From perception to execution:
Elucidation of signaling components that link initial MTI activation to local and systemic immunity in *Arabidopsis*

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Abstract

In order to resist pathogens plants have evolved so-called pattern recognition receptors (PRR) for early detection of microbe-associated molecular patterns (MAMPs). Binding of these molecules to their cognate PRR leads to the activation of plant immunity (MTI).

Recently, it has been shown that accumulation and function of the PRR EFR that recognizes the bacterial elongation factor (EF)-Tu epitope elf18, is dependent on functional endoplasmic reticulum (ER)-resident glucosidase II (GII). A weakly dysfunctional *gIIa* allele, designated *rsw3,* is impaired in late and sustained activation of defense-related genes, despite wild-type like early defense activation upon elf18 treatment. Nevertheless, *rsw3* plants exhibit a super-susceptible phenotype towards *Pseudomonas syringae* pv. *tomato* and fail to induce EFR-mediated resistance. Those findings indicate the significance of sustained MTI activation for robust defence execution. However, the mechanism linking initial MAMP detection to robust and sustained immune activation remains elusive.

A whole genome transcript analysis revealed a group of genes which failed to be induced in *rsw3* at 10 hours post treatment with elf18. *In silico* analysis provided evidence for those genes to be directly involved in defense execution in the presence of pathogens. Among those genes we identified *PBS3*. Analysis of *pbs3* plants exhibited a similar phenotype as *rsw3* plants showing impairment in sustained elf18-triggered transcriptional reprogramming, pointing to an important role of the PBS3 enzyme and its catalysed products during EFR-mediated immunity.

In addition, EFR-induced sustained *PROPEP2* and *PROPEP3* up-regulation could not be maintained in *rsw3* plants. Those genes encode for two putative precursor proteins of endogenous elicitor peptides in *Arabidopsis*. The Leu-rich repeat receptor kinases PEPR1 and PEPR2 recognize the Pep-epitopes, triggering immune outputs which are reminiscent of MAMP responses. Those findings suggest that the Pep/PEPR pathway acts as an amplifying machinery of MTI. In this respect, sustained induction of *PROPEP2* and *PROPEP3* upon elf18-elicitation might represent a mechanism of linking initial MAMP signaling activation to robust immunity.

Here we show that basal defense against hemibiotrophic pathogens as well as the generation of SAR is compromised in *pepr1 pepr2* plants, providing evidence for a role of this signaling system in plant immunity. By analyzing genome-wide transcriptional changes, we obtained commonalities and differences between EFR- and PEPR-regulated genes and pathways. Our data indicate that Pep/PEPR signaling activation facilitates co-activation of typically antagonizing salicylate and jasmonate pathways, consistent with a role of PEPRs for defenses against hemibiotrophic pathogens. Interestingly, the expression of *PROPEP2* and *PROPEP3* is predominantly detectable at local challenged sites during SAR, pointing to a role of the Pep/PEPR pathway in the generation of systemic immune signals.

In sum, the data presented in this work uncover possible novel mechanism linking MTI activation to defense execution and reveal new insights into the function of the PEPR signaling pathway during basal defense and SAR.

Zusammenfassung

Die erfolgreiche Pflanzenabwehr basiert auf der Früherkennung von pathogen-spezifischen hochkonservierten Molekülen („Microbial-associated molecular patterns"-MAMPs) durch sogenannte Mustererkennungsrezeptoren („Pattern recogniton receptor"-PRR). Die Bindung von MAMPs an ihre PRRs führt zur Aktivierung einer basalen Immunantwort („Mamp-triggered Immunity"-MTI).

Kürzlich wurde gezeigt, dass die Anreicherung und Funktionalität des PRR EFR, der ein Fragment des bakteriellen Elongationsfaktors Tu (elf18) erkennt, von der Funktionalität des Enzyms Glukosidase II des Endoplasmatischen Retikulum abhängt. Pflanzen mit schwach funktionalen *gIIα* Allel, *rsw3* genannt, verfehlen die späte und langanhaltende Aktivierung von Abwehrgenen, obwohl frühzeitig ablaufende Abwehrantworten dem des Wildtypes entsprechen. Allerdings weisen *rsw3* Planzen einen hochanfälligen Phänotyp gegenüber *Pseudomonas syringae* pv.*tomato* auf und scheitern eine erhöhte Resistenz nach vorangegangener Erkennung von elf18 zu entwickeln. Anhand dieser Daten wird die Signifikanz der langanhaltenenden MTI-Aktivierung für eine robuste Immunantwort erkenntlich. Nichtsdestotrotz bleiben die Mechanismen, die die initiale Pathogenerkennung mit einer stabilen und anhaltenden Immunantwort verknüpfen, weitestgehenst unbekannt.

Mit Hilfe einer transkriptomweiten Genexpressionsanalyse wurde eine Gruppe von Genen identifiziert, welche 10 Stunden nach elf18-Behandlung in *rsw3*-Planzen nicht induziert wurden. *In silico*-Analysen wiesen eine Verknüpfung dieser Gene mit einem aktiven Abwehrverhalten in der Gegenwart eines Pathogens auf. Ein Gen dieser Gruppe ist *PBS3,* welches die Konjugation von Aminosäuren an Salicylsäure katalysiert. Untersuchungen von *pbs3*-Pflanzen ergaben phänotypische Übereinstimmungen mit *rsw3,* welche anhand von Defiziten in der langanhaltenden Regulierung von Abwehrgenen nach elf18-Erkennung deutlich wurden. Diese Ergebnisse deuten auf die Wichtigkeit des PBS3-Enzyms und seiner Produkte während der EFR-vermittelten Immunantwort hin.

Zusätzlich konnten *PROPEP2* und *PROPEP3* als zwei weitere elf18-induzierte Gene identifiziert werden, welche in *rsw3*-Pflanzen misreguliert werden. Diese Gene kodieren für putative Vorläufer endogener Elicitoren, die von den Rezeptoren PEPR1 und PEPR2 erkannt werden. Aktivierung dieser ruft eine Abwehrreaktion hervor, die starke Ähnlichkeit zu einer MAMP-vermittelten Immunantwort aufweist. Möglicherweise basiert die langanhaltende, durch elf18-induzierte Aktivierung von *PROPEP2* und *PROPEP3* auf einen Mechnismus, bei dem das Pep/PEPR-System erste MTI-Reaktionen mit einer stabilen Immunantwort verknüpft.

In der nachfolgenden Arbeit werden Daten präsentiert, die zeigen, dass *pepr1 pepr2* Pflanzen ein geschwächtes basales Abwehrverhalten gegenüber hemibiotropher Pathogene und ein Defizit zur

Generation von systemisch erworbener Resistenz (SAR) aufweisen. Anhand von genomweiter Transkriptomanalysen konnten Übereinstimmungen und Unterschiede zwischen elf18- und Pep-induzierter Abwehrantworten bestimmt werden. Desweiteren deuten die Daten darauf hin, dass der PEPR-Signalweg auf die Koaktivierung von den sich normalerweise antagonisierender Salicylsäure- und Jasmonsäurewegen basiert, welches die Anfälligkeit von *pepr1 pepr2* Pflanzen gegenüber hemibiotrpher Pathogene erklären würde. Interessanterweise, wird dieser Signalmechanismus während der SAR-Entwicklung ausschließlich im lokalen, befallenen Gewebe der Pflanze aktiviert. Dies könnte darauf hindeuten, dass er zur Generation von SAR-Signalen im lokalen Gewebe beiträgt.

1 Introduction

Similar to animals and humans, plants are surrounded and inhabited by numerous microbes. Some microbes establish a commensal relationship whereas some even share a symbiotic life style with the plant. However, despite the cohabitation with harmless microbes, plants are exposed to a constant threat by pathogens which feed from the plant for successful replication and propagation. Some microbes enter the plants apoplastic spaces through natural openings like stomata, hydathodes or wounding sites whereas others penetrate and invade plant cells by using specialized structures. Similar to the various approaching strategies, pathogens have evolved several mechanisms to feed from the plants resources. Depending on the feeding style, they are classified into biotrophic and necrotrophic pathogens, with biotrophic pathogens being characterized to rely on living host cells whereas necrotrophs derive nutrients from dead plant tissue (Glazebrook 2005). The biphasic life style consisting of an initial biotrophic and subsequent necrotrophic phase categorizes the microbe as a hemibiotroph. Although various microbial life styles and infection strategies have been established for plant invasion, only a small number of adapted pathogens are actually able to infect the plant successfully by negating plant defense mechanisms (Nurnberger and Lipka 2005).

The inability of non-adapted pathogens to overcome plant defense responses leads to a robust resistance phenotype of the plant called non-host resistance (Thordal-Christensen 2003). Besides preformed barriers such as the cuticula and rigid plant cell wal,l this phenomenon relies on the plants ability to distinguish self from non-self structures and the subsequent initiation of defense outputs.

1.1 The plant immune system

Early recognition of invading pathogens by membrane-resident pattern recognition receptors (PRRs) is the first step to successfully restrict arising infections. These receptors bind typically evolutionarily conserved microbe-associated molecular patterns (MAMPs) and activate cell-autonomous responses leading to MAMP-triggered immunity (MTI) (Boller and Felix 2009). Adapted pathogens however evolved defense mechanism to suppress MAMP-triggered plant defense responses consequently enabling the proliferation in the host. This induced susceptibility is mainly mediated by the introduction of effector proteins into the plant cell where they interfere with the activated defense machinery or directly promote virulence thereby leading to effector-triggered susceptibility (ETS).

Consequently, plants evolved intracellular immune receptors (R-proteins) specifically recognizing the presence of these effectors, leading to a second layer of immune responses eventually terminating the pathogens growth (effector-triggered immunity; ETI). Continuing co-evolution of the parasite and the host have led to a molecular arms race of effector and R-protein acquisition to overcome the respective evolved advantage of the opponent (Chisholm, Coaker et al. 2006; Jones and Dangl 2006; Bent and Mackey 2007; Katagiri and Tsuda 2010). However, this mutual battle for maintenance of evolutionary advantages is rare and does not apply for the majority of potential host-pathogen interactions. In most cases the activation of MTI, also referred to as basal resistance, is sufficient to contain growth of the non-adapted pathogen (Nurnberger and Lipka 2005).

1.1.1 MAMP-triggered immunity

Binding of MAMPs to their cognate PRR leads to the activation of defense signaling within seconds. Molecular events following MAMP-perception have been intensively studied for many decades and involve ion fluxes across the plasma membrane (e.g. Ca^{2+} spiking), the generation of reactive oxygen species (ROS) as well as nitric oxide (NO) and the activation of mitogen-activated (MAP) and calcium-dependent protein (CDP) kinases. Those early responses become apparent 1-10 minutes after MAMP recognition. Further downstream events include the activation of hormone biosynthesis and signaling, transcriptional reprogramming and the accumulation of antimicrobial compounds. Typical late responses developing over one to several days, comprise callose deposition and growth inhibition (Gomez-Gomez, Felix et al. 1999; Asai, Tena et al. 2002; Kunze, Zipfel et al. 2004; Schwessinger and Zipfel 2008; Clay, Adio et al. 2009; Boudsocq, Willmann et al. 2010). However, the early events described represent hallmarks of MTI which have been intensively studied. Recent work by Lu et al. (2009) demonstrates a separation of MTI in a rather early and late phase. The identification of *rsw3* plants which allowed early elf18-triggered responses (ROS spiking, MAPK activation, ethylene production and the induction of early defense genes) but exhibits defects in sustained transcriptional reprogramming, allows uncoupling of those two phases. Furthermore, *rsw3* plants failed to induce resistance upon elf18 application and showed a supersusceptible phenotype towards *Pst* DC300. Those findings demonstrated the insufficiency of early responses for full MTI activation and the requirement of initial and sustained MTI signaling (Lu, Tintor et al. 2009).

Genetic studies have provided evidence that single PRR signaling branches contribute significantly to plant immunity. For example loss of FLS2 (FLAGELLIN SENSING 2) or EFR (EF-TU RECEPTOR), two most characterized PRRs in plants, enhances disease susceptibility to virulent, weakly virulent and non-

adapted bacteria (Zipfel, Robatzek et al. 2004; Hann and Rathjen 2007; Zipfel 2009). In contrast, enhanced immunity could be observed when plants were treated with MAMPs prior a following infection with *Pst* DC3000 or the fungus *Botrytis cineria* (Zipfel, Robatzek et al. 2004; Ferrari, Galletti et al. 2007). Application of the MAMPs induced MTI and rendered the plant more resistant (Zipfel 2009). In addition, this immunization response could not only be observed in leaves directly pretreated with MAMPs prior a challenge infection but also in systemic before untreated tissues (Mishina and Zeier 2007). Thus, activation of MTI induces defense responses which are capable of restricting pathogen growth in direct proximity and distant plant organs.

1.1.2 Pattern Recognition Receptors during plant immunity

Pattern recognition receptors (PRR) detect molecular structures typical of an entire microbial class (MAMPs), such as fungal chitin or bacterial lipopolysaccharides (LPS), pepditoglycans (PGN), elongation factor Tu (EF-Tu) or flagellin (Boller and Felix 2009). A major class of known plant PRRs are membrane-localized receptor-like kinases (RLKs) containing an extracellular domain, a single-pass transmembrane domain and an intracellular kinase domain. Among the best studied PRRs in plants are the leucine-rich repeat (LRR)-containing RLKs FLS2 and EFR of *Arabidopsis* (Gomez-Gomez, Felix et al. 1999; Kunze, Zipfel et al. 2004).

A highly conserved N-terminal part of bacterial flagellin (Felix, Duran et al. 1999), the 22-amino-acid peptide flg22, was shown to act as a potent elicitor in various plant species, indicating that the PRR for flagellin is evolutionarily ancient (Boller and Felix 2009). Orthologs of FLS2 with a high degree of conservation are present in all higher plants and functionality of FLS2 orthologs in tomato, tobacco and rice have been demonstrated (Hann and Rathjen 2007; Robatzek, Bittel et al. 2007).

In contrast, recognition of one of the most abundant and conserved proteins in bacteria, EF-Tu, by EFR seems to be restricted to Brassicaceae. However, similar to flg22, a short 18 amino-acid peptide corresponding to the acetylated N-terminus of EF-Tu, called elf18, triggers MAMP responses at subnanomolar concentrations (Kunze, Zipfel et al. 2004).

Importantly, perception of elf18 and flg22 by their cognate receptors seems to be highly specific, since known downstream responses are entirely dependent on EFR and FLS2, respectively. Furthermore, functionality of those receptors seems to be critical for plant immunity due to enhanced bacterial growth of both adapted and non-adapted strains in *efr* and *fls2* mutants (Zipfel, Kunze et al. 2006; Nekrasov, Li et al. 2009; Saijo, Tintor et al. 2009). Interestingly, recent studies reported provoking evidence for the additional binding ability of FLS2 for peptides different to flg22.

According to the data FLS2 responds to high concentrations of the shoot apical meristem growth regulator CLV3p resulting in activation of defense related outputs (Lee, Chah et al. 2011). In contrast to flg22, CLV3p depicts an endogenous derived signalling peptide of *Arabidopsis* (Kondo, Sawa et al. 2006). Furthermore, AxYs21-A1, a MAMP from the rice pathogen *Xanthomonas oryzae* pv. *oryzae*, was also shown to exhibit immune stimulatory functions upon FLS2 binding in *Arabidopsis* (Danna, Millet et al. 2011). Those data imply a multi-binding activity of FLS2 to various peptides for defense activation in several organs. However, cautions have been raised for the interpretation of these studies (Segonzac, Nimchuk et al. 2012). Future studies will be required to elucidate the specificity of ligand binding to PRRs in plant immunity. Nevertheless, it is possible that PRRs might have another distinct ligand than the cognate ligand, which is not necessarily highly related in the peptide sequence.

1.1.2.1 Receptor generation

In eukaryotic cells, proteins targeted to endo-membranes, the plasma membrane or the apoplast enter the secretory pathway to reach their functional sites (Anelli and Sitia 2008). The generation of trans-membrane or secretory proteins occurs through the endoplasmatic reticulum (ER) with the help of ER-resident chaperones that tightly control folding and maturation of the nascent protein. Three different pathways have been described for ER protein folding of which one relies on Asn (N)-glycosylation of the client protein. For example, this serves for folding of EFR and FLS2 (Nekrasov, Li et al. 2009; Saijo, Tintor et al. 2009). During translation of the protein into the ER lumen, N-glycosylation is catalyzed by an oligosaccaryltransferase enzyme adding a preassembled glycan chain ($Glc_3Man_9GlcNAc_2$) to the N-residues to the Asn-X-Ser/Thr motif of the nascent protein. Afterwards, the two enzymes glucosidase I (GI) and glucosidase II (GII) remove one glucose residue each from the glycan chain. The remaining mono-glucosylated glycans ($Glc_1Man_9GlcNAc_2$) on the client protein attracts ER-resident lectin-like chaperons calnexin (CNX) and calreticulin (CRT) that assist further protein folding (Williams 2006). An additional trimming of the last glucose residue finally initiates release of the client protein from the chaperon complex and the properly folded protein is forwarded to complete secretion. In case of incorrect folding, UDP-glucose:glycoprotein glucosyltransferase (UGGT) binds the client protein and reattaches a glucose residue to the sugar chain enabling reiterate binding of CNX and CRT for another round of protein folding (Taylor, Ferguson et al. 2004). Interestingly, loss of CRT and UGGT in *Arabidopsis* did not result in pleiotropic defects pointing to a difference in the function of the CRT/UGGT cycle between plants and mammals since in the latter,

deletion of individual members results in embryonic lethality or premature death (Anelli and Sitia 2008; Saijo, Tintor et al. 2009). However, severe loss of GI and GII causes lethality in plants as well (Boisson, Gomord et al. 2001; Burn, Hurley et al. 2002), pointing to the unique tolerance of plants to perturbations of the CRT/UGGT cycle.

Genetic screens have revealed several *Arabidopsis* mutants that are impaired in genes encoding key enzymes of the ER quality control (ERQC) pathway (e.g. CRT3, UGGT, GIIα and GIIβ) leading to perturbations of EFR but not FLS2 signaling (Lu, Tintor et al. 2009; Nekrasov, Li et al. 2009; Saijo, Tintor et al. 2009). Even though both receptors undergo N-glycosylation and share structural similarities their requirement for functional ER chaperons seems to be divergent (Boller and Felix 2009; Saijo 2010).

Arabidopsis plants lacking functional CRT, UGGT or GIIβ were abolished in EFR accumulation and subsequent downstream signaling (Saijo, Tintor et al. 2009). A weak allele of GIIα, designated *rsw3*, had been earlier described to show a swollen root phenotype associated with defects in cellulose biosynthesis at high temperature (Burn, Hurley et al. 2002). Even though the mutants accumulated wild-type like levels of EFR and were able to activate several early elf18-triggered responses, they were abolished in full EFR-mediated defense execution (see 1.1.1) indicating the complex and detailed requirement of ERQC for functional PRR generation.

1.1.2.2 FLS2 and EFR immune complexes

MAMP-induced activation of PRRs activates a series of defense responses which are detectable within seconds and minutes (Boller and Felix 2009). However, those cellular outputs cannot be initiated by the single action of PRRs but requires several other proteins which work in close association with FLS2 and EFR. The small LRR-RLK BAK1 protein which was found to interact and positively regulate the brassinosteroid receptor BRI1 (Clouse 2011), is also required for successful PRR function. Similar to BRI1, the protein also rapidly forms complexes with EFR and FLS2 upon MAMP recognition but is not required for ligand binding *per se* (Chinchilla, Zipfel et al. 2007; Heese, Hann et al. 2007). BAK1 belongs to a group of 5 SERK proteins of which the others are also recruited into EFR and FLS2 complexes upon ligand binding (Roux, Schwessinger et al. 2011). However, whereas FLS2 clearly favors the interaction with BAK1, EFR also binds to SERK1, SERK2 and BKK1 with high affinity (Roux, Schwessinger et al. 2011). The functional requirement of those enhancer proteins was demonstrated by almost complete insensitivity of the *bak1-5 bkk1-1* double mutant to MAMP application without

severe defects in plant development and growth (Roux, Schwessinger et al. 2011; Schwessinger, Roux et al. 2011).

Another group of membrane-associated cytoplasmic kinases including BIK1, PBS1, PBL1 and PBL2 constitutively associates with FLS2 and BAK1 (Lu, Wu et al. 2010; Zhang, Li et al. 2010). Flg22-binding initiates trans-phosphorylation of BIK1 by BAK1, which subsequently phosphorylates both FLS2 and BAK1. Phosphorylation of BIK1 has also been reported for elf18 treatment and the kinase was shown to interact with EFR in protoplasts (Lu, Wu et al. 2010; Zhang, Li et al. 2010). It is believed that this family of cytoplasmic kinases represents PRR-signaling activators since loss-of-function *bik1* and *pbl* mutants were impaired in MTI responses and showed enhanced susceptibility towards *Pst* DC3000 *hrcC*- (Lu, Wu et al. 2010; Zhang, Li et al. 2010).

Flg22- elicitation also recruits two E3-ubiquitin ligases, PUB12 and PUB13, into the FLS2-BAK1 membrane complex. The two enzymes are also phospho-activated by BAK1 in an flg22-dependent manner and subsequently poly-ubiquitnate FLS2 leading to degradation of the receptor (Lu, Lin et al. 2011). In addition, FLS2 was reported to be internalized into intracellular mobile vesicles after activation of downstream signaling (Robatzek, Chinchilla et al. 2006). Presumingly, internalization and degradation of the receptor attends to the tight control of immune activation and avoids over-induction of defense responses. However, whether receptor removal and degradation from the plasma membrane acts as a general mechanism of PRR signaling control or is unique to FLS2 remains to be determined.

1.1.3 DAMP signaling

For a rapid and successful defense response plants need to sense microbes and the danger posed by them. The identification of non-self structures alone seems to be insufficient since MAMPs are shared among pathogenic and non-pathogenic microbes. Co-incidental detection of endogenous molecular patterns that are associated with cellular homeostasis changes, termed danger-associated molecular patterns (DAMPs), together with MAMPs, has been proposed to elicit a robust defense response (Huffaker and Ryan 2007).

In animals, several proteins and molecules have been identified to act as DAMPs including heat shock proteins, high mobility group protein-1, interleuking-1α, defensins, extracellular ATP, nucleic acids, S100 molecules, hyaluronan and uric acid (Bianchi 2007). They can be either activated by pathogen-induced damage or trauma and trigger a similar set of defense responses as characterized for MAMPs (Bianchi 2007; Wise, Moscou et al. 2007).

The existence of DAMPs in plants has been proven in several studies. Disruption of plant cell wall components by insect chewing or pathogen-derived degrading enzymes releases pectins, oligogalacturonide (OG) fragments and oligosaccharides (Heil 2012). The elicitor function of OGs has been reported to involve induction of phytoalexin biosynthesis, the expression of glucanase and chitinase as well as oxidative burst (De Lorenzo, Brutus et al. 2011). Furthermore, treatment of plants with OGs protected grapevine and *Arabidopsis* leaves against infection with the necrotrophic fungus *Botrytis cinerea* (Aziz, Heyraud et al. 2004; Ferrari, Galletti et al. 2007). Despite cell wall derived components, the release of nucleic acids as well as ATP to the apoplast were reported to activate defense responses (Roux and Steinebrunner 2007; Chivasa, Murphy et al. 2009; Hawes, Curlango-Rivera et al. 2011). In addition, many JA-inducing elicitors are fragments of plant proteins that do not occur in the extracellular space of an intact plant tissue (Ryan and Pearce 2003; Pearce, Siems et al. 2007; Pearce, Yamaguchi et al. 2010; Yamaguchi, Barona et al. 2011). The Solanaceaous-specific wound-responsive peptide systemin is processed from the larger Prosystemin precursor protein and regulates many defense responses including the accumulation of protease inhibitors and other anti-nutritive proteins. The peptide also induces the biosynthesis of JA in the companion-cell-sieve element complex of the vascular bundle and thereby enhances the generation of the mobile signal for systemic resistance acquisition (Ryan and Pearce 2003). Nevertheless, neither the Prosystemin protein nor the processed systemin version contains an N-terminal secretion signal. It remains speculative whether the proteins are released through a leaderless secretion pathway or by disruption of the cell through insect feeding (Yamaguchi and Huffaker 2011). Thus, whether systemin acts as a DAMP has not yet been demonstrated.

Another group of endogenously derived peptides were recently discovered in *Arabidopsis*. Utilizing an elicitor-induced alkalinization activity assay with *Arabidopsis* suspension-cultured cells, Pep1 isolated from *Arabidopsis* leaves was identified. The 23 amino-acid long peptide derives from the C-terminal end of a larger precursor protein, called PROPEP1, which belongs to a group of seven homologs in *Arabidopsis* (Huffaker, Pearce et al. 2006). Similar to Pep1, synthesized peptides for the conserved epitope of the other 6 members also induced alkalinization in suspension-cultured cells upon binding to the Pep receptor PEPR1 (Yamaguchi, Pearce et al. 2006). In fact, two receptors, PEPR1 and PEPR2, have been identified to specifically bind Pep-peptides and activate downstream signaling reminiscent to MTI (e.g. Ca2+ spiking, ROS generation, MAPK activation, root growth inhibition, defense gene expression) (Huffaker, Pearce et al. 2006; Huffaker and Ryan 2007; Krol, Mentzel et al. 2010; Qi, Verma et al. 2010). Of note, PEPR1 and PEPR2 also belong to the group of LRR-RLKs and PEPR1 has

been shown to physically interact with BAK1 in a ligand-dependent manner (Schulze, Mentzel et al. 2010). PEPR1 and PEPR2 are expected to localize to the plasma membrane and bind their ligands in the apoplastic spaces. The *PROPEP* genes, in particular *PROPEP2* and *PROPEP3*, are induced upon MAMP application, pathogen infection, wounding and hormone treatment with methyl-JA, ethylene or SA (Huffaker, Pearce et al. 2006). Furthermore, it has been shown that treatment with Peps enhances disease resistance against *Pst* DC3000 (Yamaguchi, Huffaker et al. 2010) and that plants overexpressing *PROPEP1* and *PROPEP2* exhibited enhanced resistance against the root pathogen *Pythium irregulare* (Huffaker, Pearce et al. 2006). Based on these findings, the Pep/PEPR pathway has been proposed to act as a DAMP system to amplify MTI-induced defense responses during pathogen challenges.

1.1.4 Effector-triggered immunity

In order to colonize and propagate successfully, adapted pathogens had to evolve mechanism to overcome the bulwark of basal defense responses by the host. Plant pathogenic bacteria are able to insert effector proteins into the host cell using the type III secretion system (T3SS), a needle-like structure which penetrates the plant cell (Deslandes and Rivas 2012). In contrast fungal and oomycete pathogens form a specialized infection structure, the haustorium, which is thought to involve secretory pathways for effector delivery as well (Panstruga and Dodds 2009). Inside the plant cell those effectors interfere with the defense machinery of the plant, manipulate signaling pathways or even take advantage of the eukaryotic cell body to promote their own virulence (Feng and Zhou 2012). Consequently, plants evolved another class of immune receptors (resistance (R) proteins) specifically sensing effector proteins for prompt containment of the bacterial threat. Recognition of effectors is either mediated by direct physical interaction (gene-for-gene resistance) or indirectly by perceiving effector activity though an accessory protein (guard model) (Flor 1971; Dodds and Rathjen 2010). Effector recognition leads to a more strong immune response that is frequently associated with the development of localized programmed cell death called hypersensitive response (HR) (Greenberg and Yao 2004). However, recent publication have doubted the requirement of an HR for successful ETI by showing that pathogen growth could be restricted without inducing localized cell death (Slootweg, Roosien et al. 2010; Heidrich, Wirthmueller et al. 2011).

The phytobacterial effector repertoire has been intensively studied and allows the understanding of several virulence strategies underlying effector activity. For example, AvrPto and AvrPtoB from *Pst* have been shown to directly target several *Arabidopsis* and tomato PRRs to block MTI (Goehre,

Spallek et al. 2008; Xiang, Zong et al. 2008; Gimenez-Ibanez, Hann et al. 2009; Zeng, Velasquez et al. 2012). In contrast to *Arabidopsis,* tomato harbors two mimics of immune receptor kinases, Pto and Fen, that act as decoys for AvrPto and AvrPtoB action to trigger ETI (Loh and Martin 1995; Dong, Xiao et al. 2009). In *Arabidopsis*, no R protein has been described to recognize the actions of these two effectors.

RIN4 (RPM1-interactin protein 4) has been described as a key regulatory protein providing mechanistic links for PTI, ETS and ETI. As a defined negative regulator of MTI (Kim, da Cunha et al. 2005), RIN4 has become a popular target of effector proteins. For example AvrB or AvrRpm1 induce phosphorylation of RIN4 and thereby enhance the negative effect of RIN4 on MTI (Mackey, Holt et al. 2002). The R-protein RPM1 guards RIN4, recognizes its phosphorylation and subsequently initiates ETI (Grant, Godiard et al. 1995). Interestingly, a new generation of effectors cleaves RIN4 to prevent detection by RPM1 and induction of ETI, nicely illustrating the aforementioned arms race of effector-induced ETS and R-gene-mediated ETI (Kim, Desveaux et al. 2005).

In addition to those specific examples effectors have also been shown to interfere with MAPK signaling, biosynthesis of SA, photosynthesis, or the microtubule network (Deslandes and Rivas 2012). Furthermore, some even mimic eukaryotic transcription factors and activate specific virulence genes of the hosts genome (Bogdanove, Schornack et al. 2010).

Unlike the diverse appearance of effector proteins, most R-genes encode for a 'nucleotide-binding site plus leucine-rich repeat' (NB-LRR) class of proteins which can be further subdivided into two main classes based on their deduced N-terminal structure (Dangl and Jones 2001). The TIR-NB-LRR proteins disclose homology to the *Drosophila* Toll and mammalian interleukin 1 receptors (TIR), whereas CC-NB-LRRs harbor a characteristic coiled-coil domain at the amino-terminus.

Signal transduction downstream of activated NB-LRR receptors remains poorly understood. It is well accepted that EDS1 (ENHANCED DISEASSE SUSCEPTIBILITY 1) is required for TIR-NB-LRR responses, whereas NDR1 (NON-RACE-SPECIFIC DISEASE RESISTANCE 1) functions downstream of CC-NB-LRRs (Aarts, Metz et al. 1998; Falk, Feys et al. 1999). Furthermore, EDS1 and its two interacting partners PAD4 (PHYTOALEXIN DEFICIENT 4) and SAG101 (SENESCENCE ASSOCIATED GENE 101) are important activators of salicylic acid (SA) signaling (1.2). In addition, EDS1 has been reported to interact with the TIR-NB-LRR protein RPS4. Upon effector delivery by *Pseudomonas* bacteria carrying the effector protein AvrRps4, AvrRps4 interacts with EDS1 at the endomembrane leading to alteration of the endomembrane-associated receptor complex. In *Arabidopsis*, released soluble and potentially mobile RPS4-EDS1 signaling complexes locate to the cytoplasm and nucleus for activation of different

subcellular defense branches, possibly including SA signaling and transcriptional activation of defense genes (Heidrich, Blanvillain-Baufume et al. 2012).

R-protein-mediated responses show high overlap with signaling outputs characteristic for MTI. However, the duration and amplitude of the defense response is more prolonged and robust against pathogenic perturbations (Tsuda and Katagiri 2010). Together, MTI and ETI are interconnected parts of plant innate immunity, conferring resistance against a pathogen attack and furthermore generating alarming signal molecules to prepare distant tissue for following infections (Durrant and Dong 2004; Mishina and Zeier 2007).

1.1.5 Systemic acquired resistance

The immune response of a plant is not restricted to areas of pathogen attack but comprises a complex cell to cell communication network throughout the plant. Upon infection, effective defense activation allows successful containment of the pathogen growth and furthermore generates a systemic signal which shifts non-colonized distant tissue in an alarmed defense state, termed systemic acquired resistance (SAR) (Durrant and Dong 2004; Vlot, Klessig et al. 2008; Shah 2009). This phenomenon was discovered in 1960 when tobacco mosaic virus challenged tobacco plants exhibited an increased resistance response to secondary infections in distant tissues (Ross 1961). Further investigations revealed that induced resistance in systemic leaves can also be initiated by root-colonizing bacteria or fungi (induced systemic resistance, ISR) as well as by herbivore feeding and wounding (wound-induced resistance, WIR) (Liu, Maldonado-Mendoza et al. 2007; van Loon 2007). Independent of the trigger, systemic resistance responses are long-lasting and allow broad-spectrum disease resistance to following infections. Furthermore, they require the mobilization of a signal molecule from the infection or wounding site that travels presumably along the vascular system to systemic leaves (Durrant and Dong 2004; Vlot, Klessig et al. 2008).

Wounding or herbivore feeding induces the generation of the phytohormone jasmonic acid (JA) which contributes to defense activation in local and systemic leaves (Sun, Jiang et al. 2011). Furthermore, it has been shown that JA itself serves as the systemic signal during WIR (Lee and Howe 2003). The root-microbial induced resistance (ISR) involves activation of both JA and ethylene signaling whereas SAR largely relies on SA, another phytohormone, which constitutes a key player around the infection for the generation of a mobile signal as well as in systemic leaves for SAR generation (Shah 2009). Even though some evidence pointed to a role of SA as a mobile signal during SAR, following investigations disproved this suggestion (Dempsey, Shah et al. 1999). Alternatively, azelaic acid,

dehydroabietinal, pipecolic acid, terpenoids, green leaf volatiles or jasmonates were proposed as transmitted signal candidates (Shah 2009; Dempsey and Klessig 2012).

Induction of systemic resistance by different cues evolves through precisely evaluated hormonal blends. The assumption that SA is exclusively required for SAR generation was challenged by Truman et al. who demonstrated an additive crucial role of JA signaling during SAR and a timely separation of the two hormone branches during this process (Truman, Bennett et al. 2007). Based on their work it appears likely that SA and/or ethylene in return are involved during the establishment of ISR or WIR. Understanding the indispensable role of phytohormones during plant immunity and their crucial synergistic or antagonistic interacting modes has been a great challenge for scientists for many years.

1.2 Hormone signaling in plant immunity

SA, JA and ethylene are the three major phytohormones involved in the regulation of plant defense against pathogenic attacks (Thomma, Penninckx et al. 2001). However, their tight controlled accumulation in response to different pathogens is crucial for successful defense execution. Whereas SA becomes largely engaged in defense responses against biotrophic and hemibiotrophic pathogens, JA and ethylene have been associated with defense activation against necrotrophs (Glazebrook 2005). The phenolic compound SA can be synthesized from chorismate via two distinct enzymatic pathways involving either PAL (PHENYLALANINE AMMONIA LYASE) or ICS1/SID2 (ISOCHORISMATE SYNTHASE). Biosynthesis of SA is activated upon recognition of MAMPs, effectors or pathogens (Mishina and Zeier 2007). During PTI and TIR-NB-LRR-triggered ETI, EDS1 and PAD4 are required for the onset of SA generation (Wiermer, Feys et al. 2005), whereas NDR1 mediates SA biosynthesis downstream of CC-NB-LRR type R proteins (Bernoux, Ellis et al. 2011). SA perception and signaling is received and largely controlled by the regulatory proteins NPR1 (NONEXPRESSOR OF *PR* GENES 1) together with its paralogs NPR3 and NPR4 (NONEXPRESSOR OF PR GENES 3 / 4) (Fu, Yan et al. 2012; Wu, Zhang et al. 2012). Upon SA perception NPR1 acts as a transcriptional co-activator of a large set of defense-related genes, including several *PR* (PATHOGENESIS RELATED) genes. In the absence of a functional NPR1 protein, SA-induced transcriptional reprogramming is almost completely blocked (Fu, Yan et al. 2012), as well as the establishment of SAR which evolves from SA signaling at the site of infection to protect undamaged tissues against subsequent pathogen invasion (see 1.1.5).

In response to pathogen or insect attack the oxylipins jasmonates (JA and its derivatives) are generated from polyunsaturated fatty acids. A key step during JA biosynthesis is the production of

allene oxide by DDE2/AOS (DELAYED DEHISCENCE 2 / ALLENE OXIDE SYNTHASE) which is further converted to OPDA (12-oxophytodienoic acid) (Gfeller, Dubugnon et al. 2010). JA can be readily metabolized to methyl jasmonate or conjugated to amino acids such as isoleucine, which results in the biologically active jasmonyl-isoleucine (JA-Ile) (Fonseca, Chini et al. 2009). Recognition of JA-Ile by the key regulatory protein COI1 (CORONATINE INSENSITIVE 1) which acts as a part of the SCF^{COI1} (E3 ubiquitin-ligase SKP1-Cullin-F-box) protein complex, leads to ubiquitinylation and subsequent degradation of JAZ (JASMONATE ZIM- domain) proteins that function as repressors of JA signaling (Sheard, Tan et al. 2010; Pauwels and Goossens 2011). Therefore, activation of the JA hormone branch results in removal of JAZ proteins from transcriptional activators, which results in derepression of the JA signaling pathway and induction of a large number of JA-responsive genes (Memelink 2009). In *Arabidopsis,* generation of JA can activate two downstream signaling branches: the so-called MYC branch and the ERF branch. The MYC branch is controlled by MYC-type transcription factors, namely MYC2, MYC3, and MYC4 in *Arabidopsis*, and its outputs involve the activation of the marker gene *VSP2* (*VEGETATIVE STORAGE PROTEIN 2*) (Dombrecht, Xue et al. 2007). Regulation of the ERF branch is mediated by ERF (ETHYLENE RESPONSIVE FACTOR) family transcription factors and results in induction of marker genes such as *PDF1.2a* (*PLANT DEFENSIN 1.2a*) (Lorenzo, Piqueras et al. 2003). In contrast to the MYC branch, initiation of the ERF signaling branch additionally requires the accumulation of ethylene. In general, activation of the ERF branch is associated with enhanced resistance to necrotrophs (Berrocal-Lobo, Molina et al. 2002; Lorenzo, Piqueras et al. 2003), whereas MYC-mediated defense responses are associated with the wound-responses and defense against herbivores (Lorenzo, Chico et al. 2004; Kazan and Manners 2012).

The activation of specific hormone branches in response to different stress cues allows a precise defense response by the plant. However, simultaneous infection by several attackers on the other hand revealed a complex regulatory relationship between the hormone signaling sectors including synergistic and antagonistic actions of SA, JA and ethylene (Spoel and Dong 2008; Pieterse, Leon-Reyes et al. 2009; Robert-Seilaniantz, Grant et al. 2011). Several studies have demonstrated that accumulating SA antagonizes JA signaling (Koornneef, Leon-Reyes et al. 2008). Furthermore it was reported that the regulatory protein NPR1 is required for SA-mediated suppression of JA responses (Leon-Reyes, Spoel et al. 2009). For example, induction of SA accumulation by *Pst* suppresses JA signaling and renders the plant more susceptible to infection with a necrotizing fungus (Spoel, Johnson et al. 2007). Similarly, the expression of *PDF1.2a* and *VSP2* has been shown to be highly sensitive to suppression by exogenous application of SA (Koornneef, Leon-Reyes et al. 2008). This

strong repressing effect of SA on JA signaling is even utilized by butterflies whose leave-depositioned eggs induce SA accumulation to suppress JA-mediated defense responses that are effective against larval feeding (Bruessow, Gouhier-Darimont et al. 2010).

In contrast, bacterial pathogens producing the virulence factor coronatine are able to suppress SA-dependent defenses by the plant. Coronatine acts as a molecular mimic of the high biologically active JA-Ile and binds directly to the JA receptor COI1 (Katsir, Schilmiller et al. 2008). Interestingly, coronatine exhibits an even higher activity than JA-Ile (Yan, Zhang et al. 2009), which might be required to counteract the strong antagonistic effect of SA on JA signaling. Moreover, the bacterial virulence in systemic tissues is enhanced by local action of coronatine (Cui, Bahrami et al. 2005), exemplifying the broad action of the virulence factor in the suppression of SA-mediated defenses.

Despite the antagonistic effects, synergistic interactions have been reported as well. The composition of the hormonal blend depending on timing and concentrations of the hormones is crucial for either antagonistic or synergistic effects and each a certain mixture can be effective against different invading pathogens. Defense against the hemi-biotrophic fungus *Colletotrichum higginsianum* was associated with the simultaneous induction of *PR1* and *PDF1.2a* (Liu, Kennedy et al. 2007), which represent a downstream marker of SA- and JA signaling, respectively.

The gaseous phytohormone ethylene has been identified to act as a modulator of the SA-JA crosstalk. Exogenously applied ethylene or ethylene produced during pathogen infection can bypass the requirement of NPR1 for JA-suppression by SA, and then support the negative effect of SA signaling on JA-dependent outputs (Leon-Reyes, Spoel et al. 2009). In contrast, when the JA and ethylene sectors are fully induced prior to the SA sector, the antagonistic effect of SA on JA is abolished. Therefore, ethylene maintains JA signaling in the presence of accumulating SA. Furthermore, this specific hormonal constitution allowed the co-activation of both JA/ethylene and SA downstream targets (Leon-Reyes, Du et al. 2010).

In sum, the engagement of phytohormone signaling upon pathogen recognition allows the establishment and fine control of a precise defense response according to the pathogens encountered. However, the molecular mechanisms that coordinate the complex interactions of phytohormone signaling remain poorly understood.

1.3 Thesis aim

Numerous research groups have investigated the basis of MTI, but the complexity of the process and the various proteins involved leave many gaps of knowledge. Characterization of *rsw3* plants however, indicated a separation of MTI in two phases. Whereas recognition of elf18 and the induction of early cellular outputs remain functional in *rsw3*, the mutant fails to sustain transcriptional up-regulation of defense related genes, e.g. *WRKY* transcription factors and *PROPEPs*. Furthermore, transcriptional activation of *PR2* and *PR1* at 10 and 24 hours post treatment with elf18 was abolished. Importantly, *rsw3* plants exhibited a supersusceptible phenotype to the virulent bacterial strain *Pst* DC3000 and failed to induce resistance upon pretreatment with elf18 indicating the importance of sustained MTI activation for robust defense activation (Lu, Tintor et al. 2009). Those findings prompted us to subject elf18-treated wild-type and *rsw3* plants to whole transcriptome analysis to unconver components linking initial MTI induction to defense execution.

In addition, recent publications point to the engagement of PROPEP proteins and their cleaved variants in defense amplification during MTI (Huffaker and Ryan 2007). EFR-mediated sustained expression of *PROPEP* genes was abolished in *rsw3* plants, thus provoking the idea that the Pep/PEPR system represents a novel mechanism for linking initial MTI activation to a robust defense response.

Furthermore, we aimed to solve several remaining questions about the functionality of the Pep/PEPR pathway: Does the Pep/PEPR pathway play a role in MTI? Does it simply amplify MTI responses or does PEPR-mediated signaling induce specific cellular responses? Is the Pep/PEPR pathway involved in ETI? Where does the Pep/PEPR pathway function during plant immunity?

By using *pepr1 pepr2* double receptor mutants and molecular biological as well as biochemical approaches, the elucidation of the role of the PEPR pathway in plant immunity was aspired.

2 Results

2.1 Identification of components that link microbial recognition to robust immunity

2.1.1 Transcriptome analysis of *rsw3* plants

Pathogen recognition by PRRs leading to MTI builds the first layer of plant defense. For many years, MTI was rather associated as being a transient process since many characteristic outputs as ion fluxes, the generation of reactive oxygen species, MAPK activation as well as transcriptional reprogramming appear within seconds, minutes and hours (Felix, Duran et al. 1999; Gomez-Gomez, Felix et al. 1999; Asai, Tena et al. 2002; Kunze, Zipfel et al. 2004; Clay, Adio et al. 2009). However, recent publications point to the importance of sustained activation of MTI for successful defense execution (Tsuda, Sato et al. 2008; Lu, Tintor et al. 2009). Our group previously characterized a weak allele of the α-subunit of ER resident glucosidase-II (*rsw3*) that fails to maintain EFR signaling. Despite wild-type like steady state levels of EFR, *rsw3* plants retain responsiveness to elf18 for activation of ROS, MAPK, early defense genes and callose deposition. However, sustained up-regulation of *WRKY22 and WRKY29* genes as well as the late induction of *PR1* and *PR2* is impaired in *rsw3*. Furthermore the mutant exhibits enhanced susceptibility to *Pst* DC3000 and is abolished in elf18-induced resistance (Lu, Tintor et al. 2009). It appears that sustained EFR-triggered transcriptional reprogramming is essential for mounting robust immunity. However, which mechanisms link the initial activation to the relatively late phase of MTI remains unknown.

Since *rsw3* plants appear to be compromised in this specific transition step, the mutant seemed to be a useful tool for elucidating underlying mechanisms. Therefore, we conducted a microarray experiment comparing wild-type and *rsw3* seedlings 2, 10 and 24 hours after elf18-treatement (hpt). Genes that were at least 2 times up- or down-regulated during the time course in comparison to the 0 h time point with a false discovery rate (FDR) ≤ 0.05 were considered as differentially expressed genes.

Table 1: Analysis of EFR-triggered transcriptome-wide changes in wild-type and *rsw3* plants. Two-week old wild-type and *rsw3* plants were treated with elf18 for 2, 10 and 24 hours and subjected to microarray analysis. The 106 genes which are 2 x less up-regulated in *rsw3* were defined as "key genes" and selected for further **analysis.**

	Up (WT) (2x FDR = 0.05)	**Down (WT) (2x FDR = 0.05)**	**Differently expressed (WT vs. *rsw3*)**	**2 x less up-regulated in *rsw3***	**2x less down-regulated in *rsw3***
2h vs. 0h	664	401	0	7	11
10h vs. 0h	746	613	133	106	14
24h vs. 0h	570	298	92	46	20

As reported earlier, elf18 perception by EFR induces transcriptional reprogramming within the first hours (Zipfel, Kunze et al. 2006). In our experimental conditions, 1065 genes were differently regulated in wild-type plants after two hours upon elf18 treatment. More interestingly, also at 10 and 24 hpt a high number of differentially regulated genes (1359 at 10 hpt and 869 at 24 hpt) were identified (Table 1). This result confirmed the earlier hypothesis that MTI is not only characterized by a rapid and transient response of the cell but rather comprises a sustained activation of defense genes.

Comparing the transcriptional changes of *rsw3* with wild-type plants during EFR-triggered MTI, the above discussed findings that *rsw3* retains initial activation of elf18-induced responses but fails to sustain gene activation, were confirmed. At 2 hpt the expression pattern of *rsw3* was almost indistinguishable from that of wild-type plants. In contrast, at 10 hpt 133 genes were differently expressed between wild-type and *rsw3* plants and 106 of those were at least two times less up-regulated in *rsw3*. At 24 hours this difference was less apparent but still 92 genes were differently expressed between the two genotypes with 46 genes 2-times less induced in *rsw3* in comparison to wild-type (Table 1).

Most striking was the high number of 106 genes (called "key genes" hereafter) that were less activated 10 hpt in *rsw3* in comparison to wild-type (Supplementary Table 1, page 96). The generation of a heatmap (Figure 1) visualizing the expression values of those "key genes" using a color code illustrates the initial similarities between the two genotypes at an early stage (2 h) of defense induction and the peculiar discrepancies at later time points (10 and 24 hours).

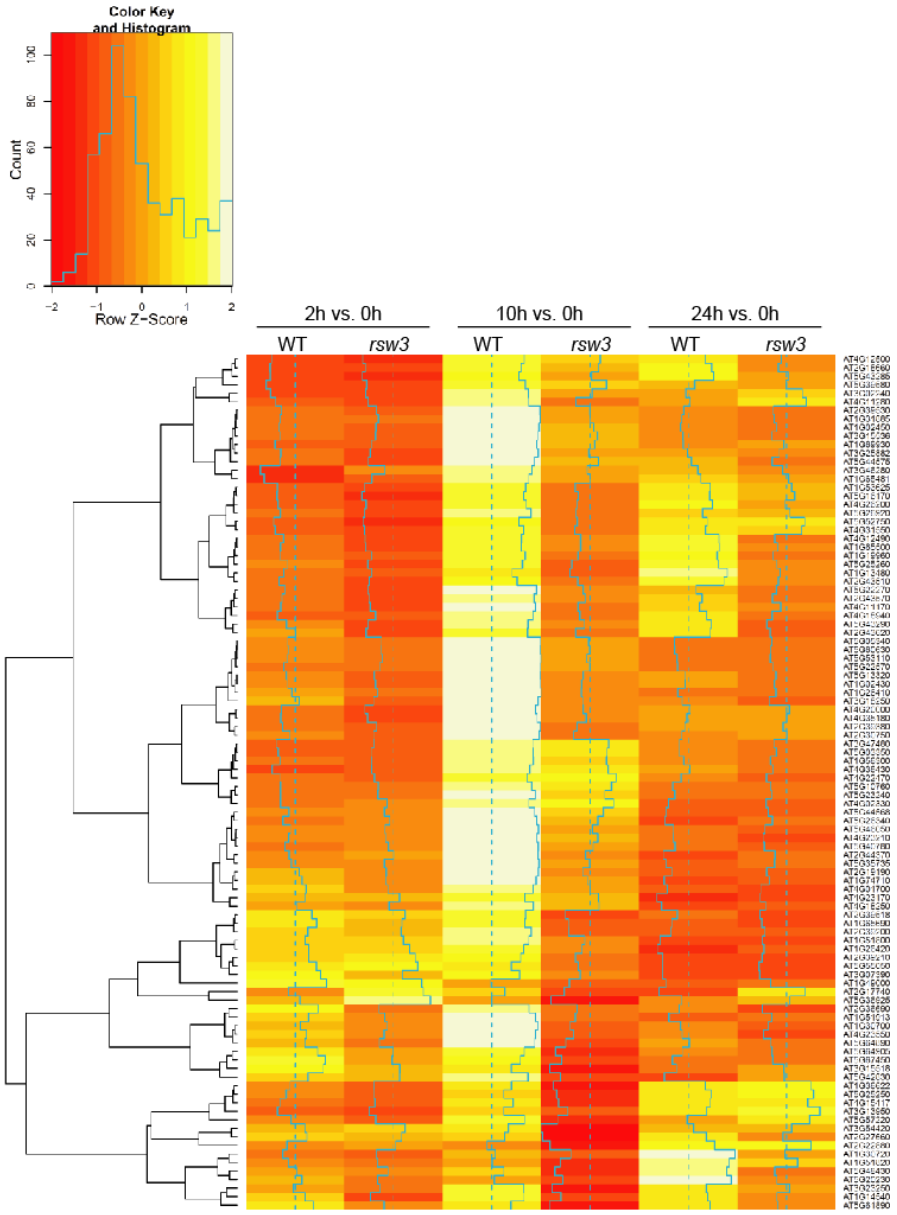

Figure 1: Heatmap of "key genes". The expression levels of the selected "key genes" at 2, 10 and 24 hpt with elf18 in wild-type and *rsw3* plants were visualized by using a color key for the expression values as indicated in the upper left corner of the figure.

To further investigate the loss of sustained gene activation in *rsw3* and to understand the resulting immune-compromised phenotype, the "key genes" were subjected to gene ontology analysis (www.http://bioinfo.cau.edu.cn/agriGO/). Indeed, terms associated with plant defense were strongly enriched including "response to stress", "response to biotic stimulus", "defense response", "response

to bacterium" and "response to fungus" indicating that the genes misregulated in *rsw3* contribute to plant defense and presumably explain the supersusceptible phenotype of the mutant (Supplementary Figure 1, page 95).

Further support could be obtained by *in silico* analysis using public available transcriptome profiling data (Genevestigator). The majority of the genes were strongly induced by a variety of pathogens (bacteria, fungi, oomycete), elicitors and as well salicylic acid. Furthermore the use of modified bacterial strains allows the dissection of specific plant defense responses. For example, a less virulent bacteria strain lacking the T3SS for effector delivery (Pst DC3000 Δ*hrcC*) triggers MTI responses whereas *Pst* DC3000 carrying the specific avirulence gene *AvrRpm1* induces ETI rapidly. According to the database, the "key genes" were strongly induced during MTI, ETI and non-host resistance. Interestingly, they remained un-induced during SAR indicating their potential involvement in defense execution upon the direct exposure to pathogens.

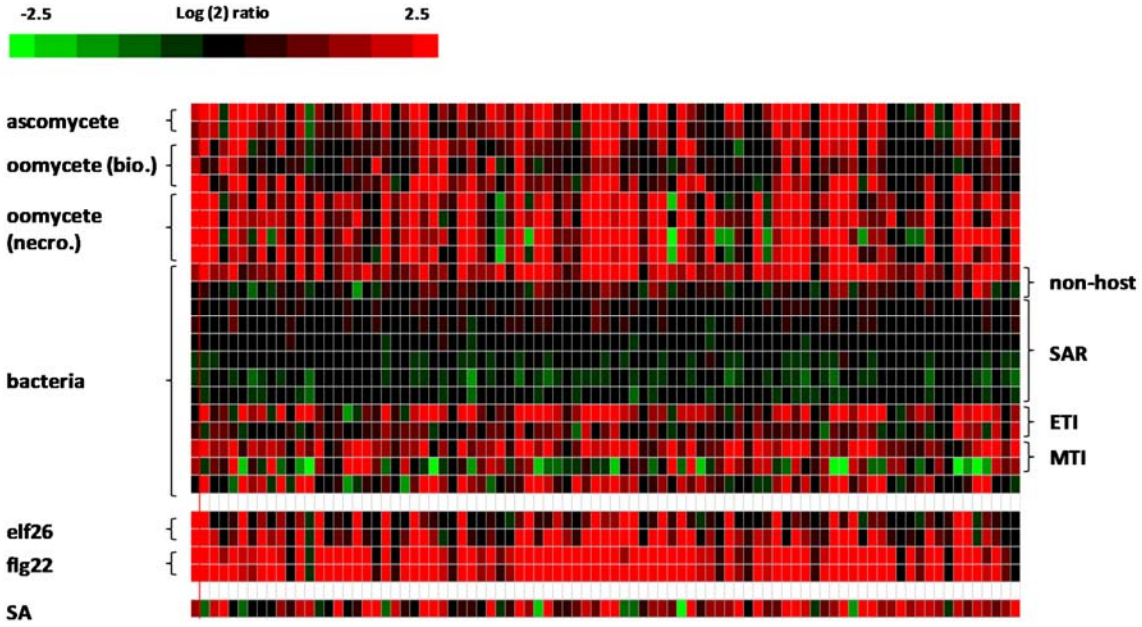

Figure 2: Expression levels of "key genes" in response to several defense related stress cues. Response of identified "key genes" to the displayed stimuli on the left site using the tool Genevestigator V3. Relative gene expression is indicated in red or green for representing up- or down-regulated gene expression, respectively (Hruz, Laule et al. 2008).

2.1.2 Characterization of mutant lines

In order to analyze the contribution of some of those "key genes" to the compromised phenotype of *rsw3,* 61 T-DNA lines covering 39 of the 106 "key genes" were ordered from NASC (European Arabidopsis Stock Centre) (Supplementary Table 2, page 98). Subsequent genotyping revealed 20 homozygous mutant lines which were conducted for further analysis together with *pbs3-1*, *pbs3-2*, *pbs3-3*, *mlo6 mlo*12 and *mlo2 mlo6 mlo12*.

Table 2: Characterization of T-DNA insertion mutants. Anthocyanin assay using the indicated concentrations of elf18 and flg22 were performed with the listed mutants and compared to wild-type as well as *rsw3* plants. – indicates suppression of anthocyanins in the presence of elf18 or flg22, + indicates anthocyanin accumulation.

		elf18		flg22	
Nr.	name/ description	0,1 µM	1 µM	0,1 µM	1 µM
1	FAD-binding domain-containing protein	-	-	-	-
4	anionic peroxidase, putative	-	-	-	-
6	similar to unknown protein [*Arabidopsis thaliana*]	+	-	+	+
7	Chitinase family protein	-	-	-	-
10	disease resistance protein (TIR-NBS-LRR), putative	-	-	-	-
15	similar to unknown protein [*Arabidopsis thaliana*]	-	-	-	-
23	vacuolar sorting receptor, putative	-	-	-	-
25	NIMIN-1/NIMIN1; protein binding	-	-	-	-
27	*ptr3-2*	-	-	-	-
28	ATPTR3/PTR3 (PEPTIDE TRANSPORTER PROTEIN 3)	-	-	-	-
30	peroxidase, putative	-	-	-	-
36	NIMIN-2 (NIM1-INTERACTING 2)	-	-	-	-
39	peroxidase, putative	-	-	-	-
41	chitinase, putative	-	-	-	-
45	oxidoreductase, 2OG-Fe(II) oxygenase family	-	-	-	-
45!	VQ motif-containing protein	-	-	-	-
46	aspartyl protease family protein	-	-	-	-
49	legume lectin family protein	-	-	-	-
58	ATTI1	-	-	-	-
61	WRKY38	-	-	-	-
	pbs3-1	-	-		
	mlo6/12	-	-		
	mlo2/6/12	-	-		

Originally, the *rsw3* mutant was characterized in a screen for elf18-insensitive mutants lacking the ability to suppress anthocyanin accumulation under high sucrose condition in the presence of elf18 (Lu, Tintor et al. 2009). Serrano et al. 2012 described a possible underlying signaling crosstalk

between MAMP-triggered immunity and abiotic stress-triggered flavonoid accumulation (Serrano, Kanehara et al. 2012). They showed that flavonoids reduce flg22-triggered defense responses and in return MTI induction suppresses anthocyanin accumulation. The inability of *rsw3* to suppress flavonoid accumulation in the simultaneous presence of high-level sucrose and the elicitor elf18 may results from the loss of sustained defense gene activation of the identified "key genes". Therefore we tested the anthocyanin-repressing function of the mutant lines in the presence of elf18 and flg22 (Table 2: Characterization Of T-DNA Insertion Mutants).

Only one of the 23 lines tested showed a compromised phenotype: line Nr. 6, encoding an unknown protein, failed to suppress flavonoid accumulation in the presence of high (1 µM) and lower doses (0.1 µM) of flg22 and lower doses of elf18 (0.1 µM) pointing to general function of this protein in the MTI- flavonoid cross talk.

In a second characterization step, the mutant lines were screened for elf18-induced resistance against *Pseudomonas* bacteria. As described by Zipfel et al., pre-treatment of plants with flg22 or elf18 reduces the multiplication of bacteria in subsequent infection assays (Zipfel, Robatzek et al. 2004; Zipfel, Kunze et al. 2006). Therefore, well-expanded leaves of four-week old plants were either pressure-infiltrated with 1 µM elf18 or water (mock) and 24 hours later syringe-inoculated with *Pst* DC3000 (Figure 3). Like wild-type plants, most of the mutants showed an enhance resistance response upon activation of EFR signaling (line Nr. 4, 10, 25, 27, 28, 39, 41, 45, 49, 58 and 61). In contrast the bacterial growth in elf18-pretreated leaves of line 1, 7 and 23 was not as strongly reduced indicating that loss of the respective gene lead to reduced EFR-triggered immunity.

Additionally, we could identify two lines (Nr.15 and 30) showing an enhanced resistance phenotype in mock-pretreated leaves. However, further resistance induction by elf18 could not be observed. Thus, basal immunity of those plants seemed to be enhanced but could not further be increased by pretreatment with elf18.

Of note was the enhanced susceptibility of mock-pretreated lines 6 and 36, even though they still acquired elf18-induced resistance. Nevertheless, both mutant lines allowed about 5 times higher bacterial growth as wild-type plants. The interrupted gene in line Nr. 36 encodes for NIMIN-2, a NPR1-interacting protein presumably contributing to SAR gene expression in *Arabidopsis* (Weigel 2001).

As already mentioned before the knock-out gene of line Nr.6 encodes for a protein of unknown function. Thus, loss of this protein does not only influence the MTI-flavonoid cross talk of the mutant but additionally allows enhanced bacterial growth.

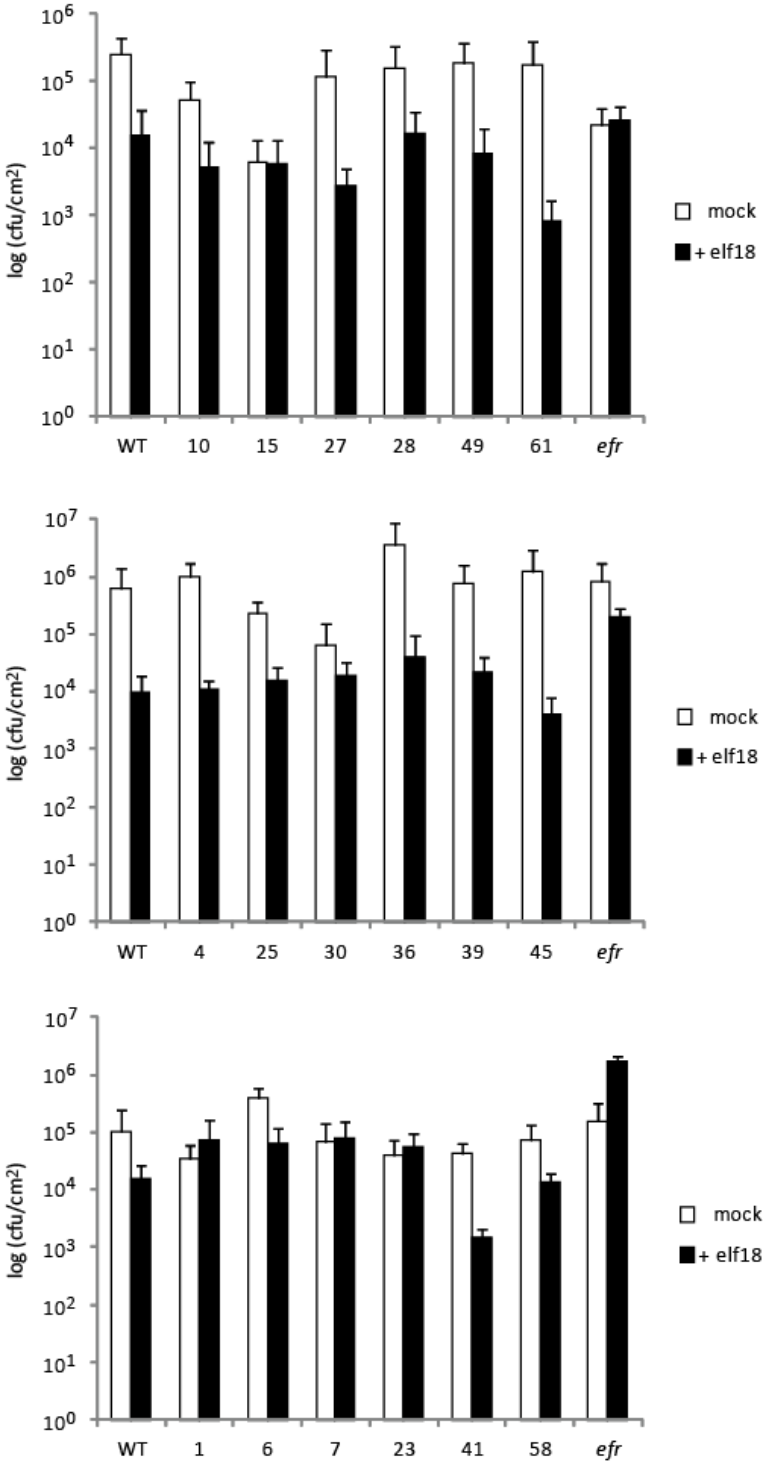

Figure 3: EFR-triggered enhanced resistance. Four-week old leaves of the indicated genotypes were infiltrated with 1µM elf18 or water 24 hours before syringe-inoculated with *Pst* DC3000. Three days later the bacterial titer was determined.

2.1.3 *pbs3* plants seems to pheno-copy *rsw3*

Among the most strongly misregulated genes by *rsw3* we found *PBS3* (AvrPphB susceptible 3). In response to elf18, *PBS3* was 27-fold induced after 10 hours in wild-type plants whereas in *rsw3* the induction fold was only 7-fold in comparison to 0 hpt. The gene has already been described as a positive contributor during defense to bacterial pathogens (Warren, Merritt et al. 1999), however a possible contribution of PBS3 in MTI remains elusive. Activation of *PBS3* gene expression in response to elf18 points to a role of the protein already during MTI. In order to test this hypothesis four-week old leaves of wild-type, *rsw3*, *pbs3-1* and *efr fls2* plants were pre-treated with elf18, flg22 or mock 24 hours before syringe-inoculation with *Pst* DC3000. After three days the bacterial proliferation was determined. As described above MAMP pre-treatment enhances the defense response of the plant and subsequently restricts bacteria growth. Lu et al. showed that *rsw3* still exhibits an enhanced resistance phenotype when pretreated with flg22 but fails to activate EFR-triggered resistance (Lu, Tintor et al. 2009). Interestingly, a similar observation was made by analyzing *pbs3-1*. The mutant was able to activate FLS2-triggered but not EFR-triggered induced resistance (Figure 4).

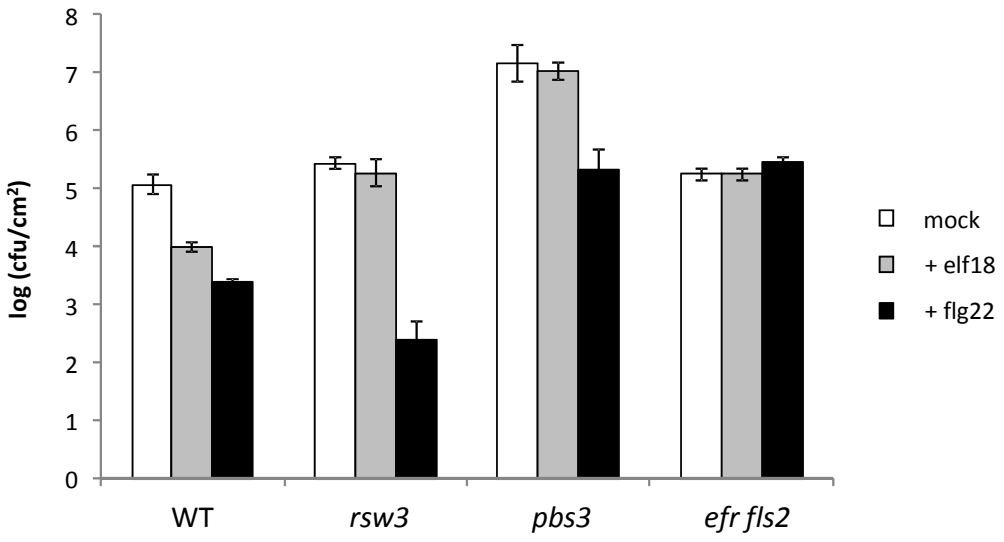

Figure 4: MAMP-induced resistance assay. Four-week old leaves of the indicated genotypes were infiltrated with 1µM elf18, 1µM flg22 or water 24 hours before syringe-inoculated with *Pst* DC3000. Three days later the bacterial titer was determined.

Furthermore, in agreement with previous reports (Warren, Merritt et al. 1999), *pbs3-1* showed a supersusceptible phenotype to *Pst* DC3000 with our without elf18 pretreatment. A similar conclusion could be obtained in an independent experiment testing two additional mutant alleles of *PBS3* (*pbs3-1, pbs3-2, pbs3-3*) (Supplementary Figure 4, page 100).

The finding that loss of PBS3 influences elf18- but not flg22-induced resistance prompted us to further investigate the differential requirement of PBS3 in specific MTI signaling pathways.

In this context we tested transcriptional changes of defense related genes in response to either elf18 or flg22. Again similar to *rsw3* the induction of *PR1 (PATHOGENESIS-RELATED 1)*, *PR2 (PATHOGENESIS-RELATED 2)*, *PROPEP3* and *FRK1* (*FLG22-RESPONSIVE KINASE 1*) at a relatively late phase after elf18-perception was strongly reduced in *pbs3-1* in comparison to the response of wild-type. As shown in Figure 5A up-regulation of *PR1* and *PR2* were almost completely abolished in *pbs3-1* as well as in *rsw3* plants. In addition both mutants failed to fully activate *PROPEP3* and *FRK1* transcripts 10 and 24 hours post treatment. Interestingly, activation of those defense genes in response to flg22 was largely remained in *pbs3-1* and *rsw3* (Figure 5B). *PROPEP2*, *PROPEP3* and *FRK1* were activated to a similar level as wild-type at a rather late time point of FLS2-signaling. However, *pbs3-1* was again almost completely impaired in *PR1* transcript accumulation and also *rsw3* could only partially activate *PR1* expression. This result allows two conclusions; first that *pbs3-1* as well as *rsw3* fails to sustain gene activation during a relative late phase of EFR signaling. Nevertheless, the mutations do not affect the late phase activation of a similar set of genes upon flg22-elicitation. Secondly, induction of *PR1* gene expression was affected in both mutant lines independent of the elicitor treatment, indicating a separate regulation of EFR-mediated defense gene expression (e.g. *FRK1*, *PROPEP2*, *PROPEP3*) depending on PBS3 and RSW3, and PBS3-/RSW3-mediated *PR1* mRNA accumulation in general. The similar phenotype of *pbs3-1* and *rsw3* plants in response to MAMP treatment leads to the assumption that loss of *PBS3* activation in *rsw3* plants strongly contributes to the *rsw3* phenotype.

To analyze whether *pbs3-1,* like *rsw3,* is rather impaired in the late phase but not in the initial induction of MTI, we tested early MTI responses upon elicitor recognition like MAPK activation or accumulation of reactive oxygen species. Both responses were found to be wild-type like in *rsw3* upon elf18 treatment.

Therefore, we treated 10-day old wild-type and *pbs3* seedlings with elf18 and flg22 and examined MAPK activation by Western blotting.

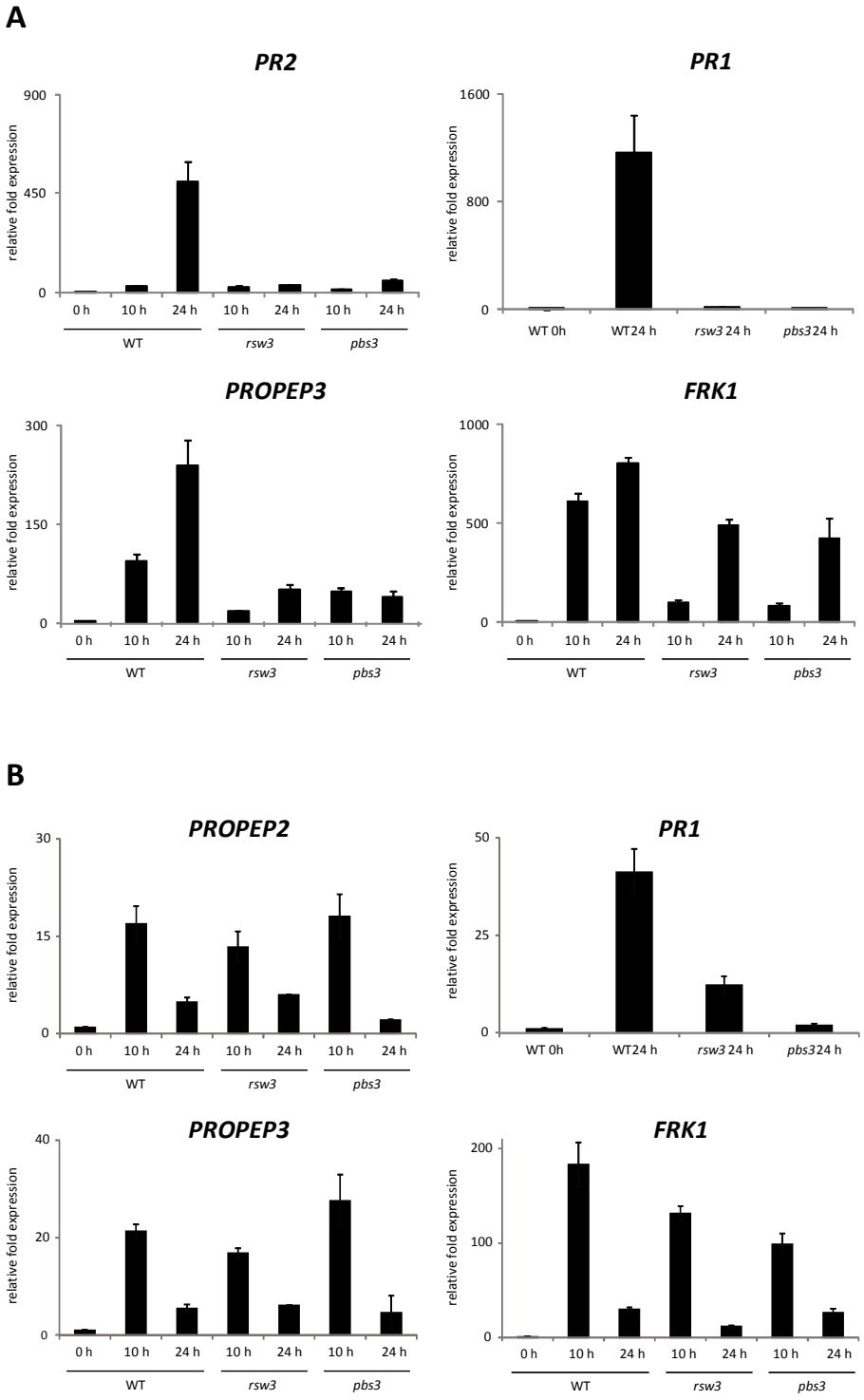

Figure 5: Transcriptional changes in response to MAMP treatment. The transcript accumulation of defense related genes in response to 1µM elf18 (A) and flg22 (B) were analyzed by qRT-PCR for the indicated mutants. The relative expression is shown in fold, with the gene/At4g26410 value at 0 h in WT plants as 1.

Already 5 minutes after MAMP application activated MPK3, MPK4 and MPK6 could be detected. The effect is transient and lasts for 30 minutes (Asai, Tena et al. 2002; Suarez-Rodriguez, Adams-Phillips et al. 2007). In comparison to wild-type plants, the MAPK activation patterns of *pbs3-1* plants were almost identical during both treatments (Figure 6).

Another early MTI output that appears within minutes upon elicitation is the generation of ROS. Preliminary results testing three different alleles of *pbs3* indicate that ROS spiking in response to MAMPs were not affected by loss of PBS3 (Supplementary Figure 6, page 101).

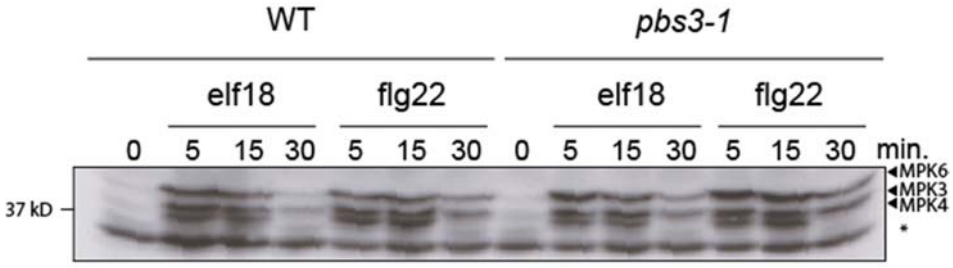

Figure 6: MAPK activation upon MAMP treatment. Ten-day old seedlings of wild-type and *pbs3-1* plants were treated with 1 µM elf18 and flg22 and subsequently harvested for protein detection 5, 15 and 30 minutes later. Positions of active MPK3, MPK4 and MPK6 forms (right) and molecular weight markers (left) are indicated.

In conclusion, it can be stated that *pbs3* exhibits almost identical defects in EFR signaling as *rsw3*. Early responses that are initiated with minutes upon elf18 perception retain wild-type like (Lu, Tintor et al. 2009) whereas the relatively late phase of EFR-signaling including sustained transcriptional reprogramming seems to be compromised, which might lead to the loss of EFR-triggered resistance. Furthermore, defects of *pbs3* appear to specifically affect EFR but not FLS2 signaling. Additionally, it could be observed that, again in concert with the *rsw3* phenotype, positive regulation of the *PR1* locus independent of the elicitor treatment is affected in *pbs3* plants. Taken together, PBS3 seems to play a dual role in regulating MAMP-induced transcriptional changes and also appears to act as a linker of initial and sustained MTI phases.

2.2 The characterization of an endogenous elicitor/receptor Pep/PEPR system in plant immunity

2.2.1 Sustained activation of *PROPEP2* and *PROPEP3* in EFR-triggered immunity

While searching for defense related genes required for the phase transition from early to a relatively late phase of MTI, Xunli Lu et al. have identified a misregulation of *PROPEP2* and *PROPEP3* expression by EFR in *rsw3*. The two genes encode for precursor proteins of putative endogenous elicitors which are thought to amplify MTI signaling and/or act as DAMPs (Huffaker and Ryan 2007; Krol, Mentzel et al. 2010). Those genes are massively up-regulated upon elf18 application and mRNA levels remain elevated for 24 hours in wild-type plants. Similar to the earlier described genes (e.g. *WRKY22* and *WRKY29*)(Lu, Tintor et al. 2009) the high level of *PROPEP2* and *PROPEP3* expression could not be maintained in *rsw3* (

Figure 7A). The result was confirmed by the data of the transcriptome analysis described in 2.1.1. *PROPEP3* could also be found among the misregulated "key genes" in *rsw3* that possibly account for the immune-compromised phenotype of the mutant. In wild-type plants the gene was already 14-fold induced 2 hours after elf18 application and the induction fold was even enhanced at 10 hpt. At 24 hours the amount of *PROPEP3* mRNA dropped but was still 10-fold higher than at 0 hours. Similar to earlier description of *rsw3 PROPEP3* was induced at 2 hours similar to wild-type like levels but the sustained high activation of the gene at 10 hpt could not be observed. Even though the expression pattern of *PROPEP2* is very similar to that of *PROPEP3,* the gene was not grouped to the "key genes" due to the high p-Value (p = 0,138183) at 10 hpt in *rsw3* (

Figure 7B). Nevertheless, the data indicate that *PROPEP2* and *PROPEP3* are among the target genes of sustained transcriptional reprogramming, which is closely associated with effective defense execution, in EFR-triggered immunity.

PROPEP2 and *PROPEP3* were proposed to be precursors of putative bioactive peptides that as endogenously generated elicitors function as an amplifying system during stress signaling (Huffaker, Pearce et al. 2006; Huffaker and Ryan 2007)(1.1.3, page 6). The Pep-peptides were shown to be recognized by the two LRR-RLK PEPR1 and PEPR2 (Yamaguchi, Huffaker et al. 2010). Activation of *PROPEP2* and *PROPEP3* might lead to the engagement of the Pep/PEPR pathway in EFR-triggered MTI. Lowered PEPR activation due to reduced induction of *PROPEP2* and *PROPEP3* in *rsw3*, to some extent,

possibly contributes to the aforementioned defects in EFR-induced resistance. Therefore we hypothesized that artificial activation of the PEPR pathway by exogenously Pep-application could rescue the loss of sustained defense gene activation in *rsw3* plants.

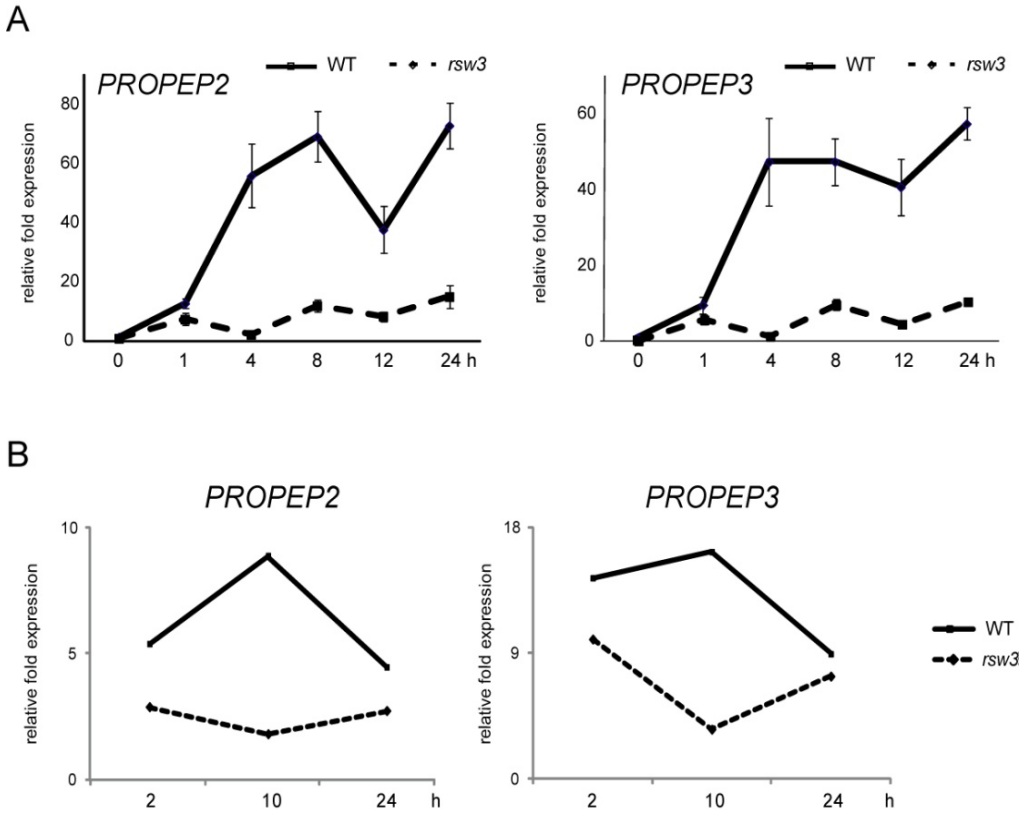

Figure 7: EFR-triggered induction of *PROPEP2* and *PROPEP3* expression. A) 10-day-old wild-type or *rsw3* seedlings were incubated with 1µM elf18 and harvested at the indicated time points for gene expression analysis by quantitative real-time PCR (qRT-PCR). The relative expression is shown in fold, with the gene/At4g26410 value at 0 h in WT plants as 1. B) Plants were treated as in A and cDNA was subjected to microarray analysis. Average expression values of three independent microarray chips for each data point are shown.

As shown in Figure 8 exogenous Pep2 application indeed stimulates wild-type like sustained activation of a subset of defense-related genes that are not durably activated upon elf18 application in *rsw3* plants. Moreover, we found that *PR1* gene activation is greatly reduced in the absence of PEPRs 24 hours after elf18-perception (Tintor, Ross et al. 2012).

Taken together these findings allow the assumption that in addition to PBS3, the Pep/PEPR pathway is required for linking initial MAMP responses to robust immunity.

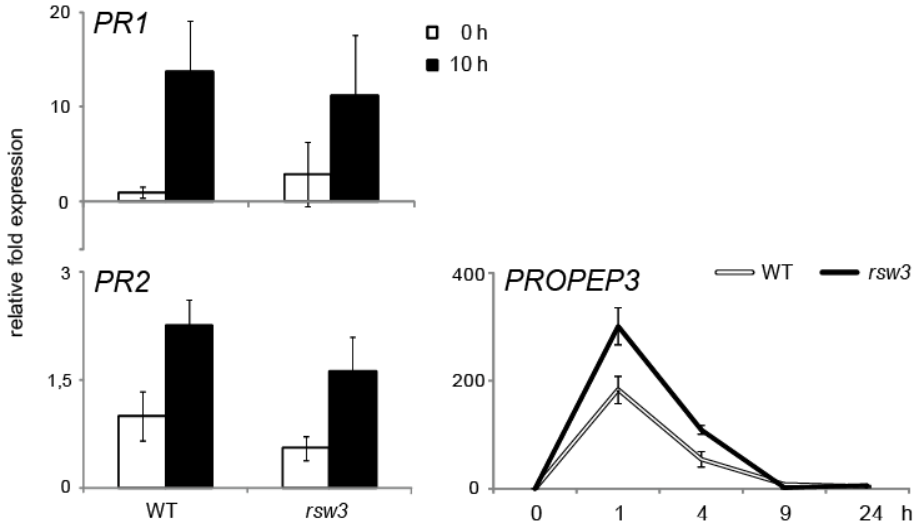

Figure 8: Exogenously applied Pep-peptide rescues defects of sustained gene activation in *rsw3*. Wild-type and *rsw3* seedlings were grown in liquid MS media for 10-days and then treated with 1µM Pep2. Samples were harvested for qRT-PCR analysis at the indicated time points. The relative expression is shown in fold, with the gene/At4g26410 value at 0 h in WT plants as 1.

2.2.2 Pep-triggered transcriptional reprogramming represents co-activation of SA- and JA-mediated immune branches by the Pep/PEPR pathway

Pep1 was first identified through purification of endogenous peptides from cell extracts that exhibit immune-stimulatory functions. As a readout, Huffaker et al. measured alkalinization of the medium of suspension cultured cells, a typical response of the cells to elicitors (Huffaker, Pearce et al. 2006). Subsequent characterization of Pep1 activity was largely based on assays applied for analysis of MTI. Interpretation of the results led to the conclusion that the endogenous Pep-signaling that appeared to be very similar to MAMP signaling, acts as an amplification loop of MTI.

In order to reveal unique and common features compared to MTI and to gain insights into the molecular basis of Pep-triggered immunity, we examined and compared genome-wide transcriptional reprogramming upon Pep2 application with that upon elf18 elicitation. To this end, we exposed 2-week-old seedlings to saturated concentrations (1 µM) of Pep2 or elf18 for 2 and 10 hours, using the corresponding receptor mutant plants (*pepr1 pepr2* or *efr*, respectively) as negative controls.

Of 15851 gene probes on the whole genome microarray (ATH1, Affymetrix), we scored 1401 and 1286 genes that were respectively up- or down-regulated at a cut-off threshold of 2-fold upon Pep2 application for 2 h in WT plants in comparison to the 0 h time point. At 10 hours post Pep2

application 234 and 164 genes, respectively, were up- or down-regulated after the normalization with *pepr1 pepr2* plants. On the other hand, WT plants exhibited an increase and a decrease of the mRNA levels for 1144 and 895 genes at 2 hour after elf18 elicitation and for 474 genes and 665 genes at 10 hour after elf18 elicitation, respectively.

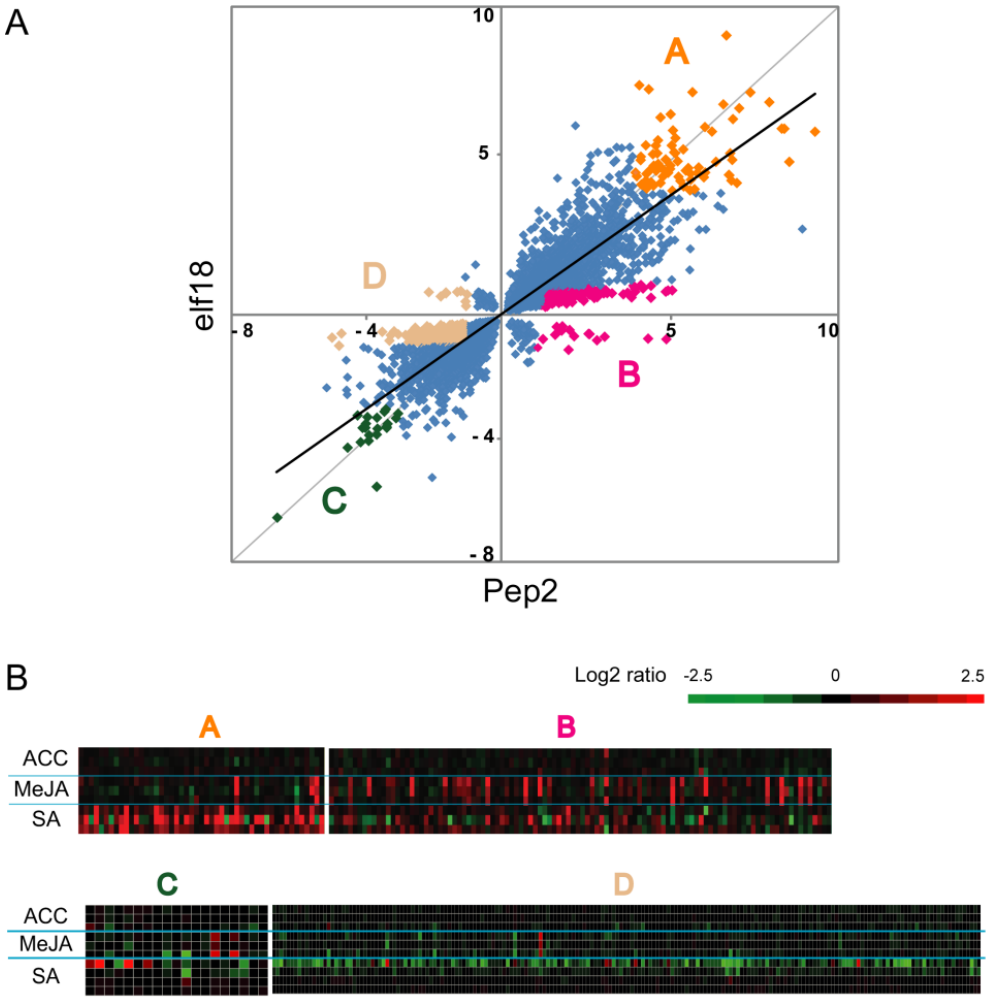

Figure 9: Transcriptome-wide comparison between elf18- and Pep2-triggered transcriptional changes. A)The log2 ration of all genes that were differentially regulated at 2 hpt ($q \leq 0.05$) with Pep2 (1µM) were plotted against the log2 ratio of all differentially regulated genes ($q \leq 0.05$) after elf18 treatment (1µM). The regression of this scatter blot is indicated by the black line. Uniquely 2-fold up-regulated genes upon Pep2 treatment (> log2 = 1 upon Pep2, < log2 = 1 for elf18, $q < 0.05$) are highlighted in pink (group B) and genes strongly up-regulated upon both treatments (> log2 = 4 for elf18 and Pep2, $q < 0.05$) are highlighted in orange (group A). Uniquely 2-fold down-regulated genes upon Pep2 treatment (< log2 = -1 upon Pep2, > log2 = -1 for elf18, $q < 0.05$) are highlighted in beige (group D) and genes strongly down-regulated upon both treatments (> log2 = -3 for elf18 and Pep2, $q < 0.05$) are highlighted in green (group C). B) *In silico* analysis of the selected subgroups using genevestigator (V3) in regard to their responsiveness to ACC, MeJA and SA are illustrated in the heatmaps below. Scale indicates the log-transformed p-values of down-(green) and up-(red) regulated genes (top).

Then we compared the transcriptome profiles between the two elicitors. In agreement with the aforementioned findings that Pep2 or elf18 perception triggers a common set of several MTI-characteristic outputs, there is an overall high correlation in the target genes between the Pep/PEPR and EFR pathways (Figure 9A).

Genes strongly up-regulated by both treatments (log 2 ≥ 4; Figure 9, group A) were cross-referenced with the genes responsive to the defense-related phytohormones SA, MeJA, and ET in the earlier described transcriptome profiles in *Arabidopsis* (Genevestigator). Of the three phytohormones, SA-responsive genes were clearly over-represented in both samples, again supporting the hypothesis that the PEPR and EFR pathways activate a largely overlapping set of immune outputs, including massive induction of defense-related genes that are responsive to SA.

As mentioned before the main goal of the microarray experiment was to identify genes unique for Pep-signaling. To this end we selected a subset of genes greatly activated upon Pep2 application (log2 ≥ 1) without significant changes upon elf18 application (log2 ≤ 1) (Figure 9, group B), suggesting that these genes represent cellular outputs that are selectively triggered by the Pep/PEPR pathway.

Table 3: Gene ontology enrichment upon elf18 and Pep2 treatment. Two-fold induced genes (log2 ≥ 1) obtained from the microarray 2 hours upon Pep2 or elf18 treatment were analyzed for gene ontology enrichment focusing on responses to phytohormones.

GO term	Elf18 (p-Value)	Pep2 (p-Value)
Response to SA	3,6 e-07	2,4 e-06
Response to ethylene	6,3 e-04	2,2 e-06
Response to JA	1,4 e-02	6,8 e-07

Cross-reference of this gene set with phytohomormone-responsive genes of the public available transcriptome profiles revealed an overrepresentation of MeJA responsive genes for Pep2-regulated genes. This result was strengthened by gene ontology analysis on all 2-fold (log2 ≥ 1) induced genes by Pep2 and elf18. A significant enrichment of JA, SA and ethylene responsive genes was identified upon Pep2 application whereas only SA-responsive genes were significantly over-represented in the EFR-regulated genes (Table 3). It seems that unlike MAMP signaling Pep-triggered immunity involves co-activation of typically antagonizing SA and JA/ethylene hormone signaling branches.

In sum, the transcriptome-wide comparison of EFR- and PEPR-signaling revealed expected similarities as well as striking differences. Whereas earlier described profiles were almost indistinguishable

between EFR- and FLS2-triggered transcriptional reprogramming, at least in the early time points tested (in 1 hour post elicitation) (Zipfel, Kunze et al. 2006), we identified notable differences between elf18- and Pep2-triggered transcriptional reprogramming at 2 hours post treatment.

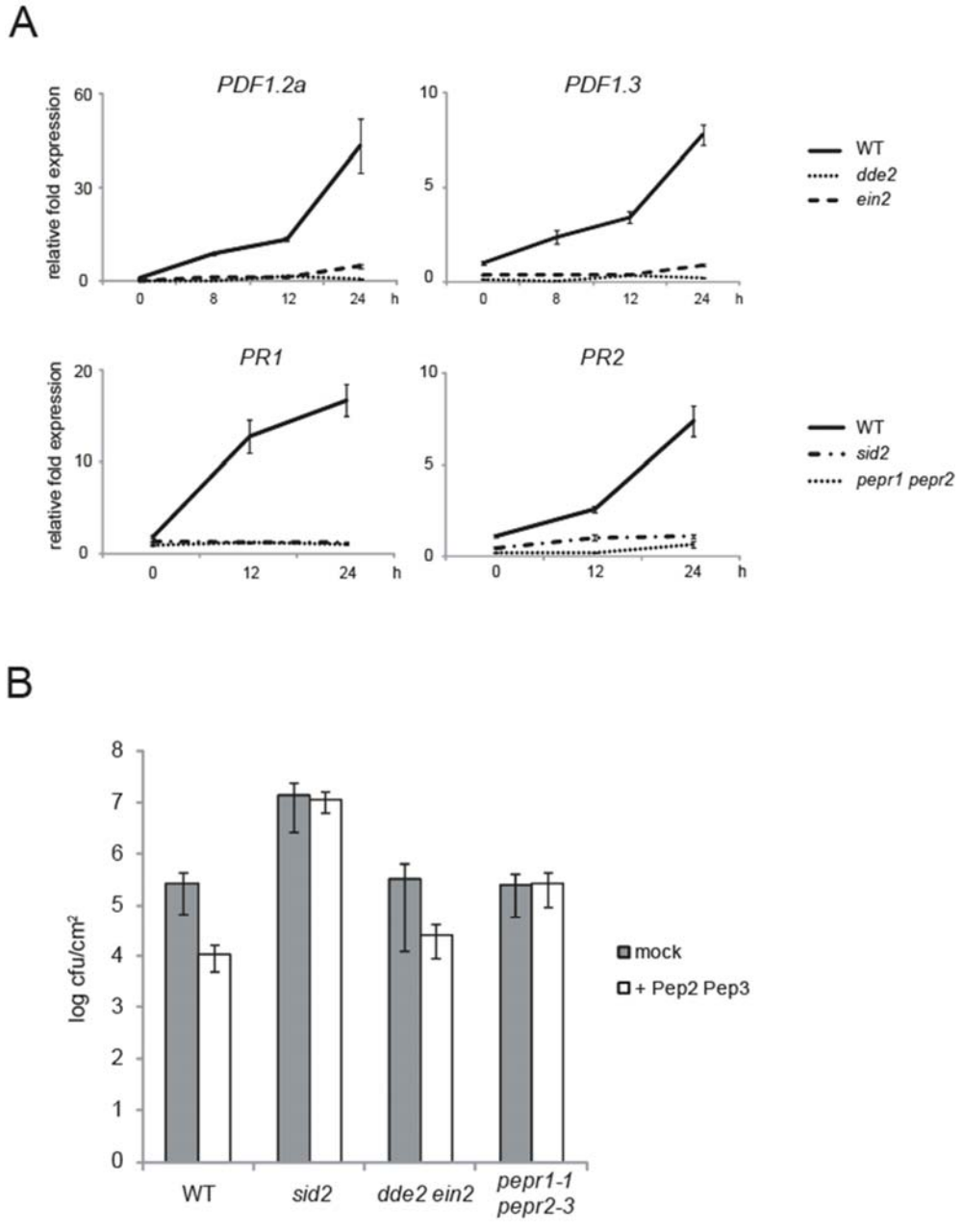

Figure 10: The Pep/PEPR pathway requires both SA and JA/ethylene signaling. A) 10-day-old seedlings were treated with 1µM Pep2 and harvested for qRT-PCR analysis of typical SA- and JA-marker genes at the indicated time points. The relative expression is shown in fold, with the gene/At4g26410 value at 0 h in WT plants as 1. B) Leaves of 4-week-old plants were infiltrated with 1µM Pep2 and Pep3 each one day before syringe-inoculation with *Pst* DC3000 (1 x 10^5 cfu). After three days the bacterial proliferation was analyzed.

It seems that EFR-triggered signaling largely relies on the activation of SA-responsive genes to confer MTI, whereas, the Pep/PEPR pathway facilitates co-activation of SA and JA/ET-mediated immune branches that might otherwise antagonize each other in a different context.

This finding was validated by quantitative reverse-transcription PCR (qRT-PCR) analysis for selected SA- and JA/ethylene responsive genes. The transcript profiles of *PR1* and *PR2*, encoding pathogenesis-related protein that have been widely used as marker genes for SA-mediated immunity and the JA/ethylene responsive genes *PDF1.2a* and *PDF1.3*, encoding small basic anti-microbial peptides called defensins (Thomma, Cammue et al. 2002; van Loon, Rep et al. 2006), were investigated upon Pep2 application. We confirmed that Pep2 application significantly activates all 4 genes in WT plants and furthermore that loss of the genes required for salicylic acid, jasmonic acid or ethylene response leads to the loss of gene induction (Figure 10A). Among others, pathogen-induced generation of salicylic acid requires isochorismate synthase 1 (SID2), jasmonic acid biosynthesis requires allene oxide synthase (DDE2) and ethylene signaling almost fully relies on an ER-membrane localized protein designated EIN2 (Alonso, Hirayama et al. 1999; Wildermuth, Dewdney et al. 2001; von Malek, van der Graaff et al. 2002). Pep-triggered activation of both *PR1* and *PR2* is impaired in *sid2* plants, whilst that of both *PDF1.2a* and *PDF1.3* is largely reduced in *dde2* and *ein2* plants (Figure 10A).

Of note, the strong activation of the JA/ethylene marker *PDF1.2a* appears to be specific to Peps since induction of the gene by flg22 or elf18 is much lesser or hardly detectable (Supplementary Figure 7A, page 102).

Numerous publications have described the antagonistic effect of JA and SA signaling (Kunkel and Brooks 2002; Thaler, Fidantsef et al. 2002; Glazebrook, Chen et al. 2003; Spoel and Dong 2008). Endogenously accumulating salicylic acid pathway strongly suppresses JA-dependent defenses including the expression of defensins (Gupta, Willits et al. 2000; Spoel, Koornneef et al. 2003; Koornneef, Leon-Reyes et al. 2008; Leon-Reyes, Spoel et al. 2009). In order to test whether potential EFR-triggered induction of defensins is suppressed by exceeding levels of SA we investigated *PDF1.2a* gene induction upon elf18 application in *sid2* and *sid2 pad4* plants. PAD4 has been shown to be important for SA accumulation in response to some SA-inducing stimuli (Jirage, Tootle et al. 1999). Surprisingly, even in the absence of SID2/PAD4-mediated SA pathway, elf18-perception did not allow *PDF1.2a* induction (Supplementary Figure 7B, page 102), again illustrating the extraordinary ability of PEPR-triggered activation of JA/ethylene simultaneously to SA signaling.

To ensure this notion, the genetic requirements for the three phytohormone pathways in Pep-induced immunity against *Pst* DC3000 were tested. For this purpose, leaves of four-week old wild-

type, *sid2*, *dde2 ein2* and *pepr1 pepr2* plants were pretreated with a mixture Pep2 and Pep3 and one day later challenged with the bacteria. Consistent with the earlier study (Yamaguchi, Huffaker et al. 2010), pretreatment with Pep-peptides results in the suppression of bacterial multiplication, which is entirely dependent on the Pep-receptors (Figure 10B). The SA biosynthesis deficient mutant (*sid2*) allowed as much bacterial growth with or without pre-treatment with Peps, demonstrating that SA is required for effective Pep/PEPR-triggered immunity at least against *Pst* DC3000. However, the decrease of Pep-induced *Pst* resistance was not consistently detected in *dde2 ein2* plants. In some cases the mutants showed a wild-type like response but in other cases they failed to induce Pep-induced resistance. The occasionally observed WT-like resistance might reflect a primary role of the SA branch for the bacterial defense that is retained in the absence of JA/ET. This would be in agreement with the finding that EFR-triggered immunity is effective against *Pst* DC3000 without discernable activation of JA-dependent defensin genes (Supplementary Figure 7, page 102). On the other hand, the failure to consistently mount effective resistance in *dde2 ein2* plants might reflect a contribution of JA/ET branches to PEPR-triggered immunity.

2.2.3 The Pep/PEPR pathway co-activates SA and JA branches with the aid of ET

In 2010 Leon-Reyes and colleagues searched for novel components involved in the regulation of SA-JA cross-talk. They screened well-characterized *Arabidopsis* mutant lines for an altered phytohormone or defense-related phenotype, namely for their inability to display SA-mediated suppression of JA-responsive defense gene expression. They found that the mutant *cev1* (Ellis and Turner 2001) that exhibits constitutively activated JA and ethylene signaling pathways is impaired in the hormonal cross talk between SA and JA. Further analysis revealed that simultaneous induction of the JA and ET pathway renders the plant insensitive to future SA-mediated suppression of JA-dependent defenses (Leon-Reyes, Du et al. 2010).

To investigate whether PEPR-triggered signaling utilizes this connectivity of the three phytohormones in order to allow co-activation of SA and JA/ethylene branches, we tested the Pep-induced expression of defensins in the absence of the hormone branch mutants *dde2*, *ein2*, *sid2* and *pad4* and combinations thereof. In Arabidopsis, defensins are encodes by 13 *PDF* genes of which some have been reported to be JA-responsive (Thomma, Cammue et al. 2002). The double mutant *dde2 ein2* allows total knock out of the JA/ethylene branch whereas *sid2 pad4* is almost completely hindered in SA accumulation.

As shown in Figure 11 *PDF1.2a* and *PDF1.3* induction were strongly inhibited in the absence of ethylene. Depletion of both JA and ethylene (*dde2 ein2*) leads to entire deficit of gene induction by Pep2 whereas the absence of the SA signaling branch did not interfere with defensin gene activation. Interestingly, even in the absence of ethylene to the simultaneous disruption of the SA branch *PDF1.2a* and *PDF1.3* expression could substantially be restored. However, the restored PEPR-triggered induction of the two genes is again abolished by the disruption of the JA branch in *dde2 ein2 pad4 sid2* plants, confirming the essential role of JA for this output activation. This result indicates that rather JA than ethylene *per se* is responsible for the induction of *PDF1.2a* and *PDF1.3*. However, in the absence of ethylene the SA branch has a strong negative effect on JA signaling and suppresses the gene expression. Thus, ethylene supports JA to overcome the repressing function of SA. It seems likely that PEPR-signaling exploits the above described interconnectivity of the three phytohormones as proposed by Leon-Reyes et al. (2010).

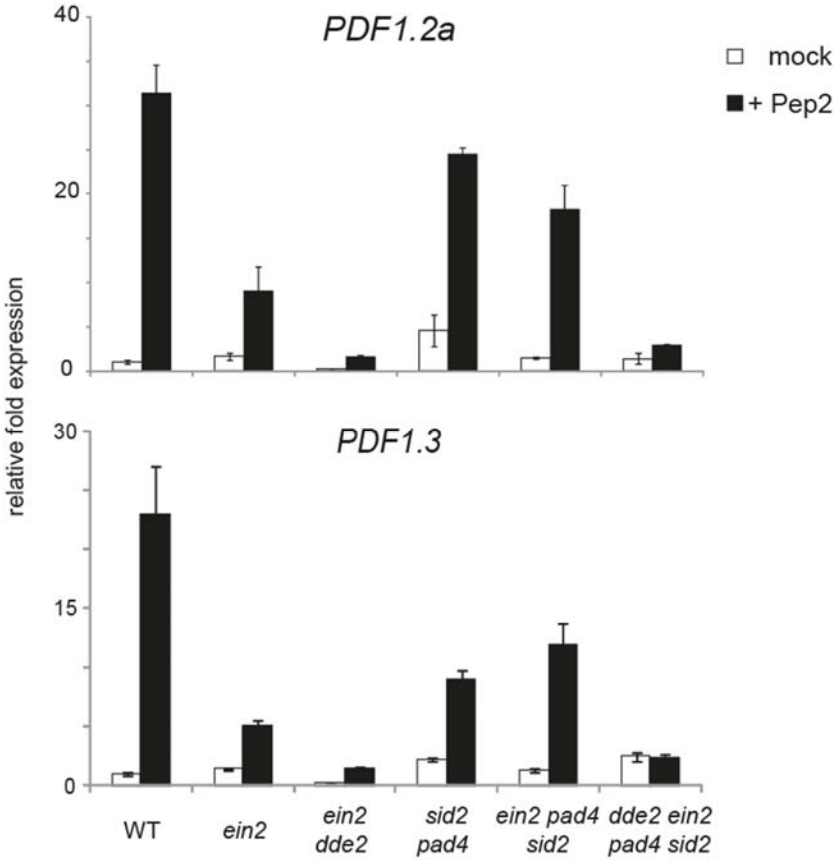

Figure 11: Pep-signaling acts through ethylene for the co-activation of SA and JA branches. 10-day-old seedlings of the indicated genotypes were treated with 1µM Pep2 and harvested for qRT-PCR analysis 24 hpt. The relative expression is shown in fold, with the gene/At4g26410 value at 0 h in WT plants as 1.

2.2.4 PEPRs contribute to basal defense against hemi-biotrophic pathogens

Earlier studies have failed to detect a significant immune compromised phenotype of *pepr1 pepr2* plants in the absence of primary exogenous Pep-application. *Pythium irregulare*, *Pst* DC3000 or *Alternaria brassicicola* could grow to the same extent on *pepr1 pepr2* as well as wild-type plants (Yamaguchi, Huffaker et al. 2010). However, given the many characteristics shared between MAMP- and Pep-triggered signaling, it remained possible that the growth of less virulent pathogen strains might be enhanced in *pepr1 pepr2* plants, as often reported with MTI-compromised mutants (Nekrasov, Li et al. 2009).

The above described finding that Pep-recognition leads to co-activation of SA- and JA-mediated immune branches motivated us to test a possible contribution of PEPRs to host immunity against hemi-biotrophic pathogens. Hemi-biotrophy is characterized by the switch from an initial biotrophic to a later necrotrophic life style of the invading microorganism (Glazebrook 2005). On this account the growth of the two less virulent hemi-biotrophic pathogens *Pst* DC3000 Δ*AvrPto* Δ*AvrPtoB* and *Colletotrichum higginsianum (Ch) path-29* were analyzed in wild-type and three independent alleles of *pepr1 pepr2* plants (Huser, Takahara et al. 2009). Infection with a less virulent strain of *Pst* DC3000 lacking the effector genes *AvrPto* and *AvrPtoB* (Lin and Martin 2005) resulted in enhanced bacterial counts in leaves of two of the double receptor mutants (*pepr1-2 pepr2-2*, *pepr1-1 pepr2-3*) and *efr fls2* in comparison to wild-type plants (Figure 12A). Additionally, all three *pepr1 pepr2* plants allowed significantly more growth of *Ch path-29* regardless of the methods applied (Figure 12B). In average the necrotic lesion sizes measured on wild-type plants were about 2 mm in diameter whereas lesions on *pepr1 pepr2* were measured 0.5 mm wider, similar to those of *pen2-1*, a supersusceptible mutant to a broad range of fungal pathogens (Lipka, Dittgen et al. 2005; Maeda, Houjyou et al. 2009; Hiruma, Onozawa-Komori et al. 2010).

In a second experiment fungal biomass was assessed three days post drop-inoculation of the spores on the surface of plant leaves. The quantitative amount of fungal growth (estimated by determining the amount of fungal *ACTIN* mRNA) is correlated with the biomass of the plant (normalization gene At4g26410). In concert with the lesion size measurement it could be observed that loss of the PEPR pathway enables the pathogen to grow more rapidly than on wild-type plants (Figure 12C).

These results demonstrate that PEPRs contribute to basal immunity against the tested hemi-biotrophic pathogens, providing evidence for the significance of the Pep/PEPR pathway in host immunity.

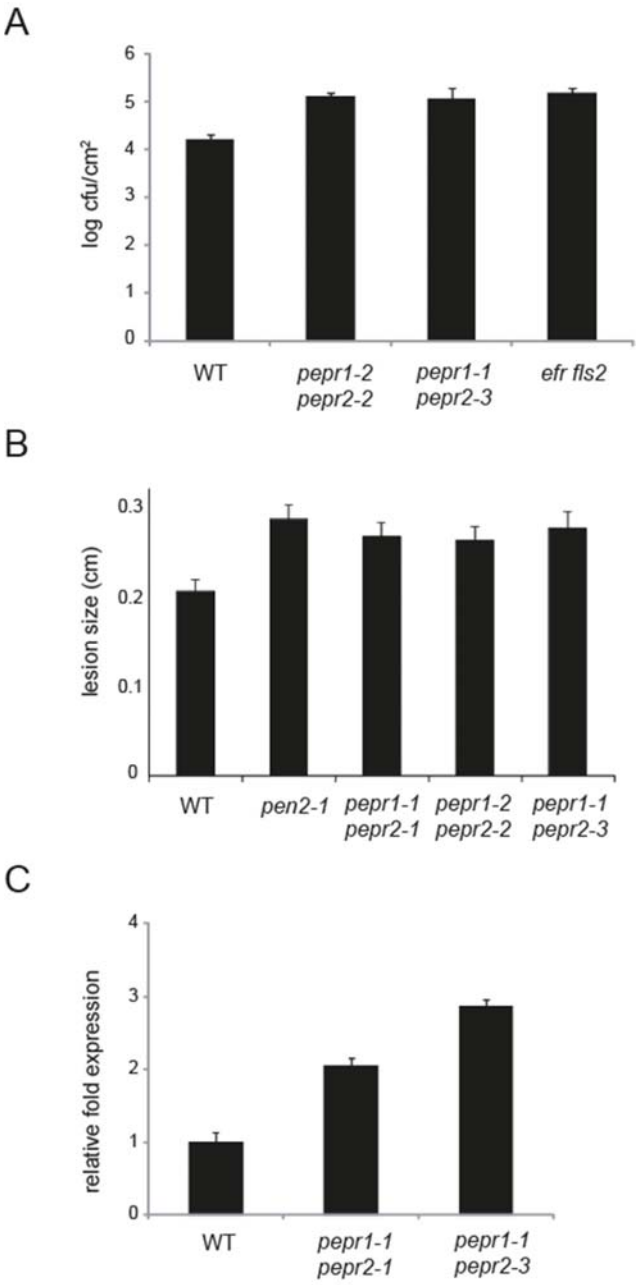

Figure 12: Basal defense to hemi-biotrophic pathogens is reduced in pepr1 pepr2. A) Four-week-old wild-type, *pepr1 pepr2* and *efr fls2* plants were infiltrated with 10^5 cfu *Pst*DC3000 (Δ*AvrPto* Δ*AvrPtoB*) and the bacterial titer was determined 3 days after inoculation. Values are the means ± SD of two biological replicates (n = 4). B) The lesion sizes (n = 30) on leaves of 4-week-old WT, *pen2-1* and *pepr1 pepr2* (three different alleles) plants were measured 5 days post drop-inoculation (5 x 10^5 spores/ml) with *C. higginsianum* (*Ch*) *path-29*. C) Biomass qRT-PCR of *Ch path-29* three days post drop-inoculation (1 x 10^5 spores/ml) on 12-day old wild-type and *pepr1 pepr2* seedlings. The relative expression is shown in fold, with *Ch*ACTIN/At4g26410.

2.2.5 The Pep/PEPR pathway promotes systemic immunity

The aforementioned findings point to a model in which Pep-triggered immunity coordinates the activation of JA- and SA-mediated immune branches during MTI. Recent publications have reported that MTI alone is sufficient to serve as a trigger for systemic acquired resistance (SAR) (Mishina and Zeier 2007). Furthermore, the establishment of SAR requires the engagement of SA- as well as JA-induced processes (Gaffney, Friedrich et al. 1993; Durrant and Dong 2004; Truman, Bennett et al. 2007). Moreover, the Pep/PEPR pathway has been associated with the wounding response since transcript levels of *PEPR1*, *PEPR2* and *PROPEP1* were enhanced upon mechanical wounding (Huffaker, Pearce et al. 2006; Yamaguchi, Huffaker et al. 2010). In rice, tobacco and tomato it was demonstrated that a systemic acquired resistance response can also be induced by wounding (Ryan 1992; Schweizer, Buchala et al. 1998; Sato, Seto et al. 2009).

Taken together these finding encouraged us to test a possible role of Pep-induced signaling during the acquirement of systemic immunity. Thus, we assessed local and systemic transcriptional changes of widely used defense markers, *PR1* and *PR2*, upon local infection with *Pst* DC3000 (*AvrRpm1*) which triggers very fast and potent ETI via the resistance protein RPM1. Wild-type like induction of both genes in locally challenged leaves of *pepr1 pepr2* was observed, even though *PR2* was only very weakly induced (2-fold) in comparison to *PR1* (~200 fold) (Figure 13A). This is in good agreement with the finding that RPM1-induced cell death, quantified by electrolyte leakage, was not altered in the double receptor mutant in local leaves (Supplementary Figure 8, page 103). However, this also implies that in contrast to basal immunity, ETI does not rely on functional PEPR signaling.

Unlike the local response, *pepr1 pepr2* plants were impaired in systemic up-regulation of *PR1* and *PR2* in distant unchallenged leaves indicating a critical role of the Pep/PEPR pathway for systemic immune signaling.

In a next step we tested whether SAR is compromised against a pathogen in *pepr1 pepr2* plants by inoculation of local leaves (expanded rosette leaves in the lower layer of the plant) with *Pst* DC3000 (*AvrRpm1*) 48 hours prior systemic (young expanded rosette leaves in the upper layer of the plant) challenge-inoculation with *Pseudomonas syringae* pv. *maculicola (Psm)*. Compared to the control mock-inoculation, locally induced RPM1-triggered ETI decreases systemic growth of *Psm* by about 10 fold in systemic leaves of wild-type plants. In contrast, pre-inoculation of local leaves of two alleles of *pepr1 pepr2* with the ETI-inducing pathogen did not alter the bacterial growth of *Psm* in systemic tissue (Figure 13B).

From both experiments it can be concluded that loss of PEPR pathway dampens the systemic acquired resistance response.

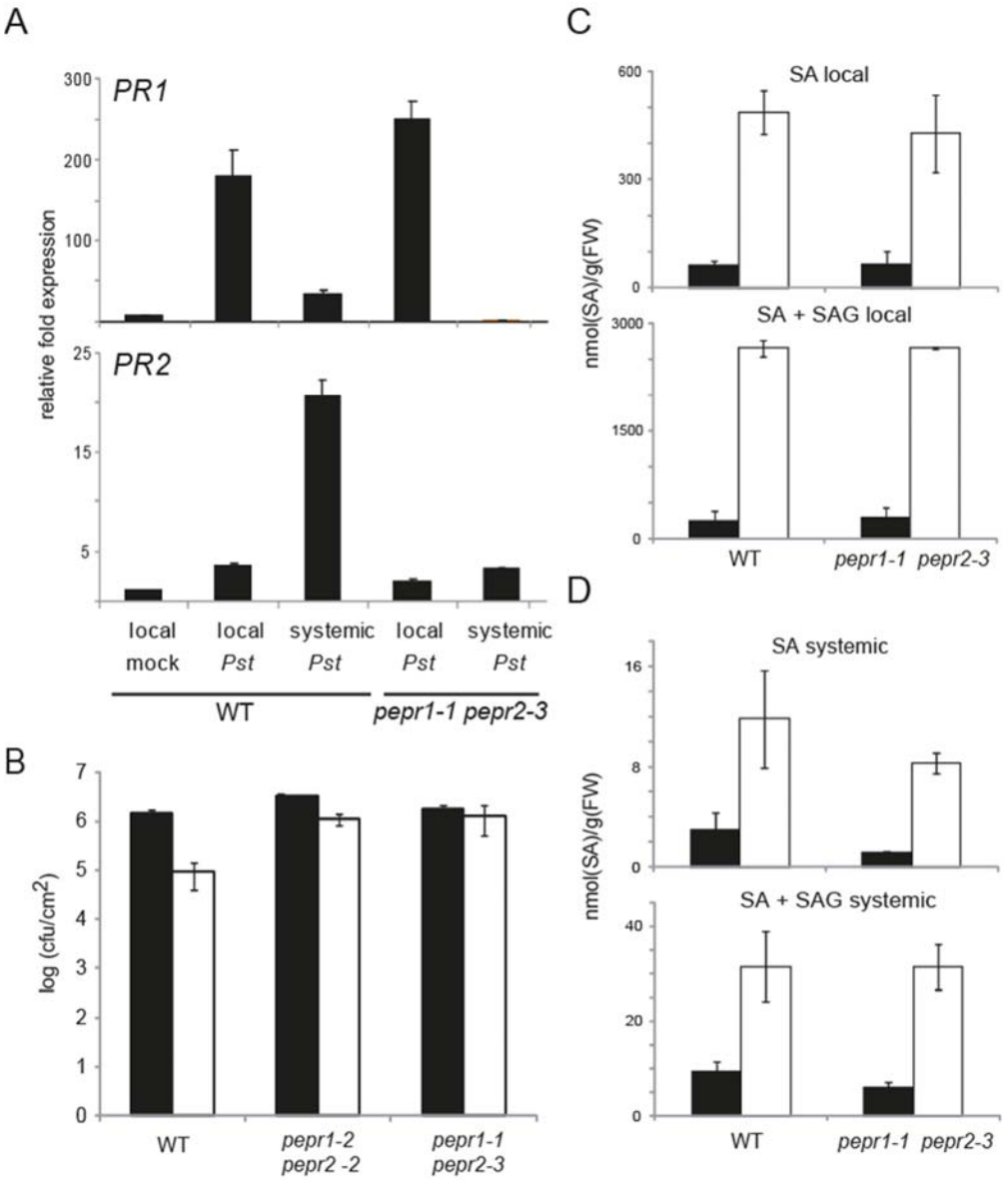

Figure 13: Systemic acquired resistance is impaired in *pepr1 pepr2* mutants despite wild-type like local and systemic SA accumulation. A) Local leaves of four-week-old WT and *pepr1 pepr2* plants were infiltrated with 10 x 10^7 cfu of *Pst* DC3000 (*AvrRpm1*) or MgCl2 as mock control. For qRT-PCR analysis local leaves were harvest 24 hpi and systemic leaves 48 hpi (n=4). The relative expression is shown in fold, with the gene/At4g26410 value as 1 in local mock treated WT leaves. B) Local leaves were treated as in A) at day 0. Systemic leaves were inoculated with 10^6 cfu *Psm* at day 2 and harvest for colony counts at day 5. Values are the means ± SD of two biological replicates (n=4). C) Local leaves were treated as in A) and harvested 24 hours later for SA measurement. Systemic leaves were collected 48 hours after inoculation of local leaves. Values are the means ± SD of three biological replicates (n=4).

Many studies have deeply investigated the molecular basis of SAR in several plant species e.g. tobacco, cucumber and *Arabidopsis* (Malamy, Carr et al. 1990; Metraux, Signer et al. 1990; Lawton, Weymann et al. 1995). It is know that the endogenous signaling molecule salicylic acid (SA) accumulates following pathogen exposure and is require for induction of SAR. Exogenous SA application induces resistance to the same spectrum of pathogens and activates the same set of genes as biological inducers of SAR (Kessmann, Staub et al. 1994). Removal of endogenous SA, due to expression of an introduced bacterial *NahG* gene encoding salicylate hydroxylase, abolishes the expression of SAR and results in other defects in resistance to pathogens (Gaffney, Friedrich et al. 1993; Delaney, Uknes et al. 1994). Thus, SA is a central component for the establishment of SAR. In order to test possible perturbations of SA accumulation in *pepr1 pepr2* plants, we conducted local pathogen challenged as well as systemic untreated leaves to SA analysis. Inoculation of local leaves with *Pst* DC3000 (*AvrRpm1*) resulted in a comparable increase of endogenous free and total (free SA plus SA 2- O-β-glucoside [SAG]) SA contents in wild-type as well as *pepr1 pepr2* plants after one day (Figure 13C). Similarly, levels of free and total SA increased in systemic unchallenged leaves in both genotypes two days after local infection. The analogy of *pepr1 pepr2* plants to wild-type with respect to pathogen induced SA accumulation allowed the conclusion that the Pep/PEPR pathway contributes to SAR without influencing SA production. Uncoupling of the immune-compromised *pepr1 pepr2* phenotypes from SA accumulation, together with the requirement for SA in Pep-triggered immunity (Figure 10), argues for a role of the Pep/PEPR pathway in the control of a SA-dependent signaling step toward transcriptional reprogramming and/or enhanced immunity in both MTI and SAR.

2.2.6 The Pep/PEPR pathway predominantly operates in pathogen-challenged local tissues to confer systemic immune activation

The genetic evidence revealed that Pep/PEPR signaling is indispensable for the establishment of SAR. However, it still remained elusive how this pathway actually contributes to this phenomenon. In 2007 Huffaker and Ryan proposed a model in which the Pep/PEPR system serves as an amplification loop of MTI signaling to enhance the defense response. This hypothesis was supported by the peptide's property of inducing expression of their own precursor genes to initiate a feedback mechanism. However, it remains elusive whether the amplifying mechanism only appears at the infection sites and/or pursues systemically.

To this end, the mRNA levels of *PROPEP2* and *PROPEP3* were quantified in local and systemic leaves during SAR, since their induction is suggestive of PEPR signaling activation given the aforementioned positive feedback regulation of the pathway. As described in 2.2.5, lower leaves were infiltrated with the avirulent *Pst* DC3000 strain carrying *AvrRpm1*. Local tissue was harvested at 24 hours and systemic untreated leaves 48 hours post inoculation (Figure 14). Surprisingly we found that significant induction of both genes was only detected in local leaves around the infection site indicating that no PEPR ligand recognition occurs in systemic leaves in the absence of direct pathogen contact.
My data that Pep2- infiltration in lower leaves of four-week old plants did not elicit systemic *PROPEP2* and *PROPEP3* gene expression also supported the above finding (Supplementary Figure 9, page 103).
Moreover, these results were also compatible with the earlier described finding that *PROPEP3*, as one of the "key genes" identified in the transcriptome wide analysis of *rsw3* in chapter 2.1.1, belongs to the genes that are induced upon defense execution but not in systemic leaves upon SAR activation (Figure 3).

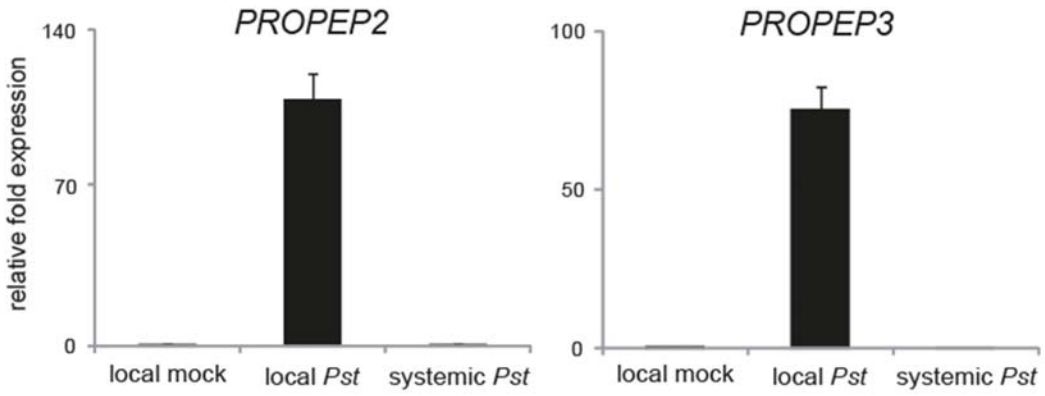

Figure 14: *PROPEP2* and *PROPEP3* are exclusively induced in local leaves. Gene expression of *PROPEP2* and *PROPEP3* in local and systemic leaves 24 hours after infiltration of 10^5 cfu of *Pst* DC3000 in local leaves of four-week-old wild-type and *pepr1 pepr2* plants. The relative expression is shown in fold, with the gene/At4g26410 in mock treated local leaves of WT as 1.

To localize the two PROPEP proteins *in vivo* during SAR defense response, several independent transgenic *Arabidopsis* lines that express a *C*-terminal Venus fusion of *PROPEP2* or *PROPEP3* under the corresponding native regulatory sequences were generated. The functionality of the fusion protein was verified in the transient gene expression system in *N. benthamiana*. (Supplementary Figure 10, page 104)
Total lysates derived from *N. benthamina* leaves expressing PROPEP2-Venus, PEPR1-Flag, and BAK1-HA were subjected to coIP analysis. It was reported that upon PEPR-ligand recognition the receptor

interacts with BAK1 and that loss of BAK1 results in reduced Pep-triggered ROS and ethylene production (Schulze, Mentzel et al. 2010; Roux, Schwessinger et al. 2011). In that aspect we reasoned that PEPR1 – BAK1 interaction can serve as a specific indicator of PEPR activation.

Specific detection of PROPEP2-Venus, apparently in the full-length form, in the co-immunoprecipitates of PEPR1 but not in the mock IP controls was observed (Supplementary Figure 10A, page 104). This interaction could be competed with exogenously applied Pep2 or Pep3 but not flg22 or elf18 validating the specificity of receptor-ligand binding (Supplementary Figure 10, page 104). Based on these data, we conclude that PROPEP2-Venus and PROPEP3-Venus constructs generate functional proteins. Thus, they were used to trace the protein abundance during SAR.

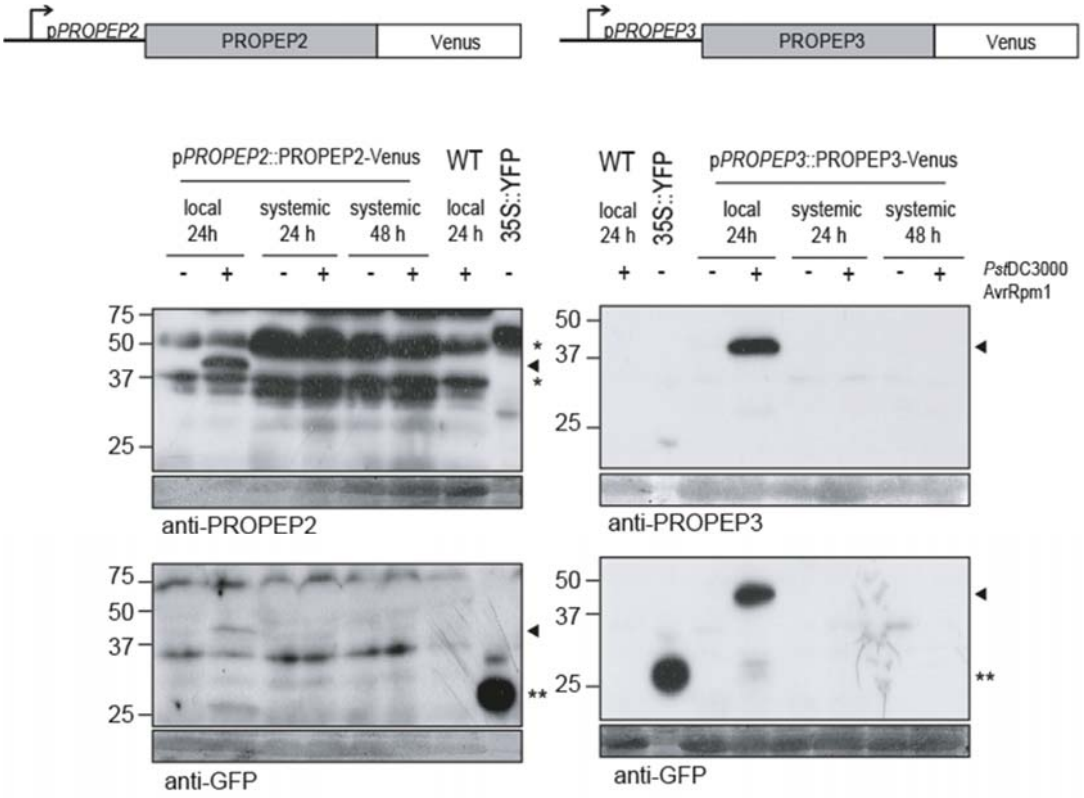

Figure 15: PROPEP2 and PROPEP3 accumulation during SAR. Wild-type Arabidopsis plants were stably transformed with a construct expressing *PROPEP2* or *PROPEP3* tagged with the Venus protein (yellow-fluorescent protein) under the control of the respective endogenous promoter. Local leaves of 4-week-old plants were infiltrated with 10^7 cfu of *Pst* DC3000 (*AvrRpm1*) and harvested 24 hours later. Systemic leaf samples were taken at 24 and 48 hours after local inoculation. Extracted proteins were subjected to immunoblot analysis using anti-PROPEP2 (α-PROPEP2) or anti-PROPEP3 (α-PROPEP3) antibodies. Non-transformed wild-type plants and plants expressing YFP only were used as controls. Errors indicate the position of protein band (◀), one star indicates unspecific bands (*) and the YFP protein band is labeled with two stars (**). The experiment was performed and the data kindly provided by Kohji Yamada.

Upon inoculation of the transgenic lines with *Pst* DC3000 *AvrRpm1* local leaves were harvest 24 hours and systemic leaves 24 and 48 hours later. By using specific antibodies for PROPEP2 and PROPEP3, respectively or anti-GFP for the detection of the Venus protein, it was possible to detect PROPEP2 and PROPEP3 fusion protein in local leaves upon the bacterial challenge. In agreement with the transcript data described above no protein was detectable in systemic leaves at both time points tested

(

Figure 15, experiment was performed by Kohji Yamada). From that it can be concluded that the elicitor-active ligands (and/or their precursors) predominantly accumulate in directly pathogen-challenged sites during SAR. It appears likely that PEPR-signaling occurs around the infection site, thereby serving for the generation of systemic signals. The lack of detectable *PROPEP2* and *PROPEP3* expression and protein accumulation in systemic leaves, which would be indicative of PEPR signaling and thus positive auto-feedback activation, makes it unlikely that the PROPEP proteins themselves could serve as the systemic signal.

Subsequent dissection of PEPR-triggered systemic immunity was focused on the influence of exogenously applied Pep-peptides in local leaves on systemic defense outputs. If the PEPR pathway act in local tissues to confer SAR, it would predict that local Pep application can activate systemic immune response. In this respect, local MAMP application leads to activation of SAR including the induction of defense genes (Mishina and Zeier 2007). Lower leaves of four-week old wild-type plants were infiltrated with Pep2 and systemic leaves were analyzed for transcriptional changes one day later. The widely used SAR marker gene *PR1* was strongly induced in systemic leaves upon the local activation of PEPR signaling. Interestingly, the JA/ethylene marker *PDF1.2a* was also highly up-regulated in systemic leaves upon local Pep-elicitation. Thus, PEPR-triggered signaling incorporates the co-activation of the typically antagonizing SA and JA/ethylene hormone branches directly at the site of Pep-perception (see 2.2.2) but furthermore helps to generate a systemic signal that allows the activation of the same phenomenon in distant tissue without involving re-activation of the Pep/PEPR pathway (Figure 16A).

Further genetic evidence proved the requirement of the Pep receptors as well as the JA/ethylene branch for the activation of Pep-induced systemic *PDF1.2a* activation. As in Figure 16B displayed *dde2 ein2* plants completely fail to express *PDF1.2a* in systemic leaves upon local Pep-application indicating that Pep-induced activation of the marker gene in systemic leaves also requires JA/ethylene-mediated signaling. Defensins belong to the only class of peptides that seems to be conserved among plants, invertebrates and vertebrates in the innate immune response (Thomma, Cammue et al. 2002). Some

of them were found to display antifungal or occasionally antibacterial activity *in vitro*. Hiruma et al. 2011 showed that over-expression of *PDF1.2a* and *PDF1.3* in the *Arabidopsis thaliana* mutant *edr1* that allows higher entry rates of non-host *Colletotrichum* species, results in enhanced resistance towards *Colletotrichum gloeosporioides* (Hiruma, Nishiuchi et al. 2011).

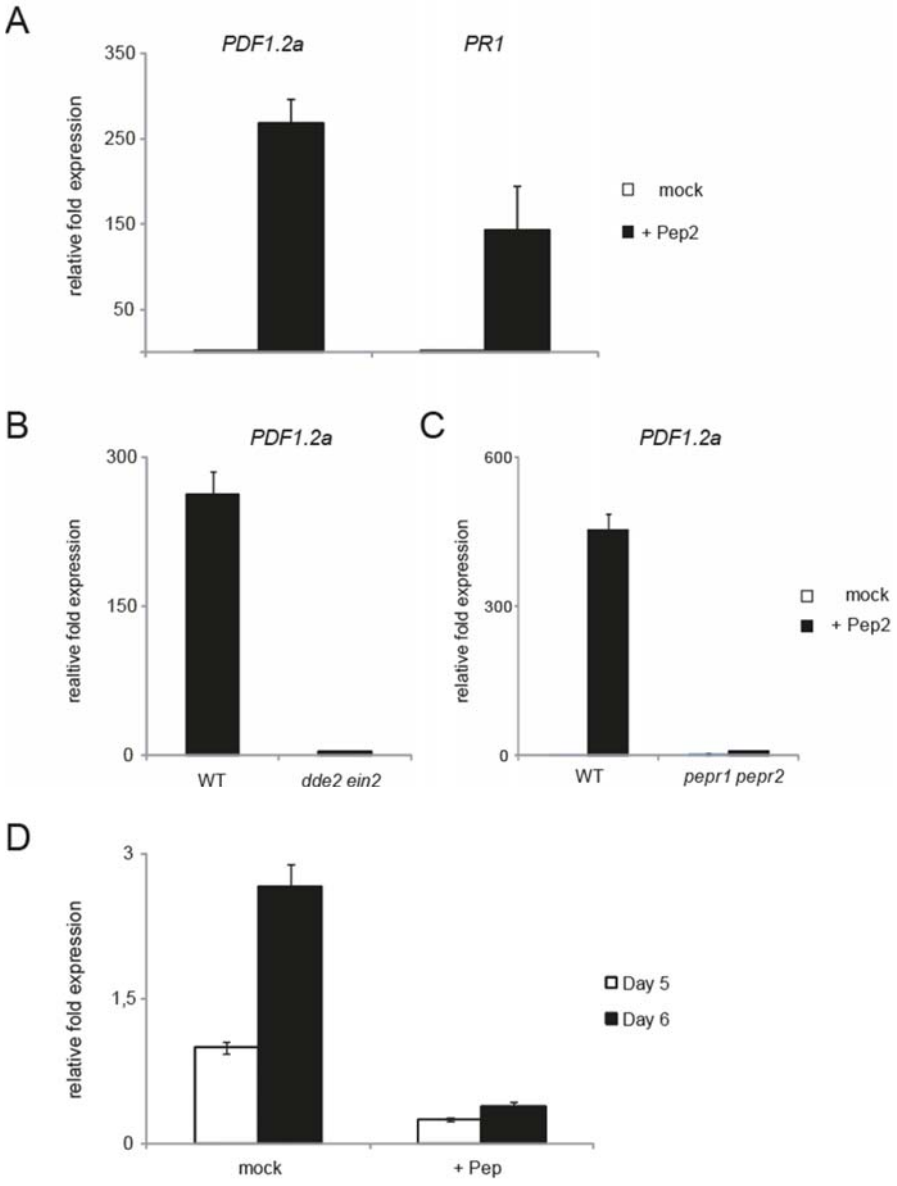

Figure 16: Exogenous application of Pep-Peptides is sufficient to induce systemic gene expression and resistance. A,B,C) Local leaves of four-week-old plants were infiltrated with 1µM Pep2 one day before systemic leaves were harvested for qRT-PCR analysis of *PR1* and *PDF1.2a*. The relative expression is shown in fold, with the gene/At4g26410 in mock treated leaves of WT as 1. D) Local leaves of four-week-old plants were infiltrated with 1µM Pep2 and Pep3 or mock 24 hours before spray-inoculation with 5 x 10^5 spores/mL *Ch path-29*. Systemic leaves were harvested at the indicated time points and subjected to qRT-PCR analysis of fungal biomass. The relative expression is shown in fold, with the *ChACTIN*/At4g26410 in mock treated leaves of WT at 5 dpi as 1.

Hence, this predicts that Pep-induced systemic defensin expression leads to enhanced resistance against *Colletotrichum higginsianum*. Indeed, fungal biomass analysis revealed lower growth rates of *Ch path-29* six days after inoculation on systemic leaves of Pep-pretreated plants in comparison to mock-pretreated ones (Figure 16D).

Taken together the data point to a model in that PEPR-triggered signaling is required for the execution of defense by incorporating the co-activation of SA and JA/ethylene-mediated defense responses in local leaves and thereby establishing systemic immune responses effective against at least hemi-biotrophic pathogens.

3 Discussion

3.1 Identification of molecular components linking initial MAMP-recognition to robust immunity

3.1.1 EFR-mediated sustained transcriptional activation is impaired in *rsw3* plants

Early cellular responses upon pathogen recognition have been studied for many decades. First reports for pathogen-induced ROS generation go back to 1983 (Doke 1983). In 2002, it was finally demonstrated that the *Arabidopsis* NADPH oxidase RBOHD is responsible for nearly all of the ROS produced in response to pathogens and that loss of the enzyme leads to enhanced susceptibility in response to *Pst* DC3000 Δ*AvrPto*/Δ*AvrPtoB* (Torres, Dangl et al. 2002; Mersmann, Bourdais et al. 2010). Another very early output of plant cells in response to MAMPs and pathogens was characterized by Asai et al. in 2002. They identified a complete MAP kinase cascade that functions downstream of the FLS2 receptor. They showed that within minutes after flg22 perception MAP kinases are phosphorylated and that this activation step positively contributes to plant immunity against bacterial and fungal pathogens (Asai, Tena et al. 2002). In the same work it was demonstrated that early induced WRKY22 and WRKY29 transcription factors initiate transcriptional reprogramming for successful defense activation.

In *rsw3* plants rapid elf18-induced responses like the aforementioned generation of ROS, the activation of MAP kinases and the up-regulation of early defense genes like *WRKY22* and *WRKY29* are retained. In addition, EFR-triggered ethylene production as well as callose deposition remain wild-type like. However, the mutant exhibits a supersusceptible phenotype to *Pst* DC3000 and fails to induce elf18-mediated resistance (Lu, Tintor et al. 2009). This finding indicates that despite their wide appreciation as the hallmark events of MTI, the activation of those early defense outputs alone is insufficient to establish a robust defense response.

Further analysis of EFR-triggered transcriptional reprogramming revealed an impairment of the mutant at a rather late phase of defense gene activation (8-24 hpt) (Lu, Tintor et al. 2009). *WRKY22* and *WRKY29* are rapidly expressed upon elf18 recognition and mRNA levels stayed elevated for at least 24 hours in wild-type plants. Interestingly, the continuous expression of the *WRKY* transcription factor genes was not maintained in *rsw3* plants. Furthermore, *PR1* and *PR2*, two typical late-responsive defense marker genes failed to be expressed almost completely at 24 hours after elicitation.

Those data demonstrated the uncoupling of initial and sustained MTI activation in *rsw3* plants. However, it remains unknown which components are responsible or which mechanisms link an initial with a relatively late phase of EFR-triggered immunity. To this end, *rsw3* appeared to be a useful tool to uncover components that are required for maintenance of MTI signaling in *Arabidopsis*.

Transcriptome-wide comparison of wild-type and *rsw3* plants at early (2 hours) and late (10 and 24 hours) stages of EFR-signaling disclosed two major findings: first, early responses to elf18-treatment remain wild-type like in *rsw3* plants and secondly, the sustained up-regulation of several defense-related genes at 10 and 24 hpt were not maintained in the mutant. (Figure 1, page 17). This datasets strengthens our earlier conclusion in Lu et al. 2009. Detailed investigation identified a set of 106 “key genes” that were characterized by at least 2-fold reduced induction in *rsw3* in comparison to wild-type 10 hpt with elf18. Following *in silico* analysis using publicly available transcriptome data revealed that those “key genes” are strongly induced by different pathogens, elicitors and SA (Figure 2, page 18). By comparing a repertoire of virulent, avirulent or disarmed *Pseudomonas* bacteria the involvement of those genes in different layers of immunity could be dissected. The majority of the “key genes” is strongly expressed during non-host resistance, MTI as well as ETI. Surprisingly, none of the 106 genes were induced in systemic non-challenged tissues during SAR pointing to a role of those genes during defense execution upon direct contact to pathogens. Activation of the immune system is costly and achieved at the expense of developmental and physiological processes (van Hulten, Pelser et al. 2006). Therefore, the organism needs to precisely evaluate the need for defense execution. In this respect, expression of these 106 defense-related genes might be associated with costly defense execution and only applicable in the presence of high threats of pathogens, which is not the case during SAR in leave tissue distant from the infection site. However, the failure of *rsw3* plants to express those genes in response to elf18 possibly causes the supersusceptible phenotype of the mutant also in response to bacterial infection.

The *rsw3* allele is predicted to harbor a point substitution within the catalytic domain of the α-subunit of glucosidase II (GIIα). GII acts in the endoplasmatic reticulum during protein quality control (ERQC) by removing glucose residues of client proteins (Anelli and Sitia 2008). As one of GIIα's client proteins EFR folding seems to occur in the ER lumen, and then the protein is transported via the trans-golgi network to the plasma membrane (Anelli and Sitia 2008; Pattison and Amtmann 2009). It remains unclear whether the *rsw3* immune-compromised phenotype is all attributed to misfolding of EFR alone in the presence of GIIα malfunction. On the one hand the receptor accumulates to wild-type like levels and remains functional indicated by induction of early defense outputs. On the other hand,

EFR-dependent ligand binding activity is reduced in *rsw3* plants suggesting that the receptor undergoes improper folding during ERQC caused by the mutation in GIIα (Lu, Tintor et al. 2009). This could influence the durability of an active state of the receptor and consequently affect the maintenance of EFR signaling. Furthermore, the mutation may change the stability of the receptor or receptor complex assembly (Lu, Tintor et al. 2009). It has been reported that EFR associates with BIK1, a receptor-like cytoplasmic kinase that is required for MTI signaling by directly acting downstream of FLS2, EFR and CERK1 (Zhang, Li et al. 2010). BIK1 belongs to the family of PBS1-like (PBL) kinases that are all thought to integrate defense signals from multiple immune receptors. So far, BIK1 has been studied most intensively: *bik1* fails to trigger several MTI responses and to establish MAMP-induced resistance to *Pst* (Zhang, Li et al. 2010). It is believed that upon MAMP recognition BAK1 trans-phosphorylates BIK1 (and probably also the other PBLs) which then phosphorylates both FLS2 and BAK1 to induce MTI signaling. Interestingly, BIK1 is dispensable for MAPK activation pointing to signal branching already at the level of PRR complexes (Feng, Yang et al. 2012). In this respect, improper protein folding of EFR leading to inadequate interaction with cytoplasmic kinases for activation of several defense branches appears likely.

A second possible explanation for the immune-compromised phenotype of *rsw3* is the existence of another client protein(s) affected by GIIα that are important for sustained gene activation during EFR signaling. Comparison of the "key genes" with public available transcript data showed that the majority of the genes are SA-responsive (Table 3, page 30) allowing the hypothetical existence of another ERQC client protein functioning in the regulation of SA-induced immune responses. This is supported by the earlier finding in our group that loss of two other ERQC proteins (UGGT and STT3A) abolishes SA-induced resistance that is independent of EFR (Saijo, Tintor et al. 2009). My data show that *Pst* DC3000-induced SA accumulation reaches wild-type like levels in *rsw3* plants (Supplementary Figure 2, page 99) but it is possible that a potential defect occurs in a SA-regulating process downstream of SA biosynthesis. The work of Kenichi Tsuda and colleagues supports the importance of SA signaling for sustained MTI signaling. According to the discovery that MAMPs induce SA accumulation in local and systemic leaves (Mishina and Zeier 2007), they focused on the interplay between MAMP-triggered and SA-mediated effects on gene expression changes and pathogen growth. They found that a set of genes becomes SA dependent at a later stage (9h) during MTI and that SA signaling contributes to resistance against *Pst* DC3000 *hrcC* and MAMP-triggered resistance to *Pst* DC3000 (Tsuda, Sato et al. 2008). In sum, it remains elusive which GIIα client protein causes the lack of sustained gene activation in the late phase of EFR signaling in *rsw3*.

However, it can be suggested that either improper folding of EFR itself results in perturbations of the signaling response or that another client protein presumable involved in the regulation of SA-mediated defense signaling is affected by the mutation of GIIα.

3.1.2 Genetic characterization of selected candidates involved in EFR-mediated responses

The global comparison of the EFR-triggered transcriptional changes in *rsw3* and wild-type plants revealed a set of "key genes" that fail to be induced in the mutant. Following *in silico* analysis highlighted close association of the expression of those genes for the establishment of robust immunity (3.1.1). We expected that critical mechanism linking initial MAMP responses to a rather late phase of MTI can be found among those 106 miss-expressed genes. In order to identify and characterize putative MTI components, homozygous mutants for 23 of the key genes were investigated for possible defects in EFR-triggered responses.

The 23 candidate mutants were screened in the same conditions for de-repression phenotypes in response to elf18 and flg22 (Table 2, page 19). One mutant (line Nr. 6), encoding for an unknown protein, displayed an insensitive phenotype to both elf18 and flg22. The other 22 mutants responded in a wild-type like manner and suppressed the accumulation of flavonoids in the presence of MAMPs indicating that those genes are not critical for the flavonoid-MAMP crosstalk (Table 2, page 19).

In a next experiment the elf18-induced resistance response of the mutants was examined (Figure 3, page 21). Three lines (Nr. 1, 7 and 23) were strongly compromised in elf18-triggered immunity, pointing to an important role of the three genes for EFR-mediated defense. Line Nr. 1 is mutated in the gene encoding for a FAD-binding domain containing protein. The protein is thought to be secreted and harbor electron carrier as well as oxidoreductase activity. However, the exact mode of action of this protein remains unknown. Similarly, not much is known about the gene that is deleted in line Nr.7. It encodes for a chitinase family protein that has not been in-depth examined yet. The third candidate (Nr. 23) is another yet uncharacterized protein with putative vascular sorting receptor activity. Taken from this first analysis, all three proteins seem to contribute to EFR-mediated immunity. However, detailed investigations in response to MAMPs have to follow in order to elucidate a role of those proteins in MTI.

During the screening procedure, it could be observed that lines 6 and 36, which still suppress bacterial growth upon elf18-pretreatment, showed a hypersusceptible phenotype to *Pst* DC300. Both lines allowed about 5 times more bacterial growth than wild-type plants. As mentioned above the disrupted gene in line 6 encodes for an unknown protein. Therefore, it remains elusive how this

protein contributes to basal resistance against *Pst* DC3000. As mentioned above loss of this unknown gene results in de-repression of anthocyanins in the presence of MAMPs. Surprisingly, this mutant line is also more susceptible than wild-type but still induces enhanced resistance when pre-treated with elf18. The characterization of the gene would contribute to the explanation of the observed phenomenon.

The gene knocked out in line 36 encodes for NIMIN-2, a kinase that was shown to physically interact with NPR1 in a yeast-two hybrid assay and is thus thought to serve for SAR gene expression (Weigel 2001). Nevertheless, to date no more information can be gathered about functionality of NIMIN-2 in plant immunity. Enhanced bacteria growth on *NIMIN-2* knock-out plants illustrates the involvement of this protein in plant basal resistance to *Pst* DC3000. Thus, it is interesting to further study the role of NIMIN-2 in basal and systemic immunity and to elucidate its functional interaction with NPR1.

3.1.3 PBS3 contributes to sustained EFR-mediated defense outputs

Among the genes which were massively upregulated upon elf18-treatment in wild-type but not *rsw3* plants was *PBS3* (Supplementary Table 1, page 96). The gene was initially identified as a requirement for R-gene mediated resistance against the avirulent strain *Pst* DC3000 (*AvrPphB*) (Warren, Merritt et al. 1999). Resistance to this strain by *Arabidopsis* accession Columbia-0 (Col-0) is mediated by the *RPS5* gene (Simonich and Innes 1995), which encodes a member of the nucleotide-binding site Leu-rich repeat (NB-LRR) family of R-proteins (Warren, Henk et al. 1998). The *pbs3* mutant shows enhanced susceptibility to the avirulent bacterial strains *Pst* DC3000 A*vrPphB* as well as to DC3000 strains expressing *AvrRpt2*, A*vrB* or *AvrRps4* and to the normally virulent strains *Pst* DC3000 and *Psm*. It was shown that SA levels in *pbs3* mutants are significantly lower than wild-type plants and that is accompanied by very weak *PR1* gene expression (Warren, Merritt et al. 1999; Jagadeeswaran, Raina et al. 2007). However, a possible role of PBS3 during MTI has not been addressed to date.

During the microarray analysis we found reduced expression of the *PBS3* gene in *rsw3* in response to elf18 which could be confirmed by quantitative qRT-PCR (Supplementary Figure 3, page 99). Based on the analysis of public available gene expression data, *PBS3* seems to be specifically induced by elf18 and only very little, if not at all, by other elicitors like flg22, LPS and OGs. Furthermore, inoculation of a disarmed *Pst* strain lacking the T3SS and therefore inducing MTI-responses alone did not induce *PBS3* transcript induction, either. This is in agreement with the earlier findings from Jagadeeswaran and colleagues who failed to detect *PBS3* mRNA accumulation one day after infiltration of *Pst* DC3000 *hrcC* or *hrcU* (Jagadeeswaran, Raina et al. 2007). The data indicate that *PBS3* is greatly induced during

EFR-triggered immunity but seems to be less in response to other MTI trigger. Loss of *PBS3* during EFR-signaling leads to a very similar phenotype as *rsw3*. The mutant fails to induce elf18-triggered resistance, shows impairment in EFR-mediated gene expression (Figure 5, page 24) but retains wild-type like early responses like MAPK activation or ROS production (Figure 6, page 25). Again, all defects were specifically observed for elf18- but not flg22-induced responses which is reminiscent of *rsw3* (Lu, Tintor et al. 2009).

PBS3 (also referred to as GH3.12) is a member of the GH3 protein family of acyl-adenylate/thioester-forming enzymes that have been shown to conjugate phytohomone acyl substrates (e.g. jasmonates) to amino acids *in vitro* (Staswick, Tiryaki et al. 2002). In the case of PBS3 4-aminobenzoate and 4-hydroxybenzoate (4-HBA) are the preferred acyl substrates that are conjugated with specific amino acids (e.g. Glutamin)(Okrent, Brooks et al. 2009). Even though PBS3 has been studied to quite some extend the actual function of the enzyme or its products in plant defense responses remains speculative. Okrent and Wildermuth suggest that PBS3 acts upstream of SA biosynthesis because exogenous application of SA can rescue the *pbs3* phenotype. Expression profiling data by the group of Jane Glazebrook supported this assumption. The comparison of wild-type plants with a variety of *Arabidopsis* mutants including *pbs3*, placed the enzyme also upstream of SA and NPR1, as many more genes were impacted by *pbs3* than by *sid2*, *eds5* and *npr1* (Wang, Mitra et al. 2008). Okrent and Wildermuth further strengthen this hypothesis by the substrate specificity of the enzyme, with PBS3 being inactive on SA. It seems likely that PBS3 functions early in the defense response when SA levels are still low. In their model PBS3 conjugates the formation of 4-aminobenzoate derivatives which signals or primes SA biosynthesis. Once SA synthesis has been sufficiently initiated the activity of PBS3 will be blocked.

On the basis of this idea it can be speculated that loss of PBS3 leads to delayed SA biosynthesis in *rsw3* that would be crucial for sustained activation of EFR signaling and finally for the establishment of immunity. However, this hypothesis is contrary to the early conclusion of potentially RSW3 clients acting downstream of SA biosynthesis (3.1.1) based on wild-type like SA accumulation upon *Pst* DC3000 infection in *rsw3* plants. Unlike single elf18-induced SA generation, SA accumulation in response to *Pst* DC3000 inoculation is assumingly initiated by several cues including MAMPs and effector recognition. To this end, it is still conceivable that less accumulating *PBS3* transcripts resulting in reduced PBS3 levels and subsequent delayed SA generation causes the EFR-compromised phenotype of *rsw3* plants. SA is an indispensable signaling hormone in plant defense against biotrophic and hemibiotrophic pathogens (Glazebrook 2005). MTI relies on the generation of SA and

linked downstream responses like the induction of *PR*-genes (Tsuda, Sato et al. 2008). Furthermore SA-signaling is essential for the activation and generation of SAR in local and systemic leaves. Conversely, inhibiting SA accumulation compromises the resistance against pathogens and abolishes SAR (Gaffney, Friedrich et al. 1993; Delaney, Uknes et al. 1994; Nawrath and Metraux 1999; Wildermuth, Dewdney et al. 2001; Durrant and Dong 2004). In *pbs3* plants, elf18-mediated *PR1* activation was abolished indicating perturbations during SA signaling by loss of PBS3. In this aspect, disruption of SA signaling by loss of PBS3 is conceivable as a cause of the *rsw3* phenotype.

Another interesting point raised by Okrent and Wildermuth is the upregulation of chalcone synthase (CHS) in *pbs3* upon *Psm* (Wang, Mitra et al. 2008), a key enzyme directing flux to flavonoid biosynthesis from 4-coumaroyl-CoA. This compound on the other side is also a precursor for 4-HBA that is incorporated into the phenylpropanoid pathway for cell wall remodeling upon pathogen recognition (Figure 17) indicating that flux of 4-coumaroyl-CoA needs to be precisely controlled (Gleitz, Schnitzler et al. 1991).

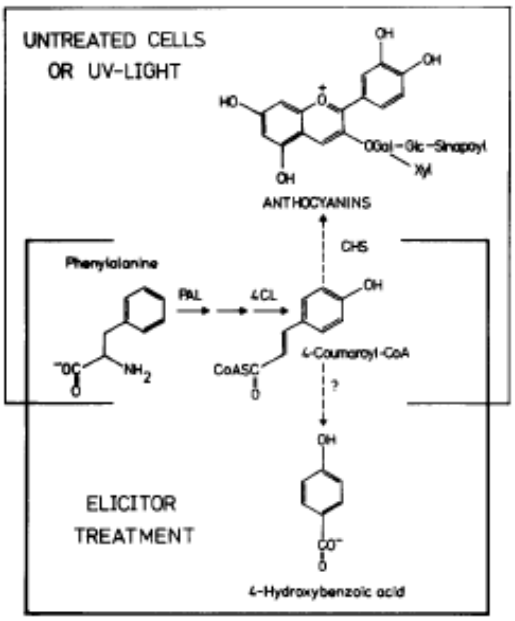

Figure 17: Proposed model of 4-coumaroyl-CoA flux between flavonoid and MTI pathways (Gleitz, Schnitzler et al. 1991).

Therefore, another possible role of PBS3 could be the coordination of pathogen-induced SA, 4-HBA, and their associated responses with 4-HBA playing a role in cell wall modifications and the direction of chorismate flux for this purpose. This model is very attractive in order to explain the de-repression of anthocyanins in *rsw3* in response to elf18 and high sucrose levels.

Inconsistently with this concept, *pbs3* plants allowed normal elf18-induced anthocyanin suppression under our experimental conditions indicating that another protein must serve as a coordinator of the MTI-flavonoid cross talk. Furthermore, *CHS* expression was indistinguishable between wild-type and *rsw3* plants during the time course after elf18 application (Supplementary Figure 5, page 100). Therefore, misregulation of *PBS3* expression during EFR-signaling in *rsw3* does not influence regulation of *CHS*. The data argue against the above proposed function of PBS3 in coordinating flux of 4-coumaroyl-CoA for the anthocyanin or phenylpropanoid pathway.

To this end, the first model is more likely as an explanation of PBS3 function in EFR-mediated immunity by priming or signaling SA biosynthesis through conjugation of 4-HBA. Future studies will be required to test this possibility.

3.1.4 Perspectives

The microarray analysis of *rsw3* plants revealed a set of "key genes" that encode for putative components important for the sustained activation of EFR-mediated MTI. Among the strongest miss-regulated genes that are highly expressed in elf18-elicited wild-type plants are two putative peroxidases. In several reports peroxidases have been described to be involved in plant defense contributing to the generation of ROS, the production of anti-microbial metabolites and the cross-linking of cell wall components to create a physical barrier (lignification, suberization, extension and ferulic acid cross-linking) (Almagro, Gomez Ros et al. 2009). The analysis of mutant line 4, 30 and 39, which are eliminated for a putative peroxidase gene, revealed no striking defects during elf18-induced resistance. However, the possible redundancy of the enzymes could lead to the loss of a detectable phenotype in EFR-mediated defense. The generation of a double mutant could uncover such redundancy and allow the elucidation of peroxidases during late phase EFR-signaling. Along the line it can be suspected that the expression of a whole gene cluster important for sustained EFR-triggered immunity is compromised in *rsw3* and that analysis of single gene knock outs is not conclusive. Moreover, the discovery of common promoter motifs among the "key genes" may allow the elucidation of putative gene clusters involved in linking initial and sustained EFR signaling. Preliminary co-expression analysis (http://atted.jp/index.shtml) also support this suggestion since the most strongly misregulated genes *PROPEP3* and the putative peroxidase At1g14540 are transcriptionally co-regulated with each other and additionally with *PROPEP2* and *MLO6,* two other strongly misregulated candidate genes. Furthermore, co-expression analysis of those and other candidate genes often disclosed a link to the phenylpropanoid pathway in *Arabidopsis* (data not shown), which is associated with the biosynthesis of SA, coumarins and lignin (Fraser and Chapple

2011). Additionally, the flavonoid pathway derives from the phenylpropanoid pathway indicating their close connection (Jiang, Wood et al. 2005). Taken together the data obtained from the transcriptome-wide comparison seem to allow further elucidation of important components and gene groups that activate sustained EFR signaling and possibly act in the MTI-flavonoid cross talk.

In regard to the involvement of PBS3 in EFR-mediated resistance it would be interesting to understand the function of the enzyme during this process and how a putative PBS3 product works as a signaling component for SA biosynthesis specifically during EFR-triggered resistance. To this end detailed biochemical analysis of derivatives of SA during MTI in response to different elicitors might be conclusive. Finally, it would be of great interest to understand the selective requirement for PBS3 in EFR-triggered immunity. In contrast to other MAMP receptors as for example FLS2 which has a high degree of conservation present in all higher plants, elf18-responsiveness can only be found in the class of Brassicacae. Therefore, it is believed that FLS2 has evolved earlier during evolution to function as a PRR. Nevertheless, previous reports demonstrated a high overlap of MTI responses shared by both pathways. Upon MAMP binding the receptors interact with BAK1 and BIK1 for activation (Monaghan and Zipfel 2012). Shortly after, Ca2+ spiking and reactive oxygen species can be detected, MAPKs are activated, ethylene is produced and transcriptional reprogramming occurs almost identically one hour after elicitation (Zipfel 2009). Additionally, pretreatment with elf18 induces an enhanced resistance response to *Pst* DC3000 and loss of EFR leads to hypersusceptibility as reported for *fls2* plants.

On the basis of those findings it was believed that MTI responses are shared by different PRRs already early after ligand binding and that the detection of multiple MAMPs enhances shared downstream responses for robust immunity.

In conflict with this assumption, the discovery of the unique requirement of ER quality control components by EFR demonstrated first evidence for separate requirements between the two PRR signaling pathways already in the generation of the receptor (Lu, Tintor et al. 2009; Saijo, Tintor et al. 2009). Another indirect evidence for differences in effectiveness of the two elicitors was the earlier observed lower potency of EFR-triggered responses to restrict pathogen growth. However, it must be noted that the artificial usage of those synthetically generated peptides might not demonstrate the true nature of those signaling cascades (Kunze, Zipfel et al. 2004)(Figure 4, page 22).

Anyhow, the specific requirement of PBS3 and GIIα suspects the involvement of several more EFR-specific signaling components. Further analysis of the transcript data obtained from the microarray may allow the elucidation of other EFR-specific signaling molecules and help the understanding of the sophisticated network of MTI.

3.2 The role of an endogenous peptide-receptor system in plant immunity

In 2006, the *Arabidopsis*-derived endogenous elicitor peptide Pep1 was discovered and shown to exhibit immune stimulatory functions. Pep1 and its 6 homologs (Pep2-Pep7) were proposed to act as an amplifying mechanism to promote MTI since many defense outputs were reminiscent to those of MAMP-elicitation (Huffaker, Pearce et al. 2006; Huffaker and Ryan 2007). Following investigations demonstrated functional similarities of the Pep-peptides to the solanaceous peptide systemin proposing a model in which Peps act as DAMPs in *Arabidopsis*. First of all, the peptides which are embedded within the C-terminal part of their precursor proteins, designated PROPEPs, are probably cleaved from their precursor protein and released to the apoplastic spaces where they are believed to be perceived by their respective receptors PEPR1 and PEPR2. However, neither the precursor proteins nor the putative bioactive peptides harbor a classical secretion signal for release to the apoplast. None the less, it was shown that the *PROPEP* genes, in particular *PROPEP2* and *PROPEP3*, were induced upon wounding, methyl-jasmonate, ethylene, pathogens and elicitors (Huffaker, Pearce et al. 2006).

In contrast to MAMP-signaling pathways it has never been shown that lack of the Pep/PEPR system diminishes plant immunity. Based on the findings so far it remains speculative whether Peps can really defined as DAMPs and whether the plant immune system requires this signaling pathway for the establishment of a robust defense response.

3.2.1 MTI and basal immunity rely on functional Pep/PEPR signaling

Previous attempts to detect a defense-compromised phenotype of the double-receptor mutant by the use of the virulent strain *Pst* DC3000 have failed in our and other labs (Yamaguchi, Huffaker et al. 2010). We found that *pepr1 pepr2* plants allowed enhanced growth of a less virulent strain of *Pst* DC3000 lacking the two effector proteins AvrPto and AvrPtoB. The effector proteins have been shown to target MAMP receptors FLS2, EFR and CERK1 to block their activity (Goehre, Spallek et al. 2008; Xiang, Zong et al. 2008; Gimenez-Ibanez, Hann et al. 2009). Furthermore AvrPtoB interferes with BAK1 kinase activity that has been shown to be required for PRR signaling (Cheng, Munkvold et al. 2011). Recognition of MAMPs activates MTI responses including the induction of *PROPEP* transcripts (Figure 7, page 27), which are thought to promote the generation of PEPR ligands. MTI-suppression by AvrPto and AvrPtoB could consequently lead to loss of PROPEP up-regulation, therefore to lack of Pep-ligands and subsequently to the abolishment of Pep/PEPR signaling.

Independent of this hypothesis, it is conceivable that AvrPto and/or AvrPtoB target the PRRs PEPR1 and PEPR2 directly to inhibit Pep/PEPR signaling as it has been already reported for EFR, FLS2 and CERK1. This could explain the observed enhanced growth of the *AvrPto*/*AvrPtoB*-deficient bacterial strain in *pepr1 pepr2* plants (Figure 12A, page 36).

Inhibition of BAK1 by AvrPtoB could serve as another explanation for PEPR signaling perturbations. Co-immunoprecipitation experiments showed ligand-induced direct physical binding of BAK1 with PEPR1 and interaction between both Pep-receptors with BAK1 during yeast-two hybrid assays (Postel, Kufner et al. 2010; Schulze, Mentzel et al. 2010). Therefore, blocked BAK1 activity could subsequently inhibit PEPR-mediated defense responses.

Consequently, it seems likely that in the presence of AvrPto and AvrPtoB Pep/PEPR singling is suppressed and that only in the absence of the effectors loss of the otherwise suppressed Pep/PEPR pathway becomes considerably required for full defense acquisition.

Interestingly, *pepr1 pepr2* plants exhibited also enhanced susceptibility towards *Colletotrichum higginsianum (Ch) path-29* (Huser, Takahara et al. 2009) (Figure 12B, page 36). Random insertional mutagenesis upon *Agrobacterium tumefaciens*-mediated transformation of *Ch* revealed 40 fungal mutants of which *path-29* was significantly less efficient in penetration and formed appressoria induced a hypersensitive response without producing visible hypha inside the affected cells. The T-DNA in *path-29* was inserted upstream of an ORF that is homologous to an ATP-binding endoribonuclease, which is possibly encoding for the pathogenicity gene missing in *path-29*. Since it is unknown how this putative virulent factor influences pathogenicity and how loss of the gene leads to an enhanced defense response by the plant in comparison to *Ch* wild-type strains, it also remains unclear how the fungal mutant grows better in *pepr1 pepr2*. It is possible that this ATP-binding endoribonuclease serves to counteract defenses mounted by the PEPR pathway.

The previously published data and models suggest the engagement of Pep-signaling during basal defense (Huffaker, Pearce et al. 2006; Huffaker and Ryan 2007). The results presented in this work (Figure 12, page 36) provide evidence for the critical contribution of the PEPR pathway for basal resistance against the two hemi-biotrophic pathogens *Pst* and *Ch* and the involvement in MAMP-signaling which is further supported by the following findings: (1) *PROPEP2 and 3* genes show robust and prolonged induction upon MAMP-treatment (Figure 7, page 27), (2) *PR1* expression is strongly reduced in *pepr1 pepr2* plants upon elf18 elicitation (Supplementary Figure 11, page 104), (3) the Pep/PEPR pathway is activated downstream of EFR in an ethylene-dependent and independent manner (Tintor, Ross et al. 2012) and (4) Pep-induced defense responses are almost identical to

MAMP-induced responses. (Huffaker and Ryan 2007; Krol, Mentzel et al. 2010; Qi, Verma et al. 2010; Yamaguchi, Huffaker et al. 2010).

3.2.2 Pep/PEPR pathway facilitates co-activation of otherwise antagonizing SA- and JA/ET-dependent immune branches

Activation of *PROPEP2* and *PROPEP3* by MAMPs placed this pathway close to MTI. Furthermore, the immune-compromised phenotype of *pepr1 pepr2* confirms the long suspected assumption that Pep-signaling contributes to basal resistance of *Arabidopsis.* Nevertheless, mechanistic aspects as well as the specific function remain elusive. We conducted a genome-wide comparison of Pep-induced and elf18-mediated transcriptional changes to reveal detailed information about similarities and differences of the two pathways. In agreement with earlier studies a large number of differentially regulated genes were shared by both treatments including the induction of typical defense markers like *FRK1* and *PROPEP3*. *In silico* analysis indicated that the majority of those genes can be induced by SA. However, specifically Pep2-induced genes exhibited high responsiveness to MeJA and ethylene pointing to the co-activation generally antagonistic SA- and JA/ethylene signaling branches during PEPR-mediated immunity (**Figure 9**, page 29). Further confirmation of this interesting finding could be obtained by GO enrichment analysis of all genes that showed ≥2-fold induction by both elf18 and Pep2 treatments. Whereas responsiveness to SA ranks on top of the hormone responses after elf18 application, Pep2 assembles all hormone-responsive enrichment terms starting with JA, ethylene and then SA (Table 3, page 30). Quantitative real-time PCR analysis provided additional evidence for this phenomenon and the requirement of the respective SA and JA/ethylene biosynthesis components (Figure 10A, page31). The hormonal cross- talk between SA and JA signaling has been intensively studied. Most publications report antagonizing effects of the two hormone branches (Bostock 1999; Felton and Korth 2000; Kunkel and Brooks 2002; Thaler, Karban et al. 2002; Glazebrook, Chen et al. 2003; Beckers and Spoel 2006; Koornneef, Leon-Reyes et al. 2008; Spoel and Dong 2008) however also synergistic effects could be obtained (Mur, Kenton et al. 2006; Liu, Kennedy et al. 2007). The composition of the hormonal blend depending on timing and concentrations of the hormones is crucial for either antagonistic or synergistic effects and each mixture can be effective against specific invading pathogens.

Defense against bacterial infections with *Pst* DC3000 have so far been associated with SA-dependent responses and it was shown that pretreatment with the SA-inducing elicitor elf18 can render the plant more resistant against the pathogen. The involvement of the Pep/PEPR pathway in basal

defense against *Pst* Δ*AvrPto* Δ*AvrPtoB* (Figure 12A, page 36) and the related SA/JA co-activation upon Pep-perception (Figure 10, page 31) prompted us to test the requirement of those hormone pathways during Pep-triggered resistance. The lack of *SID2* led to enhanced bacterial growth with or without Pep-pretreatment whereas bacterial growth could generally be restricted by Pep-application in mutants defective in JA/ethylene signaling. This also points to the importance of SA-mediated defense responses in Pep-triggered resistance against bacterial infections. However, co-activation of JA and SA signaling branches has been observed during defense activation against *Ch* which was associated with the simultaneous induction of *PR1* and *PDF1.2a* (Liu, Kennedy et al. 2007). In this respect it is possible that Pep-induced resistance against *Ch* would require both, SA and JA branches. Therefore, enhanced susceptibility of *pepr1 pepr2* towards *Ch path-29* (Figure 12B, page 36) might be also based on the lack of JA-dependent defense outputs.

As mentioned above, in contrast to antagonistic effects of JA and SA signaling, synergistic activation of the hormone branches have been rarely described. In 2010 the group of C. Pietersé presented a mechanism where synergistic effects of ethylene on JA were responsible for counteracting the antagonism of SA (Leon-Reyes, Du et al. 2010). Analysis of recently published mutants defective in several phytohormone pathways (Tsuda, Sato et al. 2009) in response to Pep2 treatment demonstrated the use of this hormone signaling connectivity by the Pep/PEPR system for co-activation of SA and JA branches. In the presence of ethylene, SA failed to inhibit JA-dependent *PDF1.2a* induction whereas loss of ethylene led to repression of *PDF1.2a* expression. However, the induction of the gene *per se* did not require ethylene since *PDF1.2a* was again induced upon simultaneous removal of both SA and ethylene signaling branches together. Therefore, the presence of JA alone was sufficient for *PDF1.2a* mRNA accumulation (Figure 11, page 34). However, the synergistic effect of ethylene is prominent for the potentiation of JA but negligible for SA signaling since loss of ethylene did not affect Pep-induced *PR1* accumulation (Tintor, Ross et al. 2012). In this respect ethylene seems to allow JA-mediated signaling to overcome the repressive function of SA during PEPR signaling. Of note, the synergistic effect by ethylene on JA does only effect activation of the ERF branch of JA signaling whereas the MYC branch stays suppressed. Transcript levels of the MYC branch marker gene *VSP2* were strongly suppressed during Pep-signaling (Supplementary Figure 12, page 105). Furthermore, *VSP2* expression was constitutively elevated in *ein2* plants even in the absence of Pep2, indicating that PEPRs require ethylene to repress the MYC branch of JA signaling while activating the ERF branch together with SA-mediated responses.

3.2.3 The PEPR pathway acts at the infection site and confers systemic acquired resistance

The defense response of plants is not limited to the side of infection. Upon pathogen attack MTI and ETI are activated to restrict pathogen growth and moreover to alarm distant tissue for subsequent infections. This systemic defense activation, called systemic acquired resistance (SAR), allows a much faster and effective immune response (Durrant and Dong 2004). Former investigations have shown that SAR can evolve from a single local stimulus like flg22 perception, the central SAR-establishing component SA and by pathogens (Ross 1961; Metraux, Signer et al. 1990; Mishina and Zeier 2007). Furthermore, generation of systemic immune responses is not restricted to pathogen responses but can also be induced by wounding or commensal root bacteria (Green and Ryan 1972; Vanpeer, Niemann et al. 1991). A unique feature shared by all systemic acquired resistance responses is the establishment of broad-spectrum resistance (Dempsey and Klessig 2012). To date it is poorly understood which molecules serve as systemic signals and how a broad-range resistance can be achieved. Our data reveal a regulatory function of the PEPR system in the generation of SAR as evident with the reduced systemic expression of defense marker genes *PR1* and *PR2* in *pepr1 pepr2* plants as well as by enhanced bacterial growth in systemic leaves following an immunizing infection in local tissue (Figure 13, page 38).

Interestingly, the abolishment of SAR in *pepr1 pepr2* plants is not coupled to reduced levels of SA. As a central signaling component SA is indispensable for the establishment of SAR in both local and systemic leaves and mutants exhibiting a SAR-deficient phenotype are often defective in SA signaling (Cao, Bowling et al. 1994; Delaney, Friedrich et al. 1995; Glazebrook, Rogers et al. 1996; Shah, Tsui et al. 1997). Thus, the PEPR pathway might represent a unique SA-independent signaling pathway for acquiring systemic plant immunity.

It is tempting to speculate that the small mature Pep-peptides could serve as a systemic signal itself. However, the reported positive feedback amplification of the PEPR pathway by perception of Pep-peptides should then allow the detection of the precursor peptides in systemic leaves (Figure 14, page 40) which could not be observed. Accumulation of *PROPEP2* and *PROPEP3* mRNA as well as the protein could exclusively be detected in local leaves (Figure 15, page 41) placing active PEPR-signaling around the infection site. Whereas SA needs to be generated in both tissue types for contribution to local and systemic defense (Malamy, Carr et al. 1990), the Pep/PEPR pathway seems to predominantly act at local sites to contribute to the generation of the systemic signal.

Activation of FLS2 signaling by exogenous flg22 application is sufficient to induce systemic SA accumulation, defense gene induction and finally systemic acquired resistance (Mishina and Zeier

2007). In analogy to this, the capability of Pep/PEPR signaling to trigger systemic responses was examined. Treatment of local leaves with Pep-peptides induced systemic gene expression of *PR1* and *PDF1.2a* that was dependent on the JA/ethylene branch for *PDF1.2a* induction (Figure 16A, page 43). Thus, PEPR-mediated defense activation involves not only the co-activation of JA/ethylene and SA branches in local leaves but also propagates this signal to distant tissues. In addition to transcriptional changes, local PEPR activation confers systemic resistance against *Ch* (Figure 16D, page 43) implying its capability for SAR generation.

For many years the SAR phenomenon has been associated with SA-dependent signaling outputs, till in 2007 the early and temporally separate induction of JA signaling was reported to be required (Truman, Bennett et al. 2007). In contrast, the activation of induced systemic resistance (ISR) by a root colonizing bacteria was shown to be JA and ethylene dependent only, whereas ISR by the plant growth-promoting fungus *Phoma* sp. GS8-3 involved the simultaneous activation of the SA and JA marker genes *PR1* and *PDF1.2,* indicating the involvement of both hormone branches during this response (Sultana F 2008). Future studies will be required to reveal whether the activation of different hormone defense pathways is simultaneously induced or temporally separated during SAR. In agreement with this idea, SAR results in the generation of a broad-spectrum resistance (Durrant and Dong 2004; Van Wees, Van der Ent et al. 2008).

The *PROPEP* genes respond to several stress stimuli including the hormones SA, JA, ethylene, MAMPs, pathogens themselves and also wounding (Huffaker, Pearce et al. 2006) and consequently activate SA- and JA/ethylene-dependent defense responses. It is conceivable that a role of this system is to amplify defense signaling to establish basal and systemic immunity and possibly contributing to the generation of broad-spectrum resistance.

Several reports have compared the endogenous Pep-peptides with the wound-responsive endogenous and solanaceous-specific peptide systemin. Systemin was found to act in local leaves during wound-induced systemic resistance (WIR) in response to herbivore feeding (Li, Williams et al. 2002). The peptide is cleaved from the C-terminal end of a larger precursor protein called prosystemin which is expressed in the phloem parenchyma (Narvaez-Vasquez and Ryan 2004). The smaller, 18- amino acid long peptide is believed to be recognized by a putative transmembrane receptor of companion cells to activate the octadecanoid pathway for JA biosynthesis. During the establishment of WIR, JA serves as the systemic signal traveling through the vascular system to distant leaves for initiation of wound-responsive defense outputs (e.g. proteinase inhibitor (PI) expression) (Fisher 1990; Farmer, Johnson et al. 1992; Zhang and Baldwin 1997; Stenzel, Hause et al.

2003). Tomato mutants (*spr1* plants) impaired in systemin signaling show stronger impairment in systemic wound-induced PI expression than local PI expression defining the role for systemin as a local mediator of JA synthesis to strengthen the systemic response (Lee and Howe 2003; Schilmiller and Howe 2005). Furthermore, grafting experiments with *spr1* plants demonstrated that PI expression was abolished in local and systemic leaves when the rootstock derived from *spr1* plants and the scion from wild-type plants, indicating the necessity of systemin signaling in local tissues. In contrast, when *spr1* scions were grafted on wild-type rootstocks, PI expression could be detected in local and systemic leaves. Those findings clearly point to a model in which systemin acts at or near the wounding site contributing to WIR (Lee and Howe 2003).

The commonalities between the systemin- and Pep- signaling pathways have led to a model in which the PEPR pathway contributes to SAR in *Arabidopsis* in a similar manner as systemin in tomato. Our findings indicate that the PEPR pathway becomes active in close proximity to the infection site (cross-ref) but which components underlie PEPR-mediated SAR activation remains elusive. Recent investigations have identified several small molecules such as azelaic acid, dehydroabietinal, and pipecolic acid which are required for SAR generation. The hormones methyl salicylate as well as jasmonic acid are also believed to function in long distant signaling of SAR (Dempsey and Klessig 2012). It will be of great interest to elucidate the components and underlying mechanisms of Pep-induced SAR.

Unlike systemin *PROPEP*-related proteins seem to be present in numerous species of dicots and monocots. In 2011 Alisa Huffaker identified *Zm*Pep1, the ortholog of *At*Pep1 in *Zea mays*. The peptide activates *de novo* synthesis of JA and ethylene, induces the expression of defense genes and activates defense to the causal agent of southern leaf blight (*Cochliobolis heterostrophus*) and anthracnose stalk rot (*Colletotrichum graminicola*). The authors propose that the Pep family has conserved functions across plant species as endogenous regulators of innate immunity (Huffaker, Dafoe et al. 2011).

3.2.4 Perspectives

The presented data of this work analyzing the function of Pep/PEPR signaling during plant immunity, evidentially illustrate the requirement of this signaling system for basal and systemic defenses. Our findings propose a model in which pathogen recognition elicits PEPR-mediated outputs, which in turn contribute to defense execution by facilitating co-activation of both SA and JA/ethylene signaling

branches in local leaves. Furthermore, Pep perception is required for the generation of systemic immunity which again is based on the dual activation of SA and JA/ethylene branches (Figure 17).

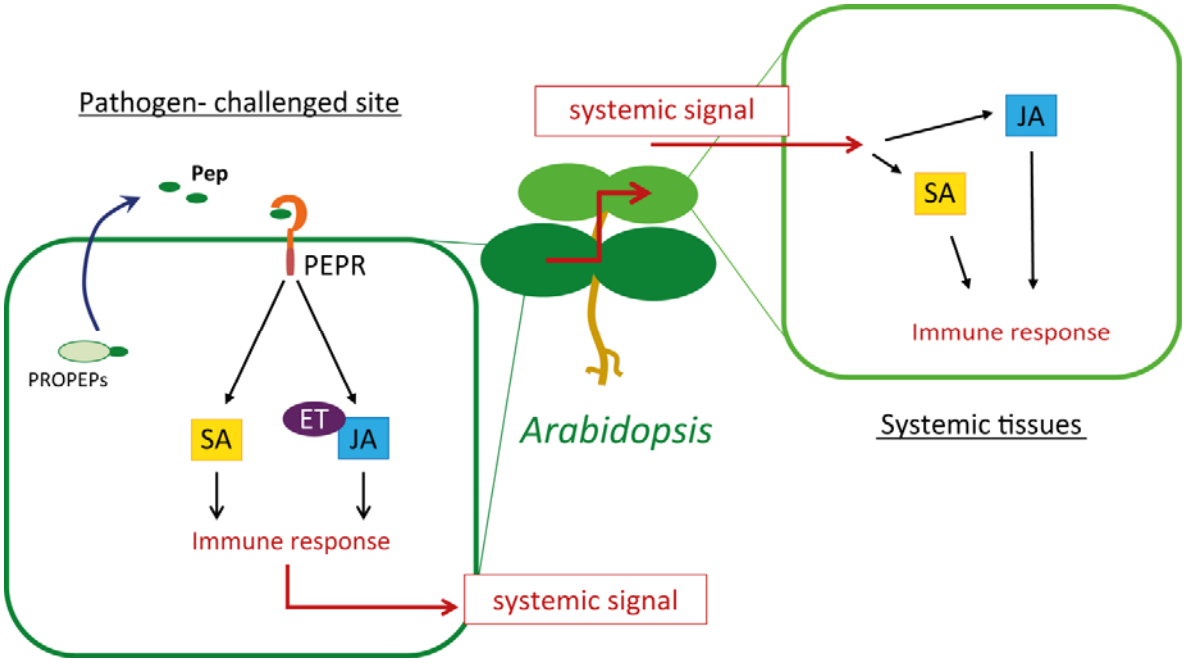

Figure 18: Model of Pep-signaling in plant immunity. The biosynthesis of PROPEPs is induced upon pathogen challenge in local leaves of the plant. After processing of the precursor proteins, the putative bioactive Pep-peptides are released to the apoplastic spaces where they bind to their cognate receptors initiating PEPR signaling. Subsequently, SA- and JA/ethylene-signaling branches are activated directly contributing to local resistance. Furthermore, PEPR-mediated signaling activation contributes to the generation of a mobile signal which in distant tissue allows an immune response based on dual activation of SA and JA/ethylene branches even in the absence of an activated PEPR system.

It can be presumed that the PEPR system which is activated not by a single stimulus but by many pathogen associated signals, acts as an amplification system for the generation of a systemic signal as is it proposed for the wound-induced endogenous peptide systemin in solanaceous plant species.

It will be of great interest to learn if the PEPR pathway is engaged in several immunizing systemic responses (e.g. WIR, ISR) and if broad-spectrum resistance relies on the functionality of this system.

The general belief of Peps acting as DAMPs can further be supported by this work. Possibly, Peps are released upon pathogen damage and consequently contribute to basal resistance and to SAR, similarly to MAMPs (Mishina and Zeier 2007). In addition, the finding that this system predominantly acts in local tissues upon direct contact with the pathogen, rather than in systemic non-challenged tissues, strengthens the model of a DAMP signaling system.

However, it still remains elusive whether and how the Pep-peptides are generated from their precursor peptides, how they are released by the cell without harboring a typical secretion signal and how they are subsequently received by their cognate receptors. First evidence argues against the need of PROPEP cleaving for the release of the mature bioactive Pep-peptide. Structural similarities between PROPEP1 and IL-13, an interleukin, have been noted. Interleukins are inflammatory peptide cytokines which or processed and exported through a leaderless secretion pathways upon MAMP recognition by Toll-like receptors (Medzhitov 2010). Furthermore, prediction models suggest export of PROPEP1 through leaderless secretion (Yamaguchi and Huffaker 2011). In addition, co-immunoprecipitation experiments in tobacco plants demonstrated the binding and functional induction of BAK1-PEPR1 interaction by the apparently full length precursor protein (Supplementary Figure 10, page 104). However, tobacco plants naturally lacking this specific Pep/PEPR system may not reflect the actual mechanism. It is imaginable that in this plant species the protein is not cleaved and that subsequently the bioactive C-terminal end as part of the whole precursor peptide can still bind to the receptors. Future biochemical and cell-biological work will hopefully elucidate the precise steps of Pep-ligand generation, detection and signaling.

4 Materials and Methods

4.1 Materials

4.1.1 Plant material

Arabidopsis thaliana (*A.th.*) wild-type Col-0 and the listed mutants in Table 4 and Table 5 were used in this study.

Table 4: Mutants used in this study

Mutant allele	Accession	Mutagen	Source/Reference
rsw3	Col-0	EMS	R.E. Williamson, Australia
efr-1	Col-0	T-DNA	C. Zipfel (2006), GB
efr-1 fls2	Col-0	T-DNA/SAIL	C. Zipfel (2009), GB
pbs3-1	Col-0	EMS	R.W. Innes, USA
pbs3-2	Col-0	T-DNA	R.W. Innes, USA
pbs3-3	Col-0	transposon insertion	R.W. Innes, USA
mlo2-5 mlo6-2 mlo12-1	Col-0	T-DNA/T-DNA/transposon	R. Panstruga, GER
pepr1-1 pepr2-1	Col-0	T-DNA	C. Ryan (2010)
pepr1-2 pepr2-2	Col-0	T-DNA	C. Ryan (2010)
pepr1-1 pepr2-3	Col-0	T-DNA	B. Kemmerling, GER
pen2-1	*gl1*	EMS	V. Lipka (2005), GER
dde-2	Col-0	transposon insertion	B. Keller (2002), CH
ein2-1	Col-0	EMS	J. Ecker (1999), USA
pad4-1	Col-0	fast neutron mutagenesis	J. Glazebrook (1999), USA
sid2-2	Col-0	fast neutron mutagenesis	F. Ausubel (2001), USA
dde2 ein2	Col-0	transposon/EMS	K. Tsuda (2009), GER
pad4 sid2	Col-0	fast neutron mutagenesis	K. Tsuda (2009), GER
ein2 pad4 sid2	Col-0	EMS/fast neutron mut.	K. Tsuda (2009), GER
dde2 ein2 pad4 sid2	Col-0	transp./EMS/fast neutr. mut.	K. Tsuda (2009), GER

Table 5: Characterized mutant lines in this study

Nr.	Accession	AGI Code	NASC Nr.	Order Nr.	Annotation
1	Col-0	AT1G30700	535214	SALK_035214	FAD-binding domain-containing protein
4	Col-0	AT1G14540	655479	SALK_044730C	anionic peroxidase, putative
6	Col-0	AT1G25400	593560	SALK_093560	similar to unknown protein
7	Col-0	AT2G43620	657631	SALK_056680C	similar to glycoside hydrolase family 19 protein
10	Col-0	AT4G11170	666727	SALK_091592C	disease resistance protein (TIR-NBS-LRR), put.
15	Col-0	AT3G10930	873312	SAIL_155b_E09	similar to unknown protein
23	Col-0	AT1G30900	873290	SAIL_338_H03	vacuolar sorting receptor, putative
25	Col-0	AT1G02450	668591	SALK_086460C	NIMIN-1/NIMIN1; protein binding
27	Col-0	AT5G46050	664402	SALK_003119C	*ptr3-2*
28	Col-0	AT5G46050	660209	SALK_138430C	ATPTR3/PTR3
30	Col-0	AT4G36430	574491	SALK_074491	peroxidase, putative
36	Col-0	AT3G25882	648447	SALK_148447	NIMIN-2 (NIM1-INTERACTING 2)
39	Col-0	AT5G39580	322914	GK-287E07	peroxidase, putative
41	Col-0	AT4G01700		GK-344H04	chitinase, putative
45	Col-0	AT2G36690	365745	GK-068E11	oxidoreductase, 2OG-Fe(II) oxygenase family
45!	Col-0	AT4G20000	835497	SAIL_793_H05	VQ motif-containing protein
46	Col-0	AT5G10760	654836	SALK_111104C	aspartyl protease family protein
49	Col-0	AT5G03350	671553	SALK_074760C	legume lectin family protein
58	Col-0	AT2G43511	662058	SALK_033910C	ATTI1
61	Col-0	AT5G22570	858252	WiscDsLox489-492C21	WRKY38

4.1.2 Pathogens

4.1.2.1 *Pseudomonas syringae*

All *Pseudomonas syringae* strains that are used in this study are listed in Table 6.

Table 6: *Pseudomonas* strains used in this study

strain	Resistance
Pseudomonas syringae pv. *tomato* (*Pst*) DC3000	Rif
Pst DC3000 Δ*AvrPto* Δ*AvrPtoB*	Rif/Kan
Pst DC3000 (*AvrRpm1*)	Rif/Kan
Pseudomonas syringae pv. *maculicola (Psm)*	Spec

The bacteria were grown on NYG medium containing the respective antibiotic(s) at 28°C.

4.1.2.2 *Colletotrichum higginsianum*

The *Colletotrichum higginsianum* strain *path-29* was used in this study ((Huser, Takahara et al. 2009)). The fungus was grown on Mathur's media at 25°C in continuous light conditions.

4.1.3 Oligonucleotides

Oligonucleotides used in this study are listed in Table 7, Table 8 and Table 9 and were purchased from Sigma (Deisenhofen, Germany), metabion international AG (Martinsried, Germany) or Eurofins (Ebersberg Germany).

Table 7: Oligonucleotides used for genotyping

Nr.	AGI-Code	Forward	Reverse
1	AT1G30700	TTTTTGTCAAATGTCAAGAATGC	TCAAAAGACCGTCAGGACAAC
4	AT1G14540	GATTCATTCACATCCCACCAC	CTCACACATTAGGGCAAGCTC
6	AT1G25400	TCCAGCTAATTGTCTTTTCCG	GTAATTACGACAGCGACTCCG
7	AT2G43620	ATGACCGACATGGAATTTCTC	AGTCAAGAGAGGGAGTCCGAG
10	AT4G11170	CCAAACTCATAACAAATTCAAAATG	TAAGGTATGCCTTGTGCTTGG
15	AT3G10930	TAAAGTTGCACCGCAATATCC	GGAACAAACTCTCCGGGTAAG
23	AT1G30900	CTGCAAGATGCTCGATTAACC	ATTTCCCCAACCAATTCATTC
25	AT1G02450	ACAAGTGGCCGACAATATGAG	TCCTTGTGATCCGAAACAAAC
27	AT5G46050	TAAGCTAGAAGCGATCGGTTG	GAACAGTGTTGGCGAAGAGAG
28	AT5G46050	AGTCCTGCATTTTGTGACTCTG	TTTTCCCAACATAGTTGATATTGG
30	AT4G36430	GTACCCGTACATCACACGGAC	ACTGGCAAACTTTTGCTTCTG
36	AT3G25882	CGACATTGACGGAAAATTCAG	AGAAGGGGAAAAACATGAAGG
39	AT5G39580	AAAGTTTAACGCCTGCTAAAACAA	TTCGATGCCAAGGAAACTCT
41	AT4G01700	ACAATAGTTCAATGCCCCATG	TTTTGTTTTGATTCCGCAGTC
45	AT2G36690	TTAATTATCTAGGGCGAGGGC	ATGGTGAATCTTGCGTGATTC
45!	AT4G20000	ATGTTAATGGCGTTGAAGCAC	GACGTTATCTCCATTTTTCGC
46	AT5G10760	TTCACCGCAACCAAAGTAAAC	GACTTGGAGTCAGTCGTCTCG
49	AT5G03350	TTGGGATGCAAAGCAAATTAC	AATTCGACAGCAAAGATGTGG
58	AT2G43511	ACAACAATGAGGCAACCAAAC	TTCCTGTGGAAACCAACAAAG
61	AT5G22570	ATTTGGTAAACCCAAATTGGC	CGATGAAGGAGGATAAGAGCC

Table 8: Oligonucleotides used for quantitative real-time PCR

Gene	AGI Code	Forward	Reverse
AOC3	AT3G25780	CTGAAAAGAGCGGTGACAGAT	CACCAGTGACAGCGAGGAAC
ExPro	AT4G26410	GAGCTGAAGTGGCTTCCATGAC	GGTCCGACATACCCATGATCC
FRK1	AT2G19190	CTTGACCCCGAGTACTATTCGAC	CCTGACATGATCACTTATATGCACCT
LOX2	AT3G45140	TACTTTCCCAACCGACCAAC	CCTGTTTCTGCGATGGGTAT
PBS3	AT5G13320	ACACCAGCCCTGATGAAGTC	CCCAAGTCTGTGACCCAGTT
PDF1.2a	AT5G44420	ACGCACCGGCAATGGTGGAA	TGCATGATCCATGTTTGGCTC
PDF1.3	AT2G26010	AAGCACCGATAATGGTGGAAGCAC	GTATAATTGGTAGTCATTGGTAGC
PR1	AT2G14610	GGTAGCGGTGACTTGTCTGG	AAGGCCCACCAGAGTGTATG
PR2	AT3G57260	ATGGGAGACACGGCCAACAT	CGACACCACGATTTCCAACG
PROPEP2	AT5G64890	AGAAAAGCCTAGTTCAGGTCGTC	CTCCTTATAAACTTGTATTGCCGC
PROPEP3	AT5G64905	GTTCCGGTCTCGAAGTTCATC	ATCTTCCTCGCTGTGTGATGAC
VSP2	AT5G24770	TCAGTGACCGTTGGAAGTTGTG	GTTCGAACCATTAGGCTTCAATATG
Ch-ACTIN	AB495246	CTCGTTATCGACAATGGTTC	GAGTCCTTCTGGCCCATAC

Table 9: Oligonucleotides for cloning of *PROPEP2* and *PROPEP3*

Gene	AGI Code	Forward	Reverse
PROPEP2	AT5G64890	ATCCTCCTTATAAACTTGTATTGCCGCG	CACCCACAGGTTGGAAGCTCTCAAAGC
PROPEP3	AT5G64905	CACCGCACTTTAAGTTACATTGTTTAGTC	ATTGTGTTTGCCTCCTTTTCTGAAC

4.1.4 Enzymes

Restriction enzymes were purchased from New England Biolabs (Frankfurt/Main, Germany) and Fermentas (St. Leon-Rot, Germany). Restriction digestions were performed following the manufacturer's recommendations, using the provided 10 x reaction buffer. Standard and quantitative RT-PCR reactions were performed using home-made Taq DNA polymerase or Taq DNA polymerase from Ampliqon (Odense, Denmark), respectively.

4.1.5 Chemicals and Antibiotics

Laboratory grade chemicals and reagents were purchased from Sigma-Aldrich (Deisenhofen, Germany), Roche (Mannheim, Germany), Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Invitrogen (Karlsruhe, Germany) and Serva (Heidelberg, Germany) unless stated otherwise.
Antibiotic stock solutions (1000x) were prepared as indicated and stored at -20°C (Table 10).

Table 10: Antibiotics used in this study

Antibiotic	Short term	Concentration	Source
Rifampicin	Rif	100 mg/mL in DMSO	DUCHEFA BIOCHEME
Kanamycin	Kan	50 mg/mL in H_2O	Sigma
Streptamycin	Strep	50 mg/mL in H_20	Sigma

4.1.6 Elicitors

As elicitor-active surrogates the peptides flg22, elf18, Pep1, Pep2 and Pep3 were used. The elicitors flg22 and elf18 were identified from bacterial flagellin and elongation factor Tu, respectively. Pep1, Pep2 and Pep3 were synthesized according to the putative bioactive sequence of *Arabidopsis* derived endogenous peptides. Peptides were synthetized by EZBiolab Inc. (Carmel, USA) or JPT Peptide Technology (Berlin, Germany) with the following sequences:
flg22 – QRLSTGSRINSAKDDAAGLQIA

elf18 – AcSKEKFERTKPHVNVGTIG

Pep1 – ATKVKAKQRGKEKVSSGRPGQHN

Pep2 - DNKAKSKKRDKEKPSSGRPGQTNSVPNAAIQVYKED

Pep3 - EIKARGKNKTKPTPSSGKGGKHN
The peptides are described in (Felix, Duran et al. 1999; Kunze, Zipfel et al. 2004; Huffaker, Pearce et al. 2006; Huffaker and Ryan 2007).

4.1.7 Antibodies

Anti-GFP antibody was purchased from Invitrogen, anti-HA from Roche and anti-Flag M2 from Sigma. PROPEP2 antibodies were generated using the two portions of PROPEP2 (the amino acid residues 36-51 CQPAKKTAPSPVTFNQ [1,72 kDa] and 85-109 TNSVPNAAIQVYKED [1,65 kDa]) as an antigen. The amino acid residues of PROPEP3 (amino acid residues 1-12 MENLRNGEDNGS [1,34 kDA] and 82-96

KTKPTPSSGKGGKHN [1,52 kDa]) were used to generate antibodies for PROPEP3. Anti-phospho p44/p42 MAPK antibody that specifically recognizes an active MAPK form was purchased from Cell Signaling Technology.

4.1.8 Media, Buffers and Solutions

4.1.8.1 Media

Media were sterilized by autoclaving at 121 °C for 20 min. For the addition of antibiotics and other heat labile compounds the solution or media were cooled. Heat labile compounds were sterilized using filter sterilization units prior to addition.

Pseudomonas syringae media

NYG broth:

Bactopeptone	5 g/l
Yeast extract	3 g/l
Glycerol	20 ml/l
pH 7.0	

For NYG agar plates 1.05% (w/v) bacto agar (Becton, Franklin Lakes, USA) was added to the above broth.

Colletotrichum higginsianum media

Mathur's broth:

Glucose	2,8 g
MgSO4 7H2O	1,2 g
KH2PO4	2,7 g
Mycological peptone	2,2 g

For Mathur's agar medium 3% (w/v) bacto agar (Becton, Franklin Lakes, USA) was added to the liquid medium.

Arabidopsis thaliana media

½ MS (Murashige & Skoog medium incl. Vitamins and MES-buffer DUCHEFA BIOCHEME #0255.0050)
Sucrose 8 g/l
pH 5.8

For MS agar plates 0.8 % (w/v) plant agar (Duchefa, Haarlem, Netherlands) was added to the above described medium.

4.1.8.2 Buffers and solutions

Buffers and solutions used in this study were prepared in dH_2O and aqueous solutions were sterilized by autoclaving at 121 °C for 20 min.

Edwards Buffer
200 mM Tris-HCl pH 7.5
250 mM NaCl
25 mM EDTA
0.5% SDS

4 x Lower buffer (1.5M Tris·HCl, pH 8.8, 0.4% SDS)
181.7 g Tris base
40 mL 10% SDS
Fill up with water to 1 L. Adjust the pH with HCl.

4 x Upper buffer (0.5M Tris·HCl, pH 6.8, 0.4% SDS)
60.6 g Tris base
40 mL 10% SDS
Fill up with water to 1 L. Adjust pH with HCl.

4 x Running buffer stock (RBS)
60 g Tris base
288 g glycine
Fill up with water to 5 L.

PBS buffer
80 g NaCl
2 g KCl
14.4 g Na_2HPO_4
2.4 KH_2PO_4
pH 7.4 (HCl)

PBST buffer
PBS buffer containing 0.1% Tween20.

Protein lysis buffer (MAPK assay)
50 mM Tris pH 7.5
200 mM NaCl
1 mM EDTA
10 mM NaF
25 mM beta-glycerophosphate
2 mM sodium orthovanadate
10 % (w/v) glycerol
0.1 mM Tween20
0.5 mM DTT
1 mM PMSF
1x complete protease inhibitor cocktail (Roche, Mannheim, Germany)

10 x PCR buffer
100 mM Tris-HCl pH 8.4
500 mM KCl
20 mM $MgCl_2$

6x loading buffer
7 mL 4x Upper buffer
3 mL glycerol
1 g SDS
0.93 g DTT
Bromphenol blue

1 x Running buffer (RB)
1 L RBS
3 L H_2O
40 mL 10% SDS

Ponceau S staining solution
ATX Ponceau S (Fulka) 1:5 in dH_20

1 x Semi-dry transfer buffer (1 L)
5,8 g Tris-Base
2,9 g Glycin
0,37 g SDS (3.7 mL 10% SDS)
200 mL Methanol

Protein lysis buffer (PROPEP detection)
1 mM DTT
20 mM HEPES pH 7.5
1 mM EDTA
13 % Sucrose
1x complete protease inhibitor cocktail (Roche, Mannheim, Germany)

4.2 Methods

4.2.1 Maintenance and cultivation of *Arabidopsis* plants

Arabidopsis seeds were germinated by sowing directly onto moist compost (Stender, Schermbeck, Germany) and grown in a controlled environment growth chamber and maintained under short day conditions (10 h light/14 h darkness, 23 °C during light period, 20 °C during darkness and 60 % humidity). For pathogen treatment plants were grown for four weeks and then used for inoculations. Subsequently they were transferred to growth chambers designated for the respective pathogen.
For elicitor-triggered gene expression assays seeds were surface sterilized by washing with 70% ethanol for 1 minute, following 1 minute washing with 1% bleach and three rounds of washing with sterilized water. After two days of stratification at 4°C seedlings were grown on ½ MS agar plates for 5 days and subsequently transferred to ½ MS liquid medium and grown under controlled conditions (10 h light/14 h darkness, 21 °C during light period, 21 °C during darkness and 70 % humidity).

4.2.1.1 Plant transformation

A genomic DNA sequence including the promoter sequence of the *PROPEP2* and *PROPEP3* locus (At5g64890 and At5g64905) were subcloned into the binary vector pAM-PAT containing the VENUS protein at the C-terminal end of the gene. The promoter sequence of PROPEP2 included 1010 base pairs (bp) upstream of the start codon, for PROPEP3 the promoter region contained 1694 bps. The constructs were subsequently transformed in Col-0 via *Agrobacterium*-mediated transformation (Clough and Bent, 1998).

4.2.2 Pathogen infection assays

4.2.2.1 *Pseudomonas syringae*

4.2.2.1.1 Bacterial growth assay

Pseudomonas syringae pv. *tomato* DC3000 (Δ*AvrPto* Δ*AvrPtoB*) bacteria were grown on NYG (Rif/Kan) liquid media over night at 28°C. Cultures were collected, washed once and resuspended in sterile 10

mM $MgCl_2$. Then 2 to 3 well-expanded leaves of 8 plants per genotype were infiltrated with the bacteria solution at a concentration of 1 x 10^5 cfu/mL. Three days after inoculation, a leaf disc (5 mm diameter) was excised from 12 representative leaves. These 12 leaf discs were separated into 3 pools and then used to determine bacterial titers as follows: leaves were ground in 10 mM $MgCl_2$. After grinding the samples were thoroughly vortex-mixed and diluted 1:10 serially. Samples (10 µL out of 1 mL) were plated on NYGA (Rif/Kan) solid medium. Plates were placed at 28°C for 2 days and thereafter the colony-forming units counted. Bacterial infections were performed in three independent experiments for each condition.

4.2.2.1.2 Elicitor-induced resistance assay

For elicitor-induced resistance assays, plants were syringe-infiltrated with 1 µM elf18, flg22, Pep2, Pep3 (a mixture of those) or water (i.e., mock) 24 h before inoculation. *Pst* DC3000 bacteria were prepared as described in 4.2.2.1.1 using the appropriate antibiotics and the suspension (1x 10^5 cfu/mL) was syringe-infiltrated into 2 to 3 leaves of 8 plants per genotype per treatment. Three days after inoculation the bacterial titer was determined as described in 4.2.2.1.1.

4.2.2.1.3 SAR assays

To assess transcriptional changes and trace SA levels during SAR, local leaves of 4-week old *Arabidopsis* plants were infiltrated with 1 x 10^7 cfu/mL *Pst* DC3000 (*AvrRpm1*) Bacteria were prepared as described in 4.2.2.1.1 using the appropriate antibiotics. The local leaves were harvested for RNA extraction and SA measurements 24 hours post inoculation (hpi) and the systemic leaves at 48 hpi.

For localization studies of PROPEPs, local leaves of wild-type plants and transformants (p*PROPEP2:PROPEP2*-Venus, p*PROPEP3:PROPE3*-Venus) were infiltrated with *Pst* DC3000 (*AvrRpm1*) at a concentration of 1 x 10^7 cfu/mL. At 24 and 48 hours after infiltration local and systemic leaves (n = 8) were harvested for protein extraction.

For bacterial growth measurement local leaves of 4-week old *Arabidopsis* plants were infiltrated with 1 x 10^7 cfu/mL *Pst* DC3000 (*AvrRpm1*) or $MgCl_2$ as mock control. Two days later 1 x 10^6 cfu/mL *Pseudomonas syringae* pv. *maculicola (Psm)* were infiltrated in systemic leaves and the bacterial titer of *Psm* was assessed three days later as described 4.2.2.1.1.

4.2.2.2 *Colletotrichum higginsianum*

Colletotrichum higginsianum (*Ch*) was grown on Mathur's agar medium for 10-14 days. Spores were collected in sterile water, centrifuged and resuspended again in sterile water to the desired concentration.

For lesion size measurements *Ch path-29* (Huser, Takahara et al. 2009) was drop inoculated (1 x 10^5 spores/mL) on fully expanded leaves of 4-week old *Arabidopsis* plants 5 days before analysis (n ~ 30 lesions).

To quantitatively assess fungal growth by qRT-PCR 12-day-old seedlings were drop inoculated with *Ch path-29* (1 x 10^5 spores/mL) three days before harvest. The quantitative amounts of *ChACTIN* mRNA in relation to At4g26410 mRNA of 12 seedlings per sample were used to determine fungal biomass by qRT-PCR analysis.

To determine fungal growth on systemic leaves, local tissue was infiltrated with either 1 µM Pep2 and Pep3 or water as mock control. After 24 hours *Ch path-29* (5 x10^5 spores/mL) was sprayed on the plants and systemic leaves (n = 8) were harvested 4 and 5 days later for RNA extraction and subsequent qRT-PCR analysis.

4.2.3 MAMP-sucrose assay

Seeds were surface-sterilized with 70% ethanol, imbibed for 1-3 days at 4°C and then grown in 0.5 x MS liquid-medium in 48 well plates for 3 days. The medium was replaced with 0.5 x MS liquid-medium supplied with 100 mM sucrose and flg22/elf18 at the concentrations indicated and seedlings were grown for further 3 days. Seedlings were grown under continuous light and 23°C.

Anthocyanin isolation and measurement was performed as in (Teng, Keurentjes et al. 2005). Anthocyanins were isolated by incubating seedling material from ca. 10 seedlings 1 % (v/v) hydrochloric acid in methanol for 12 h. The mixture was centrifuged at in 13.000 rpm for 5 minutes and the absorbance of the supernatant was measured at 530 and 657 nm. Relative anthocyanin concentrations were calculated with the formula (A530-A657/4) / g FW.

4.2.4 ROS assay

For ROS assays, leaf discs (5 mm diameter) were excised from 4-week-old plants and kept overnight on water before they were transferred to 50 µL fresh water. Subsequently, ROS production was induced as described by Felix et al., 1999; by application of 100 µM elf18 or flg22, in a reaction

mixture containing 50 µL water, 20 µM luminol (Fluka, Deisenhofen, Germany) and 1 µg horseradish peroxidase (Sigma-Aldrich, Deisenhofen, Germany). Luminescence was measured by a luminometer (Centro LB 960 microplate luminometer, Berthold Technologies, Wildbach, Germany).

4.2.5 Ion leakage measurement

Leaves of four-week old plants were infiltrated with 10^8 cfu *Pst* DC3000 (*AvrRpm1*). Leaf discs were excised at 1, 3, 5, 6, 8, 10 and 22 hours post infiltration, washed in water for 30 minutes and then transferred to 2ml H_2O. From the water 60 µL were removed and the electrolyte accumulation was measured with a conductometer.

4.2.6 Elicitor-induced gene expression

10-day old seedlings grown in liquid ½ MS liquid media were treated with the indicated peptides (if not stated otherwise with a final concentration of 1 µM) for the indicated time points. Subsequently, the seedlings were shortly dried on a paper towel and directly frozen in liquid nitrogen.

4.2.7 SA measurement

Leaf material (100 to 200 mg fresh weight) was extracted with aqueous methanol (Bednarek, Schneider et al. 2005). Leaf extracts were hydrolyzed with ß-glucosidase (EC 3.2.1.21; Sigma-Aldrich), and released SA was re-extracted as described (Lee and Raskin 1998). HPLC analyses were performed on an Agilent 1100 HPLC system.

4.2.8 Molecular biological methods

4.2.8.1 Genotyping

Ordered T-DNA lines from NASC (National Arabidopsis Stock Centre; www.arabidopsis.info) were analyzed for homozygous T-DNA insertion by genotyping PCR. Therefore, at least 20 plants of each obtained seed stock were grown for 2-3 weeks. One leave was excised from the plant and genomic DNA was isolated as described by (Edwards, Johnstone et al. 1991). *Arabidopsis* leaf tissue was ground in Edwards buffer and centrifuged at 13,000 rpm for 5 min. The supernatant was collected and DNA was precipitated with isopropanol and centrifuged. The pellet was washed with 70% ethanol, dried and re-suspended in sterile water. Subsequently the DNA was used for standard PCR

using the primers provided by http://signal.salk.edu/tdnaprimers.2.html and home-made Taq-Polymerase.

standard PCR reaction mix:
1 µL Primer (For and Rev)
1 µL dNTPs
0.2 µL Taq
2 µL template
2 µL 10x PCR buffer
13.8 µL H_2O

standard PCR program:			
Initial denaturation	94 °C	3 min	
Denaturation	94 °C	30 sec	30 x
Annealing	55 °C	30 sec	
Extension	72 °C	1 min	
Final Extension	72 °C	5 min	

4.2.8.2 RNA extraction using TRI reagent

Frozen leave samples were homogenized in liquid nitrogen with mortar and pistil and RNA was isolated using the TRI Reagent (purchased by Ambion) following the manufacturer´s protocol. Concentration and quality was determined using a NanoDrop photometer (PeqLab). RNA with a 260/280 and 160/230 ratio of ~2.0 was used for cDNA synthesis.

4.2.8.3 cDNA synthesis

According to the manufacturer's protocol 5µg RNA were applied (ROCHE: Transcriptor Reverse Transcriptase) to synthesize cDNA using oligo(dT)-primer. cDNA was solved in 200 µl water and subsequently used for quantitative real-time PCR.

4.2.8.4 Quantitative real-time PCR (qRT-PCR)

RNA extraction and cDNA synthesis were performed as described above. Quantitative RT-PCR was performed on the IQ5 real-time PCR Thermocycler (Bio-Rad, Hercules, USA) using the primers listed in Table 8. A typical PCR reaction mix and thermal profile is shown below. Expression of the genes of interest were normalized to the reference gene At4g26410, an expressed protein that has been shown to exhibit a very constant expression profile throughout various biotic and abiotic stresses (Czechowski, Stitt et al. 2005). The results were analyzed using the comparative cycle threshold (ΔΔCt) method (Libault et al., 2007). If not stated otherwise fold-changes were calculated relative to wild-type samples at 0 hours post treatment set to 1. Data were shown as the mean +/- standard deviation (SD) from three technical replicates.

Reaction mix	
cDNA (1:10)	1 µL
PCR buffer (10x)	2.5 µL
dNTPs (10 mM)	0.5 µL
Forward Primer	1 µL
Reverse Primer	1 µL
SYBR Green*	1.25 µL
Fluorescin**	1.2 µL
Glycerol (50 %)	4 µL
DMSO (100%)	0.75 µL
Taq (Ambion)	0.5 µL
H_2O	Ad to 25 µL

*1:3000 diluted with H2O;
** 1:1000 diluted with 1xTE buffer

PCR programme			
Initial denaturation	95 °C	2 min.	
Denaturation	95 °C	20 sec.	40x
Annealing	55 °C	30 sec.	
Extension	72 °C	25 sec.	
	95 °C	1 min.	
	55 °C	1 min.	
Melting curve (81 x)	55-95 °C	10 sec; à 0.5 °C	

4.2.8.5 Microarray analysis

4.2.8.5.1 ATH1 Genome Array – comparison of elf18- with Pep2-triggered signaling

Seedlings were treated with 1 µM elf18 or Pep2 and harvested in liquid nitrogen at 0, 2 and 10 hpt. Total RNA was isolated with RNeasy Mini kit supplied with RNase-Free DNase set (Qiagen) according to the manufacturer's instructions. RNA quality was assessed with RNA Nanochips on a Bioanalyzer (Agilent). Biotinylated cRNA was prepared according to a standard Ambion protocol from 1 µg total RNA (MessageAmp II-Biotin Enhanced Kit; Ambion). After amplification and fragmentation, 12.5 µg of cRNA was hybridized for 16 h at 45°C on GeneChip ATH1-121501 Genome Array. GeneChips were washed and stained with Fluidics Script FS450-004 in the Affymetrix Fluidics Station 450 and scanned using a GeneChip Scanner 3000 7G. The data were analyzed with Affymetrix GeneChip Operating Software version 1.4 using Affymetrix default analysis settings and global scaling as normalization method.

Probe signal values were subjected to the quantile normalization (Bolstad, Irizarry et al. 2003) and summarization using the GeneChip robust multi-array average (GC-RMA) algorithm (Wu and Irizarry 2004) to obtain the expression level values of the genes. Results were analyzed by the following linear model using the lmFit function in the limma package in the R environment: log2 (expression level value)~ sample + replicate. The eBayes function in the limma package was used for variance shrinkage

in calculation of the p-values and the Storey's q-values were calculated using the q-value function in the q-value package from the p-values (Storey and Tibshirani 2003).

In order to select candidate genes all genes that were at least two-fold differentially regulated in comparison to 0 time point and exhibited a q-Value ≤ 0.05 were used for the analysis. Then the expression values between the two genotypes were further analyzed.

Genes differentially regulated 10 hpt (2-fold up or 2-fold down, q-Value ≤ 0.05) were additionally normalized to the respective receptor mutant (*efr*, *pepr1 pepr2*). Analysis of overrepresented gene ontologies was carried out by using the online tool AgriGO (http://bioinfo.cau.edu.cn/agriGO/) (Du, Zhou et al. 2010). Genevestigator V3 (https://www.genevestigator.com/gv/index.jsp) was used for meta-analysis of gene expression (Hruz, Laule et al. 2008).

4.2.8.5.2 Tiling Genome Array – identifying components that link initial MAMP activation to robust immunity (comparing rsw3 *to wild-type plants)*

Seedlings of wild-type and *rsw3* plants were treated with 1 µM elf18 and harvested at 0, 2, 10 and 24 hpt. High quality total RNA was isolated using RNeasy Mini kit supplied with RNase-Free DNase set (Qiagen) according to the manufacturer's protocol. The quality was assessed with RNA Nanochips on a Bioanalyzer (Agiland) and subsequently reverse-transcribed into doublestranded cDNA. Then *in vitro* the RNA was transcribed in the presence of biotin-labeled nucleotides using the Affymetrix GeneChip 3' IVT Express Kit (Affymetrix, Santa Clara, USA) including polyA controls as recommended by the manufacturer. Quantity and quality of the biotinylated cRNA was determined by using NanoDrop ND 1000 (PeqLab, Erlangen, Germany) and Bioanalyzer 2100 (Agilent, Böblingen, Germany). 15 µg of biotin-labeled cRNA samples were fragmented randomly to 35-200 bp at 94°C in Fragmentation Buffer (Affymetrix, P/N 901229). Array hybridization was carried out by mixing biotin-labeled cRNA samples with 300 µl Hybridization Mix (Affymetrix, P/N 900720) containing Hybridization Controls and Control Oligonucleotide B2 (Affymetrix, P/N 900454). Afterwards, samples were hybridized onto Affymetrix AGRONOMICS1 Arabidopsis tiling arrays for 16 h at 45°C. Hybridized arrays were washed using an Affymetrix Fluidics Station 450 running the FS450_0004 protocol. The Affymetrix GeneChip Scanner 3000 was used to measure fluorescence intensity emitted by the labeled target.

Microarray data processing was performed by Emiel Ver Loren van Themaat at the MPIPZ in Cologne. Normalization and statistical analysis of the data was carried out as described by Irizarry and colleagues and Smyth (Irizarry, Bolstad et al. 2003; Smyth 2004). Fold-changes were calculated by

comparing expressed genes at 2, 10 and 24 hours to the 0 hour time point. 2-fold induced or repressed genes with a False discovery rate (FDR) ≤ 0.05 were further analyzed.

4.2.9 Biochemical methods

4.2.9.1 MAPK assay

4.2.9.1.1 Protein lysis

To analyze the activation status of MAPKs upon MAMP treatment, 10-day old seedlings were treated with 1µM of the elicitor and harvested at the indicated time points in liquid nitrogen and ground to a fine powder. Proteins were thawed in 150 µL MAPK lysis buffer, mixed and centrifuged for 15 minutes at 15000 rpm. The supernatant was recovered in a new tube, 6x SDS samples buffer was added and the sample either frozen at -20°C or directly used for analysis.

4.2.9.1.2 SDS page

The samples were heated to 95°C for 3-5 minutes und subsequently loaded on the SDS page.

resolving gel (10 %)	8ml/gel (1.5 mm)
4 x lower buffer	2 mL
30 % acryl amid	2.7 mL
H2O	3.3 mL
10 % APS	40 µL
TEMED	2.5 µL

stacking gel (3 %)	3ml/gel
4 x upper buffer	0.75 mL
30 % acryl amid	300 µL
H2O	1.89 mL
10 % APS	60 µL
TEMED	1.5 µL

30-40 µl of the sample and 7.5 µl of a prestained molecular-weight marker (Precision plus protein standard dual color; BioRad) were loaded into the resolving gel containing a 3% stacking gel. Gels were run in electrophoresis tanks (Mini-Protean 3 Cell; BioRad) in 1x running buffer for ~60 min at 30 mA/gel (constant) until the marker suggested a sufficient separation of the proteins.

4.2.9.1.3 Western Blot (semi-dry blotting)

Proteins were transferred from SDS-gels to PVDF-membranes using the Semidry-blotting system of Biorad (Trans-Blot SD Semi-Dry Transfer Cell) using 1 x semi-dry buffer. The membrane was pretreated with MeOH for 30 seconds and subsequently washed in semi-dry blotting buffer. Whatman paper and the gels were also washed in semi-dry buffer before blotting. The blot was arranged from bottom to top in the following order: 1. 3 layers of buffer-soaked Whatman paper,

membrane, gel, 3 layers of buffer-soaked Whatman paper. Blotting was performed at 100 mA/gel (constant) for 90 minutes at room temperature.

Afterwards the membrane was incubated in TBST + 5% milk powder for one hour and then transferred to PBST buffer containing the first antibody and incubated at 4 °C over night.

At the next day membranes were washed three times 5 min in TBST and incubated with the secondary antibody in TBST for 1 hour. After washing twice in TBST, an ECL (enhanced chemi-luminescence) detection assay was performed according to the manufacturer's instructions (Chemiluminescence detection using the SuperSignal® West Pico Chemimuminescent kit (Pierce, Rockford, USA)'), poured over the transfer membrane and incubated for 5 minutes. Luminescence was detected on a light sensitive film (Kodak).

4.2.9.2 Immunoblot analysis and Co-IP

Leaves were ground to fine powder in liquid nitrogen and extraction buffer [50 mM Tris-HCl pH 7.0; 2% SDS; 2 mM DTT; 10% glycerol; 1 mM AEBSF (Sigma), 1% (v/v) P9599 protease inhibitor cocktail (Sigma)] was added. Samples were cleared by centrifugation at 15000 rpm for 15 min. at 4°C and separated in polyacrylamide gel (4.2.9.1.2). Immunoblot analysis was performed using anti-PROPEP2, anti-PROPEP3 or anti-GFP antibody.

To detect protein-protein interaction, leaf samples were ground to fine powder in liquid nitrogen and extraction buffer [50 mM Tris-Hcl pH 7.5; 150 mM NaCl; 10% glycerol; 5 mM DTT; 2 mM EDTA; 1 mM PMSF (Sigma), 1% (v/v) P9599 protease inhibitor cocktail (Sigma), 0,5% (v/v) IGEPAL CA-630] was added. Samples were cleared by centrifugation at 10000 rpm for 15 minutes at 4°C and subsequently filtrated using a 75 µm mesh. 1 µL Anti-Flag antibody was added to the sample. After 10 minutes incubation on ice 100 µL washed beats were added and the mixture was then rotated for 1 hour at 4°C. Following two additional washing steps, 2x SDS sample buffer was added and the sample was then boiled for 10 minutes before separation in a polyacrylamide gel (4.2.9.1.2) and detection using anti-Flag, anti-HA or anti-PROPEP antibody.

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6 Abbreviations

%	percent
°C	degree Celsius
A	Ampere
ABA	abscisic acid
Arabidopsis	*Arabidopsis thaliana*
Avr	avirulence
bp	basepair(s)
C	carboxy-terminal
CC	coiled-coil
cDNA	copied DNA
cfu	colony forming unit
Ch	*Colletotrichum higginsianum*
COI1	CORONATINE INSENSITIVE 1
Col-0	*Arabidopsis thaliana* ecotype Columbia-0
CNX	calnexin
CRT	calreticulin
DAMP	damage associated molecular pattern
DDE2	DELAYED-DEHISCENCE 2
Dpi	days post induction
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
dNTP	deoxynucleosidetriphosphate
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDS1	ENHANCED DISEASE SUSCEPTIBILITY 1
EDTA	ethylenediaminetetraacetic acid
Ein2	ethylene insensitive 2
Elf18	18-amino acid sequence of bacterial EF-Tu
EFR	EF-Tu receptor
EF-Tu	elongation factor Tu
EMS	ethyl methanesulfonate
ER	endoplasmatisches reticulum
ERQC	ER quality control
ET	ethylene
ETI	effector-triggered immunity
EtOH	ethanol
Fig.	figure
Flg22	22-amino acid sequence of bacterial flagellin
FLS2	FLAGELLIN SENSING RECEPTOR 2
FRK1	FLG22-RESPONSIVE KINASE 1
g	gram
g	gravity constant (9.81 ms-1)
GIIα	glucosidase II alpha
GFP	green fluorescent protein

h	hours
hpi	hours post inoculation
HPLC	high performance liquid chromatography
Hpt	hours post treatment
HR	hypersensitive response
ICS1	ISOCHORISMATE SYNTHASE 1
Ile	isoleucine
ISR	induced systemic resistance
JA/JAs	jasmonic acid/jasmonates
JA-Ile	JA-isoleucine
JAZ1-12	JASMONATE-ZIM-DOMAIN PROTEIN 1-12
kb	kilobase(s)
kDa	kilo Dalton
L	liter
LOX2	LIPOXYGENASE 2
LPS	lipopolysaccharides
LRR	leucine rich repeats
m	milli
M	molar (mol/l)
µ	micro
MAMP	microbe-associated molecular pattern
MAPK	mitogen activated protein kinase
MeJA	methyl-jasmonate
MeOH	methanol
$MgCl_2$	magnesium chloride
min	minutes
MLO	MILDEW RESISTANCE LOCUS O
mRNA	messenger ribonucleic acid
MTI	MAMP-triggered immunity
MW	molecular weight
n	nano
N	amino-terminal
NaCl	sodium chloride
NB	nucleotide binding
NDR1	NON-RACE-SPECIFIC DISEASE RESISTANCE 1
NPR1	NONEXPRESSER OF PR GENES 1
OD	optical density
OG	oligogalacturonides
ox	overexpressor
p35S	35S promoter of CaMV
PAD4	PHYTOALEXIN DEFICIENT 4
PAGE	polyacrylamide gel-electrophoresis
PBS	phosphat buffered saline
PBS3	AvrPphB susceptible 3
PCR	polymerase chain reaction
PDF1.2a	PLANT DEFENSIN 1.2a
PEN2	PENETRATION 2
Pep	C-terminal part of PROPEP

PEPR	Pep-receptor
pH	negative decimal logarithm of H+ concentration
PR1	PATHOGENESIS-RELATED 1
PR2	PATHOGENESIS-RELATED 2
PROPEP	precursor of Pep
PRR	pattern recognition receptor
Pst	*Pseudomonas syringae* pv. *tomato*
pv.	pathovar
P value	probability value
qRT-PCR	quantitative real-time PCR
R	resistance
RLK	receptor-like kinase
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	rounds per minute
RPM1	RESISTANCE TO P. SYRINGAE PV. MACULICOLA 1
RSW3	RADIAL SWOLLEN ROOT 3
RT	room temperature
SA	salicylic acid
SAG101	SENESCENCE-ASSOCIATED GENE 101
SAR	systemic acquired resistance
SB	sample buffer
SD	standard deviation
SDS	sodium dodecyl sulphate
sec	seconds
SID2	SALICYLIC ACID INDUCTION DEFICIENT 2
T3SS	(bacterial) type three secretion system
Taq	*Thermophilus aquaticus*
T-DNA	transfer DNA
TEMED	N,N,N',N'-Tetramethylethylenediamine
TF	transcription factor
TIR	toll/interleukin-1 receptor
TLR	toll-like receptor
TRIS	tris-(hydroxymethyl)-aminomethan
U	unit
UGGT	UDP-glucose:glycoprotein glucosyltransferase
UV	ultraviolet
V	volt
vir	virulence
VSP2	VEGETATIVE STORAGE PROTEIN 2
WIR	wound induced resistance
wt	wild-type

7 Supplementary information

Supplementary Figure 1: Gene ontology analysis of the 106 selected "key genes" using agriGO (http://bioinfo.cau.edu.cn/agriGO/index.php).

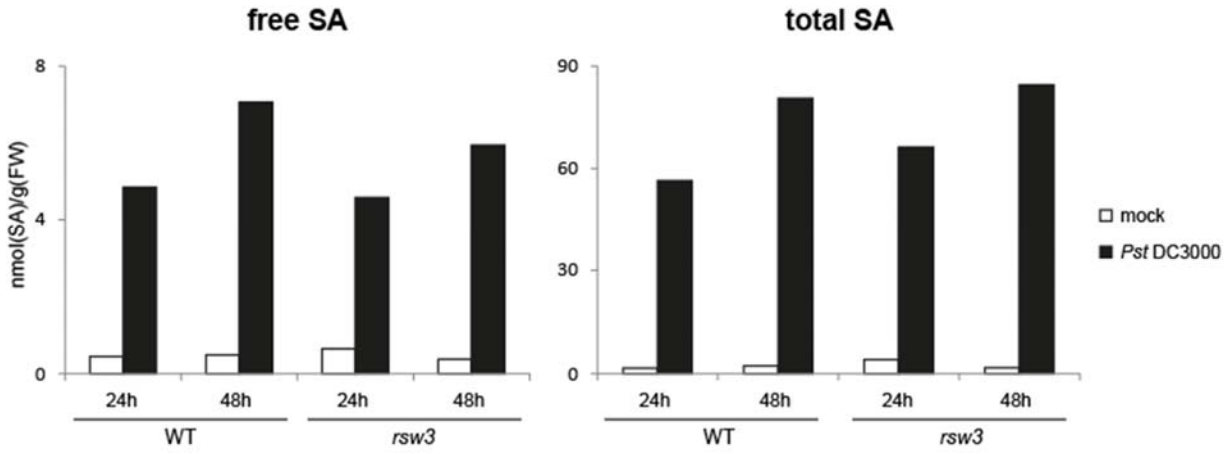
Supplementary Table 1: At least 2-fold less-induced genes in *rsw3* in comparison to wild-type 10 hours after elf18 application.

		Fold change						
		WT			*rsw3*			
Nr.	AGI code	2h vs. 0h	10h vs.0h	24h vs. 0h	2h vs. 0h	10h vs.0h	24h vs. 0h	rsw3 (10vs0) / WT (10vs0)
1	AT1G13480	4,183598545	12,69703041	17,5148283	1,364343462	1,891278599	5,509954434	0,148954404
2	AT1G14540	11,21738877	16,18786377	13,33910914	3,907810559	2,60685371	6,871334991	0,161037537
3	AT5G64905	14,3737776	16,24885682	8,942206568	9,994248144	3,549496713	7,345543094	0,218445934
4	AT5G05340	7,304376497	49,24347687	2,635795203	4,143880471	11,97675646	1,905591623	0,243215086
5	AT5G13320	6,401473258	27,32554261	8,461121115	2,678524535	7,185276689	5,741681283	0,262950924
6	AT1G26410	5,950750666	17,73574197	4,330549217	3,026261303	4,684396037	4,228713042	0,264121797
7	AT1G02430	1,722769348	5,531350344	2,030656625	1,008681559	1,659542041	1,417712565	0,300024757
8	AT5G22570	1,204422836	8,451321891	1,304902266	0,906124282	2,558280221	1,007419525	0,302707701
9	AT5G61890	6,135723532	6,217302601	6,386791841	4,140607235	1,886564232	4,178183978	0,303437737
10	AT4G19970	1,343690509	5,05181257	1,617957823	0,908405736	1,578172031	1,560539636	0,312397186
11	AT1G30720	8,594010382	16,65700896	38,36022621	6,142538978	5,217947239	13,94105236	0,313258356
12	AT5G20230	7,677660184	6,98038327	13,44634145	3,987956417	2,221967536	5,00736305	0,318315979
13	AT5G25260	13,66020625	29,17396021	28,9170204	6,231151004	9,406541785	12,76513845	0,322429376
14	AT1G30700	8,945186532	15,35433267	6,920553912	7,165254454	5,044411669	5,23563257	0,328533436
15	AT3G10930	1,743879254	4,82321474	4,093631824	1,405938529	1,593014074	2,874766018	0,330280562
16	AT3G23250	15,17988734	22,3173133	19,32668411	12,59681582	7,3866418	16,1326276	0,330982574
17	AT4G28420	1,889522018	5,232283995	1,355389862	1,474963399	1,782739161	1,212584763	0,340719113
18	AT4G11170	2,097286262	6,723520475	4,145076212	1,395525444	2,301876572	2,786500106	0,3423618
19	AT4G18940	1,495737737	5,894214984	4,192453799	1,468112148	2,070654925	1,744676677	0,351302918
20	AT5G39670	3,731373216	5,236042506	6,855595732	1,870456436	1,839596284	4,384789889	0,351333336
21	AT2G39380	4,264633128	11,78785391	5,416159191	3,745288768	4,241372885	5,409488539	0,359808742
22	AT4G11280	2,094987981	7,049855257	3,943417499	1,561484575	2,536787452	5,532456352	0,359835395
23	AT2G19190	28,1461424	56,67616287	14,37700216	20,61552244	20,52510655	12,32901418	0,362147074
24	AT3G25882	2,087852707	11,45271424	4,147743953	0,747009128	4,148540088	3,219351107	0,362232044
25	AT5G60630	3,662504725	13,29636895	2,56606941	2,829777182	4,828804676	2,148787067	0,363167169
26	AT5G22270	4,935492991	15,24618188	8,29150383	2,076407458	5,563606551	4,913029771	0,364918023
27	AT1G53625	17,67932696	52,64280627	44,36370689	10,30679714	19,57617834	33,18891314	0,371868062
28	AT1G31885	1,313063207	7,628181731	2,174245744	1,306155227	2,847508616	1,616252825	0,373287989
29	AT5G67450	9,64609868	9,491610284	4,864675659	6,359798588	3,581782695	5,100868769	0,377363017
30	AT1G02450	1,27729536	13,46739131	2,911314323	0,992489878	5,119171251	2,48257424	0,380116025
31	AT1G13540	1,83545726	4,301196279	1,346242464	1,253215603	1,640319442	1,264561103	0,381363541
32	AT5G48430	16,82965418	19,61694223	26,23127982	15,04937656	7,539979946	13,4946107	0,384360613
33	AT4G23550	7,58251889	11,33584172	5,574826466	5,169739044	4,36735712	3,590080729	0,385269769
34	AT5G53110	4,507784364	13,53071078	3,933990131	3,923142381	5,22118606	3,103374308	0,385876703
35	AT1G61560	3,067776069	4,118886044	2,919473619	2,416058344	1,594745392	2,86874814	0,387178809
36	AT2G43570	2,842573721	9,988129811	6,346367888	1,35793523	3,936258199	2,714669136	0,394093617
37	AT4G35180	5,730890844	21,66384223	9,162686923	2,654917944	8,591495527	9,587695773	0,396582261
38	AT5G64810	1,447936031	4,333271535	2,583094949	1,138584148	1,735939884	1,521840158	0,400607225
39	AT3G18250	30,80428913	57,75345829	24,32836605	15,55986718	23,39330132	16,99739203	0,405054554
40	AT1G29860	1,840271262	4,751911333	2,177977508	1,026756968	1,938580346	1,555190241	0,407958022
41	AT1G74710	4,931649487	9,336699128	1,242388006	3,184137836	3,817084557	2,045079795	0,408825914
42	AT4G39670	2,952084369	5,594001377	10,20269419	2,02114851	2,28852806	8,671435055	0,40910395
43	AT5G13080	2,226053384	5,712585292	3,689445571	1,445098734	2,343376288	1,614443316	0,410212919
44	AT2G35930	5,147211109	3,673343108	4,497073794	3,966884549	1,523561192	3,721392997	0,414761471
45	AT2G39530	3,579029707	13,83749741	5,077378762	2,999308794	5,80011003	3,662670859	0,419158888
46	AT3G13950	4,555692919	9,099457911	10,06384562	3,4877782	3,873307061	12,37053708	0,425663495
47	AT3G50260	2,577417258	5,49766941	6,297693369	1,631166635	2,345244059	3,293692277	0,426588775
48	AT2G31335	5,680208722	5,969957132	2,802977718	2,686494781	2,555070916	1,524486991	0,427988151
49	AT2G42060	2,058721737	4,250752042	3,083555486	1,715675423	1,831219142	2,124599822	0,430798862
50	AT1G12940	2,541843819	5,361366849	2,140829123	2,387463514	2,311312462	1,443182859	0,431105076

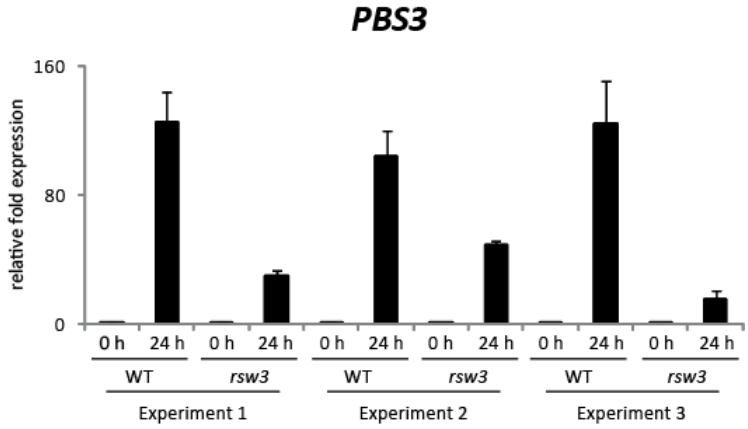
		Fold change						
		WT			*rsw3*			
Nr.	AGI code	2h vs. 0h	10h vs.0h	24h vs. 0h	2h vs. 0h	10h vs.0h	24h vs. 0h	rsw3 (10vs0) / WT (10vs0)
51	AT1G78000	1,403663678	5,242655985	4,240561638	1,455187354	2,272154622	4,686255843	0,433397619
52	AT1G65610	1,13730758	3,245188505	2,570488477	1,09265599	1,410928079	2,154117234	0,434775384
53	AT1G80820	3,550526618	4,447707546	3,452884264	3,36266642	1,937621257	3,491111539	0,435644933
54	AT4G14450	3,270756843	6,318124056	7,534863252	2,279161384	2,755883878	9,109100612	0,436187048
55	AT5G03700	5,125788539	6,547177397	7,730144592	4,773735414	2,868751556	5,350194119	0,438166156
56	AT4G08040	2,801017536	6,652731836	4,248949262	2,630115466	2,919913966	2,249756012	0,438904504
57	AT5G52750	3,466493557	10,85730444	9,604774472	2,088203188	4,807112525	9,475821367	0,442753775
58	AT1G27730	2,484341394	6,59136764	4,696241924	2,064297987	2,922152879	5,718573521	0,443330283
59	AT5G42830	9,19823292	11,46997258	5,621840939	8,565008968	5,086413791	7,708221833	0,443454747
60	AT4G12490	5,508673781	13,28269841	13,37396804	2,353465193	5,915594092	5,490944824	0,445360868
61	AT2G43620	8,008671021	13,24416317	11,19432144	4,01663982	5,903485113	5,594919222	0,445742403
62	AT1G51913	19,46985964	27,34958793	11,09279509	13,7445377	12,25001941	12,27824711	0,447905081
63	AT5G04340	2,608844834	3,823538083	2,435663286	1,720541346	1,715506605	1,890912091	0,448669941
64	AT2G39420	1,385883538	3,089598657	1,495781752	1,445219377	1,395629304	1,450317258	0,45171864
65	AT1G25400	2,796337839	4,616872803	3,450106867	1,610541647	2,091097548	1,853860193	0,452925094
66	AT3G55150	2,399932073	3,561382495	5,475243692	2,562671238	1,615802504	5,253400047	0,453700917
67	AT3G44720	1,064146133	1,333182189	1,712557991	0,74889526	0,605430174	1,383780506	0,454124109
68	AT4G08555	2,817843685	5,703175549	5,379166974	1,429163013	2,598813748	5,072354224	0,455678372
69	AT1G33840	2,073472599	4,491282626	2,386911862	2,442629354	2,049313098	1,295073236	0,456286827
70	AT1G69930	4,725171858	38,09128049	12,46326654	3,581814784	17,42667338	13,56847	0,457497704
71	AT2G33380	0,500340546	0,237631024	2,780559905	0,294137004	0,108722902	1,311332594	0,45752823
72	AT3G52450	8,364460561	6,578858605	8,081271405	8,742199382	3,01551355	7,870124845	0,458364244
73	AT2G36210	2,355843599	3,40151729	2,131120075	2,170087459	1,560235138	1,7553408	0,458687993
74	AT3G46690	2,606443093	4,530147284	3,675981371	2,185435145	2,083929855	3,278580311	0,460013709
75	AT1G65500	4,164725969	10,75556041	10,90318092	2,219776676	4,948663767	4,993320168	0,460102828
76	AT1G73810	1,4118841	3,369210316	1,947287147	1,159168732	1,555683735	1,817332585	0,461735418
77	AT4G20000	7,115348795	18,28121085	10,16123276	4,28845972	8,472183647	10,31670873	0,463436679
78	AT1G51820	9,684444316	11,11425288	19,14079365	7,863183348	5,162812588	13,24565717	0,464521785
79	AT5G24530	0,852238788	3,946868604	2,717164653	0,526683508	1,834414222	1,528788216	0,46477712
80	AT3G21781	4,936239778	4,651023315	3,353220649	2,182066193	2,166553956	2,370155409	0,465823069
81	AT2G18680	4,078130653	4,283810049	8,193899198	3,011538201	1,996143224	11,26110699	0,465973795
82	AT5G41040	0,535557011	1,495098763	0,575471126	0,648710929	0,697281004	0,781931306	0,466377888
83	AT3G15536	2,874752767	18,84058353	5,500392948	2,185027427	8,806263768	2,985917544	0,46740929
84	AT1G64400	6,655732556	6,75628052	1,975965129	5,157233501	3,16143008	2,116340223	0,467924633
85	AT5G16170	2,839906568	7,267156593	6,317502879	1,61425962	3,405793605	5,161285554	0,468655596
86	AT1G13530	1,41691781	3,422524548	3,014029463	1,205136443	1,604736378	2,09981195	0,468875053
87	AT1G24140	2,48345825	5,110478734	6,179523016	1,513590297	2,397257395	5,535077101	0,469086659
88	AT1G65690	13,60637853	15,30492826	7,92829752	11,64867773	7,195115931	6,725870656	0,470117586
89	AT4G37390	1,120481143	3,365070045	1,073949624	1,223943366	1,582972923	0,970680954	0,470413068
90	AT2G43510	3,786435123	9,198367306	10,54060132	2,748170079	4,329820432	4,830453969	0,470716192
91	AT1G02940	2,460782583	5,111648696	1,634818529	1,996172659	2,416470713	0,971089342	0,472738026
92	AT5G26920	5,644084458	12,02340282	9,112009345	4,007409489	5,756107731	7,81836872	0,478741985
93	AT4G39950	1,573934575	3,405978791	2,939457245	1,200745932	1,633254794	2,793554428	0,479525826
94	AT4G15417	5,509147931	8,661453775	8,681594239	5,266103313	4,160665127	8,643502658	0,480365679
95	AT5G24760	2,605856148	3,455632537	1,808194861	2,360794302	1,672474948	1,601382734	0,483985184
96	AT2G47130	3,408729513	3,647824241	2,504230703	1,986495856	1,768007228	1,961002775	0,484674456
97	AT5G55050	8,981682559	8,728045267	2,234283703	9,378089535	4,246748722	1,960927366	0,486563554
98	AT3G15518	11,49993886	10,70422144	7,840178876	9,504408411	5,227268538	8,660108334	0,488337108
99	AT1G08830	1,573986942	3,01235175	3,508772034	1,047447469	1,473079643	1,85571828	0,489013158
100	AT1G51890	3,530192514	4,834467226	2,808126954	2,642946737	2,375501668	2,69175117	0,49136783
101	AT2G39200	18,48185291	23,74403207	12,49554548	17,43192338	11,68862108	11,6942048	0,492276166
102	AT1G66090	2,561742109	4,048422205	7,693601633	1,575169671	2,000444269	7,168721479	0,494129359
103	AT5G67340	2,605247165	3,505923816	2,515026636	2,392075113	1,735144795	2,396509582	0,494917998
104	AT3G09520	1,927256125	2,775785227	5,854743946	1,42485512	1,376139719	5,176003883	0,495765921
105	AT1G66480	3,679695506	3,245200871	2,295499286	2,618067059	1,609269274	2,557552709	0,495892038
106	AT1G18300	4,628268466	4,207891164	2,708976384	3,313575928	2,087977062	2,54293439	0,496205102

Supplementary Table 2: T-DNA lines and genotypic characterization.

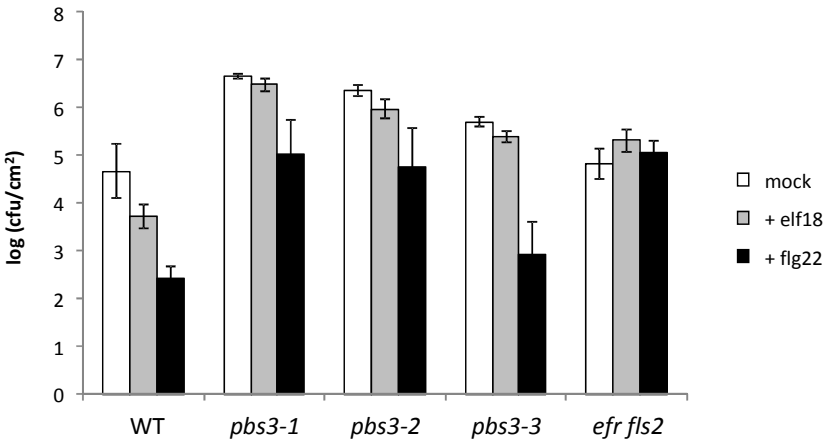
Nr.	NASC Code	Name	ABI Code		homo genotyping
1	535214	SALK_035214	AT1G30700	FAD-binding domain-containing protein	x
2	374643	GK-650C11	AT1G30720	similar to FAD-binding domain-containing protein	
3	629387	SALK_129387	AT5G53110	similar to zinc finger family protein	
4	655479	SALK_044730C	AT1G14540	anionic peroxidase, putative	x
5	633276	SALK_133276	AT5G48430	aspartic-type endopeptidase/ pepsin A	
6	593560	SALK_093560	AT1G25400	similar to unknown protein [Arabidopsis thaliana]	x
7	657631	SALK_056680C	AT2G43620	Chitinase family protein	x
8	656839	SALK_105828C	AT2G43620	Chitinase family protein	
9	871985	SAIL_193_G09	AT1G13480	similar to unknown protein [Arabidopsis thaliana]	
10	666727	SALK_091592C	AT4G11170	disease resistance protein (TIR-NBS-LRR class)	x
11	674117	SALK_023944C	AT4G11170	disease resistance protein (TIR-NBS-LRR class)	
12	678257	SALK_024857C	AT1G08830	CSD1 (COPPER/ZINC SUPEROXIDE DISMUTASE 1)	
13	879450	SAIL_439_G04	AT1G08830	CSD1 (COPPER/ZINC SUPEROXIDE DISMUTASE 1)	
14	760577	GK-437B08	AT3G10930	similar to unknown protein [Arabidopsis thaliana]	
15	873312	SAIL_155b_E09	AT3G10930	similar to unknown protein [Arabidopsis thaliana]	x
16	616511	SALK_116511	AT5G60630	unknown protein	
17	320792	GK-118H04	AT5G60630	unknown protein	
18	581257	SALK_081257	AT5G05340	peroxidase, putative	
19	677511	SALK_151762	AT5G39580	peroxidase, putative	
20	598399	SALK_098399	AT3G25882	NIMIN-2 (NIM1-INTERACTING 2)	
21	677394	SALK_148447C	AT3G25882	NIMIN-2 (NIM1-INTERACTING 2)	
22	619574	SALK_119574	AT1G13110	CYP71B7; oxygen binding	
23	873290	SAIL_338_H03	AT1G30900	vacuolar sorting receptor, putative	x
24	680584	SALK_004977C	AT4G36430	peroxidase, putative	
25	668591	SALK_086460C	AT1G02450	NIMIN-1/NIMIN1; protein binding	x
26	554065	SALK_054065	AT5G36925	similar to unknown protein [Arabidopsis thaliana]	
27	664402	SALK_003119C	AT5G46050	*ptr3-2*	x
28	660209	SALK_138430C	AT5G46050	ATPTR3/PTR3; transporter	x
29	655589	SALK_067396C	AT5G22270	similar to unknown protein [Arabidopsis thaliana]	
30	574491	SALK_074491	AT4G36430	peroxidase, putative	x
31	514421	SALK_014421	AT4G36430	peroxidase, putative	
32	502841	SALK_002841	AT1G13110	CYP71B7; oxygen binding	
33	586460	SALK_086460	AT1G02450	NIMIN-1/NIMIN1; protein binding	
34	562537	SALK_062537	AT5G36925	similar to unknown protein [Arabidopsis thaliana]	
35	651762	SALK_151762	AT5G39580	peroxidase, putative	
36	648447	SALK_148447	AT3G25882	NIMIN-2 (NIM1-INTERACTING 2)	x
37	525186	SALK_025186	AT5G46050	ATPTR3/PTR3; transporter	
38	303158	GK-131H12	AT5G46050	ATPTR3/PTR3; transporter	
39	322914	GK-287E07	AT5G39580	peroxidase, putative	x
40	623810	SALK_123810	AT4G12500	protease inhibitor/LTP family protein	
41	-	GK-344H04	AT4G01700	chitinase, putative	x
42	543012	SALK_043012	AT4G35180	LHT7 (LYS/HIS TRANSPORTER 7)	
43	406298	GK-066E10	AT4G35180	LHT7 (LYS/HIS TRANSPORTER 7)	
44	678291	SALK_027342C	AT1G26410	FAD-binding domain-containing protein	
45	365745	068 E 11	AT2G36690	oxidoreductase, 2OG-Fe(II) oxygenase family	x
45	835497	SAIL_793_H05	AT4G20000	VQ motif-containing protein	x
46	654836	SALK_111104C	AT5G10760	aspartyl protease family protein	x
47	678133	SALK_014781C	AT2G22880	VQ motif-containing protein	
48	681857	SALK_147734C	AT2G22881	VQ motif-containing protein	
49	671553	SALK_074760C	AT5G03350	legume lectin family protein	x
50	385939	100H11	AT5G03351	legume lectin family protein	
51	643675	SALK_143675.50.70.x	AT2G43570	chitinase, putative	
52	322172	600G05	AT2G43570	chitinase, putative	
53	673254	SALK_115555C	AT5G40780	LHT1 (LYSINE HISTIDINE TRANSPORTER 1)	
54	681393	SALK_083700C	AT5G40781	LHT1 (LYSINE HISTIDINE TRANSPORTER 1)	
55	872938	SAIL_102_E08	AT3G54420	ATEP3 (Arabidopsis thaliana chitinase class IV)	
56	663313	SALK_091655C	AT5G10760	aspartyl protease family protein	
57	659224	SALK_124829C	AT2G43510	ATTI1 (AT TRYPSIN INHIBITOR PROTEIN 1)	
58	662058	SALK_033910C	AT2G43511	ATTI1 (AT TRYPSIN INHIBITOR PROTEIN 1)	x
59	512310	SALK_012310	AT5G20230	ATBCB; copper ion binding	
60	557903	SALK_057903	AT5G20231	ATBCB; copper ion binding	
61	858252	WiscDsLox489-492C21	At5g22570	WRKY38	x



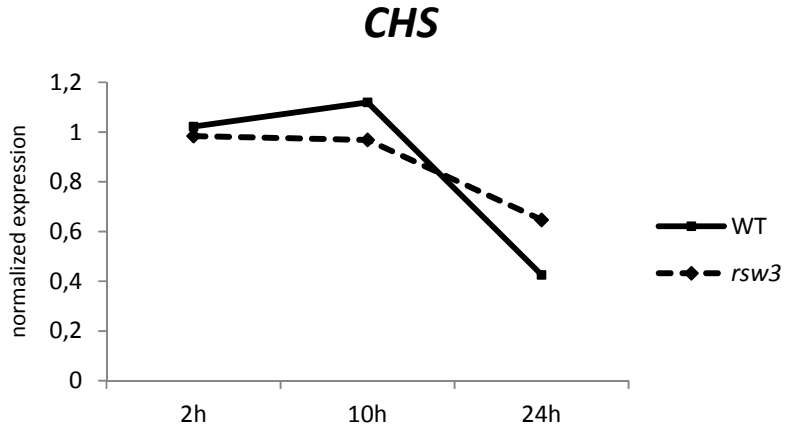
Supplementary Figure 2: Pathogen-induced SA accumulation. Leaves of four week-old wild-type and *rsw3* plants were infiltrated with 1 x 10^7 cfu *Pst* DC3000 and harvested for SA measurement 24 and 48 hours later.



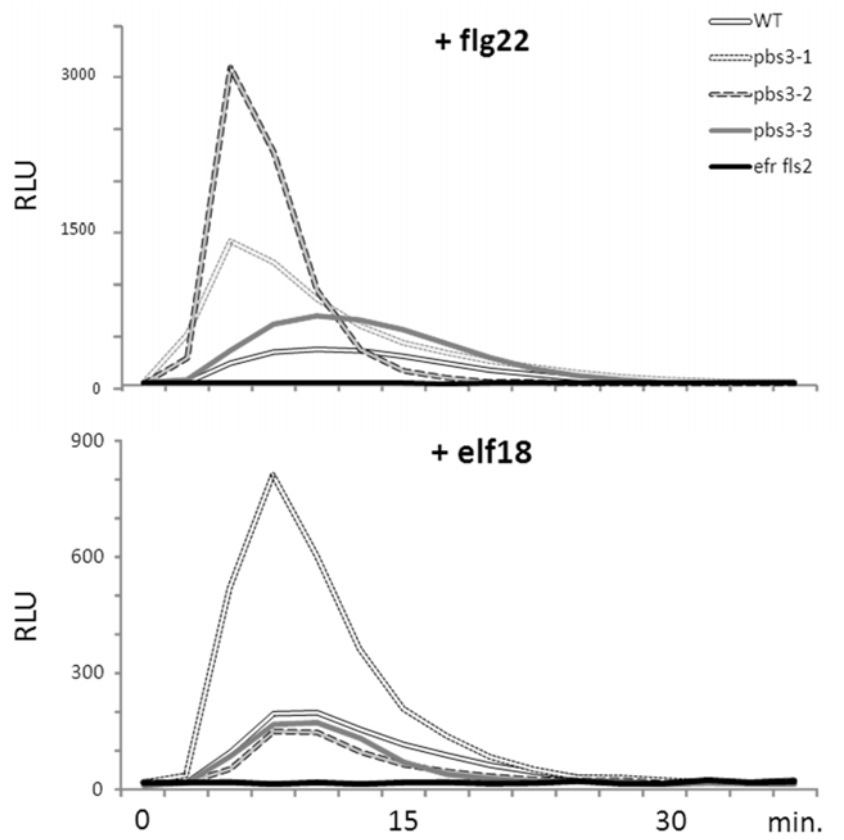
Supplementary Figure 3: *PBS3* expression in *rsw3* plants. 10-day-old wild-type and *rsw3* seedlings were treated with 1µM elf18 and subjected for qRT-PCR analysis at 0 and 24 hours after treatment. The relative expression is shown in fold, with the gene/At4g26410 value at 0 h in WT plants as 1.



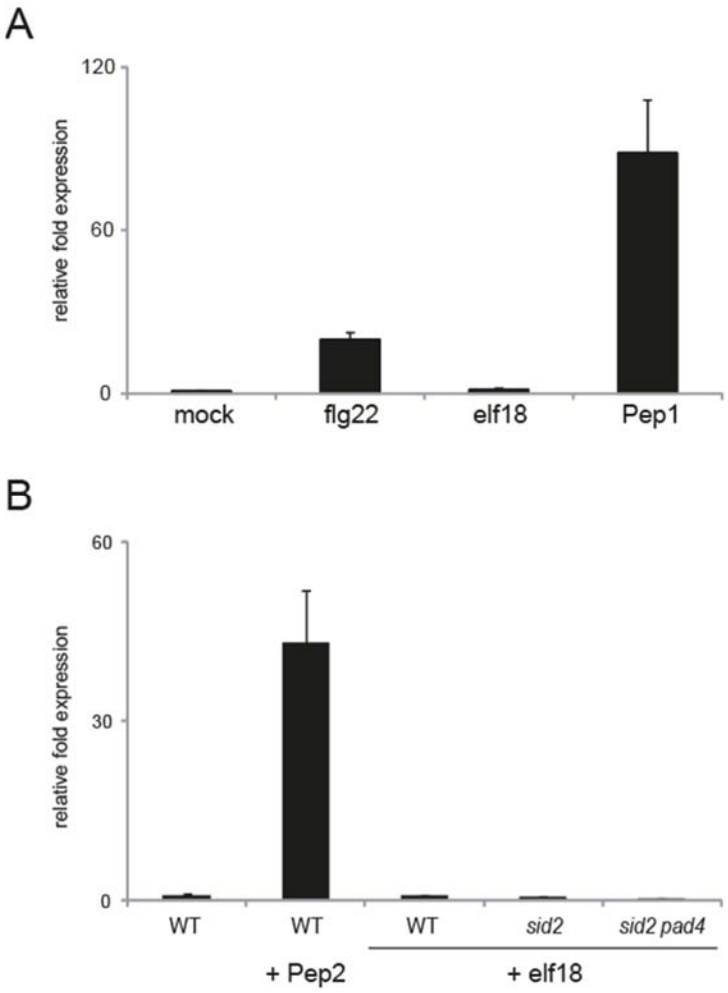
Supplementary Figure 4: MAMP-induced resistance assay. Leaves of 4-week-old plants of the indicated genotypes were infiltrated with 1µM elf18 or flg22 one day before syringe-inoculation with *Pst* DC3000 (1 x 10^5 cfu). After three days the bacterial proliferation was analyzed.



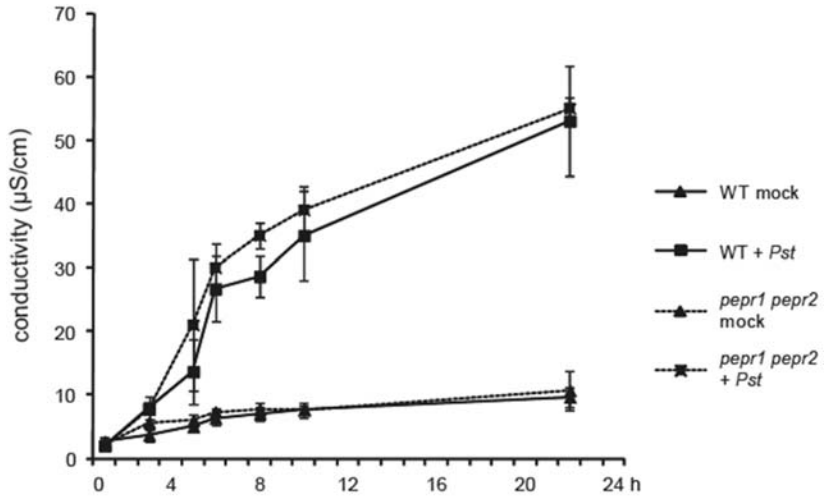
Supplementary Figure 5: Transcript abundance of *CHS* upon elf18 treatment in wild-type and *rsw3* plants. Average expression values of *CHS* from three biological replicates in comparison to time point 0 of the respective genotype upon treatment with 1 µM elf18 to ten-day old seedlings. The data were withdrawn from microarray analysis using Affymetrix AGRONOMICS1 *Arabidopsis* tiling array.



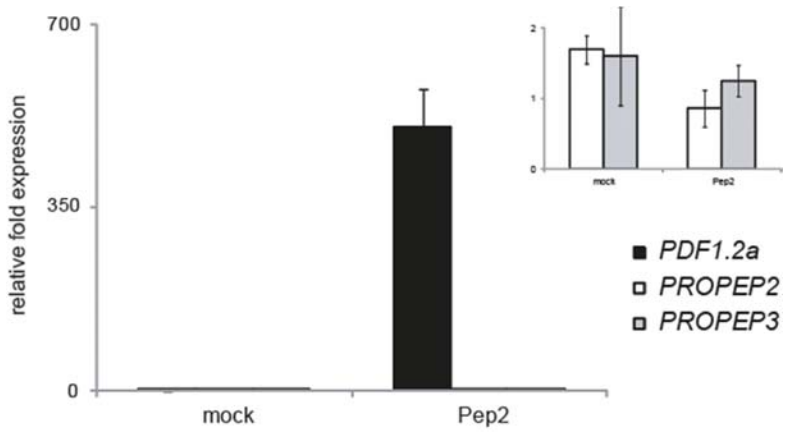
Supplementary Figure 6: MAMP-induced ROS spiking. Leaf discs of four-week old plants of the indicated genotypes were treated with 1 µM flg22 or elf18. Relative light units were measured over 34 minutes.



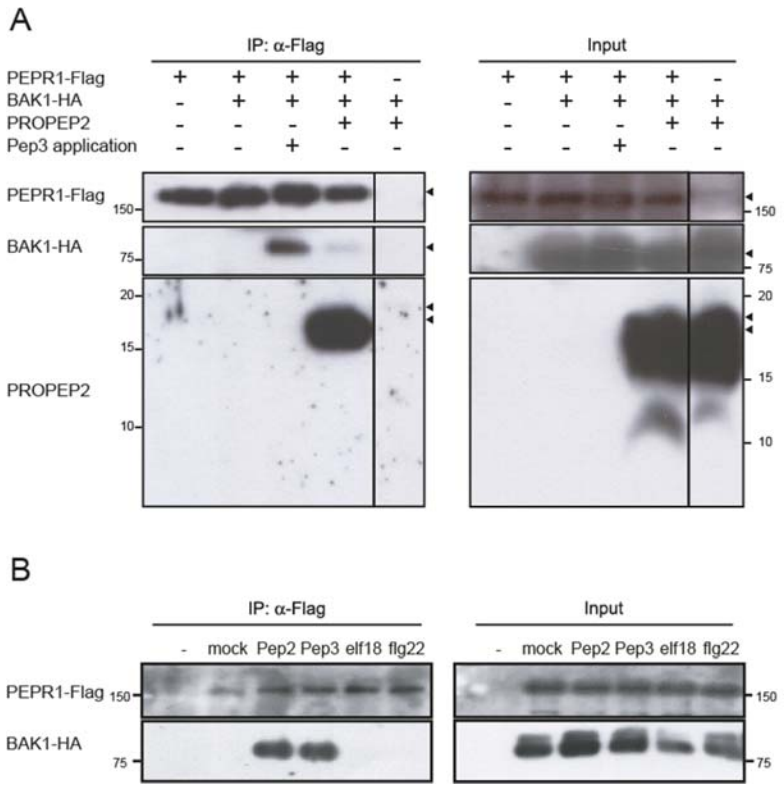
Supplementary Figure 7: Defensin gene expression is specifically activated by Pep-elicitation. A) 10-day-old wild-type seedlings were treated with the indicated elicitors and harvested at 24 hpt for *PDF1.2a* transcript analysis. The relative expression is shown in fold, with the *PDF1.2a*/At4g26410 value after mock treatment in WT plants as 1. B) 10-day-old seedlings of the indicated genotypes were treated and analyzed as described for A).



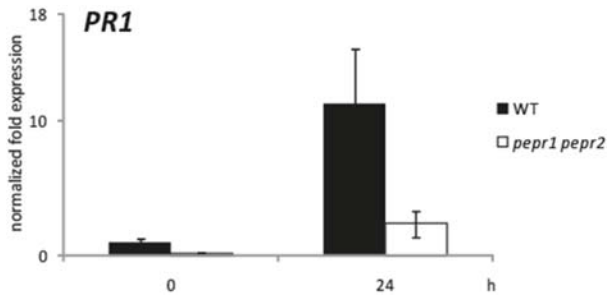
Supplementary Figure 8: RPM1-induced cell death. Four-week-old plant leaves were infiltrated with *Pst* DC3000 (*AvrRpm1*) at a concentration of 10^8 cfu or 10 mM $MgCl_2$ as mock control. At 1,3,5,6,8,10 and 22 hours post infiltration samples were subjected to ion leakage measurements.



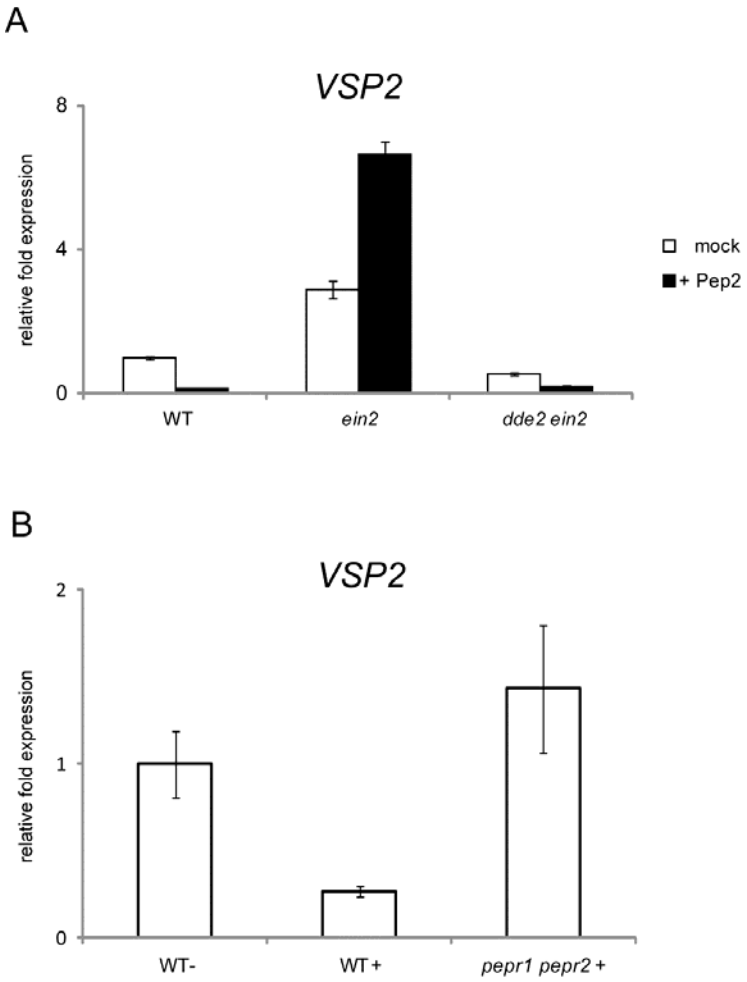
Supplementary Figure 9: Pep2-induced systemic gene induction. Four-week old wild-type plants were infiltrated with 1µM Pep2 in lower leaves and systemic untreated leaves were harvested 24 hours later for transcript analysis of *PDF1.2a*, *PROPEP2* and *PROPEP3*. The relative expression is shown in fold, with the gene/At4g26410 value after mock treatment in WT plants as 1.



Supplementary Figure 10: PEPR1 co-immunoprecipitates with BAK1 in a ligand-specific manner. A) Leaves of *N. benthamiana* were transiently co-transformed with PEPR1-FLAG, BAK1-HA and PROPEP2 constructs. After three days leaf samples were incubated in water containing Pep3 for 30 minutes and subsequently used for Co-IP experiments. Errors indicate the position of protein band (◂). B) Leaves of *N.benthamiana* transiently expressing PEPR1-FLAG and BAK1-HA for 2 days were infiltrated with 200 nM the indicated peptides and 15 minutes later harvested for Co-IP. Experiments were performed and data kindly provided by Misuzu Yamada.



Supplementary Figure 11: *PR1* expression requires functional Pep-signaling. *PR1* transcript induction in 10-day old seedlings treated with 1 µM elf18 for 10 h. The relative expression is shown in fold, with the *PR1*/At4g26410 value after at 0 hpt in WT plants as 1.



Supplementary Figure 12: Repression of the MYC2 branch during Pep-signaling. A) 10-day-old wild-type seedlings were treated with 1 µM Pep2 and harvested at 24 hpt for *VSP2* transcript analysis. The relative expression is shown in fold, with the *VSP2*/At4g26410 value after mock treatment in WT plants as 1. B) 10-day-old seedlings of the indicated genotypes were treated and analyzed as described for A).

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„And thanks to Paul for giving me the opportunity to work in his department"……..
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Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegt worden ist, sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde.
Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Dr. Yusuke Saijo und Prof. Dr. Paul Schulze-Lefert betreut worden.

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