sterilized using filter sterilization units, respectively.

2.1.8 Media and antibiotics

Media were sterilized by autoclaving at 121 °C for 20 min. Table 2.7 shows the composition of used media. For the addition of antibiotics (as listed in Table

2.8) and other heat labile compounds the solution or media were cooled down to 55 °C. Heat labile compounds were sterilized using filter sterilization units prior to addition.

Table 2. 7 Media used in this study

Medium	Ingredients per 1 liter	Species
LB	10 g Bacto-Tryptone, 5 g NaCl, 5 g Yeast extract (YE)	*E.coli*
King's B	20 g glycerol, 40 g Proteose Pepton 3, after autoclaving addition of 0.1 % (v/v) $MgSO_4$ and KH_2PO_4	*Pseudomonas syringae*
PDB	24 g PDB (Potato Dextrose Broth, Duchefa), pH 5.8 (NaOH)	*Botrytis cinerea*
½ MS	2.2 g MS (Duchefa), pH 5.7 (KOH)	*Arabidopsis thaliana*

Table 2. 8 Antibiotics used in this study

Antibiotics	Con.(mg/ml)	Solvent
Ampicillin (Amp)	50	H_2O
Spectinomycin (Spe)	50	H_2O
Kanamycin (Kan)	50	H_2O
Rifampicin (Rif)	5	H_2O

2.2 Methods

2.2.1 Growth of *E.coli*

E. coli strains were inoculated overnight at 37 °C either on LB-plates or in liquid LB medium at 200 rpm. Antibiotics were added into the media according to the resistance genes the plasmid DNA was harboring.

2.2.3 Growth of *Agrobacterium tumefaciens*

A. tumefaciens strains were inoculated for 48 hours at 28 °C on LB-plates or

liquid LB medium at 200 rpm. Antibiotics were added into the media according to the resistance genes the plasmid DNA was harboring.

2.2.3 Maintenance and growth of plant material

Arabidopsis seeds were germinated by sowing directly onto GS90-soil (Gebr. Patzer GmbH) mixed with vermiculite. Seeds were cold treated by placing the pots after sowing on a tray with a lid and incubating them in the dark at 4 °C for 2 days. Pots were subsequently transferred to a controlled growth chamber, covered with a lid and maintained under short day conditions (8 h photoperiod, light intensity of approximately 200 µEinstein m^{-2} sec^{-1}, 22 °C and 50-60% humidity). The lids were removed when seeds had germinated. After three weeks, seedlings were separated by transferring one seedling per pot. For harvesting seeds, some plants were transferred to long day conditions (16h photoperiod) to allow early bolting and setting of seeds. To collect seeds, aerial tissue was enveloped with a paper bag and sealed with tape at its base until siliques shattered.

Nicotiana benthamiana and *Solanum lycopersiucum* plants were grown on a mixture of soil and sand containing 0.1% (v/v) Confidor in the greenhouse (13 h light, 11 h darkness).

For culture of *A. thaliana* on MS-Medium, the desired quantity of seeds was aliquoted in a fresh Eppendorf tube for surface sterilization. Open tubes with seeds were placed inside a desiccator containing a 100 ml beaker with 50 ml of 12% sodium-hypochloride solution (chlorine bleach). After addition of 1.5 ml of 37% HCl into the sodium hypochloride solution, the lid of the desiccator was immediately closed and sealed with Parafilm. Seeds were surface-sterilized by generated chlorine gas for 3-16 h. After opening of the desiccator, sterilized seeds were taken out and left in opened tubes under the sterile workbench for 15 min to allow the remaining chlorine gas to evaporate. Sterilized seeds were directly plated out on suitable culture media. Plates were sealed with Parafilm

and stored in the fridge, and transferred to light 2-3 days later.

2.2.4 Culture and maintenance of fungi

Three pieces of agar covered with fungal mycelium were cut from grown fungal plates and transferred to fresh Maltose-peptone plates (10 g malt, 2.5 g peptone, 15 g agar/l). Fungi were incubated at room temperature (RT) until a well growing mycelium was observed (about 2-3 three weeks). Three pieces of sub-cultured fungi were again cut and transferred to Maltose-peptone liquid medium (200 ml medium in a 500 ml flask) and fungi were incubated at RT with or without shaking for 2-3 weeks.

2.2.5 Long term storage of fungi

Each 10 ml Moser B medium (Macro-elements (g/l): Glucose (10), Sucrose (10), Maltose (10), Malt extract (10), Peptone (2), K_2HPO_4 (0.15), KH_2PO_4 (0.35), NH_4NO_3 (1), $NaNO_3$ (0.3), $CaCl_2$ (0.1), $MgSO_4 \cdot 7H2O$ (0.5); Micro-elements (mg/l): Thiamine (50), Biotine (1), Inositol (50), $ZnSO_4$ (1), $FeCl_3$ (10); Agar (15 g/l)) was prepared in 20 ml McCartney bottles. The agar has left to set by propping the bottle at a 40 degree angle without letting the sloping agar reach the neck of the bottle. A piece of fungus from Maltose peptone plate was cut and put on the agar surface. Fungal cultures were incubated at RT until grown well (about 2-3 three weeks) and afterwards stored at 4 °C.

2.2.6 Harvest of fungal culture medium and mycelium

Culture medium was filtered with a 75-mm nylon mesh and mycelium was washed once with distilled water. Liquid medium or mycelium was separately dry frozen for 2-3 day. After freeze-drying, material was stored in an air-tight bottles until processing.

2.2.7 Bio-assays

2.2.7.1 Luminol-based detection of Reactive Oxygen Species

Leaves from 4-5 weeks old *Arabidopsis* plants were cut into small pieces, and floated on water in Petri dishes at RT overnight. Leaf pieces (one piece / well) were placed in wells of 96-well plates (CELLSTAR[®] 96-well white plate (Greiner bio-one)) containing 100 µl of solution (10 ng/ml peroxidase (horseradish peroxidase; Applichem), 20 µM luminol). Different elicitor solutions were added to the plate. Light emission was measured as relative light units in a 96-well luminometer (Mithras LB 940; Berthold Technologies). The plate was measured every 1-2 min for 150 min.

2.2.7.2 Histochemical dection of Reactive Oxygen Species

For histochemical detection of the production of reactive oxygen species, H_2O_2 accumulation was stained with DAB (3,3′-Diaminobenzidine) (Sigma-Aldrich) using a modified method (Thordal-Christensen et al., 1997). Briefly, about 100 µl of different elicitor solutions were infiltrated individually into mature rosette leaves of 4-5 weeks old *Arabidopsis* plants using a 1 ml needleless syringe. Detached leaves were vacuum infiltrated for 2 min with a DAB solution (1 mg/ml, pH 3.8) and afterwards incubated in a humid atmosphere for at least 6 hours at room temperature. The staining solution was decanted and leaves were subsequently washed once in H_2O and then boiled for 10 min in 96% (v/v) ethanol to extract the chlorophyll. Destained leaves were stored in fresh 96% ethanol. DAB is polymerized locally in the presence of H_2O_2 giving a visible brown stain.

2.2.7.3 Ethylene measurement

Leaves from 4-6 weeks old *Arabidopsis* plants were cut into 4 mm pieces, and floated on water in Petri dishes at RT overnight. Three leaf pieces (about

0.007g fresh weight) were transferred to 6 ml glass tubes containing 0.5 ml water. The appropriate elicitor was added to the tubes and mixed thoroughly. Vials were closed with rubber septa. 1ml ethylene accumulating in the free air space was measured by gas chromatography (GC-14A, Shimadzu, Japan) after 2-3 h of incubation.

2.2.7.4 Histochemical staining for GUS activity

Different elicitor solutions were infiltrated individually into mature rosette leaves of 4-5 weeks old *Arabidopsis* plants using a 1 ml needleless syringe. Detached leaves were placed in a 1.5 ml Eppendorf-tube filled with 1 ml GUS-staining solution (50 ml: 50 mM sodium phosphate buffer (pH 7.0), 10.6 mg $K_4Fe(CN)_6$, 8.2 mg $K_3Fe(CN)_6$, 100 µl 0.5 M EDTA (pH 8.0), 25 mg X-Gluc (previously dissolved in 250 µl DMF), 250 µl Triton X–100 (added at the end!)) or enough solution to completely cover the tissue. The tissue was vacuum infiltrated with the aid of a vacuum-pump. Then, the tissue was incubated at 37 °C overnight in an oven to ensure even warming. Chlorophyll was removed from the tissue afterwards by washing with 70% (v/v) ethanol. The solution was changed several times until the tissue was colorless. The transparent tissue was subsequently scanned.

2.2.7.5 Elicitation of transcriptome changes

For elicitation of mature plants, leaves of 4-5 week old plants were infiltrated with solutions of PAMPs using a needleless syringe and harvested after indicated time points. For treatment of seedlings, they were first cultivated on sterile ½ MS plates for 6-7 days in long-day growth conditions. Then seedlings were transferred into ½-strength liquid MS medium supplemented with 1% (w/v) sucrose (3-4 seedlings in 300 µl medium/well, CELLSTAR® 24-well plate (Greiner bio-one)) and incubated overnight. After addition of the PAMPs, the seedlings were incubated with gentle shaking and harvested at indicated time

points for RNA extraction (chapter 2.2.10.4).

2.2.8 *Arabidopsis* infection assays

2.2.8.1 Fungal infection

For plant infections, *S. sclerotiorum* strain 1980 was used and cultures were freshly prepared on potato dextrose agar (Sigma) from a -80 °C stock (Guo and Stotz, 2007). Prior to inoculation, *S. sclerotiorum* was grown on minimal medium to reduce *Sclerotinia* aggressiveness as previously described (Guo and Stotz, 2007). An agar plug (5 mm in diameter) containing actively growing mycelium was placed on the adaxial surface of rosette leaves of 4-5 week old *Arabidopsis* plants. Plants were afterwards maintained at high humidity and disease development scored at 2-3 days post inoculation.

B. cinerea isolate BO5-10 was grown on synthetic media and used for infection assays on *Arabidopsis* leaves of 4-5 week old plants as described previously (Kemmerling et al., 2007).

2.2.8.2 Fungal DNA quantification

For fungal DNA quantification four *S. sclerotiorum* or *B. cinerea* infected leaves per genotype were harvested and pooled after two or three days of inoculation, respectively. The samples were frozen in liquid nitrogen and ground to powder. Total DNA was isolated using CTAB buffer (1.4 M NaCl, 20 mM EDTA (pH 8), 100 mM Tris-HCl (pH 8), 2% CTAB). Fungal biomass was determined by qRT-PCR using the SYBR Green qPCR Master Mix (Fermentas). The relative concentration of *Sclerotinia* internal transcribed spacer (ITS) or *Botrytis Actin* genomic DNA levels to *Arabidopsis Rubisco* (large subunit) levels was used to quantify fungal biomass (Fradin et al., 2011). Specific primers are listed in Table 6.3.

2.2.8.3 Histochemical analysis of fungal growth and plant cell death

Trypan blue staining was used to visualize *Sclerotinia* mycelium and developing plant cell death after infection with *S.sclerotinia*. Leaf material was placed in a 6-well plate (CELLSTAR® (Greiner bio-one)) and immersed in a trypan blue solution (10 ml lactic acid, 10 ml 100% glycerol, 10 ml Aqua-Phenol, 10 ml ddH$_2$O, 80 ml EtOH and 300 mg trypan blue). The plate was placed into a boiling water bath for 1-2 min followed by destaining in 5 ml chloral hydrate solution (1 mg/ml in water) for 2 h and a second time overnight on an orbital shaker. The stained leaf material was examined under a light microscope (Nikon Microscope eclipse 80i).

2.2.9 Fungal MAMP purification

2.2.9.1 Protein extraction from culture filtrate and mycellium

Freeze-dried culture filtrate and mycellium (see chapter 2.2.5) was dissolved at 0.5 g/5 ml in 100 mM MES buffer (pH 5.4, for cation exchange chromatography) or 100mM Hepes buffer (pH 7.4, for anion exchange chromatography). The sample was centrifuged at 13000 g for 15 min at 4 °C. The supernatant was transferred to a new tube. This step was repeated once. The supernatanat was then dialysed against 50 µM MES or HEPES buffer for 2 h at 4 °C in a dialysis cassette (Slide-A-Lyzer Dialysis Cassettes, Pierce). After dialysis, the protein solution was applied to the respective ion exchange column (chapter 2.2.9.2).

2.2.9.2 Small-scale purification (one step purification)

For small-scale purification, a 1 ml HiTrap Q FF column (cation exchanger, GE) or a 1 ml HiTrap SP FF column (anion exchanger, GE) were used in this study. A 5 ml syringe was filled with the extraction buffer. After the stopper was removed, the column was "drop to drop" connected to the syringe (with the provided adaptor) to avoid introducing air into the column. The snap-off end at

the column outlet was removed. The preservatives were washed out with 5 column volumes of extraction buffer at a speed of 1 ml/min. Then, the column was washed with 5 column volumes of elution buffer (Mes or Hepes buffer + 0.5 M KCl). Finally, the column was equilibrated with 5-10 column volumes of extraction buffer. 2 ml sample solution (chapter 2.2.9.1) was loaded onto the column at a speed of 1 ml/min by using a syringe. After flow through of 0.5 ml dead volume, 2 ml flow-through solution was collected. The column was washed with at least 5 column volumes with extraction buffer (Mes or Hepes buffer), followed by an elution with 5 column volumes of elution buffer. After a flow through of 0.5 ml dead volume, a 2 ml elution fraction was collected. After the completed elution, the column was regenerated by washing with 5 column volumes of elution buffer. After a subsequent washing with 5-10 column volumes of extraction buffer, the column was prepared for a new sample. The column was stored with 20% ethanol. The elicitor activity of fractions was identified by measurement of their ability to trigger ethylene biosynthesis in *A. thaliana* leaves.

2.2.9.3 Partial purification of SsE1 by chromatography (two-step purification)

To obtain concentrated culture filtrate, dried material was re-suspended in 100 mM Mes buffer, pH 5.4 (6 ml/g dry weight) and centrifuged twice for 20 min at 14000 g and 4 °C to remove insoluble particles. The resulting supernatant was used for the isolation of SsE1. Around 200 ml cleared concentrated culture filtrate was subjected to cation exchange chromatography in a two-step approach using an ÄKTA Explorer FPLC system (GE Healthcare) kept at 4 °C. In a first step, culture filtrate was loaded onto a XK16 column (GE Healthcare) packed with Sepharose SP FastFlow matrix (GE Healthcare) to a bed volume of 15 ml and equilibrated with buffer A (100 mM Mes buffer, pH 5.4). After washing with buffer A, elution was performed with buffer B (100 mM Mes buffer,

pH 5.4, 500 mM KCl). A single fraction corresponding to the elution peak monitored with OD_{280} nm was collected manually. In a second step, total eluate was diluted 10 times in buffer A and loaded onto a Source 15S 4.6/100 PE column (GE Healthcare) equilibrated with buffer A. After washing with buffer A SsE1 was eluted with a linear gradient of buffer B (0% to 60% in 40 column volumes). 500 µl fractions corresponding to the whole elution peak monitored with OD_{280} nm were collected using automated fractionation. Active fractions containing SsE1 were identified by measurement of their ability to elicit ethylene production in *A. thaliana* leaves.

2.2.10 Molecular biology

2.2.10.1 Isolation of plasmid DNA from *E.coli*

Plasmid was extracted from 2-5 ml overnight inoculated cell culture.Plasmid was isolated and column-purified using the QIAprep Spin MiniPrep Kit (Qiagen) according to the manufacturer's introduction

2.2.10.2 Isolation of genomic DNA from plants

One little leaf piece was put in a 1.5 ml Eppendorf tube and briefly crunched with a blue pestle. 200 µl Edwards buffer was added into the tube and samples were completely homogenized at room temperature. The samples were centrifuged for 5 min at 13000 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) or 45 minutes (small leaf piece), and was centrifuged for 10 min at 4 °C with 14000 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 13000 rpm, the supernatant removed and the pellet air dried. Finally, the DNA pellet was dissolved in 50 µl (small leaf piece) or 100 µl (large leaf

piece) ddH$_2$O overnight at 4 °C or alternatively heated for 10 minutes at 65 °C.

2.2.10.3 Isolation of genomic DNA from fungi

A small piece of fungi from Maltose-peptone agar was put into an Eppendorf tube without any agar. The DNeasy plant kit (Qiagen) was used to extract fungal genomic DNA according to the manufacturer's instructions. Finally, fungal DNA was dissolved in 50 µl H$_2$O.

2.2.10.4 Isolation of RNA from plants

50-100 mg of fresh tissue (seedling or leaf material) was placed into a 1.5ml Eppendorf tube and frozen in liquid N$_2$, the samples can be stored at -80 °C. For RNA extraction, the pestles and samples were cooled in liquid N$_2$ and the tissue was ground to fine powder with a cold pestle in a pre-cooled rack. Subsequently, 1 ml Trizol (Chomczynski and Sacchi, 1987) was added, the sample was carefully mixed by vortexing and incubated for 10 min or longer at RT. After addition of 200 µl chloroform and 10 seconds of vortex-mixing, the samples were incubated for 10 min or longer at room temperature and centrifuged for 10 min with 13000 rpm at RT. The upper aqueous phase (about 500 µl) was transferred into a new tube and 1 volume (about 500 µl) of isopropanol was added followed by mixing. RNA precipitated for at least 15 min at RT (overnight at -20 °C results in higher yield) was collected by centrifugation for 10 min at 14000 rpm and 4 °C. The RNA pellet was washed with 1 ml 70% (v/v) EtOH. Finally the air dried RNA pellet was dissolved in 20-40 µl fresh sterile ddH$_2$O.

2.2.10.5 Reverse transcription

The RNA concentration was determined using a Nanodrop 2000 (Peqlab Biotechnologie GmbH). For the reverse transcription with M-MuLV RT RevertAid (200 U/µl, Fermentas), 2-5 µg RNA in 10 µl H$_2$O was denatured at

70°C for 10 min, and immediately cooled on ice. Afterwards 10 µl RT mix (4 µl 5x RT buffer, 2 µl 30 µM oligo-dT, 2 µl 2.5 mM dNTPs, 1 µl reverse transcriptase, 0.5 µl RNAse inhibitor (RiboLock, 40 U/µl, Fermentas), 0.5 µl H$_2$O) was added. The reaction mixture was incubated at 42 °C for 90 min in a PCR machine, followed by enzyme deactivation at 70 °C for 5-10 min.

2.2.10.6 Polymerase chain reaction (PCR)

Standard PCR reactions were performed by using home-made Taq DNA polymerase while for cloning of PCR products Phusion hot start DNA polymerase (Fermentas) were used according to the manufacturer instructions. All PCRs were carried out in a PTC-200 Peltier thermal cycler (MJ Research).

2.2.10.6.1 PCR using Taq DNA polymerase

A typical PCR reaction mix and thermal profile is shown below.

Reaction mix (total 20 µl volume)

Component	Volume
Template DNA	0.1-20 ng
10X reaction buffer	2 µl
dNTP mixture (together 2.5 mM)	1 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
Taq DNA polymerase	0.5 µl (1U)
ddH$_2$0	up to 20 µl

PCR program:

Stage	Temperature (°C)	Time period	No. of cycle
Initial denaturation	95	3 min	1
Denaturation	95	30 sec	

Annealing	50-60	30 sec	30-39
Extension	72	1 min per kb	
Final extension	72	10 min	1
		End	

2.2.10.6.2 PCR using Phusion hot start II DNA polymerase

A typical PCR reaction mix and thermal profile is shown below.

Reaction mix (total 50 µl volume)

Component	Volume
Template DNA	10-50 ng
5X reaction buffer	10 µl
dNTP mixture (together 10 mM)	1 µl
Forward primer (10 µM)	2.5 µl
Reverse primer (10 µM)	2.5 µl
DNA polymerase	0.5 µl (1U)
DMSO (optional)	1.5 µl
ddH_2O	up to 50 µl

PCR program:

Stage	Temperature (°C)	Time period	No. of cycle
Initial denaturation	98	30 sec	1
Denaturation	98	10 sec	
Annealing	50-60	20 sec	30-39
Extension	72	20 sec per kb	
Final extension	72	10 min	1
		End	

2.2.10.7 Real-time PCR

For quantitative Real-time PCR, cDNA from the reverse transcription reaction (2.9.5) was diluted 5 times with fresh sterile water, and 1 µl diluted cDNA was used in a 20 µl reaction. For fungal quantification, a 100-fold dilution of genomic DNA was used (chapter 2.2.8.3). SYBR Green Supermix is a product of Fermentas.

Reaction mix (in a 20 µl volume)

Component	Volume
Template cDNA	1 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
2x SYBR Green Supermix	10 µl
ddH$_2$0	up to 20 µl

PCR program:

Stage	Temperature (°C)	Time period	No. of cycle
Initial denaturation	95	15 min	1
Denaturation	95	15 sec	
Annealing	58	15 sec	40
Extension	68	10 sec	real time detection
Denaturation	95	1 min	1
Annealing	50	2 min	1
Melting curve	55-95	10 sec	81
	+ 0.5 °C per cycle		
	End		

2.2.10.8 Genotyping of fungi

The following primers and touch-down PCR program were used to amplify the sequencing fragment from 1 µl extracted fungal DNA (chapter 2.9.3).The PCR reaction was performed as described in chapter 2.9.6.

The primer sequences were described previously (Garnica et al., 2009):

ITS1f : 5'-AGTTCTGCCCAGTCTCAAATA-3'

NL4 : 5'-GGTCCGTGTTTCAAGACGG-3'

PCR program

Stage	Temperature (°C)	Time period	No. of cycle
Initial denaturation	95	3 min	1
Denaturation	95	30 sec	
Annealing	60	45 sec	9
Extension	72	1.15 min	
-1 °C per cycle			
Daturation	95	30 sec	
Annealing	50	45 sec	25
Extension	72	1 min	
Final extension	72	1 min	1
End			

Amplified PCR products were purified using the QIAquick protocol (Qiagen). Cycle sequencing was accomplished using the ABI PRISM Dye-Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems, Foster City, CA, USA) with 50 ng purified PCR product. Sequencing was performed with an ABI 3100 automated sequencer (Applied Biosystems). Forward primer ITS1F and reverse primer NL4 were used for sequencing. Forward and reverse sequences were assembled and edited using Sequencher version 4.1 (Gene Codes Corporation, Ann Arbor, MI, USA**).**

2.2.10.9 Restriction endonuculease digestion of DNA

Restriction digests were carried out using the manufacturer's recommended conditions. Digestion was performed in 20 µl reaction volume with 1 U enzyme/µg DNA at 37 °C for 1h. For preparative amount, DNA was digested overnight at 37 °C in a volume of 100 µl.

2.2.10.10 Isolation of DNA fragments from agarose gels

PCR products and enzyme digest fragments need to be purified for further cloning. DNA fragments separated by 0.8-1.5 agarose gel electrophoresis were excised from the gel with clean razor blade and extracted using the GeneJet™ Gel Extraction Kit (Fermentas) according to manufacturer's protocol.

2.2.10.11 DNA ligation

Typically, DNA ligation was carried out overnight at 16 °C in a total volume of 10 µl containing 1 µl T4 DNA ligase (1 U/µl; Fermentas), ligation buffer (supplied by the manufacturer), 50 ng vector and 3 to 5 fold molar excess of insert DNA for sticky and blunt end ligations. In some cases ligations were performed at 4 °C overnight or at room temperature for 1 - 3 h.

2.2.10.12 Site-specific recombination of DNA in Gateway compatible vectors

The pCR8/GW/TOPO Cloning kit (Invitrogen) was used for directionally cloning of PCR products into pCR8/GW/TOPO to generate an entry clone for entry into the Gateway system according to the manufacturer's instructions. To transfer the fragment of interest into gene expression constructs, an LR reaction between the entry clone and a Gateway destination vector was perform as following:

LR reaction buffer (5x)	1 µl
Entry clone	100-150 ng
Destination vector	100-150 ng
LR clonase^{TM} II enzyme mix	1 µl
ddH$_2$O to	10 µl

Reactions were incubated at 25 °C overnight.

2.2.10.13 Transformation of chemically competent *E. coli* cells

A 60 µl aliquot of chemically competent cells (Inoue et al., 1990) was thawed on ice. 5 µl from a 10 µl ligation reaction or recombination reaction was added to the cell aliquot and incubated on ice for 30 min. The mixture was heat-shocked for 1 min at 42 °C and immediately put on ice for 1 min. 500 µl of LB medium was added to the Eppendorf tube and incubated at 37 °C for 1 h on a rotary shaker. 200 µl transformed cell culture was plated onto selective LB media plates.

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

The desired *Agrobacterium* strain was streaked out onto LB agar plate containing adequate antibiotics and grown at 28 °C for two days. A single colony was picked and a 5 ml LB liquid medium, containing appropriate antibiotics, was grown overnight at 28 °C. The whole overnight culture was added to 500 ml LB and grown to an OD$_{600}$ of 0.5-1.0. Subsequently, the culture was chilled on ice for 15 - 30 min. From this point onwards bacteria were maintained at 4 °C. Bacteria were centrifuged at 4500 rpm for 15 min at 4 °C and the pellet was resuspended in 200 ml of ice-cold sterile water. Bacteria were again centrifuged at 4500 rpm for 15 min at 4 °C. Bacteria were resuspended in 100 ml of ice-cold sterile water and centrifuged as described above. The bacterial pellet was resuspended in 4 ml of ice-cold 10% glycerol and centrifuged as described above. Bacteria were resuspended in 1-1.5 ml of

ice-cold 10% glycerol. 40 µl of aliquots were frozen in liquid nitrogen and stored at - 80 °C.

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

100 ng of plasmid DNA was mixed with 40 µl of electro-competent *A. tumefaciens* cells (stored previously at -80 °C and prepared according to chapter 2.2.10.14), and transferred to an electroporation cuvette on ice (1 mm electrode distance). The cells were pulsed once with 1500 V for 5 ms (Eppendorf, Hamburg), the cuvette was put back on ice and immediately 500 µl of LB medium was added to the cuvette. Cells were quickly resuspended by slowly pipetting up and down and then transferred to a 1.5 ml Eppendorf tube. The tube was incubated for 1.5 h in a rotary shaker at 28 °C. A 100 µl aliquot of the transformation mixture was plated onto selective LB agar plates.

2.2.10.16 Sequencing

Sequencing of the generated constructs and PCR products was performed by GATC Biotech AG (Konstanz). The sequence analysis was performed using the BioEdit and Vector NTI 11 (Invitrogen) software.

2.2.10.17 Map-based cloning

To identify the locus for SsE1 sensitivity, the insensitive accession Lov-1 was crossed to Col-0. The F2 population was screened for their response to SsE1. The insensitive plants were selected for mapping the locus for SsE1 perception. The receptor for SsE1 was mapped to a 1 Mb interval on chromosome 3 by virtue of 270 F2 plants. The markers used for mapping were designed based on different polymorphisms between Lov-1 and Col-0 (http://signal.salk.edu/atg1001/index.php). Primer sequences of markers used for mapping are shown in Table 6.1.

2.2.11 Protein biochemistry

2.2.11.1 Protein extraction

Leaves or pooled seedlings (50-100 mg) were put in 2 ml or 1.5 ml Eppendorf tubes, respectively, and ground to fine powder with a metal pistil (pre-cooled in liquid N_2). Total protein was extracted in homogenization buffer containing 50 mM Tris-Cl (pH 7.6), 100 mM NaCl, 10% (v/v) glycerol, 0.1% (v/v) Tween-20, 20 mM 2-mercaptoethanol and the EDTA-free complete miniprotease inhibitor cocktail (Roche). After addition of 100 µl extraction buffer, samples were collected on ice. The samples were centrifuged at 14000 rpm and 4 °C for 20 min. The supernatant, without any pellet, was transferred to a fresh tube and kept on ice. The protein concentration was determined by the Bradford-System. 10 µl protein sample was added to 990 µl Bradford-Solution. After 5 min at RT, OD_{595} was monitored. Based on a BSA-standard curve, the protein concentration can be estimated using the following formula:

protein concentration [mg/ml] = OD_{595}/(0.0283 x used volume).

2.2.11.2 Laemmli SDS-PAGE

Denaturing SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by using the Mini–PROREAN 3 system (Biorad) and discontinuous polyacrylamide gels (Laemmli, 1970). Resolving gels were poured between two glass plates and overlaid with Millipore water. After gels had polymerized for 30-45 min, the overlaying water was removed and the gel surface carefully dried with filter paper. The stacking gel was poured on top of the resolving gel. A comb was inserted and the gel was allowed to polymerize for 30 min. In this study, a 10-15% resolving gel was used and the concentration of the overlaid stacking gel was 4%. Gels were 1.0 mm in thickness. Protein extracts (approximately 30 µg) prepared from plant samples (chapter 2.2.11.1) were mixed with 3X SDS sample buffer (10 ml: 3 ml Glycerol, 2.4 ml 5% (w/v) SDS,

0.15 mg Bromphenol blue, 3.75 ml 0.5 M (pH 6.8) Tris-HCl). Then, the samples were heated for 5 min in 95 °C, and centrifuged for one minute. The samples were loaded on the gels and SDS-PAGE was performed using 1x SDS-running buffer (25 mM Tris base, 192 mM Glycine, 0.1% (w/v) SDS).

2.2.11.3 Tricine SDS-PAGE

Compared to the conventional Laemmli SDS-PAGE system, the Tricine-SDS PAGE is more suitable for the separation of small proteins (Schagger, 2006). Tricine SDS-PAGE was carried out by using the Mini–PROREAN 3 system (Biorad) and discontinuous polyacrylamide gels were prepared as described (Schagger, 2006). In this study, a 12% running gel and a 4% stacking gel were used. The upper buffer chamber was filled with cathode buffer (500 ml: 6.055 g Tris base, 8.96 g Tricine and 0.5 g SDS), and the lower chamber was filled with anode buffer (500 ml: 12.11 g Tris base, pH 8.9). Protein extracts prepared from SsE1-fractions after chromatographic purification (chapter 2.2.9) were mixed with an equivalent volume of NOVEX 2X Tricine-SDS sample buffer (10 ml: 3.0 ml 3.0 M Tris-HCl, pH 8.45, 2.4 ml glycerol, 0.8 g SDS, 1.5 ml 0.1% Coomassie Brilliant Blue G-250, 0.5 ml 0.1% Phenol Red). Then, the samples were heated for 5 min at 95 °C, and centrifuged for one minute. The samples were electrophoresed at 15 mA constant current until they had migrated through the stacking gel. 30 mA constant current was used for running the rest of the gel until the tracking dye had migrated out of the gel.

2.2.11.4 Coomassie Brilliant blue staining

The SDS-PA gel was washed with ddH$_2$O for 5 min repeating at least two more times. The gel was covered with Coomassie blue solution (0.125% (w/v) Coomassie Brilliant Blue R-250, 50% (v/v) MeOH, 10% (v/v) acetic acid)) with gently shaking for 45 minutes. The gel was rinsed with ddH$_2$O for 5 min repeating at least two more times. The gel was destained overnight with 10%

acetic acid. The gel image was acquired by a scanner.

2.2.11.5 Silver staining

The SDS PA gel was incubated for at least one hour in a fixing solution (50% (v/v) MeOH, 12% (v/v) acetic acid and 0.0185% (v/v) formaldehyde), washed with 50% (v/v) EtOH for 3 times 20 minutes and treated with fresh 0.02% (w/v) $Na_2S_2O_3$ solution for one minute. Subsequently, the gel was washed with water (3 times 20 seconds), incubated for one hour with an impregnation solution (0.2% (w/v) $AgNO_3$ and 0.028% (v/v) formaldehyde) and then repeatedly washed with water. In a final step the gel was treated for 10-15 minutes with a developer solution containing 6% (w/v) Na_2CO_3, 0.0185% (v/v) formaldehyde and 0.4% (w/v) $Na_2S_2O_3$. The staining of the proteins was stopped with washing the gel with water for 2 times 2 min and then treating it with 50% (v/v) MeOH and 12% (v/v) acetic acid for 10 minutes.

2.2.11.6 Protein elution from a Tricine-SDS PA gel

Fractions with elicitor activity were pooled and lyophilized. The protein samples were resolubilized with 35 µl 1X NOVEX Tricine-SDS sample buffer. 5 µl and 30 µl samples were separately run in the Tricine-SDS PAGE side by side (chapter 2.211.3). The gel lane with the 5 µl sample was stained with silver (chapter 2.2.11.5). The other gel lane containing the 30 µl sample was left unstained and was cut into 1 mm segments. These slices were placed in 100 µl 0.1% (w/v) SDS. After incubation for 1 h at 70 °C and 16 h at 37 °C, 15 µl supernatant was assayed for ethylene-inducing activity.

2.2.11.7 Western blot

The proteins were transferred from the Laemmli SDS-PA gel onto a Hybond nitrocellulose membrane (GE Healthcare) in 1x transfer buffer (25 mM Tris base, 192 mM Glycine,1% (w/v) SDS, 200 ml methanol per litter) using a

Mini–PROREAN 3 system (Biorad) for approximately 1 h at 100 V. 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. The membrane was then incubated with TBST (10 mM Tris base pH7.5, 150 mM NaCl , 0.1% Tween-20) containing 5% (w/v) dried milk for 1 h at RT to block unspecific binding sites. The membrane was washed with 15 ml TBST for 3 times each 5 min at RT. The membrane was incubated with the desired primary antibody over night at 4 °C in 10 ml TBST containing 5% (w/v) dried milk. After additional washings with 15 ml TBST for 3 times each 5 min at RT, the membrane was incubated with the respective secondary antibody for 1-2 h in 10 ml TBST containing 5% (w/v) dried milk at RT. Then the membrane was washed with 15 ml TBST for 3 times each 5 min at RT. Chemiluminescent substrate (ECL; GE Healthcare) was applied before exposure to an x-ray film (CL-XPosure, Thermo Scientific).

2.2.11.8 Determination of MAPK kinase activation

For the detection of MAPK activiation, total plant proteins were extracted (chapter 2.2.11.1) and subjected to a 12% SDS-PAGE with 30 µg/lane, transferred to a nitrocellulose membrane and probed with a primary antibody raised against phosphor-p44/42-MAPK and secondary antibody (Table 2.13).

2.2.11.9 Transient protein expression and co-immunoprecipitation

Agrobacterium tumefaciens strain C58C1 containing the indicated construct were grown overnight in LB medium supplemented with appropriate antibiotics. Cultures were spun down and resuspended in 10 mM $MgCl_2$ and 150 µM acetosyringone to OD600 = 1.0. The indicated cultures were mixed 1:1 and syringe-infiltrated into 4 week old *N. benthamiana* leaves. Leaves were again syringe-infiltrated with elf18 or SsE1 at 40-48 h post-inoculation and harvested after further 5 min by freezing in liquid nitrogen. For total protein extracts, leaves were ground in liquid nitrogen and extraction buffer (50 mM Tris-HCl pH

7.6, 150 mM NaCl, 10% glycerol, 10 mM DTT, 10 mM EDTA, Protease Inhibitor Cocktail (Roth), and 1% NP40 (AppliChem) was added at 2 ml/g tissue. After 20 min centrifugation at 13000 rpm and 4 °C, supernatants were adjusted to 2 mg/ml protein and incubated for 4 h at 4 °C with 30 μl anti-HA affinity matrix (Roche) or GFP-Trap-A beads (Chromotek) in 1.5 ml tubes. Following incubation, beads were washed four times with extraction buffer containing 0.5% NP-40. For elution, 30 μl of 2x protein-loading buffer was added to the beads which were then heated at 95 °C for 10 min. The samples were subjected to 10.5% Laemmli SDS-PAGE, transferred to a nitrocellulose membrane and probed with primary and secondary antibody (chapter 2.2.11.5 and Table 2.13).

Table 2. 9 Antibodies used for immunoblot detection

Primary antibodies

Antibody	Source	Dilution	Reference
α-p42/44 MAPK	rabbit	1:2000	Cell Signaling Technology
α-HA	rabbit	1:3000	Sigma-Aldrich
α-myc	mouse	1:6000	Sigma-Aldrich
α-GFP	goat	1:4000	Acris Antibodies

Secondary antibodies

Antibody	Feature	Dilution	Reference
α-mouse IgG	HRP conjugated	1:10000	Sigma-Aldrich
α-goat IgG	HRP conjugated	1:10000	Sigma-Aldrich
α-rabbit IgG	HRP conjugated	1:10000	Sigma-Aldrich

2.2.11.10 Protein expression in *E. coli*

Candidate genes of SsE1 were amplified and cloned into pMAL-p5x with an N-terminal MBP tag (NEB). The C-terminus of RLP30 was amplified and cloned into pDEST15 with an N-terminal GST-tag (Invitrogen). BAK1-KD was

cloned into pET22b (+) with a C-terminal His tag (Novagen). All these plasmids were transformed into *E. coli* strain BL21 (DE3). For the induction of the expression of recombinant MBP-tagged proteins bacterial cultures were treated with 0.3 mM isopropyl β-D-thiogalactopyranoside (IPTG) and the expression of the other recombinant proteins with His- or GST-tag were induced by adding 0.2% (w/v) L- Arabinose when the bacterial culture reached an OD_{600} of 0.6 at 37 °C. The protein was extracted after inducing the cultures for 2-3 hours at 28 °C. Recombinant protein purification was done according to the following procedure. Bacterial cells from ~50 ml LB medium were collected by centrifugation at 4 °C at 8000 rpm for 10 min and resuspended in 5 ml HEPES buffer (50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT) for vectors pDEST12 and pET22b or 5 ml Mes buffer (100 mM Mes buffer, pH 5.4) for vector pMAL-p5x. The resupended cells were sonicated on ice 3 times for 10 sec or more if the cells are not completely disrupted using Sonopuls hd 3100 (Bandelin, Germany). The cells were centrifugated at 13000 rpm for 20 min at 4 °C. The supernatant was used for the next steps. MBP-tagged fusion proteins were directly used to test elicitor activity in different dilutions. GST-tagged fusion proteins were purified and enriched using Glutathione Sepharose Fast Flow (GE Healthcare) according to the manufactures' protocol. His-tag fusion proteins were purified using Ni-NTA-Sepharose 6 Fast Flow (GE healthcare) according to the manufacturers' instructions. After elution the fusion proteins were adjusted to the same concentration in 10% (v/v) glycerol solution and stored at -20 °C until usage.

2.2.11.11 *In vitro* kinase assay

The fusion proteins were incubated in 30 µl kinase buffer (50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 10 µM ATP and 1 µCi of [γ-^{32}P] ATP) for 1 h at 37 °C. The reaction was terminated by adding an equal volume of 2x SDS sample buffer, and then heated at 95 °C for 5 min. The samples were then

separated on a 10% (w/v) SDS-PA gel, the gels were stained with Coomassie Brilliant Blue (chapter 2.2.11.4) and dried on thick filter paper for 90 min at 80 °C. For autoradiography, the gel was exposed to an imaging plate (2025, 18 x 24 cm) in BAS cassettes (FUJI FILM) at -80 °C overnight. Imaging and analyses was performed using a phosphorimager (FMBIO III, HITACHI). In transphosphorylation assays 1 mg of each fusion protein or MBP (myelin basic protein, Fluka) was used.

2.2.12 Microscopy

The Trypan blue stained leaf material (chapter 2.2.8.4) was examined under a light microscope (Microscope eclipse 80i, Nikon). The images were taken using the 20x/0.50 objective (Plan Fluor) and digital camera (DIGITAL-SIGHT DS-U1). The images were processed with the Lucia Image software.

The visualization of fluorescence in samples was done using confocal laser scanning microscopy (TCS SP2, Leica). The images were taken by using a 40x oil immersion objective. The Software LCS Lite Version 2.61 was used for the processing of the images.

2.2.13 Statistics

All data were analyzed with a two-tailed, unpaired Student's t-test. P values of less than 0.05 were considered significant.

3. Results

3.1 MAMP purification

3.1.1 Genotyping of used fungi

For the purification of novel fungal MAMPs eight major fungal pathogen strains were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). The pathogens obtained were *Ustilago maydis* (maize corn smut); *Magnaporthe oryzae* (rice blast); *Mycosphaerella graminicola* (STB on wheat); *Fusarium graminearum* (wheat headblight); *Cercospora beticola* (beet leafspot); *Sclerotinia sclerotiorum* (stem rot on soybean and Brassica); *Rhizopus oryzae* (post-harvest decay); *Rhizoctonia solani* ("damping off" in many plants). These pathogens were selected because their genomes were or are currently sequenced which represents an advantage when it comes to PAMP/MAMP identification and further, when it comes to performing functional studies of the PAMP/MAMP. The fungal genetic background of each fungus firstly needed to be confirmed to rule out any fungal contaminations before fungal cultures were used for MAMP isolation. Because of its high degree of variation, the sequence of internal transcribed spacer 1 (ITS1) and ITS2 and 5.8S rDNA was used for the identification of fungi (Ferrer et al., 2001; Teun Boekhout, 2010). Sequencing of rDNA is relatively straightforward because universal PCR primers are available (Garnica et al., 2009). Six fungi out of eight were sequenced and the BLAST results are summarized in Table 3.1. For all sequences there was at least 99% identity between fungal queries and fungus hits (Table 3.1).

Table 3. 1 Summary of BLAST result of six fungi

Query	Hit*	Query coverage	Max identity
Cercospora beticola	*Cercospora beticola* strain CPC 11557	100%	99%
Fusarium graminearum	*Fusarium* sp. NRRL 45833	100%	100%
Rhizoctonia solani	*Rhizoctonia solani* isolate RT 7-1	90%	99%
Rhizopus oryzae	Rhizopus oryzae	100%	100%
Sclerotinia sclerotiorum	*Sclerotinia sclerotiorum* isolate 2005	100%	99%
Ustilago maydis	*Ustilago maydis* strain XA0609	100%	99%
Magnaporthe oryzae	n.d.		
Mycosphaerella graminicola	n.d.		

*For the BLAST search, the database of nucleotide collection (nr/nt) of other organisms was selected. The Megablast (Optimize for highly similar sequences) program was used from the NCBI website

3.1.2 Screen of fungal PAMPs

Increased ethylene biosynthesis is among the earliest responses to MAMPs. An increased activity of 1-aminocyclopropane-1-carboxylate (ACC) synthase activity can be detected within 10 min of treatment with MAMPs (Spanu et al., 1994; Boller and Felix, 2009). The ethylene assay has been successfully used as a robust, rapid, quantitative and highly sensitive method for the identification of MAMPs like flg22, elf18 and Eix (Avni et al., 1994; Felix et al., 1999; Kunze et al., 2004). Hence, the induction of ethylene production in leaves was used as a bioassay to monitor elicitor activities throughout the

different purification steps. Ethlyene-inducing activities of the culture medium filtrate and soluble mycelium extract of the eight selected fungi are shown in Table 3.2.

Table 3. 2 Ethylene production induced by fungal extracts

Fungal material	filtered culture medium				soluble mycelium extract			
IEX	anion		cation		anion		cation	
Fraction	FT	elution	FT	elution	FT	elution	FT	elution
Fusarium graminearum	-	+	++	-	-	-	-	-
Magnaporthe oryzae	-	-	-	+	-	-	-	-
Mycosphaerella graminicola	-	-	-	+	-	-	+	+
Sclerotinia Sclerotioru	++	-	++	+++	++	-	++	+++
Cercospora beticola	+	+	+	+	-	-	++	-
Rhizoctonia solani	-	-	-	++	-	+++	++	++
Ustilago maydis	-	+	-	-	+	+	-	-
Rhizopus oryzae	-	-	++	++	+	+	++	++

Arabidopsis leaf discs were treated with culture filtrate or soluble mycelial extract from eight fungal species. After 3 hours, ethylene production was measured by gas chromatography. This table showed the results from two independent experiments. The strength of the response to the extracts is indicated from - (production similar to non-treated control) over + and ++ (low to medium production) to +++ (high production). IEX: ion exchange column; FT: flow-through

We observed the highest activity with *Sclerotinia sclerotiorum* culture filtrate and mycelial extracts. When performing cation exchange chromatography, most of the activity bound to the column and was collected in the elution fraction for both the culture and the mycelium extract (Table 3.2).

Table 3.2 also showed that extracts from *Rhizoctonia solani* caused a very high ethylene response. The elicitor activity bound to the cation exchange column in the case of the culture filtrate and to both ion exchange columns in the case of the mycelium extract. For *Rhizopus oryzae* extracts, just like for *Sclerotinia sclerotiorum*, a prominent elicitor activity was detected in the eluate from the cation exchange chromatography in both the culture filtrate and the mycelium extract. Extracts from *Cercospora beticola* and *Ustilago maydis* presented a lower elicitor activity and extracts from *Fusarium graminearum* presented only an activity in the culture filtrate and this activity was bound to the anion exchange column but not to the cation exchange column. For *Magnaporthe oryzae* and *Mycosphaerella graminicola* we observed only a minor activity in fungal extracts (Table 3.2).

Because of their lower complexity, we first used culture filtrates as the elicitor source. The culture filtrate of *Sclerotinia sclerotiorum* showed the highest activity compared to the other fungi. Thus, *S. sclerotiorum* was selected for further MAMP purification.

3.1.3 Identification of a protein elicitor from *Sclerotinia sclerotiorum*

Approximately 100 mg of proteins were obtained from 10 L of culture filtrate from *S. sclerotiorum*. In a multi-step approach combining two separations on cation-exchang chromatography columns (Figure 3.1A), we partially purified a single elicitor containing fraction that we called SsE1 (Figure 3.1B & C).
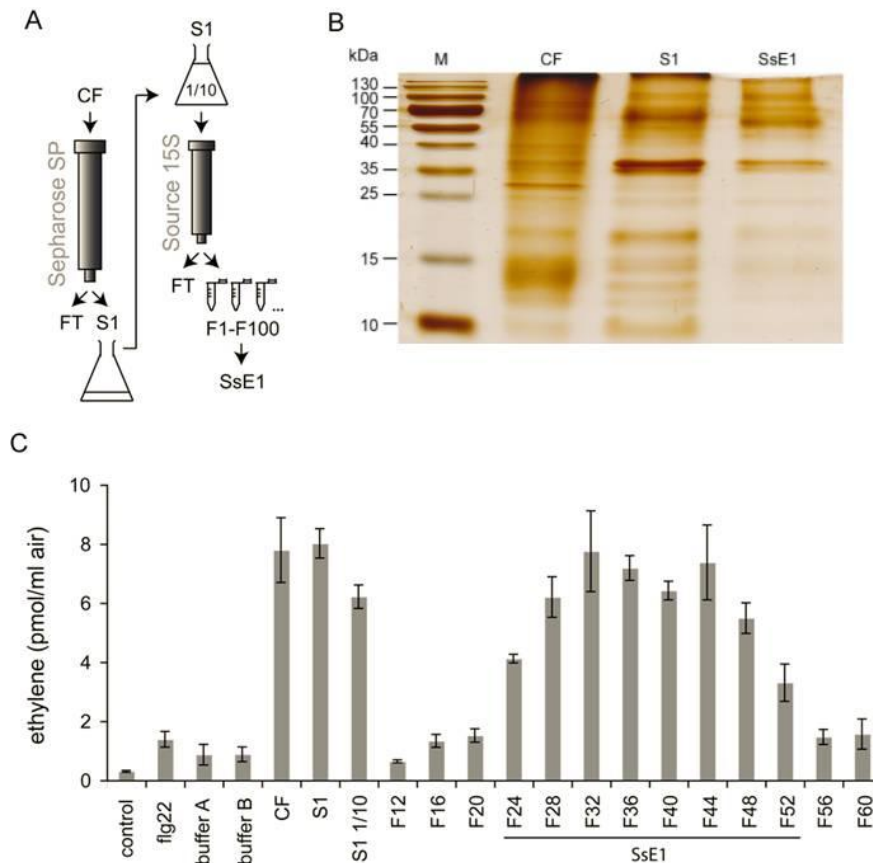
Figure 3. 1 Two-step chromatographic fractionation of *S. sclerotiorum* culture filtrate to obtain semi-purified SsE1

(A) Purification scheme of SsE1. Briefly, crude culture filtrate (CF) was loaded onto a Sepharose SP cation exchange chromatography column. The eluate (S1) was diluted 10-fold and loaded onto a Source 15S cation exchange chromatography column. Elution was performed with a linear gradient of 0 to 0.5 M KCl and 100 fractions (F1 - F100) of 0.5 ml were collected. FT = flow-through. (B) Tricine-SDS-PAGE of 2.5 µg *S. sclerotiorum* culture filtrate proteins (CF), 0.4 µg proteins eluted from a Sepharose SP cation exchange chromatography (S1), 0.3 µg proteins re-chromatographed on Source 15S cation exchange FPLC (SsE1). Proteins were visualized by silver staining. M = Standard Protein Molecular Mass Marker.(C) Ethylene response in *Arabidopsis* Col-0 to SsE1-containing fractions eluted from a Source 15S cation-exchange chromatography column. *Arabidopsis* Col-0 leaf pieces were treated with 15 µl of the fractions that were eluted from the Source 15S column. 15 µl buffer A (100 mM Mes pH 5.4) and 15 µl buffer B (100 mM Mes pH 5.4, 0.5 M KCl) were used as negative controls. 500 nM flg22, 15 µl undiluted culture filtrate (CF) as well as 15 µl from the active Sepharose SP fraction (S1), both undiluted and 10-fold diluted (S1 1/10), were used as positive controls for ethylene production. Bars represent average values ± S.D. (n=2).

SsE1 showed high elicitor activity in inducing ethylene biosynthesis. Moreover, SsE1 seemed to trigger the production of higher amounts of ethylene than 500 nM flg22 does (Figure 3.1C).
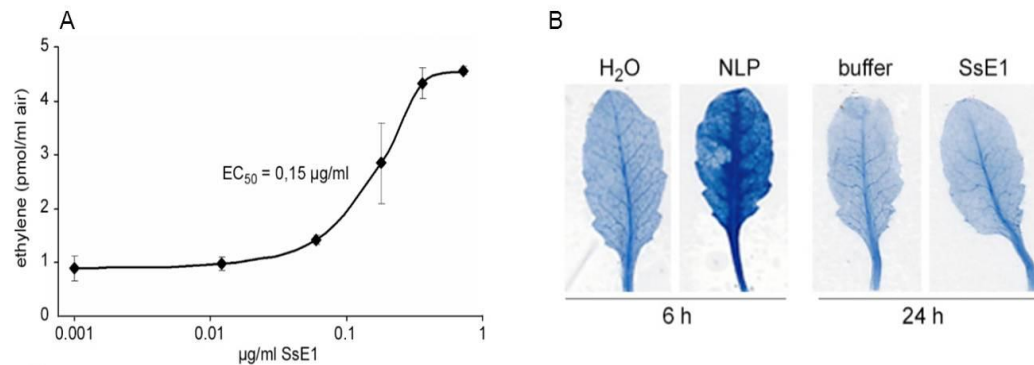
Figure 3. 2 Ethylene-inducing activity of SsE1

(A) Dose-response relationship for SsE1- induced ethylene production in *Arabidopsis* Col-0 leaves. Represented are average values ± S.D. (n=2). (B) The SsE1-containing fraction was tested for cell death inducing activity. 0.5 µg/ml SsE1 or 3 µM purified NLP from *P. parasitica* were infiltrated into *Arabidopsis* Col-0 leaves. Cell death was visualized by trypan blue staining.

Dose-response analysis of the SsE1-containing fraction revealed a concentration of ~ 0.15 µg/ml to be sufficient to trigger half-maximal ethylene production (Figure 3.2A). Hence, SsE1 appears to be a very potent elicitor, active at comparable concentrations than *bona fide* MAMPs.

S. sclerotiorum produces phytotoxic proteins such as necrosis- and ethylene-inducing-like proteins (NLPs), which act as virulence factors and induce MAMP-like responses, possibly through the release of danger-associated molecular patterns (DAMPs) caused by the disruption of cellular integrity (Qutob et al., 2006; Ottmann et al., 2009). A toxic function of SsE1 could however be ruled out, as it did not trigger any cell death upon infiltration into *Arabidopsis* leaves, in contrast to NLP from *Phytophthora parasitica*, a functional homolog of the *S. sclerotiorum* NLPs (Liberti et al., 2008) (Figure 3.2B).
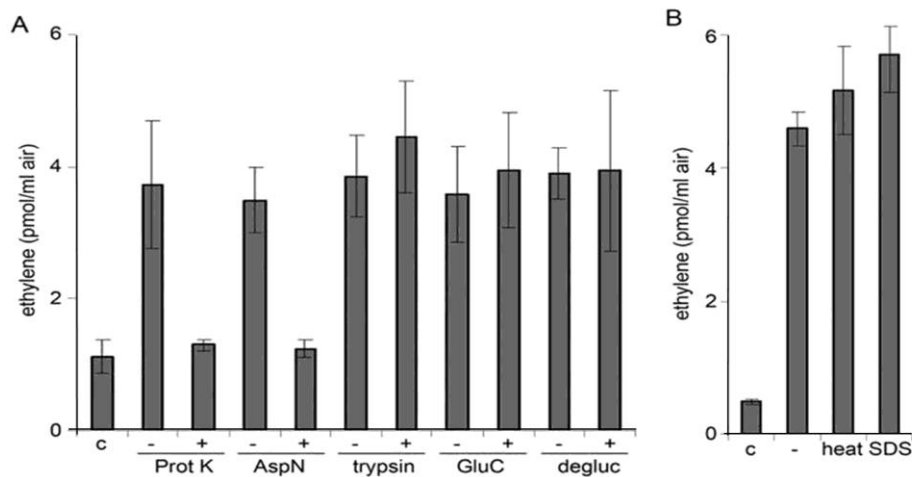
Figure 3. 3 The SsE1-activity is derived from a peptide-epitope

(A) SsE1 (0.5 µg/ml) was incubated with different proteases (+) such as proteinase K (Prot K), AspN, trypsin, GluC, or a commercial deglycosylation mixture (degluc) or the respective enzyme buffers (-) and tested for ethylene-inducing activity on *Arabidopsis* Col-0 leaf pieces. Untreated leaf pieces were used as a control (c). Bars represent average values ± S.D. (n=2). (B) Before addition to *Arabidopsis* Col-0 leaf pieces, SsE1 was either left untreated (-), heated for 10 min at 95 °C (heat) or incubated in 0.1% SDS for 1 h at room temperature (SDS). Untreated leaf pieces were used as a control (c). Bars represent average values ± S.D. (n=2).

Next, we investigated the physical properties of SsE1 and treated active preparations with proteinase K, a broad-spectrum protease, or AspN, an endopeptidase that cleaves peptide bonds N-terminal to Asp residues. Both treatments resulted in a complete loss of elicitor activity in the ethylene assay (Figure 3.3A). This result also indicated that the core motif of SsE1 activity includes the amino acid aspartic acid. In contrast, the endoproteinases GluC (cleavage C-terminal to Glu residues) or trypsin (cleavage C-terminal to Lys or Arg residues) did not affect elicitor activity (Figure 3.3A). SsE1 was also resistant to heat and SDS treatment (Figure 3.3B), suggesting that the elicitor activity is most likely associated with a linear peptide motif acting as an immunogenic epitope and that protein folding or enzymatic activity is not important. Still, we can rule out that SsE1 is a glycopeptide since deglycosylation did not affect elicitor activity (Figure 3.3A).
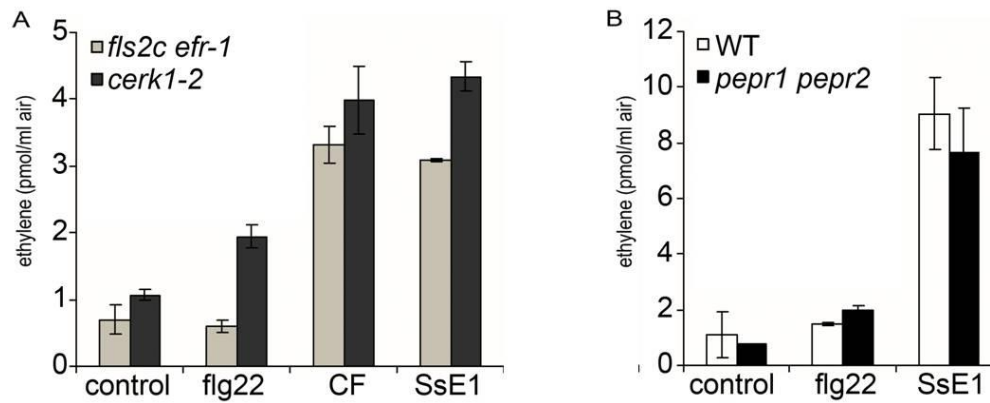
Figure 3. 4 SsE1 activity does not depend on known MAMP receptors or the DAMP receptors PEPR1 and PEPR2

(A) SsE1 (0.5 μg/ml) was tested for ethylene-inducing activity on leaf pieces from the *cerk1-2* single mutant or the *fls2c efr-1* double mutant. (B) Leaf pieces of wild type Col-0 plants and *pepr1pepr2* double mutant with treatment of SsE1. Leaf pieces left untreated (control) or treated with *S. sclerotiorum* culture filtrate (CF) and 500 nM flg22 served as controls. Bars represent average values ± S.D. (n=2).

Moreover, a contamination of the SsE1 activity with flg22 or elf18 or, more likely, fungal chitin can be excluded, as *fls2c efr-1* and *cerk1-2* mutant plants responded normally to SsE1 (Figure 3.4A). In addition, SsE1 activity did also not depend on the generation of danger-associated molecular patterns (DAMPs) such as the Pep peptides (Krol et al., 2010; Yamaguchi et al., 2010), as the *pepr1 pepr2* receptor mutants displayed a wild-type response to SsE1 (Figure 3.4B).

3.1.4 SsE1 triggers multiple immune responses in *Arabidopsis*

Early cellular events following the perception of MAMPs such as flg22 include the induced production of reactive oxygen species (ROS) and the post-translational activation of mitogen-activated protein kinase (MAPK) cascades (Boller and Felix, 2009). To find out to which extent the set of cellular changes triggered by SsE1 overlaps with the well-characterized early MAMP signal transduction, comparative studies with flg22 upon treatment of *Arabidopsis* leaves was performed. MAPK activity was analyzed by

immunoblot assays using the p44/42 antibody raised against phosphorylated MAPKs. As shown in Figure 3.5A, SsE1 strongly activated the three defense-associated MAPKs MPK3, MPK4/MPK11 and MPK6, which was indistinguishable from the induction pattern obtained with flg22. Application of SsE1 or flg22 to *Arabidopsis* leaves resulted in a significant increase in ROS production as detected by *in situ* staining of H_2O_2 using diaminobenzidine (Figure 3.5B). In a luminol based assay a significant increase in ROS production was observed within 45 minutes, although the kinetic and intensity of the oxidative burst differed from the treatment with flg22 (Figure 3.5C). A typical late plant response to pathogen infection or treatment with MAMPs is the induction of the synthesis of pathogenesis-related (PR) proteins such as PR-1 (Ferreira et al., 2007). To visualize PR-1 expression, we used a transgenic *pPR-1:GUS* reporter line, in which the *PR-1* promoter is fused to the *uidA* gene from *E. coli* coding for a-glucuronidase (GUS) (Shapiro and Zhang, 2001). As shown in Figure 3.5D, treatment with SsE1 resulted in a strong GUS expression, indicating that SsE1 induces the expression of *PR-1*.
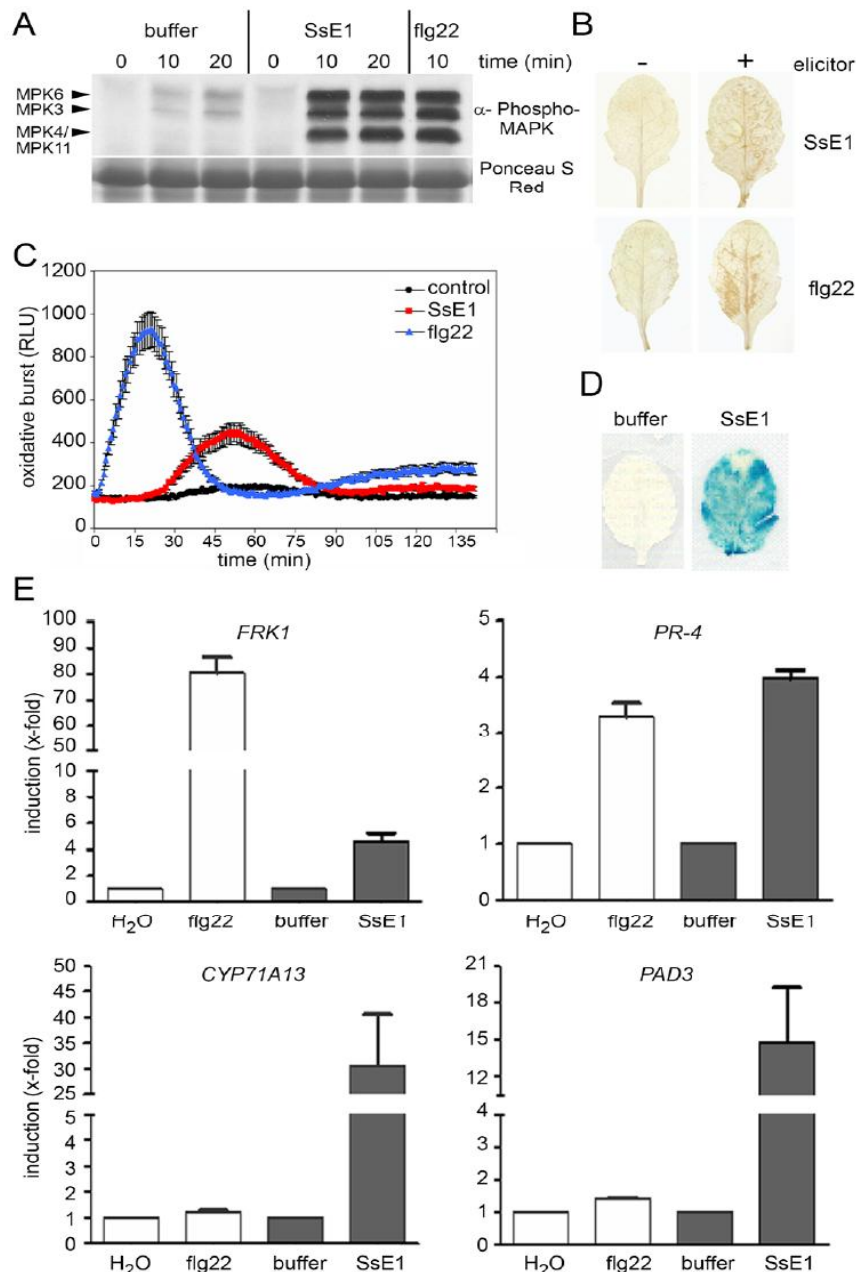
Figure 3. 5 SsE1 generates typical immune responses in *Arabidopsis*

(A) Immunoblotting of activated MAPK with anti-phospho p44/p42 antibody in *Arabidopsis* Col-0 leaf extracts. Leaf samples were collected at the indicated time after infiltration with buffer (control), 0.5 µg/ml SsE1 or 100 nM flg22. Ponceau S Red staining served as a loading control. Arrowheads indicate the position of MAP kinases 3, 4/11 and 6. (B) 0.5 µg/ml SsE1 or 100 nM flg22 were infiltrated into Col-0 leaves and harvested after 8 h. Accumulation of hydrogen peroxide was detected with diaminobenzidin (DAB). (C) Oxidative burst triggered by 0.12 µg/ml SsE1 (red) or 100 nM flg22 (blue) in *Arabidopsis* Col-0 leaf discs, measured in relative light units (RLU) in a luminol-based assay. Results are means ± S.E.M. (n = 12). (D) GUS activity in *pPR-1:GUS* transgenic *Arabidopsis* Col-0 plants. Leaves were infiltrated with 0.5 µg/ml SsE1 or buffer and collected 24 h later for histochemical GUS staining. (E)

Transcriptional profiling of defense-related genes by quantitative real-time PCR (qRT-PCR). *Arabidopsis* Col-0 leaves were infiltrated with water, 100 nM flg22 or with 0.5 μg/ml SsE1 and the corresponding buffer and collected 6 h after treatment. Expression of the indicated genes was normalized to the levels of *EF-1a* transcript and is presented as fold induction compared to the respective control. Error bars, S.E.M. (n=3).

Plant adaptation to biotic stresses promotes transcriptional up-regulation of large sets of genes that are involved in disease resistance (Navarro et al., 2004; Zipfel et al., 2004; Zipfel et al., 2006). The expression profile of typical early MAMP-regulated genes after SsE1 application revealed that the expression of *PR-4* (jasmonate/ethylene-dependent) is similarly induced 6 h after flg22 or SsE1 treatment (Figure 3.5E). However, quantitative differences were observed with the up-regulation of *FRK1* expression (salicylic acid-dependent), which is stronger in response to flg22. The opposite was observed for the gene expression of *CYP71A13* and *PAD3* (camalexin biosynthetic pathway), which is higher with SsE1 than with flg22 at the investigated time point (Figure 3.5E). The reason for this is unclear but it may reflect kinetic differences in gene expression and dose-dependent effects between SsE1 and flg22 rather than being the consequence of activation of different signaling branches. Together, these results suggest that despite the quantitative differences in the ability of SsE1 and flg22 to trigger defense responses, the two elicitors induce qualitatively similar changes.

3.1.5 SsE1 elicitor activity can be eluted from SDS-PA gels

The elicitor activity of SsE1 was stable after heat and SDS-treatment (Figure 3.3B). This result indicated that SsE1 activity should be retrievable from SDS-PA gels, an approach that has been proven successful for the identification of flg22 and elf18 (Felix et al., 1999; Kunze et al., 2004).
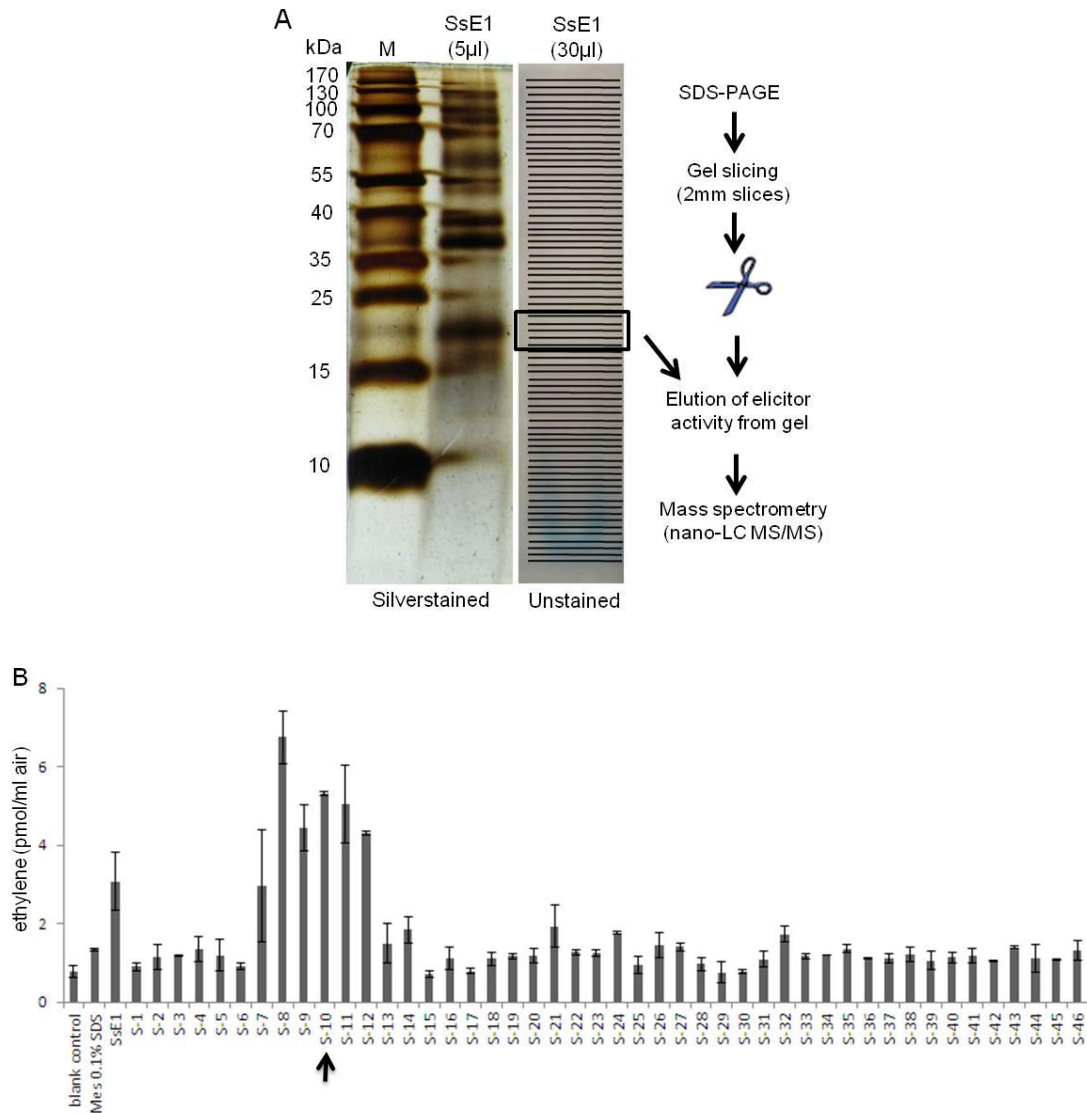
Figure 3. 6 Elicitor activity of SsE1 can be purified form SDS-PAGE

(A) Separation of the elicitor inducing ethylene biosynthesis. Four active fractions (F33-F36, Figure 3.1C) were pooled together and freeze-dried. The protein samples were re-solubilized with 35 µl 1X NOVEX Tricine-SDS sample buffer. 5 µl and 30 µl samples were separately run on a Tricine-SDS PA gel side by side. The lane with the 5 µl sample was stained with silver for visualization. As indicated in the cartoon on the right side, the other lane containing 30 µl of the sample was left unstained and cut into 2 mm segments. Each segment was immersed in 100 µl Mes buffer containing 0.1% SDS for protein elution. (B) 20 µl elution solution was used to test ethylene-inducing activity on *Arabidopsis* Col-0 leaf pieces. The arrow indicated the sample that was analyzed by mass spectrometry. Leaf pieces either left untreated (blank) and treated with Mes/ 0.1% SDS buffer were used as negative controls and SsE1 (0.5 µg/ml) was used as a positive control. Bars represent average values ± S.D. (n=2).

In this study, Tricine–SDS-PAGE was used instead of traditional Laemilli-SDS-PAGE. Tricine–SDS-PAGE is commonly used to separate proteins in a mass range from 1 to 100 kDa and is the preferred electrophoretic system for the resolution of proteins smaller than 30 kDa (Schagger, 2006). After separation of SsE1 on a Tricin-SDS-PA gel (Figure 3.6A), the unstained gel lane was cut into 2 mm segments and eluted proteins were tested for ethylene-inducing activity (Figure 3.6A). There were five gel slices showed high ethylene-inducing activity (Figure 3.6A & B). Accordingly, sample S10 that co-migrated with a major polypeptide band with an apparent molecular mass of 20 kDa (Figure 3.6A & B) was analyzed by mass spectrometry using nano-LC MS/MS (B. Macek, Proteome Centre Tübingen). By this approach, several proteins were detected within the sample (Table 3.3).

Table 3. 3 Mass spectrometry analysis revealed six candidate proteins for the SsE1 elicitor

Protein ID	Protein name	Peptides	Mascot score	MW (kDa)	pI
A7E6R4	Cytochrome C	10	370.22	12.0	4.3
A7F941	N-acyltransferase	9	363.95	19.3	5.7
A7ET57	Rho-GDP dissociation inhibitor	7	269.73	22.5	5.1
A7E4E9	Polyubiquitin	5	183.40	34.3	7.4
A7EDH2	Protein disulfide isomerase	3	105.35	39.4	7.2
A7EXV0	Pectin esterase	2	115.36	34.2	7.3

SsE1 fraction S10 was subjected to nano-LC MS/MS analysis. Protein hits with a peptide number ≥ 2 and a Mascot score sum ≥ 37 were retained. The Mascot score is defined as $S = -10*log (P)$ with P = probability that the match is a random event. Identification number (ID) from the *Sclerotinia* proteome database, molecular weight (MW) and predicted isoelectric point (pI) of the candidate proteins are indicated.

The top three candidates were selected for further cloning of the

corresponding genes from a *S. sclerotiorum* cDNA library, followed by their expression as recombinant proteins with an N-terminal MBP (maltose binding protein) tag in *E. coli* and testing of their potential to induce ethylene responses.

We successfully cloned and expressed soluble MBP-tagged Cytochrome C and Rho-GDP inhibitor in *E.coli* (Figure 3.7). Unfortunately, N-acyltransferase could not be cloned from *S. sclerotiorum* cDNA.

Bacterial lysates for each expression construct was used to test ethylene-inducing activity on *fls2c efr-1* double mutant plants in different dilutions. No ethylene production was induced except when non-diluted lysates were added to the leaves, however, this background activity was derived from bacteria and was observed in every sample, even in the empty vector control (data not shown).
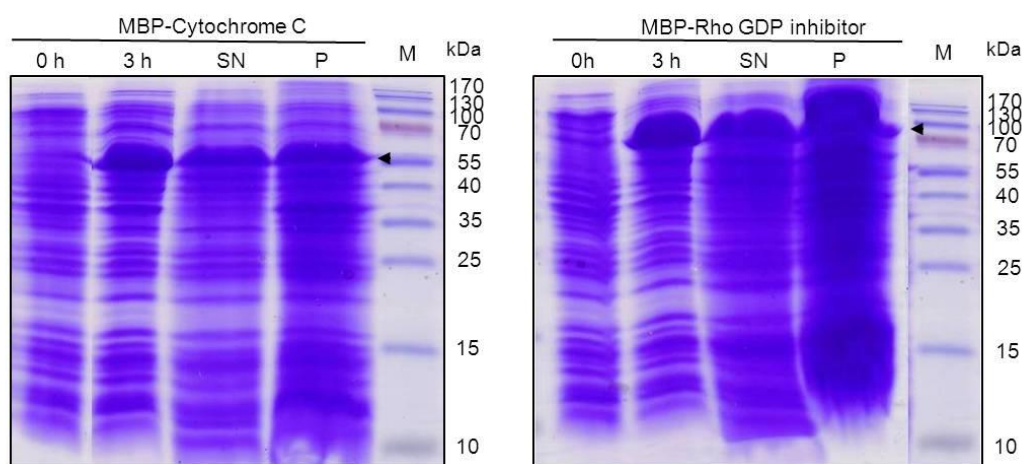
Figure 3. 7 Expression of MBP-tagged S. sclerotiorum Cytochrome C and Rho GDP inhibitor in E. coli BL21 (DE3)
Coomassie blue-stained SDS-PAG showing MBP-Cytochrome C production (left panel) or MBP-Rho GDP inhibitor production (right panel) in *E. coli*. Recombinant proteins were induced with 0.3 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 3 hours at 28 °C. Samples were taken from non-induced (0h) or induced (3 h) bacterial cultures to prepare soluble (SN) and insoluble (P) protein extracts. Approximately 40 µg proteins were loaded on the SDS-PA gel. The position of recombinant proteins is designated by an arrowhead on the right.

No elicitor activity was obtained from recombinant proteins, possibly because

the heterologous *E. coli* systems was used to express fungal proteins. Hence, these genes should not yet be excluded from the candidate list for SsE1. It is currently impossible to determine unambiguously which protein on the list corresponds to SsE1 (Table 3.3). Further approaches should include protein expression in eukaryotic expression systems such as *Saccharomyces cerevisiae* or *Pichia pastoris* or *in planta* expression.

3.2 Identification of receptor and signaling adaptors

3.2.1 Forward genetic screening identifies the receptor-like protein RLP30 as being required for SsE1 perception/sensitivity

Although the protein/peptide corresponding to SsE1 could not yet be identified, preliminary results indicate that the elicitor activity is associated with one molecular pattern rather than a mixture of different MAMPs. This hypothesis was the prerequisite to carry out a forward genetic approach for the identification of *Arabidopsis* genes required for SsE1 perception or signal transduction. Natural genetic variation between different *Arabidopsis* ecotypes may reveal accessions that are partially or fully insensitive to SsE1 and thus enable the cloning of, for instance, the SsE1 receptor. A good example for the successful employment of this approach is given by the identification of the flg22-insensitive ecotype Ws-0 that lacks a functional FLS2 receptor (Gomez-Gomez and Boller, 2000). We tested the ethylene response to SsE1 treatment in 70 different *Arabidopsis* accessions (Nordborg collection, Nottingham *Arabidopsis* Stock Centre) and found the five ecotypes Br-0, Lov-1, Lov-5, Mt-0, and Sq-1 to be fully insensitive to SsE1 (Figure 3.8). These ecotypes were not impaired in their ability to produce ethylene since they retained full responsiveness to flg22 (Figure 3.9A).
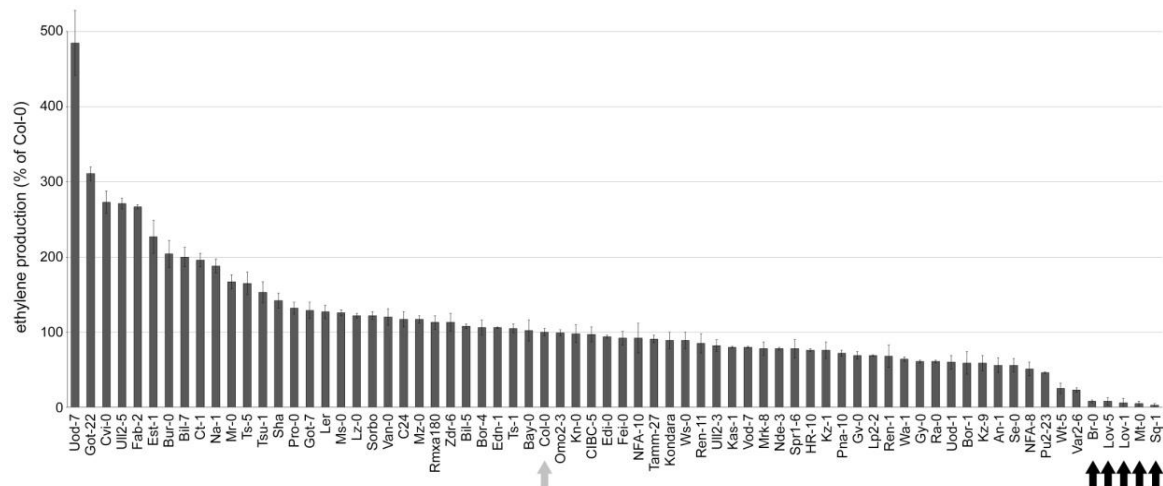
Figure 3. 8 SsE1-induced ethylene response in different *Arabidopsis* ecotypes

SsE1 (0.25 µg/ml) was tested for ethylene-inducing activity on leaf pieces of indicated *Arabidopsis* ecotypes. Bars represent relative ethylene production of the respective ecotype compared to Col-0, which was set to 100% (grey arrow). SsE1-insensitive ecotypes are indicated with black arrows. Shown are means of two replicates ± S.D.

The F1 progeny of the Col-0 ecotype crossed with the three insensitive accessions Lov-1, Mt-0 or Sq-1 displayed a normal response to SsE1 (Figure 3.9A and Table 3.4) indicating the recessive nature of this trait. Crossings between the insensitive ecotypes Lov-1, Mt-0 and Sq-1 and analysis of the resulting F1 progeny indicated that the non-responsiveness to SsE1 is allelic in all three ecotypes (Figure 3.9A & Table 3.4).

Table 3. 4 SsE1 sensitivity is controlled by a single recessive gene locus in different *Arabidopsis* ecotypes

Plants	Sensitivity	Insensitivity
F1 (Mt-0 X Col-0)	10	0
F1 (Sd-1 X Col-0)	10	0
F1 (Lov-1 X Col-0)	10	0
F1 (Lov-1 X Mt-0)	0	10
F1 (Lov-1 X Sd-1)	0	10
F2 (Lov-1 X Col-0)	206	64

SsE1 was tested for ethylene-inducing activity on leaf pieces of indicated *Arabidopsis* plants (number of plants as shown). Sensitivity: induction level is above the untreated control. Insensitivity: induction level is similar to the untreated control.

F2 populations from the Lov-1 x Col-0 cross showed a segregation ratio of 3:1 (206 sensitive versus 64 insensitive plants, Table 3.4), suggesting that the SsE1 insensitivity phenotype in the Lov-1 ecotype is controlled by a single recessive nuclear gene. We further used ethylene production as a trait to map the locus involved in sensitivity towards SsE1 in the Lov-1 x Col-0 cross. Crude mapping located this locus on the upper arm of chromosome 3 in a region between markers F11A12 and MSJ11 (Figure 3.9B). Further fine-mapping narrowed the SsE1 sensitivity locus to a region of about 1 Mb between markers F21O3 and T9J14 containing four genes encoding eLRR-RLPs (RLP30-33) but no members of the RLK protein family (Figure 3.9B). The choice of these candidates was driven by our knowledge about the well-established function of eLRR ectodomains in perception of proteinaceous MAMPs, as known for FLS2/flg22 and EFR/elf18.

Independent T-DNA insertion lines for *RLP30* (*At3g05360)*, *RLP31* (*At3g05370*) and *RLP33* (*At3g05660)* were obtained from the collection described in Wang et al. (Wang et al., 2008a) and tested for ethylene production in the presence of SsE1. The T-DNA line with an insertion in *RLP32* (*At3g05650*, FLAG_588C11) did not grow and could not be included in the assay. The results showed that only the *rlp30* mutants were affected in SsE1-dependent ethylene production whereas the knockout lines corresponding to *RLP31* and *RLP33* displayed a normal response to SsE1 (Figure 3.10A). As the predicted T-DNA insertion in the *rlp30-3* line was not found, the plants were still sensitive to SsE1 treatment (Figure 3.10A). Importantly, all the *rlp30* mutants remained fully responsive to flg22 (Figure 3.10A). Altogether, these data demonstrate a specific involvement of *RLP30* in SsE1-mediated perception or signaling.
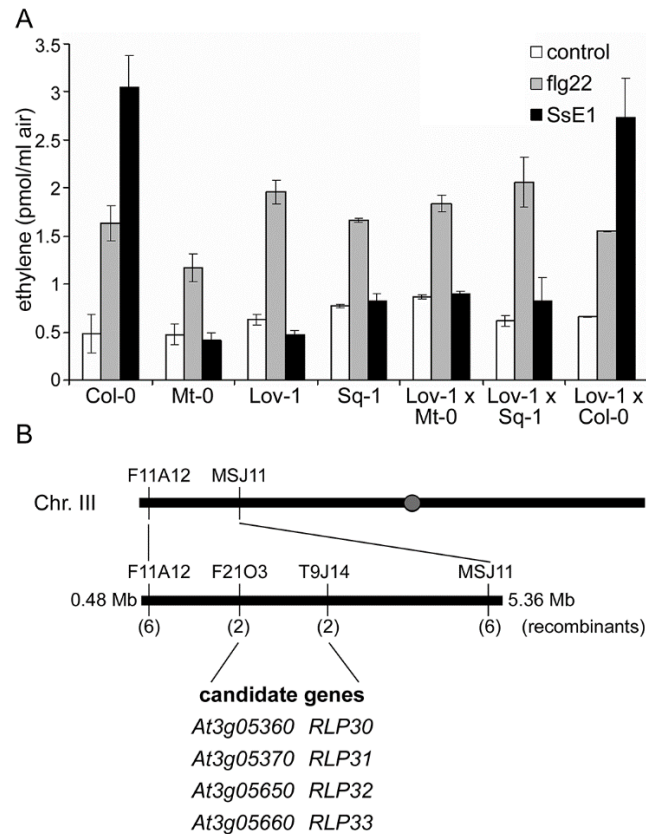
Figure 3. 9 SsE1 sensitivity locates to *Arabidoposis* Chromosome III
(A) SsE1 (0.25 µg/ml) was tested for ethylene-inducing activity on leaf pieces from *Arabidopsis* Col-0, the accessions Mt-0, Lov-1 and Sq-1 and the F1 population of the crosses Lov-1 x Mt-0, Lov-1 x Sq-1 and Lov-1 x Col-0. Leaf pieces left untreated (control) or treated with 500 nM flg22 served as controls. Bars represent average values ± S.D. (n=2). (B) Map-based cloning strategy for identifying the gene required for SsE1 perception from F2 mapping populations of the Lov-1 x Col-0 cross. Available SSLP and RFLP markers on chromosome 3 (F11A12 and MSJ11) and RFLP markers developed in this study (F21O3 and T9J14) are indicated. The number of recombinants from the mapping population of 270 F2 plants is shown in parentheses. The SsE1 insensitivity locus was mapped to a 1 Mb region between markers F21O3 and T9J14. Candidate genes within this region are shown at the bottom.
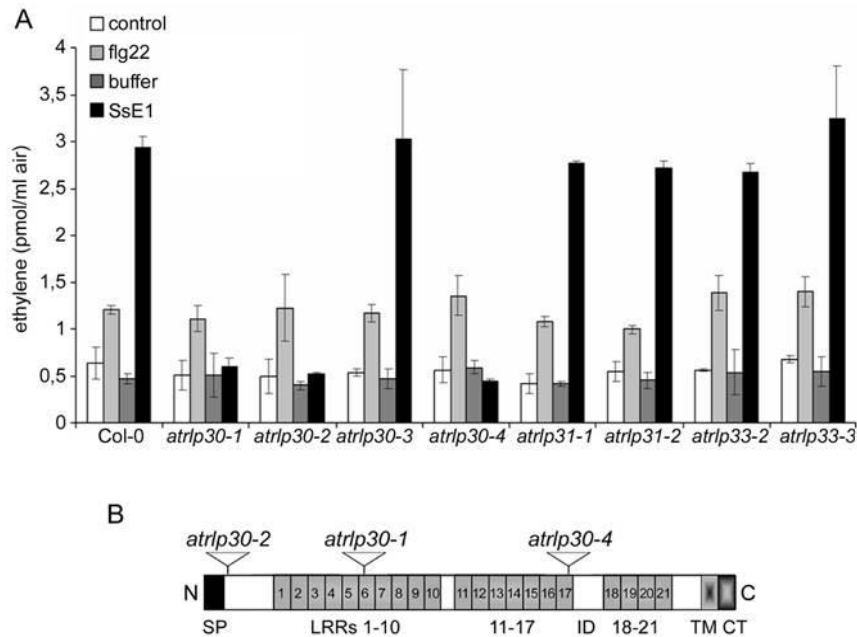
Figure 3. 10 SsE1-perception depends on the receptor-like protein RLP30

(A) Ethylene assay in *Arabidopsis* Col-0 plants compared to *rlp* mutants representing candidate genes in the chromosome region mapped for SsE1 sensitivity. Leaf pieces were treated with 0.25 µg/ml SsE1 or as a control with 500 nM flg22 prior to measurement of ethylene production. Bars represent average values ± S.D. (n=2). (B) Schematic representation of the RLP30 protein organisation. Indicated are the positions of the predicted signal peptide (SP), leucine-rich repeats (LRRs) 1 to 21, which are interrupted by an island domain (ID), the C-terminal transmembrane domain (TM) and the short cytoplasmic tail (CT). The positions of the three *atrlp30* T-DNA insertions are indicated.

3.2.2 SsE1 perception does not require ETI components or subfamily XII LRR-RLKs

RLP30 presents all the hallmarks of a cell surface-located RLP with an N-terminal signal peptide, an extracellular domain containing 21 LRRs that possibly act as SsE1 binding site, a single transmembrane domain and a short cytoplasmic tail of 25 amino acid residues (Figure 3.10B). It has been previously published that RLP30 localizes to the plasma membrane and that its expression is strongly induced by MAMP treatment or upon pathogen infection (Wang et al., 2008a). However, the absence of a cytosolic signaling domain indicates that RLP30 is most likely part of a heteromeric receptor

complex and that additional components are necessary for intracellular signaling. It has been shown that *EDS1* (ENHANCED DISEASE SUSCEPTIBILITY 1) is genetically required in ETI triggered by the RLPs *Cf4* and *Ve1* in tomato (Gabriëls et al., 2007; Fradin et al., 2009). In addition, *PAD4* (PHYTOALEXIN DEFICIENT 4) and *NDR1* (NON-RACE SPECIFIC DISEASE RESISTANCE 1) are known to be crucial in *Arabidopsis* for ETI mediated by TIR-NB-LRR and CC-NB-LRR type of resistance proteins (Hammond-Kosack and Parker, 2003).
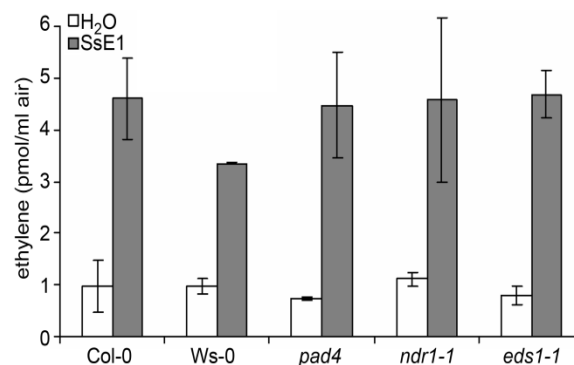
Figure 3. 11 SsE1 activity does not depend on known ETI components
Leaf pieces of *pad4*, *ndr1-1* plants (in the Col-0 background) or the *eds1-1* single mutant (in the Ws-0 background) were treated with 0.25 µg/ml SsE1 or water as a control prior to measurement of ethylene production. Bars represent average values ± S.D. (n=2).

Therefore, SsE1 was applied to *eds1*, *pad4* and *ndr1* mutants but none of them displayed an altered ethylene response, suggesting that these genes are not involved in RLP30-triggered signal transduction (Figure 3.11).

FLS2 and EFR belong to the subfamily XII of LRR-RLKs containing eight additional members of unknown function, some of them being induced during infection or MAMP treatment (Figure 3.12A) (Zipfel et al., 2006; Postel et al., 2010). It was next tested whether members of this family might play a role in the signaling events triggered by SsE1. Homozygous mutant lines were generated for seven members of this family (kindly provided by Freddy Boutrot and Cyril Zipfel, The Sainsbury Laboratory, UK), six of them in the *fls2 efr1*

cerk1 background (except for *xii6* which represents a single mutant and is thus still responsive to flg22), and tested for sensitivity to SsE1 in the ethylene assay. All the lines answered normally to SsE1, ruling out a role for proteins of the LRR-XII family in SsE1 signaling (Figure 3.12).
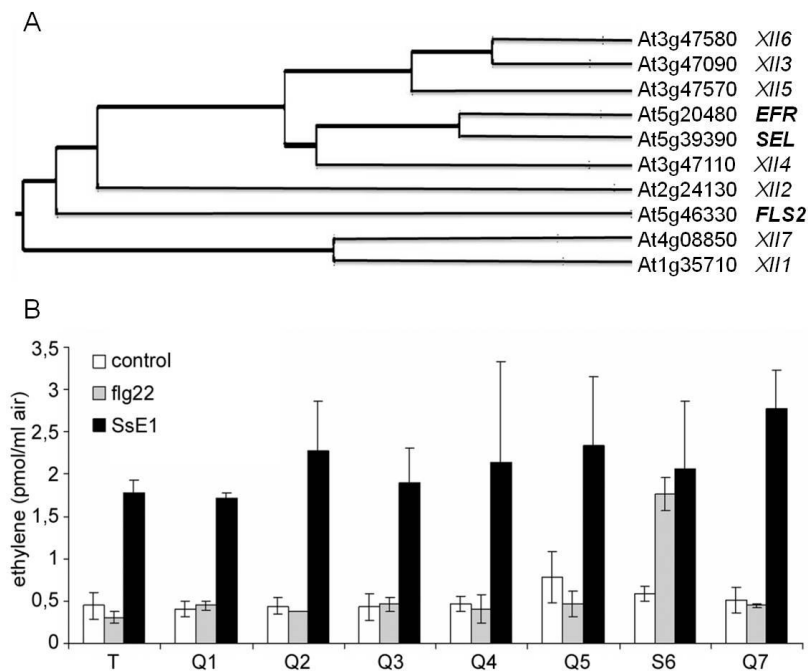
Figure 3. 12 SsE1 perception does not require LRR-RLK XII family members
(A) Polygenetic tree of subfamily XII of LRR-RLKs. Nomenclature of the LRR-RLK XII family members was according to (Shiu and Bleecker, 2001). (B) SsE1 (0.8 μg/ml) and 500 μM flg22 was tested for ethylene-inducing activity on leaf pieces of indicated *Arabidopsis* mutants. The mutants were the following: T (*fls2 efr cerk1* triple mutant), Q1 (*fls2 efr cerk1 xii1* quadruple mutant), Q2 (*fls2 efr cerk1 xii2*), Q3 (*fls2 efr cerk1 xii3*), Q4 (*fls2 efr cerk1 xii4*), Q5 (*fls2 efr cerk1 xii5*), S6 (*xii6 single mutant*), Q7 (*fls2 efr cerk1 xii7*). Bars represent average values ± S.D. (n=2).

3.2.3 SsE1 perception is dependent on BAK1

The eLRR-RLK BAK1 has been demonstrated to be a key regulator of immune- and developmental signaling pathways. BAK1 physically interacts with various other eLRR-RLKs both in a ligand-dependent and -independent manner, suggesting a role as adapter or co-receptor in plant heteromeric receptor complexes (Nam and Li, 2002; Chinchilla et al., 2007; Heese et al.,

2007; Roux et al., 2011). In solanaceous plants, where the function of RLPs in MTI and ETI is more intensively studied, virus-induced gene silencing approaches have shown that both Ve1-mediated resistance to wilt disease and EIX1/2-triggered signaling require BAK1, although the underlying molecular mechanism remains elusive (Fradin et al., 2009; Bar et al., 2010). In a reverse genetic approach, it was next investigated whether BAK1 is necessary for the SsE1-mediated immune responses. Several mutant alleles of *BAK1* exist: *bak1-3* and *bak1-4* insertional mutants are partially impaired in brassinosteroid signaling, MAMP-triggered responses and in cell death control (Li et al., 2002; Nam and Li, 2002; Chinchilla et al., 2007; Heese et al., 2007; Kemmerling et al., 2007), whereas the newly described *bak1-5* allele, encoding a BAK1 protein with an altered kinase activity, has a dominant-negative effect that affects only MAMP signaling but not brassinosteroid responses and cell death (Schwessinger et al., 2011). While the ethylene-response in *bak1-3* and *bak1-4* plants was only partially abolished, *bak1-5* plants were fully insensitive to SsE1 treatment (Figure 3.13A). The partial insensitivity to SsE1 in *bak1* mutants (*bak1-3* and *bak1-4*) might be explained by functionally redundant genes such as *BAK1-LIKE 1* (*BKK1*). Therefore, SsE1- induced ethylene responses were also measured in the *bkk1-1* and *bak1-5 bkk1-1* mutant plants. While the response toward SsE1 (and flg22) was not significantly affected in *bkk1-1*, ethylene production was completely abolished in the *bak1-5 bkk1-1* double mutant (Figure 3.13B) suggesting that *BAK1* is more important than *BKK1* for SsE1-mediated immune responses, as previously observed for flg22/FLS2 (Roux et al., 2011).
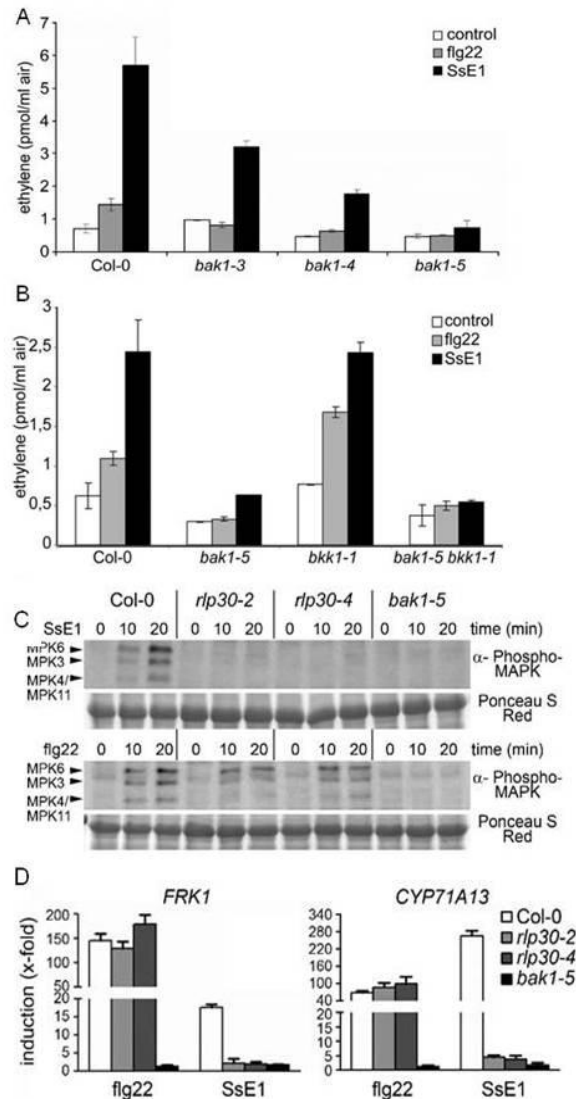
Figure 3. 13 SsE1-mediated immune responses are dependent on BAK1

(A) & (B) SsE1-induced ethylene accumulation in *bak1-3*, *bak1-4* and *bak1-5* (A) and *bkk1-1* or *bak1-5 bkk1-1* mutants (B). Leaves were treated with 0.25 µg/ml SsE1 or 500 nM flg22. Bars represent average values ± S.D. (n=2). (C) Immunoblotting of activated MAPK with anti-phospho p44/p42 antibody in *Arabidopsis* seedling extracts. Seedlings were collected 0, 10 or 20 minutes after treatment with 1 µg/ml SsE1 (upper panels) or 100 nM flg22 (lower panels). Ponceau S Red staining served as a loading control. The identity of individual MAP kinases as determined by size is indicated by arrowheads. (D) Transcriptional profiling of the defense-related genes *FRK1* and *CYP71A13* by quantitative real-time PCR (qRT-PCR). *Arabidopsis* seedlings were treated with 100 nM flg22 or 1 µg/ml SsE1 and collected 6 h after treatment. Gene expression was normalized to the levels of *Ef-1a* transcript and is presented as fold induction compared to the respective control as described in Figure 3.5E. Error bars, S.E.M. (n=3).

In the next step, we wanted to get a more detailed view on the role of *BAK1* in SsE1-mediated early signaling. We found that post-translational MAP kinase activation by SsE1 was, like upon treatment with flg22, absent in *bak1-5* plants (Figure 3.13C). Parallel assays performed with two *rlp30* mutant alleles (*rlp30-2* and *rlp30-4*) confirmed that MAPK 3, 4/11 and 6 were not activated anymore by SsE1 but responded normally to flg22 (Figure 3.13C). Further, the expression of MAMP-induced marker genes was equally dramatically reduced in *bak1-5* plants upon flg22 or SsE1 treatment, whereas *rlp30* mutants were specifically affected in their response to SsE1 (Figure 3.13C). Altogether, these data clearly demonstrated that both FLS2 and RLP30-mediated immune responses share BAK1 and that SsE1 is specifically sensed by RLP30.

3.2.4 RLP30 is phosphorylated by BAK1 *in vitro*

To investigate a direct physical interaction between RLP30 and BAK1, we transiently expressed HA-tagged RLP30 and myc-tagged BAK1 in *N. benthamiana.* Crude protein extracts were first subjected to immunoprecipitation using anti-HA agarose bead and co-immunoprecipitates were analysed for the presence of BAK1-myc. However, no direct interaction, even in the presence of SsE1 could be detected (Figure 3.14A) indicating that these two proteins are likely not to form a direct physical interaction. In a control assay, we observed a clear elf18-dependent recruitment of EFR-GFP into the complex of BAK1 and EFR (Figure 3.14B) as previous reports (Roux et al., 2011; Schwessinger et al., 2011).
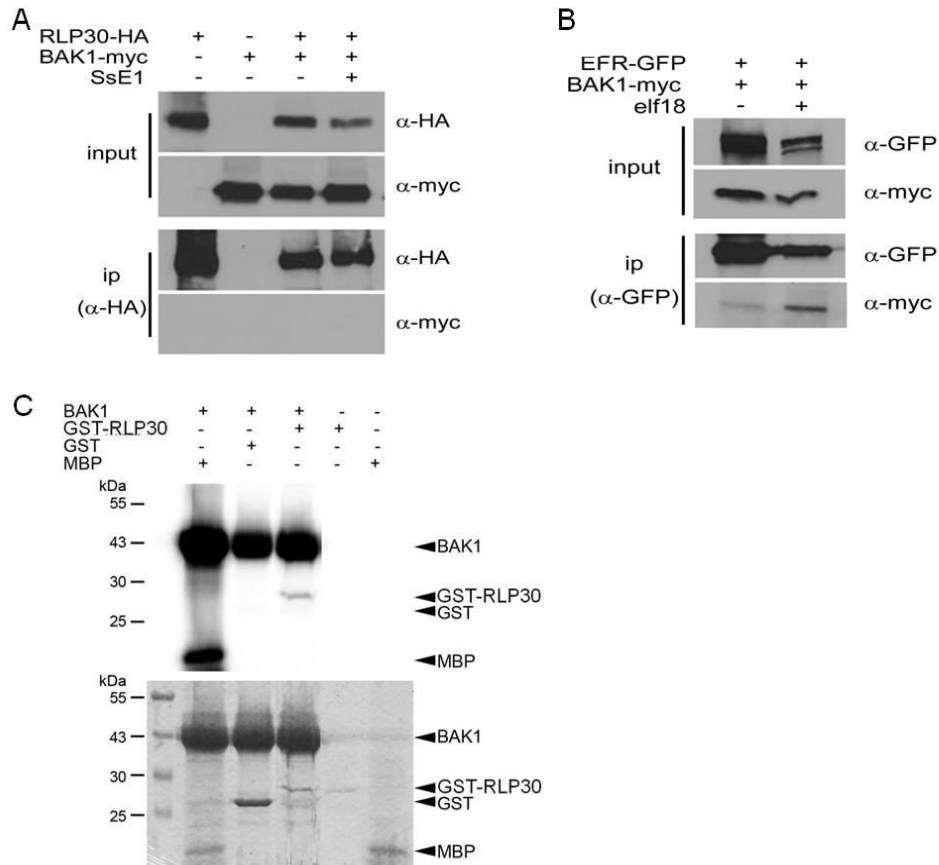
Figure 3. 14 RLP30 is phosphorylated by BAK1 *in vitro*

(A) RLP30 shows no direct interaction with BAK1 in *N. benthamiana*. Co-immunoprecipitation of leaves expressing RLP30-HA and BAK1-myc. Leaves were treated or not with 1 µg/ml SsE1 for 5 min, respectively. Total protein extracts were subjected to immunoprecipitation with anti-HA agarose beads followed by immunoblot analysis using either anti-HA or anti-myc antibodies. (B) BAK1-myc shows an elf18-dependent interaction with EFR-GFP in *N. benthamiana*. Co-immunoprecipitation of leaves expressing EFR-GFP and BAK1-myc. Leaves were treated or not with 100 nM elf18 for 5 min. Total proteins (T) were subjected to immunoprecipitation (IP) with GFP-Trap beads followed by immunoblot analysis using either anti-GFP or anti-myc antibodies. (C) Phosphorylation of RLP30 by BAK1 *in vitro*. *In vitro* kinase assay incubating C-terminal His-tagged BAK1 with N-terminal GST-tagged RLP30-C-terminus or a GST control, respectively. MBP was used as positive control for transphosphorylation. Autoradiogram, upper panel; Coomassie blue stained gel, lower panel. The identity of different proteins is indicated by arrowheads.

It has been described that the BRI1-BAK1 heteromerization results in sequential reciprocal receptor trans-phosphorylation which ultimately

increases the kinase activity of BRI1 to enhance downstream signalling outputs (Wang et al., 2008b). A similar scenario was reported for the FLS2/BAK1 interaction (Schulze et al., 2010), although different phosphorylation sites may be involved. Since AtRLP30 does not contain a cytoplasmic kinase domain but only a very short cytoplasmic tail, the RLP30 C-terminus was expressed as a GST fusion and the purified protein was assayed for phosphorylation by BAK1 in an *in vitro* kinase assay. A clear phosphorylation of the RLP30 C-terminus by BAK1 was observed, in addition to autophosphorylation of BAK1 as well as transphosphorylation of MBP by BAK1, which served as a control (Fig. 3.14C). The latter result is in disagreement with our Co-IP result that RLP30 cannot directly interact with BAK1 when transiently expressed in the heterologous system *N. benthamiana*. However, due to the current lack of a specific anti-RLP30 antibody that could be used for immunoprecipitation experiments, it cannot be excluded that RLP30 and BAK1 can interact *in vivo* in *Arabidopsis.*

3.2.5 RLP30 and BAK1 contribute to resistance against necrotrophic fungal infections

To test whether RLP30 is required for basal resistance against *S. sclerotiorum*, the knockout mutants *rlp30-2* and *rlp30-4* were challenged with the wild-type *S. sclerotiorum* strain 1980. As shown in Figure 3.15A, inoculation of both *rlp30* mutant lines with *S. sclerotiorum* mycelium resulted in increased disease symptoms and cell death compared to *Arabidopsis* wild-type Col-0. Microscopic examination of infected leaves after trypan blue staining revealed that mycelium growth is restricted to the necrotizing zone in Col-0 plants while it spreads beyond this zone in the *rlp30* mutants (Figure 3.15B). Fungal growth, assessed by measuring *S. sclerotiorum* genomic DNA levels by quantitative real-time PCR (qRT-PCR), was higher in *rlp30* plants than in Col-0 (Figure 3.15C).
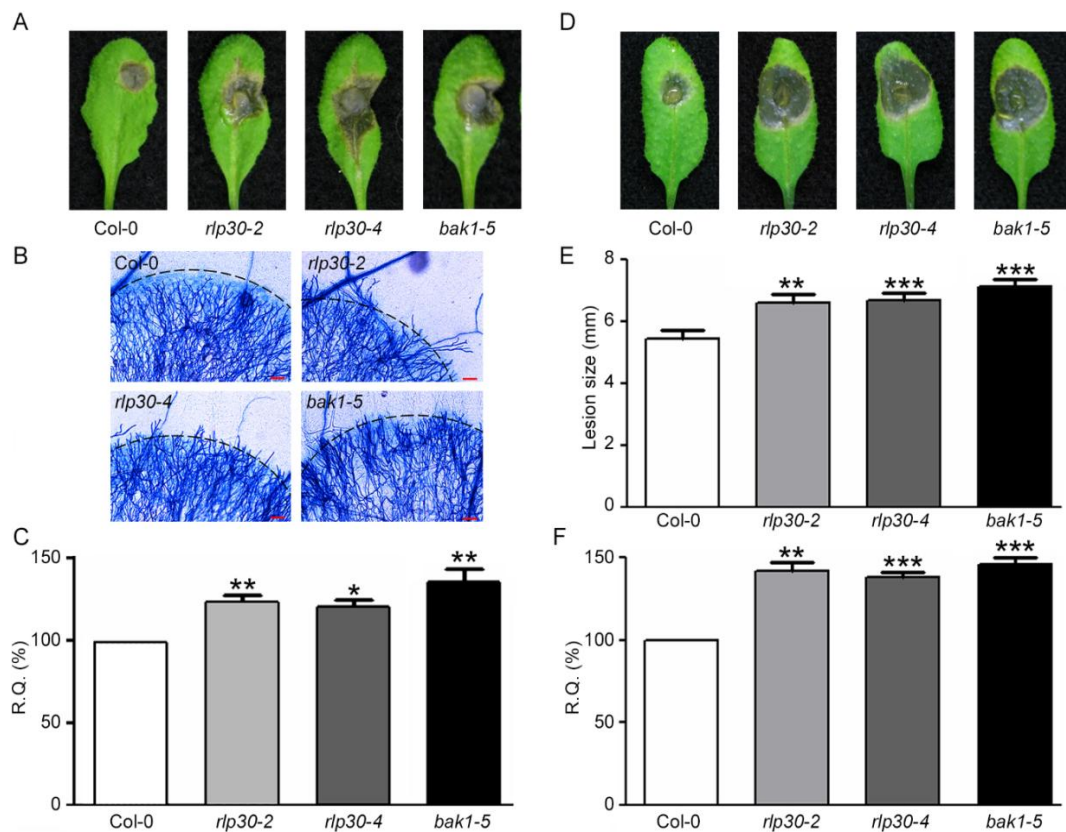
Figure 3. 15 AtRLP30 and BAK1 are required for resistance towards necrotrophic fungal pathogens

Infections with (A-C) *Sclerotinia sclerotiorum* or (D-F) *Botrytis cinerea* (A) Symptom development on Col-0 plants or *rlp30-2*, *rlp30-4* and *bak1-5* mutant plants 2 days after inoculation with *S. sclerotiorum* strain 1980. (B) Trypan blue staining of fungal hyphae 2 days after inoculation. A ring of blue-stained dead plant cells is indicated by a black line; scale bars in red indicate 100 μm. (C) Fungal biomass determined by quantitative real-time PCR (R.Q., relative quantity) 2 days after *Sclerotinia* infection in wild type Col-0 plants or the indicated mutant line. *Sclerotinia* ITS genomic DNA levels are shown relative to *Arabidopsis* Rubisco levels, Col-0 wild type is set to 100% R.Q. Shown are means ± S.E.M. (n = 4). (D, E) Col-0 plants or *rlp30-2*, *rlp30-4* and *bak1-5* mutants were drop-inoculated with 5 μl of a 2 x 10^6 spores/ml solution of *B. cinerea* and symptom development (D) or lesion sizes (E) were determined 3 days after inoculation. Results represent means ± S.E.M. (n = 20). (F) Fungal biomass at 3 days post-inoculation was determined using *Botrytis* Actin genomic DNA levels relative to *Arabidopsis* Rubisco levels as described in (C). Asterisks indicate significant differences when data sets were compared with Col-0 (*P < 0.05; **P < 0.01; ***P < 0,005, using Student's t-test).

Likewise, *B. cinerea* spore inoculation of *rlp30* plants resulted in increased cell

death and lesion size when compared to Col-0 plants (Figure 3.15D and 3.15E). Enhanced *B. cinerea* growth observed visually was paralleled by an elevated fungal DNA content in infected *rlp30* leaves (Figure 3.15F).

It has been shown that BAK1 controls plant programmed cell death and immunity to necrotrophic fungi and that the insertional mutants *bak1-3* and *bak1-4* are more susceptible to *B. cinerea* and *A. brassicicola* (Kemmerling et al., 2007), but a clear conclusion on the role of BAK1 in mediating immunity to these necrotrophic fungi was impeded by the enhanced cell death phenotype of these alleles. However, the SsE1-insensitive *bak1-5* line, which does not display increased cell death (Schwessinger et al., 2011), was also more susceptible to *S. sclerotiorum* and *B. cinerea* (Figure 3.15), revealing that BAK1 indeed plays a critical role mediating basal immunity to these pathogens. Altogether our results indicate that RLP30, together with BAK1, plays a role in defense against a broad spectrum of fungal pathogens that is not restricted to the recognition of race-specific elicitors.

3.2.6 SOBIR1 - a third receptor protein involved in SsE1 perception

RLPs like RLP30 lack a cytoplasmic kinase domain and these receptors have long been anticipated to recruit signaling partners that do contain a kinase domain. Previous reports already showed that the two RLPs involved in developmental processes, TMM and CLV2, can form functional receptor complexes with RLKs (Bleckmann et al., 2010; Guo et al., 2010; Lee et al., 2012). Although RLP30 can be phosphorylated by BAK 1 in the *in vitro* phosphorylation assay, no direct physical interaction between RLP30 and BAK1 could be detected so far (Figure 3.14A).

In search for additional RLP30 signaling partners we mined available databases of membrane protein interactors (http://www.associomics.org/Associomics/Home.html). Out of a total of 66 putative interactors several RLKs were found to interact with RLP30 in this

yeast-two-hybrid based dataset (Appendix Table 6.6 & Table 3.5).

Table 3. 5 RLP30-interacting receptor-like kinases as determined by the Membrane-based Interactome Network Database (M.I.N.D.)

AGI ID	Functional Description
AT1G21240	WAK3, wall associated kinase 3 RLK/Pelle
AT2G31880	SOBIR1, EVR, Leucine-rich repeat protein kinase family protein
AT2G37050	Leucine-rich repeat protein kinase family protein
AT4G20790	Leucine-rich repeat protein kinase family protein
AT5G59650	Leucine-rich repeat protein kinase family protein

For three of the five RLP30-interacting RLKs T-DNA insertion lines were obtained from the Nottingham *Arabidopsis* Stock Centre (NASC) and tested for ethylene production in the presence of SsE1. Only the *sobir1* mutants (*SUPPRESSOR OF BIR1-1*, At2g31880, also known as EVR, Evershed) were affected in SsE1-dependent ethylene production whereas the knockout lines corresponding to the other RLKs displayed a normal response to SsE1 (Figure 3.16A). Next, we tested the response of two T-DNA insertion lines of *SOBIR1* (N550715:*sobir1-12*, N509453:*sobir1-13*) to other elicitors. The mutants showed a normal response to elicitation with flg22 compared to wild type and *rlp30-4* plants (Figure 3.16B). Col-0 and *rlp30-4* plants also showed similar ethylene production in response to treatment with another newly described PAMP, EMAX (Jehle et al., 2013) (Figure 3.16B). In contrast, *sobir1* mutant plants displayed a loss of EMAX-triggered ethylene production similar to what was observed after SsE1 treatment (Figure 3.16B). Hence, SOBIR1 seems to also have a function in EMAX perception, which depends on the LRR-RLP RLP1 (Jehle, 2012). In contrast, genetic inactivation of *SOBIR1* did not affect MAMP responses to flg22 and efr18 (perceived by the LRR-RLKs FLS2 and EFR) or PGN and chitin (perceived by the LysM-RLPs and LysM-RLKs LYM1, LYM3 and CERK1) (data not shown), indicating that SOBIR1 is not involved in MAMP perception via LRR-RLKs or LysM-domain proteins. Hence, SOBIR1

might be a novel co-receptor for PRRs of the LRR-RLP-type, such as RLP30 (recognizing SsE1) and RLP1 (recognizing EMAX, Jehle et al., 2013).
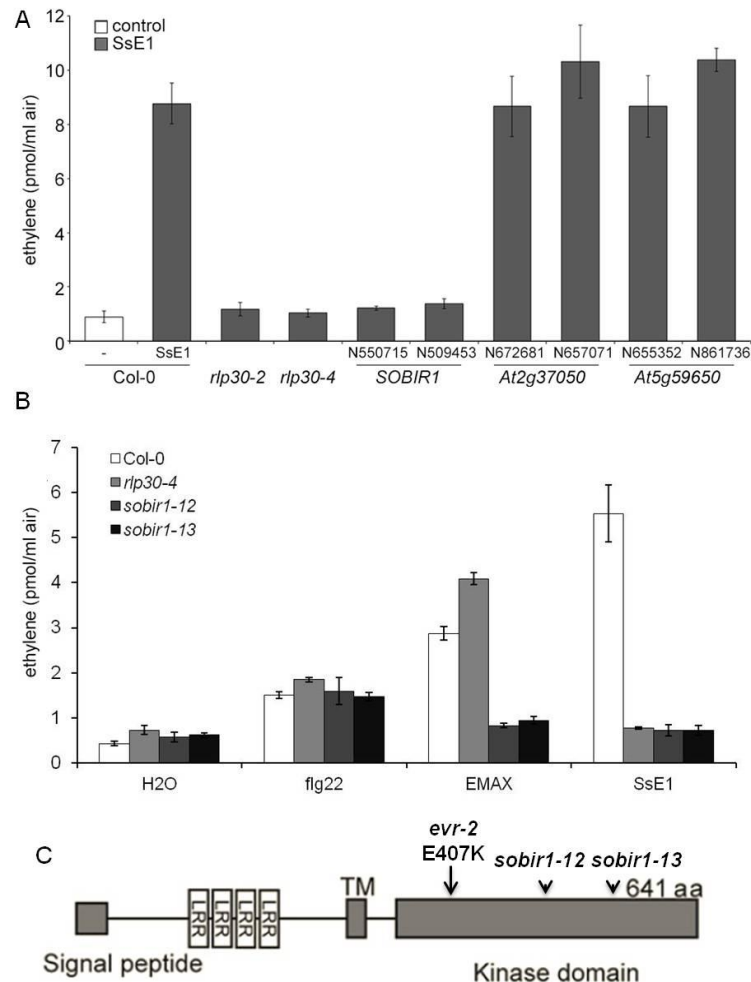
Figure 3. 16 *sobir1* mutants are impaired in the perception of SsE1 and EMAX
(A) Ethylene response to 0.25 µg/ml SsE1 in *sobir1* mutants in comparison to other RLK mutants. Each two independent T-DNA insertions lines were tested, two *rlp30* mutants served as positive control. (B) Wild type and indicated mutant plants were treated with 0.25 µg/ml SsE1, 1 µg/ml EMAX and 500 nM flg22 or left untreated (control). Leaf discs of 5 weeks old *Arabidopsis* plants were treated and ethylene production was measured by gas chromatography after 3 hours incubation,. Bars represent average values (n=2) ± S.D. (C) Diagram of the EVR protein. The regions corresponding to the signal peptide, leucine-rich repeats (LRRs), transmembrane domain (TM) and kinase domain are indicated. Point mutations are marked by arrows, and T-DNA insertions by arrowheads.

3.2.7 SOBIR1 physically interacts with RLP30

SOBIR1 encodes a typical LRR-RLK containing four extracellular LRRs, a single transmembrane domain and a cytoplasmic serine/threonine protein kinase domain (Figure 3.16C). SOBIR1 was first identified as a suppressor of cell death triggered by a mutation in the LRR-RLK BIR1 (BAK1-interacting receptor-like kinase 1, a negative regulator of multiple plant resistance signaling pathways), and *SOBIR*1 overexpression was demonstrated to activate cell death and defense responses (Gao et al., 2009). Independently, SOBIR1 was ascribed a function as an inhibitor of abscission and called EVR (Evershed) (Leslie et al., 2010).
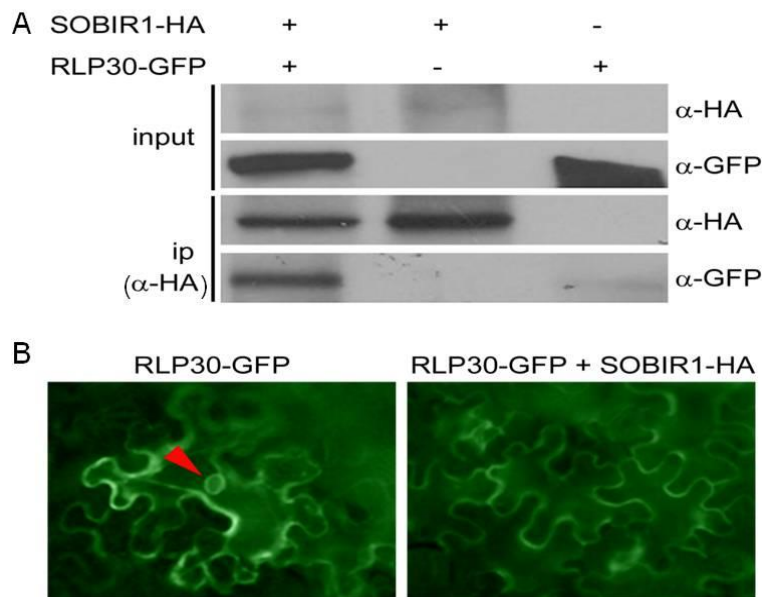
Figure 3. 17 SOBIR1 physically interacts with RLP30 and drives RLP30 to the plasmamembrane
N. benthamiana leaves transiently expressing RLP30-GFP and SOBIR1-HA were used for co-immunoprecipitation (A) or localization studies (B). (A) Total proteins (input) were subjected to immunoprecipitation with anti-HA agarose beads followed by immunoblot analysis using either anti-HA or anti-myc antibodies. (B) The same leaves were used for fluorescence microscopy, revealing a GFP signal for RLP30 expressed without SOBIR1 around the nucleus (red arrow head) and at the cell periphery, whereas co-expression with SOBIR1 lead to GFP signals exclusively found at the plasma membrane.

Since the *sobir1* mutant plants showed the same phenotype as *rlp30* with respect to a loss of SsE1 responsiveness (Figure 3.16), it was next investigated whether SOBIR1 might form a signaling complex with RLP30. To test a direct physical interaction between SOBIR1 and RLP30, we performed co-immunoprecipitation analyses of both proteins using transient co-expression of epitope-tagged SOBIR1 and RLP30 constructs in *Nicotiana benthamiana*. We observed that SOBIR1 physically interacts with RLP30 in a ligand-independent manner (Figure 3.17A). Interestingly, in leaves only expressing RLP30-GFP the fluorescence signal was found at the plasma membrane and also as a ring around the cell nucleus, whereas in RLP30- and SOBIR1-co-expressing leaves the GFP-signal was only found at the cell periphery (Figure 3.17B). Hence, SOBIR1 seems to be required for exclusive plasma-membrane localization of RLP30 (Figure 3.17B). In conclusion, SOBIR1 and RLP30 are capable of forming a ligand-independent heterooligomer in *N. benthamiana.*

3.2.8 SOBIR1-kinase activity is required for its function in SsE1 perception

Although 20% of *Arabidopsis* RLKs are predicted to be kinase inactive (Castells and Casacuberta, 2007), SOBIR1 has been demonstrated to have *in vitro* protein kinase activity (Leslie et al., 2010). Furthermore, the function of SOBIR1 in abscission depends on its kinase activity (Leslie et al., 2010).
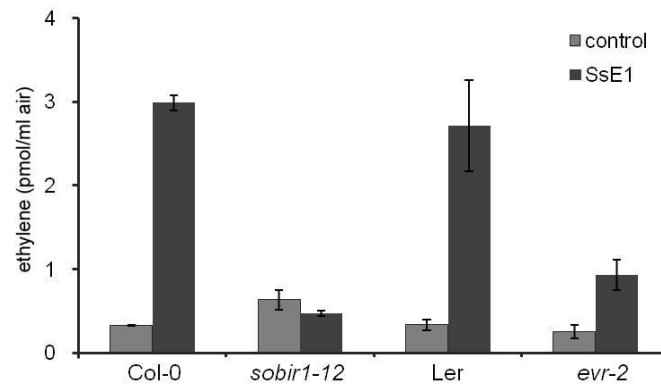
Figure 3. 18 SsE1 signal transduction relies on kinase activity of SOBIR1
Leaf pieces of Col-0 and *sobir1-12* plants (in the Col-0 background) or Ler and *evr-2*
(in the Ler background) were treated with 0.25 µg/ml SsE1 or water as a control prior
to measurement of ethylene production. Bars represent average values ± S.D. (n=2).

Likewise, SsE1-triggered ethylene production was strongly reduced in the
evr-2 mutant (Leslie et al., 2010) which contains a point-mutated,
kinase-inactive allele of *SOBIR1* (Figures 3.16C and 3.18). This result
indicates that SOBIR1-mediated phosphorylation events also play a vital role
in SsE1 signal transduction.

3.2.9 SOBIR1 is required for resistance towards fungal pathogens

The similar phenotype of mutants of *SOBIR1* and *RLP30* with respect to
SsE1-sensitivity also suggested that *SOBIR1* could play a role in plant defence
to fungal pathogens (Figure 3.16 & 3.18). As shown in Figure 3.19A, mutant
lines of *SOBIR1* incubated with *S. sclerotiorum* mycelium resulted in increased
disease symptoms and cell death compared to *Arabidopsis* wild-type Col-0. In
addition, *sobir1-12* showed hypersusceptibility towards infections with *B.
cinerea* similar to *rlp30-2* mutant plants (Figure 3.15D and 3.15E). These
results revealed a role for SOBIR1 in resistance to necrotrophic fungal
pathogens.
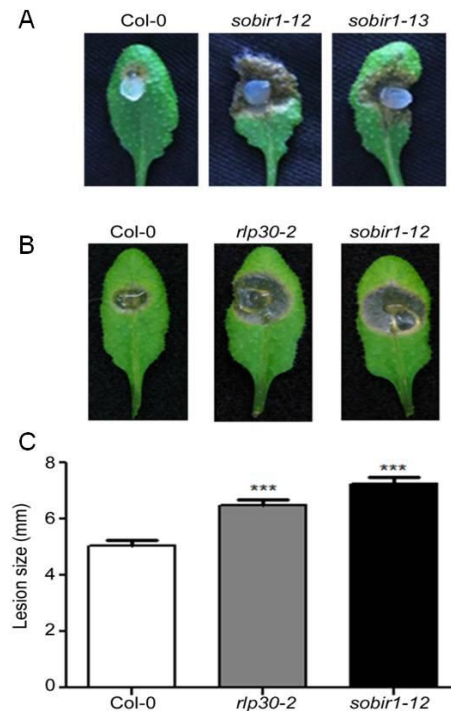
Figure 3. 19 SOBIR1 is required for resistance towards fungal pathogens
(A) Symptom development on Col-0 plants and *sobir1* mutant plants 2 after inoculation with *S. sclerotiorum* (B, C) Col-0 plants or *rlp30-2* and *sobir1-12* mutants were drop-inoculated with 5 µl of a $2x10^6$ spores/ml solution of B. cinerea and symptom development (B) or lesion sizes (C) were determined 3 days after inoculation. Results represent means ± S.E.M. (n = 20). Asterisks indicate significant differences when data sets were compared with Col-0 (***P < 0,005, using Student's t-test).

3.2.10 Co-expression of RLP30 and SOBIR1 confers SsE1 responsiveness in *N. benthamiana*.

RLP30 is required for fungal resistance in *A. thaliana* and thus interspecies transfer of this PRR could offer a novel strategy for improving fungal resistance in important crop plant species. Pioneering work was performed by Lacombe *et al.* (2010) showing that *Arabidopsis* EFR conferred responsiveness to bacterial EF-Tu in the solanaceous plants *Nicotiana benthamiana* and tomato and made them more resistant to a broad range of phytopathogenic bacteria (Lacombe et al., 2010). EF-Tu perception is normally limited to plants of the Brassicaceae family and the transfer of EFR in plant families that are insensitive to EF-Tu appears to be a key aspect toward engineering durable

bacterial resistance. Likewise, a transfer of RLP30 would only be justified if the acceptor plant does not have an equivalent perception complex for SsE, otherwise the probability is high that an adapted-pathogen has already evolved the capability to subvert SsE1-mediated immunity. We first analysed the response of *N. benthamiana* and tomato to SsE1. In contrast to flg22, SsE1 cannot trigger ethylene production in these two plants (Figure 3.20A). Using agro-infiltration in *N. benthamiana*, transient expression of RLP30 alone, however, did not result in SsE1 responsiveness. Importantly, only co-expression of RLP30 together with SOBIR1 conferred responsiveness to SsE1 which is not obtained by expressing either protein separately (Figure 3.20B). Thus, these results proved that SOBIR1 and RLP30 function as a receptor complex to transduce the SsE1 signal.
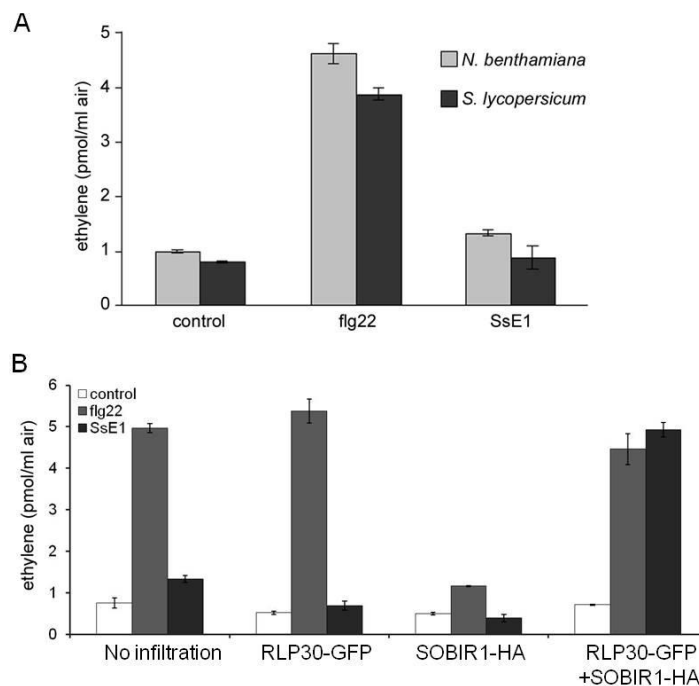
Figure 3. 20 Co-expression of RLP30 and SOBIR1 confers SsE1 responsiveness in *N. benthamiana*

(A) The SsE1 perception system is absent in solanaceous plants. Leaf pieces of *Nicotiana benthamiana* or *Solanum lycopersicum* plants were treated with 0.25 µg/ml SsE1 or 500 nM flg22. (B) Epitope-tagged RLP30 and SOBIR1 proteins were transiently produced in *N. benthamiana* alone or in combination. 2 days after agroinfiltration, leaf pieces were treated with 0.25 µg/ml SsE1, 500 nM flg22 or left

untreated (-). Ethylene production was measured by gas chromatography after 3 hours incubation. Bars represent average values ± S.D. (n=2).

4. Discussion

Plant diseases seriously limit the production of cereals and crops in world agriculture. The diseases can be controlled by chemicals, but ways to reduce chemical inputs are being sought to benefit the environment. Security and sustainable agricultural methods are therefore placing increased emphasis on the genetic potential of plants to control pathogens.

Resistance (R) genes have been widely used in plant breeding to control diseases for decades (Gurr and Rushton, 2005; Gust et al., 2010). However, the genes involved in host-pathogen interactions are highly diversified among related pathogen species that attack different plants and pathogens readily mutate or even lose effector molecules, leading to rapid breakdown of R gene-mediated resistance in the field (McDowell and Woffenden, 2003; Allen et al., 2004; Bent and Mackey, 2007). Thus, an alternative, more durable form of resistance is required in sustainable agriculture.

In contrast to effector proteins, MAMPs are highly conserved microbial molecular signatures and play pivotal functions for the micro-organism, and therefore are difficult to mutate or delete (Zipfel, 2008; Zipfel, 2009). PRRs as MAMP receptors therefore offer the prospect of durable, broad-spectrum resistance to a range of economically important pathogens.

4.1 SsE1 is a novel fungal MAMP

Up to know, only a few pairs of fungal MAMPs/PRRs have been identified (Monaghan and Zipfel, 2012; Schwessinger and Ronald, 2012). This study aimed to identify and characterize additional novel fungal MAPMs and their corresponding perception systems in *Arabidopsis*. Data generated in this study will be discussed further to integrate them into the current knowledge of plant innate immunity for a better understanding of interaction between plants and

microbes.

In the present work, a novel SsE1 elicitor from *S. sclerotiorum* was partially characterized that induces MAMP-like immune responses in *Arabidopsis* (Figure 3.1 & 3.5). SsE1 is most likely a peptide with immunogenic properties and most importantly, SsE1 does not act as a necrosis-inducing toxin, a common weapon of necrotrophic pathogens to colonize host plants (Figure 3.2, 3.3 & 3.4). SsE1 was also tested for elicitor activity in *Nicotiana benthamiana* and *Solanum lycopersicum* (Figure 3.20A), *Vitis vinifera* and *Brassica napus* (data not shown, in collaboration with Prof. Alain Pugin and Dr. Chris Ridout). These assays showed that only *Arabidopsis* was responsive to SsE1. High priority was given to the molecular identification of the protein elicitor corresponding to SsE1. Accordingly, SsE1 samples were analysed by mass spectrometry using nano-LC MS/MS (B. Macek, Proteome Centre Tübingen). By this approach, several proteins were detected within the sample (Figure 3.6 & Table 3.3). Although two candidates were successfully cloned and expressed in *E.coli*, the recombinant proteins did not shown any elicitor activity (Figure 3.7). It is currently impossible to determine unambiguously which protein on the list corresponds to SsE1 (Table 1). Secretion of recombinant proteins in *P. pastoris* presents the advantage of an eukaryotic expression system that is closer to the conditions in which SsE1 is expressed in *S. sclerotiorum*. Hence, future studies will use recombinant proteins expressed in the *Pichia* system. In parallel, SsE1 should be purified to homogeneity by performing two-dimensional SDS-PAGE after partial purification by column chromatography. The fact that the SsE1 elicitor activity is preserved in the presence of SDS will allow eluting the protein from single gel spots to test for elicitor activity and for subsequent identification by mass spectrometry analysis. Finally, as the SsE1 activity could not be destroyed by trypsin digestion (Figure 3.3), trypsin digested SsE1 preparations could be used for purifying active peptides via HPLC (using for instance C18 columns) and subsequent MS-analysis.

In a complementary approach, recombinant AtRLP30 protein could be expressed, either in *E. coli* or in *P. pastoris*, and purified protein could be used as a bait to bind SsE1 from *S. sclerotiorum* culture filtrate in a single step of affinity chromatography. AtRLP30 interactor(s) could be identified by mass spectrometry analysis and would help to narrow down the list of SsE1 candidates.

The identification of immunogenic peptides like flg22 or elf18 that can easily be synthesized in large amounts confers a huge advantage for the genetic and biochemical dissection of receptor-mediated perception and signalling pathways regulating plant defence responses. Once identified, we will determine the minimal structural motif within SsE1 that is required for AtRLP30-dependent immune activation. Identification of the molecular nature of SsE1 would also be required for further analysis of receptor activation.

Flg22 and elf18, as well-documented MAMPs, have vital roles in the bacterial lifecycle (Felix et al., 1999; Kunze et al., 2004). Whether SsE1 fulfils the criteria that are required for classification as a MAMP will only be answered after elucidation of the molecular nature of SsE1 and its taxonomic distribution. However, independently of being a PAMP/MAMP or not, it will be interesting to find out whether SsE1 plays an essential role in microbial physiology or pathogenicity by testing the *S. sclerotiorum* gain- and loss-of-function mutants for their fitness and pathogenicity.

4.2 Application of fungal extracts containing SsE1 as plant strengtheners

It is known that exogenous application of MAMPs can activate defence responses in plants and renders them more resistant towards pathogen infections. The application of MAMP as protective agent is exemplified by harpin. Harpins are glycine-rich, acidic proteins produced by gram-negative plant pathogenic bacteria and were firstly identified as elicitors of HR when applied to plant leaves as purified proteins (Wei et al., 1992). Application of

difference harpins or harpin-like proteins triggers defense responses in plants and provides broad-spectrum resistance against different kinds of phytopathogens when supplied in commercial formulations or transgenically expressed proteins in various plants (Strobel et al., 1996; Dong et al., 1999; Shao et al., 2008; Tampakaki et al., 2010). Furthermore, harpin application also can promote plant growth (Jang et al., 2006; Chen et al., 2008) and improve plant drought tolerance (Dong et al., 2005; Zhang et al., 2011) and is therefore also called plant "strengthener". Another study on plant strengthener is performed by the Spanish company PLANT RESPONSE, who has run trials for three consecutive years with selected proprietary MAMP formulations in both greenhouse and open field in multiple crops (i.e. pepper, garlic, onion grapevine, etc) with 10-30% yield increase in weight (personal communication (Marisé Borja, PLANT RESPONSE)). Therefore, also the suitability of SsE1 as a biological priming agent should be investigated in further experiments.

4.3 Signaling pathways triggered by SsE1

Upon MAMP perception an early Ca2+ influx, production of reactive oxygen species (ROS) and ethylene production occur with the subsequent activation of downstream signaling networks controlled by Mitogen-Activated Protein Kinase (MAPKs) and Calcium-activated (dependent) PK (CDPKs) cascades (Zipfel et al., 2004; Boller and Felix, 2009; Boudsocq et al., 2010; Tena et al., 2011). Plant-pathogen recognition also triggers the biosynthesis of phytohormones, such as salicylic acid (SA), ethylene (ET), jasmonic acid (JA) and abscisic acid (Glazebrook, 2005; Robert-Seilaniantz et al., 2011). The balance and interplay among these hormones and others such as auxins, giberellins and brassinosteroides plays a pivotal role in the expression of resistance to particular pathogens and pests (Robert-Seilaniantz et al., 2011). The purification to homogeneity and identification of SsE1 is not mandatory for application-oriented plant immunity research although it would be necessary to

uncover molecular mechanism of different responses triggered by different MAMPs. Results within this thesis showed the apparent quantitative differences between SsE1 and flg22 in ethylene production, oxidative burst and gene induction (Figure 3.5). Defense against *S. sclerotiorum* in *Arabidopsis* is complex and depends essentially on the plant hormones jasmonate and ethylene (JA/ET), although salicylic acid (SA) appears also to be involved (Guo and Stotz, 2007; Perchepied et al., 2010). The general view is that the JA/ET and SA pathway act antagonistically with a prominent role of JA/ET in resistance against necrotrophs, while SA appears to be more important for resistance against biotrophs (Glazebrook, 2005). Also camalexin, the most common phytoalexin produced by *Arabidopsis*, plays an important role in resistance against necrotrophs (Denby et al., 2005). Interestingly, we observed that the expression of JA/ET-regulated genes and camalexin biosynthetic genes is more strongly induced by SsE1 than by flg22. In addition, EDS1, PAD4 and NDR1, which are essential for SA-mediated resistance (Hammond-Kosack and Parker, 2003), are not involved in SsE1-induced ethylene production (Figure 3.11). Therefore, it is tempting to hypothesize that SsE1-mediated signaling preferentially activates the JA/ET branch of the immune system and a comprehensive dose-response analysis with pure SsE1 of global -omic (metabolomic, transcriptomic and proteomic) changes may help to answer whether and how the SsE1 signal is integrated differently to the flg22 signal.

4.4 The novel PRR RLP30 is required for plant immunity to necrotrophic fungi

Screening of different *Arabidopsis* accessions revealed five ecotypes with SsE1 responsiveness below 10% of the wild-type and for three of them (Mt-0, Lov-1 and Sq-1) (Figure 3.8 & Table 3.4), the loss of response maps to the RLP30 locus (Figure 3.9). It is not known yet whether the two other ecotypes

(Br-0 and Lov-5) are allelic or whether another locus or loci is/are involved. Moreover, several lines showed a lower ethylene response (25-50% of the wild-type) in the presence of SsE1 (Figure 3.8), a trait that could be used to map other components that participate in RLP30-mediated signal transduction. A recent study revealed that the differences in flg22 responsiveness observed in natural accessions of *Arabidopsis* could be explained by differences in changes of FLS2 protein abundance or flg22-binding affinity (Vetter et al., 2012). As there is currently no RLP30 antibody available, it cannot be ruled out that RLP30 protein levels are responsible for the differences in SsE1 sensitivity in the different ecotypes. Moreover, SsE1-binding affinities might differ in the various RLP30 alleles, However, to clarify this point and to analyze direct binding between SsE1 and different RLP30 versions, it will be necessary to identify SsE1 as mentioned above.

In general, plant resistance against necrotrophic pathogens is quantitative and multi-layered rendering its genetic dissection difficult. Recent work by Perchepied et al.(Perchepied et al., 2010) highlighted the existence of a large natural resistance variation toward *S. sclerotiorum* infection among the more than 50 *Arabidopsis* ecotypes that were tested. With respect to SsE1 perception, ecotypes responding normally to SsE1 would be distributed in both hyper-susceptible and resistant categories. Among the five SsE1-insensitive ecotypes identified in this thesis, only Mt-0 was in the list of the authors and the ecotype displayed intermediate resistance to *S.sclerotiorum* (Perchepied et al., 2010). This indicates that like in the case of MTI, where direct genetic evidence for the contribution of a single receptor to plant resistance has proven to be extremely challenging, redundant perception systems involving other receptors than RLP30 seem to contribute to quantitative resistance against *S. sclerotiorum* and *B. cinerea*.

In this thesis it was observed that *rlp30* mutants showed an enhanced susceptibility to *S. sclerotiorum* and *B. cinerea* (Figure 3.15). In a previous report, Wang et al. (Wang et al., 2008a) did not notice any altered resistance

towards necrotrophic fungi including *S. sclerotiorum* and *B. cinerea* in the *rlp30* mutants. A possible explanation is that the use of *S. sclerotiorum* isolates with a different level of aggressiveness and different experimental settings of the patho-assay may account for discrepancies in the results. Interestingly, in the same report, *rlp30* mutants were shown to be more susceptible towards infection with the non-adapted bacterial bean pathogen *Pseudomonas syringae* pv. *phaseolicola* (*Psp*) strain 1448A (Wang et al., 2008a), however, increased resistance towards *Psp* was not as pronounced as the hyper-susceptibility we observed towards fungal infections. Moreover, in my own experiments, I could not see altered resistance towards infection with either *Pto*DC3000 or *Psp* (data not shown). Interestingly, a sequence blast of the newly identified fungal MAMP Ave1 from race 1 *Verticillium* spp. revealed that homologous proteins were not only found in other fungal pathogens but was also found in the bacterial plant pathogen *Xanthomonas axonopodis* (de Jonge et al., 2012). Combining these results, it can be assumed that SsE1 does not only exist in fungal pathogens but might also be present in bacterial pathogens. However, sequence comparisons with putative SsE1 homologs in other species will only be possible after the nature of SsE1 has been identified. Still, RLP30 might be a promising candidate to enhance pathogen perception and to improve resistance in crop plants, lacking a perception system for SsE1, against diseases not only caused by fungi such as *Sclerotinia* and *Botrytis* species but also by bacteria.

4.5 RLPs as novel tools for improving plant disease resistance

With the identification of RLP30 participating in resistance against necrotrophic fungal pathogens and the identification of REMAX/RLP1 that is important for sensing a novel bacterial elicitor from *Xanthomonas* (Jehle, 2012), the first two *Arabidopsis* RLPs have been associated with specific functions as potential PRRs. The potential use of PTI in crop protection has not been explored in

detail, but it is known that constitutive overexpression of *Arabidopsis* PRRs, such as EFR, in crops (e.g. tomato and tobacco) confers resistance to bacterial pathogens (Lacombe et al., 2010). The identification of RLP30 and RLP1 as novel PRRs also demonstrates the value of *Arabidopsis* as a genomic resource for isolating and exploiting new genes that could be transferred into economically important crop plants lacking an obvious ortholog of the *Arabidopsis* locus to confer disease resistance. The completion of the genome sequencing of more than 400 *Arabidopsis* ecotypes (http://1001genomes.org/), the existence of large collections of F2 segregating mapping populations and knock-out lines for nearly all the putative *Arabidopsis* receptor-encoding genes permits high-throughput identification of receptors for microbial elicitors. Gene presence/absence, deletion/insertion and single nucleotide polymorphisms increase the degree of genetic variability among the different accessions (Atwell et al., 2010; Cao et al., 2011; Gan et al., 2011). A simple calculation with the more than 600 RLKs present in *Arabidopsis* Col-0 multiplied by the factors due to polymorphisms yields an impressive number of receptors with potentially different specificities awaiting their exploitation for biotechnological applications.

4.6 SOBIR1 and BAK1 are co-receptors for RLPs

RLPs represent a unique class of cell-surface receptors, as they lack a functional cytoplasmic domain. RLP30 is a protein with 21 eLRRs and a very short cytoplasmic domain (Figure 3.10B). However, the absence of a cytosolic signaling domain indicates that RLP30 is most likely part of a heteromeric receptor complex and that additional components are necessary for intracellular signaling. Current models suggested that eLRR-RLPs recruit RLKs to form heteromeric receptor complex for signaling transduction (Bleckmann et al., 2010; Guo et al., 2010; Lee et al., 2012). To identify other signaling components required for SsE1-triggered immune responses, we

performed a reverse genetic screening. The eLRR-RLK BAK1 has been demonstrated to be a core regulator of immune signaling pathways (Chinchilla et al., 2007; Heese et al., 2007; Chinchilla et al., 2009). BAK1 physically interacts with other PRRs in a ligand-dependent manner (Chinchilla et al., 2007; Heese et al., 2007; Roux et al., 2011). BAK1 is also genetically required for RLP30-induced signal transduction (Figure 3.13) and the *bak1* mutant was hyper-susceptible to infection with *S. sclerotiorum* and *B. cinerea* (Figure 3.15). The contribution of BAK1 in RLP-mediated signaling seems to be complex. It was shown that BAK1 interacts with LeEix1 (but not LeEix2), which acts as a decoy receptor and attenuates EIX induced internalization and signaling of the LeEix2 receptor (Bar et al., 2010). Therefore, BAK1 seems to act as a negative regulator in EIX signaling, which is in contrast to its positive effect on SsE1-triggered or Ve1-mediated signalling (Fradin et al., 2009). So far, we did not detect any direct interaction between RLP30 and BAK1 with or without SsE1 treatment in the *N.benthamiana* transient expression system (Figure 3.14A). However, the *in vitro* kinase assay suggested that the RLP30 cytoplasmic domain can be phosphorylated by the BAK1 kinase domain, when both recombinant proteins were purified from *E. coli* (Figure 3.14B). Hence, these somewhat contradictory results need to be addressed in the future by analyzing a putative RLP30 and BAK1 physical interaction in *Arabidopsis* instead of *N. benthamiana* and by testing if the putative BAK1 phosphorylation sites of RLP30 play a role in SsE1 signal transduction.

In addition to BAK1, the eLRR-RLK SOBIR1 was identified as being required for SsE1 perception and/or signaling (Figure 3.16). SOBIR1 was first identified as suppressor of cell death triggered by the *bir1* mutation from a genetic screen and found itself to be a positive regulator of cell death (Gao et al., 2009). A more recent study suggested that SOBIR mediates floral shedding in *Arabidopsis* probably through regulating membrane trafficking during abscission (Leslie et al., 2010). Results presented in this thesis revealed that SOBIR1 also plays a role in plant innate immunity. SsE1 signal transduction

was dependent on SOBIR1 and the *sobir1* mutants showed less resistance to the fungal pathogens *S. sclerotiorum* and *B. cinerea* (Figure 3.16 & 3.19). BIR1 (BAK1 interacting receptor-like kinase 1) belongs to the LRR-RLKs with 5 extracellular LRRs domain (Gao et al., 2009). The biochemical data showed that BIR1 as active protein kinase can physically interact with BAK1 in the plasma membrane (Gao et al., 2009). BIR1 negatively regulates two parallel defense pathways, one dependent on PAD4 and the other one dependent on SOBIR1 (Gao et al., 2009). All the genetic and biochemical data suggested that BAK1 and SOBIR1 are involved in SsE1 signal transduction, hence it should be investigated if also BIR1 is required for SsE1 perception. As genetic inactivation of *BIR1* leads to extensive cell death, activation of constitutive defense responses and seedling lethality phenotype at 24 °C, so far the *bir1* mutants could not be included in our assays. Hence, it remains to be demonstrated whether BIR1 has a role in SsE1 signal transduction.

Importantly, EMAX signal transduction pathways also depend on *SOBIR1* (Figure 3.16B), and similar to RLP30, RLP1 also physically interacts with SOBIR1 in a ligand-independent manner (Figure 3.17A and data not shown). Gene expression of *SOBIR1* was also up-regulated after MAMP or pathogen treatment (Kemmerling et al., 2011). The kinase activity of SOBIR1 is vital for its role in transmitting the SsE1 signal (Figure 3.18). Hence, it can be assumed that SOBIR1 acts as a novel co-receptor for PRRs of the RLP-type, such as RLP30 (recognizing SsE1) and RLP1 (Jehle, 2012). SOBIR1 would thus resemble BAK1 (which also has only few extracellular LRRs), which is involved in RLK signalling and is required for the function of the PRRs FLS2 and EFR. Most importantly, co-expression of SOBIR1 and RLP30 can restore responsiveness to SsE1 in *N.benthamiana* (Figure 3.20) which was not obtained by expressing either protein alone. Hence, co-transformation of a receptor complex (e.g. RLP30 and SOBIR1) could be used as novel genetic tool for the generation of fungus-resistant crops.

Proteins must be targeted to the appropriate compartment to ensure proper

function. Understanding protein subcellular localization is important to help understanding the function of a protein. Previous studies suggested that RLP30 is localized at the plasma membrane in a transient expression system (Wang et al., 2008a). Besides membrane localization of RLP30, we also detected the fluorescence of RLP30-GFP around the cell nucleus in the *N.benthamiana* transient expression system (Figure 3.17B). Interestingly, co-expression of RLP30-GFP and SOBIR1-HA resulted in a localization of RLP30-GFP mainly at the plasma membrane (Figure 3.17B). Protein localization is important for the establishment of functional complexes, and for instance co-expression of CLV2-GFP and CRNmCherry caused a relocation of both proteins from the ER to the PM (Bleckmann et al., 2010). Importantly, the relocalization of CLV2 and CRN plays a vital role for perceiving CLV3 signal (Bleckmann et al., 2010). In this thesis, only co-expression of SOBIR1 and RLP30 together can restore responsiveness to SsE1 in *N.benthamiana* and leads to plasma-membrane localization of RLP30 (Figure 3.20). All these results suggest that SOBIR1 might have a dual role in SsE1 signal transduction. One function of SOBIR1 is to phosphorylate downstream targets and/or other components of the SsE1 perception machinery, and another function in supporting the right localization of RLP30 to the plasma membrane to sense SsE1.

Although RLP30, BAK1 and SOBIR1 all have been proven to be indispensable for SsE1 signaling transduction, the relationship between these three proteins still needs to be uncovered. Future experiments need to elucidate if for instance BAK1 is recruited to the preformed RLP30/SOBIR1 complex in a SsE1-dependent manner of if SsE1 triggers dissociation of the RLP30/SOBIR1 complex. Moreover, the role of SOBIR1 in RLP30 protein localization, stability, trafficking needs to be addressed in detail.

4.7 Conclusion

In conclusion, the identification of SsE1 and RLP30 as potential elicitor-receptor partners is an important step toward understanding the molecular mechanisms underlying plant resistance to necrotrophic pathogens, a field that so far remained largely uncovered. The prediction is that basic fractionation of microbial extracts as it was performed in the presented case of SsE1 will be sufficient for successful identification of many more receptors directed against all types of pathogens in a relatively short period of time. The wealth of genetic resources available in *Arabidopsis* offers a major advantage over other plant systems to increase our fundamental understanding of receptor-dependent immunity. RLP30, along with RLP1, would be the first RLPs from *Arabidopsis* having a role in immunity control rather than developmental processes. Unraveling the mode of action of SsE1 and RLP30 will help to gain insight into the specific mechanisms that coordinate different signalling and metabolic pathways to ensure proper plant development and response to environmental changes or stresses. The identification of SOBR1 as co-receptor for PRRs of the RLP-type gives us a new clue to turn surface receptors, used individually or stacked, into tools to engineer durable, broad-spectrum resistance in agricultural crops (Gust et al., 2010; Lacombe et al., 2010).

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6. Appendix

Table 6. 1 Primers used in map based cloning

Marker name	Polymorphism Col-0/Lov-1	Primer name	Primer sequence (5' – 3')
F11A12	RsaI diget	F11A12-F	GATATGCAGCTGATTGCAGAAG
		F11A12-R	TCACGTCATCGACTAGCTGTTT
MSJ11	442bp/262bp	MSJ11-F	GTGCGACGTGCAAAAACTTAAAAG
		MSJ11-R	GAGTTGTAGATAGAGACATCATGG
F21O3	AluI digest	F21O3-F	GGTGAGTTTTCATCACCAACAT
		F21O3-R	ATCAAATGGCCGTCTTTGTG
T9J14	ScrFI digest	T9J14-F	CGGAGCTGATCTCGAATTGT
		T9J14-R	ATCGGTGGTCTCTGATGGAC

Table 6. 2 Primers for RLP30 and SOBIR1

Gene name	Primer name	Primer sequence (5' – 3')
RLP30 (At3g05360)	RLP30-CDNA-F	ATGATTCCAAGCCAATCTAATTCC
	RLP30-CR	TCAACGAGCACTTGTGGTGAC
	RLP30-CR (no stop)	ACGAGCACTTGTGGTGACTAC
	RLP30-S1F	CACATTGTTGCAAGGTTTTG
	RLP30-S2F	AGCAATCAGTTCACATTGG
	RLP30-S3F	ATTGGCTTAAAGGGCTAGTTC
	RLP30cyt-F	ACTGCAACAAAACACGAG
	RLP30cyt-R	TCAACGAGCACTTGTGGTG
	RLP30cyt-Fmut	**g**CTGCACACAAACACGAG
	RLP30cyt-Rmut1	TCAACAGGCACTTGTGG**c**G
	RLP30cyt-Rmut2	TCAACGAGCACTTG**c**GGTG
	RLP30cyt-Rmut3	TCAACGAGCA**gc**TGTGGTG
	RLP30cyt-Rmut-all	TCAACGAGCA**gc**TGcGGcGACTAC
	RLP30-T761A-F	TATCTTCTTT**g**CTGCACACGGGCACGAG

	RLP30-T761A-R	CTCGTGTTTGTGTGCAG**c**AAAGAAGATA
	RLP30-T783A-R	ACGGCACTTGTGG**c**GACT
	RLP30-T784A-R	ACGGCACTTG**c**GGTGACT
	RLP30-S785A-R	ACGGCA**gc**TGTGGTGACT
	RLP30-Rmut-all-R	ACGAGCA**gc**TG**c**GG**c**GACTACTCT
SOBIR1 (At2g31880)	SOBIR1-CF	ATGGCTGTTCCCACGGGAAG
	SOBIR1-CR (no stop)	GTGCTTGATCTGGGACAACATG
	SOBIR1-SR	CATGTACTCGTAAACGAGG
	SOBIR1-KinF	TTAGCTTCTCTGGAGATCATAGG
	SOBIR1-K377E-F	AAGATCATAGCTGTG**g**AGAAAGTGATCC
	SOBIR1-K377E-R	TGGATCACTTTCT**c**CACAGCTATGATCT
	SOBIR1-Kin-R	CTAGTGCTTGATCTGGGACAACATG
Chimeric receptor (RLP30 +SOBIR1)	SOBIR1-Kinas-F	GTAGTCACCACAAGTGCTCGT**TTAGCTTCTCTGGAGATCATAG**
	SOBIR1-Kinas-R	**CTATGATCTCCAGAAAGCTAA**ACGAGCACTTGTGGTGACTAC
	SOBIR1-TMD-F	CCAGAAGAACAAGTGATTAAC**GTAGCGGCATGGATCTTAGGG**
	SOBIR1-TMD-R	**CCCTAAGATCCATCCATGCCGCTAC**GTTAATCACTTGTTCTTCTGG
	SOBIR1-LRRCT-F	CTCGAGATCTTGGTAGC**CTCATCAAGCTTCAGACATCTCC**
	SOBIR1-LRRCT-R	**GGAGATGTCTGAAGCTTGATGAG**GCTACCAAGATCTCGAG

Table 6. 3 Primers used in qPCR analysis

Gene name	Primer name	Primer sequence (5' – 3')
At2g19190	FRK1-100-F	AGCGGTCAGATTTCAACAGT
(FRK1)	FRK1-100-R	AAGACTATAAACATCACTCT
At3g26830	PAD3-F	CTTTAAGCTCGTGGTCAAGGAGAC
(PAD3)	PAD3-R	TGGGAGCAAGAGTGGAGTTGTTG
At3g04720	PR4-F	GCAAGTATGGCTGGACCGCCT
(PR-4)	PR4-R	CCAAGCCTCCGTTGCTGCATTG
At2g30770	CYP71A13-F	GTGCTTCGGTTGCATCCTTCTC
(CYP71A13)	CYP71A13-R	CGCCCAAGCATTGATTATCACCTC
Atcg00490	AtRubisco-QF	GCAAGTGTTGGGTTCAAAGCTGGTG
(Rubisco)	AtRubisco-QR	CCAGGTTGAGGAGTTACTCGGAATGCTG
At1g07920/30/40	EF1a-F	TCACATCAACATTGTGGTCATTGG
(EF1a)	EF1a-R	TTGATCTGGTCAAGAGCCTACAG
Sclerotinia	Scl-qPCR-F	GGATCTCTTGGTTCTGGCAT
ITS	Scl-qPCR-R	GCAATGTGCGTTCAAAGATT
Botrytis	Bc_actin_qF	CCTCACGCCATTGCTCGTGT
Actin	Bc_actin_qR	TTTCACGCTCGGCAGTGGTGG

Table 6. 4 Primers used for genotyping *fls2 efr cerk1 xii* mutants

Gene name	Primer name	Primer sequence (5' – 3')
FLS2	FLS2-LP	GGAGACAGAACACCTTCAAGT
(At5g46330)	FLS2-RP	TGACCAGATTCCTCAATAGTC
EFR	EFR-LP	CCATCCCTCGCTTACATAGATTTGTC
(At5g20480)	EFR-RP	GCTGCAGCCACATATCCAGAC
CERK1	CERK1-LP	AGCAACTCGGGGTGCAATGGGT
(At3g21630)	CERK1-RP	CCGGCCGGACATAAGACTGACTAAATCTTCG
XII1	XII1-LP	GCTTTTCGAAACGTTGGAGT
(At1g35710)	XII1-RP	CGCCAGGAGTAGCAAACTCT
XII2	XII2-LP	TCAAGTTACACACGCGTTCAC
(At2g24130)	XII2-RP	ACTGAAGTTGTGGCATCTTGC
XII3	XII3-LP	CCTGCAAAGTTTGAGAAGAAC
(At3g47090)	XII3-RP	GGGAGCAATTAGTCAAAGCATC
XII4	XII4-LP	ACCCCTTCCGTTGGCACAGC
(At3g47110)	XII4-RP	GGTACCATTCCAGAGACACTTTCCA
XII5	XII5-LP	TAAACGAGATGAATGCTTCTTCACCACAGA
(At3g47570)	XII5-RP	GAGAACAATCTGACAGGAAGTATTCCTAC

XII6	XII6-LP	AATGCTTTGTCTCCACTGGTG
(At3g47580)	XII6-RP	AATGGTGGGTCTTGGGTTATC
XII7	XII7-LP	AACGGATCGATTCCTTCTGA
(At4g08850)	XII7-RP	TTTTGCCTGATAGCCGATTC
SALK	LBb1.3	ATTTTGCCGATTTCGGAAC
SAIL	LB3SAIL	TAGCATCTGAATTTCATAACCAATCTCGATACAC
GABI	o8409	ATATTGACCATCATACTCATTGC

Table 6. 5 Primers used for cloning candidate genes of SsE1

Gene name	Primer name	Primer sequence (5' – 3')
CytoC	Cytoc-F	ttttggatccATGGGTTTCAAGCCAGGAGACGC
(A7E6R4)	Cytoc-R	tttttaagcttCTACTTGGTCTCCTCCTTCAAGTG
Rho-GDP	Rho-GDP-F	tttggatccATGGCGGACCAACAAGATAATGAC
(A7ET57)	Rho-GDP-R	tttttaagcttCTACCAGTCCTTGGTGATGTCAAAAG

Table 6. 6 Interacting protein of AtRLP30 (66 interaction)

(Original data from the Membrane-based Interactome Network Database,

http://www.associomics.org/Associomics/Home.html)

AGI ID	Functional Description
AT1G07860	Unknown: BEST *Arabidopsis thaliana* protein match is: Protein kinase superfamily protein (TAIR:AT1G07870.2)
AT1G12670	Unknown protein
AT1G13770	RUS3, Protein of unknown function, DUF647
AT1G14020	O-fucosyltransferase family protein
AT1G14360	ATUTR3, UTR3, UDP-galactose transporter 3
AT1G17280	UBC34, ubiquitin-conjugating enzyme 34
AT1G19570	DHAR1, ATDHAR1, DHAR5, dehydroascorbate reductase
AT1G21240	**WAK3, wall associated kinase 3 RLK/Pelle**
AT1G21870	GONST5, golgi nucleotide sugar transporter 5
AT1G23300	MATE efflux family protein
AT1G27290	Unknown protein
AT1G29060	Target SNARE coiled-coil domain protein
AT1G31812	ACBP6, ACBP, acyl-CoA-binding protein 6
AT1G34640	peptidases
AT1G45145	ATTRX5, ATH5, LIV1, TRX5, thioredoxin H-type 5
AT1G63110	GPI transamidase subunit PIG-U
AT1G63120	ATRBL2, RBL2, RHOMBOID-like 2

AT1G65690	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family
AT1G77350	Unknown protein
AT1G78240	TSD2, QUA2, S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
AT1G78380	ATGSTU19, GST8, GSTU19, glutathione S-transferase TAU 19
AT2G22425	Microsomal signal peptidase 12 kDa subunit (SPC12)
AT2G26180	IQD6, IQ-domain 6 (Calcium binding)
AT2G27290	Protein of unknown function (DUF1279)
AT2G28315	Nucleotide/sugar transporter family protein
AT2G31880	**SOBIR1, EVR, Leucine-rich repeat protein kinase family protein**
AT2G36305	RCE1, ATFACE-2, ATFACE2, FACE2, farnesylated protein-converting enzyme 2
AT2G37050	**Leucine-rich repeat protein kinase family protein**
AT2G41490	GPT, UDP-glcnac-adolichol phosphate glcnac-1-p-transferase
AT3G01360	Family of unknown function (DUF716)
AT3G03210	Unknown protein
AT3G10640	VPS60.1, SNF7 family protein
AT3G11550	Uncharacterised protein family (UPF0497)
AT3G12180	Cornichon family protein
AT3G13175	Unknown protein
AT3G17000	UBC32, ubiquitin-conjugating enzyme 32
AT3G17210	ATHS1, HS1, heat stable protein 1
AT3G18800	Unknown protein
AT3G20600	NDR1, Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family
AT3G25805	Unknown protein
AT3G26020	Protein phosphatase 2A regulatory B subunit family protein
AT3G57650	LPAT2, lysophosphatidyl acyltransferase 2
AT3G62560	Ras-related small GTP-binding family protein
AT3G66654	Cyclophilin-like peptidyl-prolyl cis-trans isomerase family protein
AT4G14455	ATBET12, BET12, ATBS14B, BS14B, Target SNARE coiled-coil domain protein
AT4G20790	**Leucine-rich repeat protein kinase family protein**
AT4G23010	ATUTR2, UTR2, UDP-galactose transporter 2
AT4G23400	PIP1D, PIP1;5, plasma membrane intrinsic protein 1;5
AT4G27780	ACBP2, acyl-CoA binding protein 2
AT4G29330	DER1, DERLIN-1
AT4G30500	Protein of unknown function (DUF788)
AT4G30850	HHP2, heptahelical transmembrane protein2
AT4G37370	CYP81D8, cytochrome P450, family 81, subfamily D, polypeptide 8
AT4G37680	HHP4, heptahelical protein 4
AT4G38690	PLC-like phosphodiesterases superfamily protein

AT4G39890	AtRABH1c, RABH1c, RAB GTPase homolog H1C
AT5G06320	NHL3, NDR1/HIN1-like 3
AT5G11890	Unknown: BEST *Arabidopsis thaliana* protein match is: Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family (TAIR:AT1G17620.1)
AT5G21920	YLMG2, ATYLMG2, YGGT family protein
AT5G35460	Protein of unknown function DUF2838
AT5G42980	ATTRX3, ATH3, ATTRXH3, TRXH3, TRX3, thioredoxin 3
AT5G49540	Rab5-interacting family protein
AT5G52240	MSBP1, membrane steroid binding protein 1
AT5G52420	Unknown protein
AT5G59650	**Leucine-rich repeat protein kinase family protein**
AT5G63030	Thioredoxin superfamily protein

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