An Endogenous Danger Detection System in *Arabidopsis thaliana*: The *At*Pep Peptides and their Receptors

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Summary

Plants use a plethora of sophisticated detection systems to recognize a variety of attackers and to subsequently initiate defense responses. A well-known paradigm in this context is the perception of microbe-associated molecular patterns (MAMPs) by pattern recognition receptors (PRRs), a process referred to as pattern triggered immunity (PTI). Additionally, plants also recognize endogenous molecules to induce similar defense responses. These molecules are believed to be released upon enemy attack and are therefore referred to as danger-associated molecular patterns (DAMPs). The best-investigated DAMP so far is systemin, a short peptide capable of inducing defense responses in tomato and required for full-strength defense against insect herbivores.

More recently, a family of eight peptides has been discovered in Arabidopsis, named *Arabidopsis thaliana* danger peptides (*At*Peps) 1-8. These *At*Peps have been shown to be capable of inducing PTI-like responses and to be expressed upon the detection of various biotic stresses, therefore being considered as DAMPs. Moreover, two PRRs, named Pep-Receptor 1 (PEPR1) and Pep-Receptor 2 (PEPR2) have been identified to perceive *At*Peps and to induce defense responses upon receptor-ligand interaction. Despite of eliciting PTI responses and being expressed upon the detection of biotic stress, no direct beneficial involvement of the *At*Pep-PEPR system to plant defense against attackers has been described so far.

Taking advantage of a mutant deficient in both PEPRs and thus fully impaired in *At*Pep-PEPR signaling, we investigated the potential contribution of a functional *At*Pep-PEPR system to plant defense responses.

In a first approach, we investigated the potential interplay between MAMP and DAMP signaling, especially in the context of DAMPs being believed to act as endogenous amplifiers of MAMP-induced PTI. Doing so, we identified that the *At*Pep-triggered production of reactive oxygen species (ROS) is strongly enhanced upon previous MAMP detection, indeed indicating a role of the *At*Pep-PEPR signaling system as an enhancer of MAMP-triggered defense signaling.

In a second approach, we compared the *At*Pep-PEPR system to systemins – well described DAMPs in tomato with generally similar molecular features to *At*Peps. Following up the lead that systemins are important mediators of defense responses against herbivorous insects, we checked whether a similar role would apply to the *At*Pep-PEPR system. Here, we could show that the *At*Pep-PEPR system is indeed induced by herbivore feeding and strongly interacts with the plant hormone jasmonic acid (JA) to orchestrate defense responses. Accordingly, mutants deficient in *At*Pep-PEPR signaling are strongly impaired in defense responses against the generalist herbivore *Spodoptera littoralis*, underlining the importance of *At*Pep signaling in plant defense against herbivores.

Thirdly, we followed up a lead that the expression of some *At*Peps as well as both PEPRs is induced upon virus infection. Assessing the potential contribution of the *At*Pep-PEPR system to plant defense against viruses, we could not observe an increased susceptibility of plants deficient in both PEPRs. However, mutants in BAK1 (BRI1 Associated Kinase 1), a co-receptor required for full-strength *At*Pep-triggered signaling and many other PRRs, showed a clearly increased susceptibility to all viruses tested. Therefore, we established a first potential line of evidence for a role of PTI in plant defense against viruses.

All in all, we provide several lines of evidence that show the contribution of a functional *At*Pep-PEPR signaling system to plant defense. Therefore, we underline the pivotal importance of DAMP signaling to plant immunity against a plethora of biotic invaders.

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1. General Introduction

Green plants are the main source of energy to terrestrial ecosystems, which depend on the plant's ability to turn sunlight, CO_2 and water into sugar and thus make carbon available for other organisms. As a primary food source, plants are constantly being attacked by various microbial pathogens and eaten by numerous animals, the so-called "herbivores". Moreover, given their sessile nature, plants cannot avoid danger by simply moving away. Still, despite this superficially apparent deficit in self-protection and the plethora of potential enemies, plants prevail in most environments. Indeed, as a key to success, plants have evolved very reliable defense systems, consisting of several layers of constitutive and inducible responses to deter approaching enemies (Thordal-Christensen 2003; Jones and Dangl 2006; Howe and Jander 2008).

Amongst the constitutive defense systems, physical barriers, such as a waxy cuticle covering the leaf surface, thorns, trichomes, as well as lignified cell walls and secondary metabolites potentially harmful to the invader deter most organisms from attacking the plant (Thordal-Christensen 2003).

In the unlikely case that potential invaders overcome these physical barriers, they are confronted with a very sophisticated plant immune system specifically recognizing molecules derived from the attacking organism and from already attacked plant cells. The recognition of these molecules then induces a set of immediate and long-term defense responses, both locally and systemically (Boller and Felix 2009; Dangl, Horvath et al. 2013).

Intriguingly, similar signaling systems are also used by the plant to orchestrate non-defense physiological processes, such as development and reproduction (Murphy, Smith et al. 2012). Given the high similarities between the signaling processes mentioned, we will discuss the most important mechanisms and paradigms of these systems but will remain focused on the initiation and integration of defense signaling against biotic invaders.

1.1 The Concept of Innate Immunity

Unlike higher animals, plants lack a mobile immune system integrated by cells specialized in enemy detection and destruction, circulating in the bloodstream and therefore accessing most parts of the organism. Instead, each plant cell has to have an individual danger detection system, the capacity to induce defense responses, and the ability to initiate signaling cascades to render other parts of the plant alert to the imminent attack (Schilmiller and Howe 2005; Jones and Dangl 2006). In most cases, the initial detection of an abundant microbial pathogen or herbivorous animal is achieved by membrane bound receptors, so-called pattern recognition receptors (PRRs), which recognize specific structures of the invading organism or modified-self molecules to subsequently initiate downstream signaling and defense responses (Boller and Felix 2009). The concept of this detection system is referred to as pattern-triggered immunity (PTI) and is strongly similar to the concept of innate immunity in the animal field (Medzhitov and Janeway 2000; Jones and Dangl 2006: Boller and Felix 2009).

Know your Enemy: The Detection of Biotic Danger

To detect a big diversity of potential microbial pathogens and initiate PTI, PRRs specifically recognize highly conserved structures common amongst entire classes of microbes, so-called microbe-associated molecular patterns (MAMPs). As most of these structures are vital for the microbial life style, they underlie a negative selection pressure and cannot easily be amended by the microbes to evade recognition (Boller and Felix 2009; Monaghan and Zipfel 2012). Therefore, PTI is regarded as an evolutionary old defense system, which is also underlined by the before mentioned conservation between innate immunity in higher plants and animals and the fact that many PRRs are conserved amongst higher plants (Boller and Felix 2009).

Similar detection and signaling systems to PTI have been described in the context of herbivore recognition and the recognition of endogenous signaling molecules. Both will briefly be discussed in the subsequent chapters.

Many Candidates for a Plethora of Tasks: Pattern-Recognition Receptors

Membrane-bound PRRs can be broadly categorized into receptor-like kinases (RLK) and receptor-like proteins (RLP). RLKs structurally consist of an extracellular receptor domain, a membrane-spanning domain and an intracellular kinase domain, which is missing in RLPs (Morillo and Tax 2006; Tor, Lotze et al. 2009). In Arabidopsis, more than 600 RLKs have been identified in the genome, not only fulfilling roles in danger detection, but also other plant physiological processes, such as growth and reproduction (Shiu and Bleecker 2001; Shiu, Karlowski et al. 2004). Additionally, the 57 identified RLPs are also brought into connection with a vast variety of physiological processes, including defense (Tor, Lotze et al. 2009; Jehle, Lipschis et al. 2013). However, since RLPs are lacking an intracellular kinase domain, they are believed to rely on the interaction with other RLKs or with cytoplasmic protein kinases to propagate the signal induced by receptor-ligand binding (Tor, Lotze et al. 2009).

Depending on the structure of their extracellular receptor domain, RLKs can further be classified into 21 structural classes (Shiu and Bleecker 2001). Here, the family characterized by LRRs (Leucine-Rich Repeats) in the receptor domain (LRR-RLKs), is being the largest with roughly 235 members (Shiu, Karlowski et al. 2004; Lehti-Shiu, Zou et al. 2009). Also, LRR-RLKs are not solely involved in the detection of exogenous elicitor molecules and PTI, but also in the detection of endogenous signals, both in the context of defense and other physiological processes. Figure 1 provides an overview of some of the LRR-RLKs best known so far, their identified ligands in Arabidopsis and the physiological processes they are involved in.

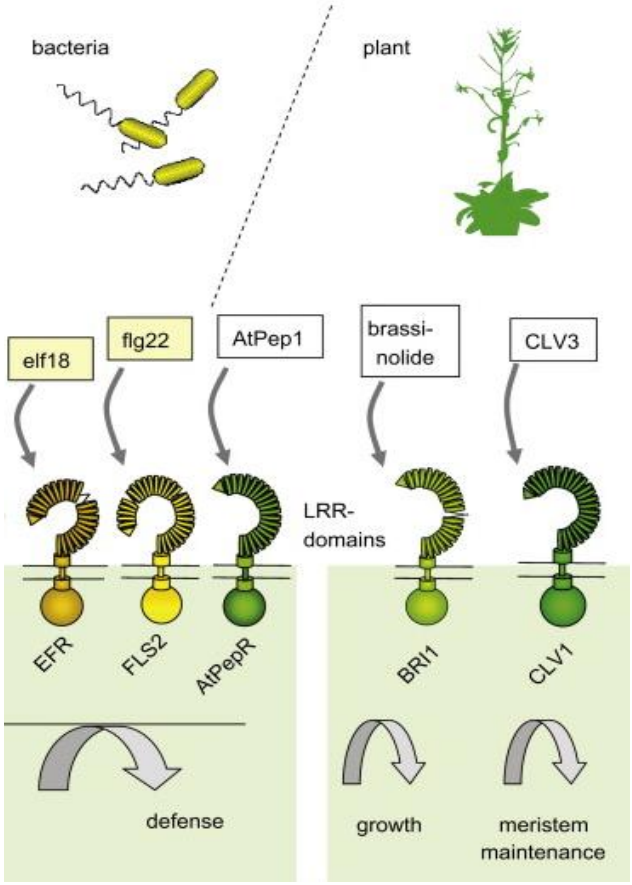

Figure 1. An overview of some well-characterized LRR-RLKs with identified ligands and their role in plant physiological processes. LRR-RLKs were shown to be involved the recognition of both, exogenous and endogenous ligands, mediating several plant physiological processes, including defense. EFR and FLS2 detect proteinaceous MAMPs derived from bacteria (Felix, Duran et al. 1999; Gomez-Gomez and Boller 2000; Kunze, Zipfel et al. 2004) and *At*PEPRs endogenous peptides (Yamaguchi, Pearce et al. 2006; Krol, Mentzel et al. 2010), all initiating PTI responses upon receptor-ligand interaction. In contrast, BRI1 and CLV1 perceive the steroid hormone brassinolide and the small endogenous peptide CLV3, respectively (Clark, Running et al. 1995; Wang, Seto et al. 2001), facilitating developmental processes. This overview features not all RLKs with identified ligands in Arabidopsis. For a full list, please refer to table 1.

Adapted from Albert et al. (2010)

LRR-RLKs can be further divided into 13 subfamilies based on copy number and arrangement of the LRR-motifs in the extracellular domains (Shiu and Bleecker 2001). Of particular interest to this work are the LRR-RLKs of the subgroups X-XII, which contain the so-far best described PRR interaction partners for several MAMPs as well as the receptors for several endogenous ligands (Ryan, Huffaker et al. 2007) (Figure 2).

Moreover, RLKs can also be grouped into RD and non-RD kinases according to specific properties of their intracellular kinase domain (Shiu and Bleecker 2003; Tor, Lotze et al. 2009). The abbreviation “RD” stands for a specific pattern in the amino acid sequence of the kinase domain, indicating the abundance of an Arginine (R) in front of an Aspartate (D) in the catalytic loop. Non-RD kinases are impaired in autophosphorylation, an important step of signal transduction. To overcome this, non-RD kinases require a co-receptor with which they

immediately dimerize after ligand binding (Dardick and Ronald 2006). Interestingly, up to date, all RLKs with known interaction partners and characterized as non-RD kinases have been shown to be involved in the recognition of microbial pathogens, which gave rise to speculations whether this would be a common motif for MAMP detection (Schwessinger and Ronald 2012).

One for Many: BAK1 as an Interaction Partner for PRRs

A well characterized co-receptor for some non-RD and even RD kinases is BRI1-associated kinase1 (BAK1), which was originally discovered to dimerize with BRI1 (Brassinosteroid-Insensitive 1), the receptor for the plant hormone brassinosteroid (BR) (Wang, Seto et al. 2001; Li, Wen et al. 2002). BAK1 is a member of the SERK (Somatic-Embryogenesis Receptor-like Kinase) family containing five members with potentially redundant functions as interaction partners for PRRs (Roux, Schwessinger et al. 2011). All SERKs are characterized by a typical serine and proline rich motif after a truncated extracellular LRR domain and belong to the LRR-RLK subclass II (Hecht, Vielle-Calzada et al. 2001; Albrecht, Russinova et al. 2008).

In the context of PTI, the two non-RD receptor kinases FLS2 (Flagellin-Sensing 2) (Gomez-Gomez and Boller 2000) and EFR (Elongation Factor-TU Receptor) are amongst the best-known interaction partners for BAK1 (Gomez-Gomez and Boller 2000; Kunze, Zipfel et al. 2004; Zipfel, Kunze et al. 2006; Chinchilla, Zipfel et al. 2007; Roux, Schwessinger et al. 2011). Since BAK1 mutants display an impaired defense response upon the perception of certain microbial structures, BAK1 is believed to be a positive regulator of the according PRRs (Chinchilla, Shan et al. 2009; Roux, Schwessinger et al. 2011). This is assumed to be achieved by direct BAK1-PRR interaction and transphosphorylation of each other's kinase domain (Schulze, Mentzel et al. 2010; Schwessinger, Roux et al. 2011).

However, other BAK1 interaction partners, such as receptors for endogenous signaling peptides and even receptors for negative regulation of defense have been identified in recent years, as reviewed by Kemmerling and Halter (2011). Also, apart from BAK1, a raising number of direct

interaction partners with PRRs have been discovered. An overview of these partners is for instance featured in a review by Schwessinger and Ronald (2012).

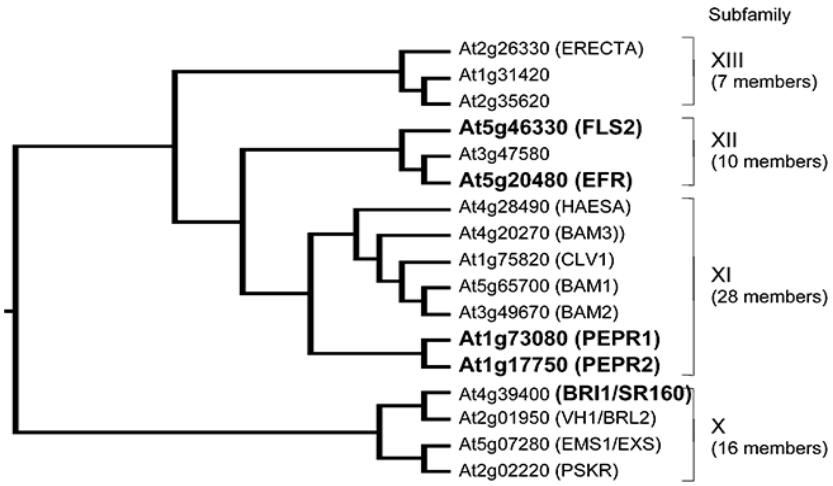

Figure 2. The phylogenetic alignment of LRR-RLK subfamilies X-XIII. Names of receptors (where named) are listed in brackets after the gene annotation codes. Shown interaction partners of BAK1 are listed in bold.

Adapted from Ryan et al. (2007)

A First Layer of Defense: PRRs Involved in the Recognition of Microbial Pathogens

As the first LRR-RLK to be involved in PTI, FLS2 was shown to recognize flagellin, the building block of the bacterial flagellum (Felix, Duran et al. 1999; Gomez-Gomez and Boller 2000). Even at subnanomolar concentrations, the recognition of flagellin by FLS2 induces a set of defense responses which eventually lead to an enhanced resistance against bacterial pathogens (Gomez-Gomez and Boller 2002; Zipfel, Robatzek et al. 2004; Chinchilla, Bauer et al. 2006). In the plant kingdom, the perception of flagellin is widely distributed, with FLS2 homologues being identified in the genomes of all higher plants analyzed so far (Boller and Felix 2009).

A second well-characterised receptor is EFR, which recognizes a conserved N-terminal part of the bacterial elongation factor EF-Tu, referred to as elf18 or elf26 (Kunze, Zipfel et al. 2004). As for flagellin-FLS2 interaction, the recognition of EF-Tu by EFR induces a similar set of defense responses which eventually render the plant more resistant to infections by bacterial pathogens (Zipfel, Kunze et al. 2006). In contrast to flagellin perception, EF-Tu is only perceived by species of the Brassicaceae plant family (Zipfel, Kunze et al. 2006). However, tomato plants can be made sensitive to elf18 application by transgenically expressing Arabidopsis EFR,

indicating a highly conserved and highly homologous PRR-independent downstream signaling cascade (Lacombe, Rougon-Cardoso et al. 2010).

Both, FLS2 and EFR are non-RD kinases belonging to the subfamily XII of LRR-RLKs and depend on BAK1 for the initiation of full-strength defense responses. Moreover, all so-far characterized members of subfamily XII have been associated with MAMP detection (Schwessinger and Ronald 2012).

However, despite the claim of non-RD kinases being exclusively involved in MAMP detection, some PRRs recognizing microbial structures are also found amongst the RD-kinases (Schwessinger and Ronald 2012). Amongst those, CERK1 (Chitin Elicitor Receptor Kinase 1) is required for the detection of chitin in Arabidopsis, a major component of fungal cell walls (Miya, Albert et al. 2007; Wan, Zhang et al. 2008). Interestingly, recent evidence suggests that CERK1 might also act as a receptor or co-receptor for the recognition of bacterial peptidoglycan, therefore being involved in both, the detection of fungal and bacterial pathogens (Willmann, Lajunen et al. 2011).

A Second Layer of Defense: Effector-Triggered Immunity (ETI)

Despite of PTI being a generally very efficient defense system, certain pathogens or pathogen strains have adapted to it by introducing virulence effectors into the host, which inhibit specific steps of pathogen detection or subsequent downstream signaling processes. This process, basically inhibiting PTI, is referred to as effector-triggered susceptibility (ETS) (Jones and Dangl 2006; Boller and He 2009; Dodds and Rathjen 2010). In this context, the interaction between Arabidopsis and the pathogen *Pseudomonas syringae* (strain DC3000), is well studied (Guttman, Vinatzer et al. 2002). *Pseudomonas* was shown to introduce several effectors into plant cells using a type III secretion system, which is widely common amongst pathogenic bacteria (Abramovitch, Janjusevic et al. 2006; Cunnac, Lindeberg et al. 2009). For instance, the *Pseudomonas* effectors AvrPto and AbrPtoB directly interact with PRRs involved in MAMP detection to inhibit the initiation of downstream signaling processes (Göhre, Spallek et al. 2008; Shan, He et al. 2008). However, not all effectors directly target PTI signaling or MAMP recognition as for instance HopU1 interferes with RNA-binding proteins and thus potentially transcriptional reprogramming after pathogen attack (Fu, Guo et al. 2007) whereas others seem to target the hormonal integration of defense responses (Da Cunha, Sreerekha et al. 2007). Similarly to bacteria, pathogenic funghi also introduce their effectors into the host cell through the haustorial interface. However, the exact mechanisms of fungal effector translocation into plant cells remain yet unclear (Panstruga and Dodds 2009).

To adapt to effectors, plants have in turn developed a second layer of immunity, referred to as effector-triggered immunity (ETI), formerly and famously known as gene-for-gene resistance (Flor 1971). This defense mechanism is based on surveillance systems either directly detecting secreted effectors or indirectly detecting a modified self signal originating from effector attack (Boller and He 2009). These surveillance systems rely on so-called *resistance* (*R*) genes, which mostly code for intracellular NB-LRR proteins (Nucleotide Binding Proteins with Leucine-Rich Repeat domains) (Jones and Dangl 2006). Upon the recognition of effectors or effector-caused modified self, these *R*-genes initiate a defense response that is generally regarded to be more rapidly induced, longer lasting, and more severe than PTI, frequently culminating in the

hypersensitive response (HR), the apoptosis of the infested and surrounding cells (Greenberg and Yao 2004; Jones and Dangl 2006; Truman, Zabala et al. 2006; Tsuda and Katagiri 2010).

Whereas PTI is mainly based on the detection of conserved and hardly changeable microbial structures, ETI is regarded as an evolutionarily dynamic process with plants and pathogens constantly adapting their effector and *R* gene repertoire to be one step ahead of the opponent. An underlining fact to support the dynamics of these adaptions is that whereas PTI interactions are frequently mediated in a non-host manner, ETI interaction is often highly specific between even a particular plant cultivar and a pathogen race (Dangl and Jones 2001). The evolutionary context of this ongoing "arms race" between plants and pathogens to avoid and achieve detection is nicely outlined in the zigzag model proposed by Jones and Dangl (2006) (Figure 3).

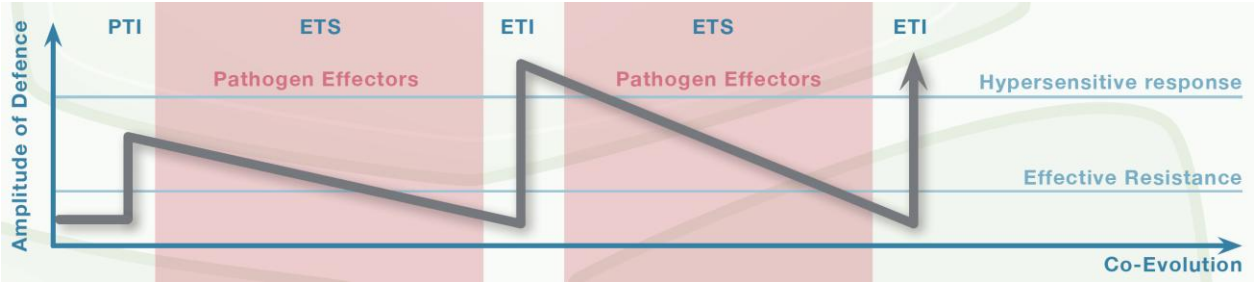

Figure 3. An illustration of the zigzag model as proposed by Jones and Dangl (2006) to describe the evolutionary background of ETS and ETI. Certain adapted pathogens have developed the ability to evade PTI by the secretion of effectors, leading to ETI. In response, the recognition of these pathogen-specific effectors by the plant can lead to an enhanced immune response, referred to as ETI and underlining the recognition of imminent pathogenic danger. This ongoing detection and evasion of detection constitutes the paradigm of Jones' and Dangl's zigzag model of ETS/ETI.

More Trouble Abound: Mechanisms Involved in the Detection of Herbivores

The detection of herbivorous insects is believed to be achieved by the detection of two separate events: a) the detection of mechanical damage caused by chewing insects b) the detection of elicitors of either plant and/or insect origin (Mithöfer and Boland 2008). To go in hand with the nomenclature for microbial elicitors, herbivore-derived elicitors have been termed herbivore-associated molecular patterns (HAMPs) (Mithöfer and Boland 2008).

Several HAMPs have been identified so far, with the most famous being volicitin, a fatty acid – amino acid conjugate found in the oral secretions (OS) of the beet armyworm (Turlings, McCall et al. 1993; Alborn, Turlings et al. 1997). Other well-known HAMPs include the bruchins, fatty-acid molecules derived from insect eggs after oviposition on plant leaves (Doss, Oliver et al. 2000) and inceptins, peptide fragments of plant-derived ATP-synthases broken down in the insect gut (Schmelz, Carroll et al. 2006).

Whereas the exact recognition mechanisms of HAMP detection still remain to be investigated, there is evidence that as for PTI, inceptin and volicitin recognition is mediated by membrane-bound receptors (Truitt, Wei et al. 2004; Maischak, Grigoriev et al. 2007). Moreover, recent reports describe a decreased defense response upon herbivore attack and OS application in BAK1-silenced *Nicotiana attenuata* plants (Yang, Hettenhausen et al. 2011). Therefore, it is tempting to speculate that similarly to the detection of MAMPs, the detection of HAMPs could also be mediated by RLKs that at least partially require BAK1 as a co-receptor.

1.2 Plants Striking Back: Physiological Responses to Biotic Danger

As already mentioned, after the perception of biotic threat, plants activate a set of immediate and long term responses as well as downstream signaling cascades to adapt to the imminent danger. This set of responses has been most thoroughly studied in the context of microbial pathogen detection and PTI. Here, I would like to outline the most important plant responses to biotic enemies, their hormonal integration and potential differences between the defense against different classes of pathogens and feeding enemies.

Direct Immune Responses

Immediately after the detection of an elicitor molecule by a PRR, a cascade of downstream responses is initiated. Most of the downstream responses listed here are also inducible by the exogenous application of MAMPs and therefore provide robust readouts for innate immunity responses. The temporal progression of these direct PTI responses is illustrated in Figure 4.

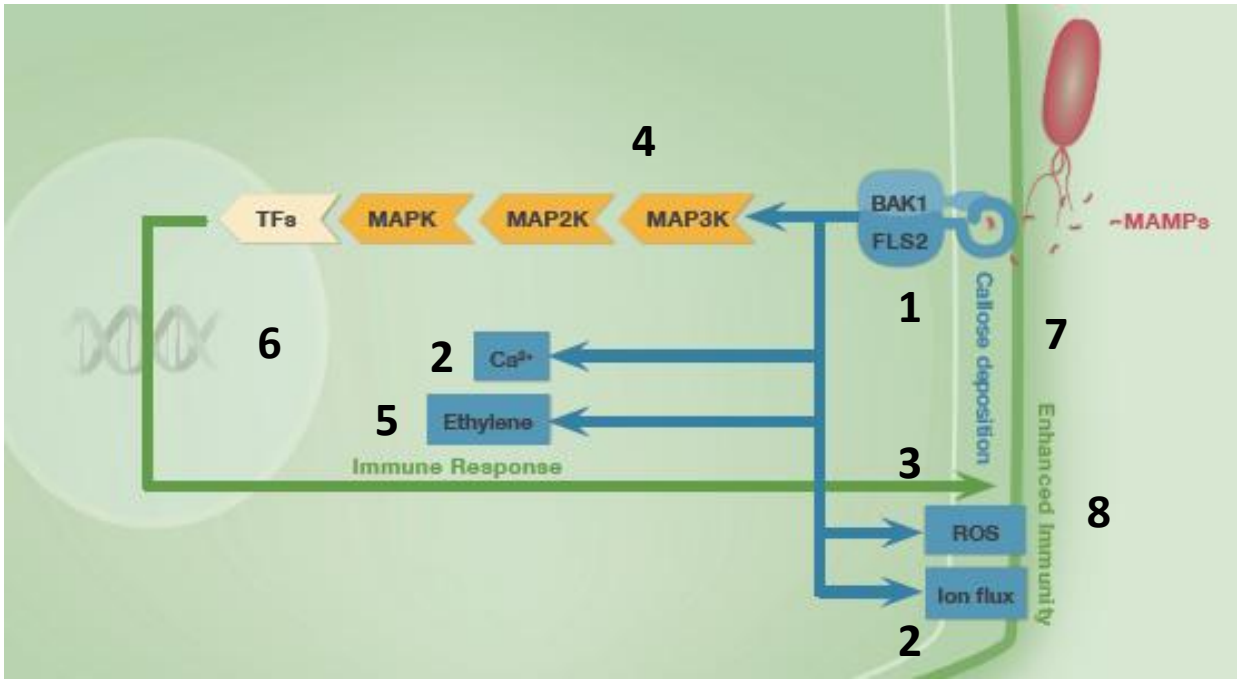

Figure 4. The spatial and temporal arrangement of direct PTI responses. Upon the recognition of ligands, many PRRs interact with co-receptors to initiate kinase activation, potentially leading to cross-phosphorylation and phosphorylation of downstream targets (1). Subsequently, a set of immediate responses is induced, such as the initiation of cross-membrane ion fluxes (2), the production of ROS (3), and the activation of MAP Kinase cascades (4). Also, ethylene biosynthesis (5), the expression of defense genes (6), as well as the deposition of callose at a later stage is initiated by the plant (7). Altogether, the signaling cascades induced by the activated PRRs and the resulting, quantitatively measurable immune responses lead to an enhanced immunity to pathogen attack (8).

Cross-Phosphorylation with Interaction Partners

Almost instantaneously after flg22 treatment, FLS2 associates with BAK1, which leads to de novo phosphorylation of both receptors (Chinchilla, Zipfel et al. 2007; Schulze, Mentzel et al. 2010). This rapid heteromerization with BAK1 was also observed for EFR and the hormone receptor BRI1, which therefore might represent a common feature in LRR-RLK signaling responses (Schulze, Mentzel et al. 2010).

Ion Fluxes

Upon the detection of MAMPs, plant cells initiate cross-membrane ion fluxes within minutes, namely an H^+ and Ca^{2+} influx into the cell and an anion efflux. This activation of ion fluxes can best be observed in suspension cultured cells, where they lead to a pH-Shift in the liquid growth medium (Boller 1995). The increase of intracellular Ca^{2+} is considered to be of particular interest since Ca^{2+} is known to be a second messenger in diverse cellular processes (Lecourieux, Raneva et al. 2006). Moreover, in Arabidopsis, four calcium-dependent protein kinases (CDPKs) were recently identified as Ca^{2+} sensors involved in regulating innate immunity (Boudsocq, Willmann et al. 2010). Additionally, CDPKs were proposed to be important for the regulation of the production of reactive oxygen species by directly phosphorylating the ROS producing enzyme NADPH oxidase (Kobayashi, Ohura et al. 2007).

Production of Reactive Oxygen Species (ROS)

The production of reactive oxygen species is another rapid response upon pathogen recognition, normally being detectable roughly 5-10 mins after MAMP application by using a luminol-based assay (as in Chinchilla et al. (2007)). Upon MAMP detection, ROS are mainly produced by plasma-membrane-bound NADPH-oxidases, referred to as respiratory-burst homologues (Rboh) (Torres, Dangl et al. 2002; Torres, Jones et al. 2006). In plant defense against pathogens, several functions for ROS are proposed, such as directly harming the invading pathogen, as reported for animal phagocytes and lymphocytes (Apel and Hirt 2004), or cross-linking of cell walls to strengthen physical barriers (Lamb and Dixon 1997). More recent evidence also suggests ROS to play a role in intracellular and systemic signaling events (Miller, Schlauch et al. 2009; Mittler, Vanderauwera et al. 2011), which is also supported by the fact

that some bacterial effectors directly block the ROS production machinery (Göhre, Spallek et al. 2008; Gimenez-Ibanez, Hann et al. 2009).

Activation of MAP Kinase Cascades

In many eukaryotic organisms, the activation of mitogen-activated protein (MAP) Kinases is a key element in the transduction of external stimuli into intracellular responses (Dong, Davis et al. 2002). MAP Kinase cascades typically start with the phosphorylation of a MAP Kinase Kinase Kinase (MAPKKK), which phosphorylates a MAPKK, which in turn phosphorylates a MAPK. Finally, MAPKs themselves can target various proteins for phosphorylation, either in the nucleus or the cytoplasm. In Arabidopsis, a MAP kinase cascade is induced by activated PRRs, resulting in the activation of MPK3 and MPK6 within 2-10 mins after the application of MAMPs like flg22 and thus provide a good readout for immediate immune responses (Nühse, Peck et al. 2000; Chinchilla, Zipfel et al. 2007).

Ethylene Production

Ethylene is a gaseous plant hormone that is involved in many aspects of plant development as well as adaptions to biotic and abiotic stress. In defense, ethylene signaling was shown to be a critical player in many separate events. It is produced within the first hours upon MAMP detection (Felix, Duran et al. 1999; Kunze, Zipfel et al. 2004) with ACS (ACC Synthase), the rate limiting enzyme involved in ethylene biosynthesis being activated within minutes (Spanu, Grosskopf et al. 1994). The accumulation of ethylene can be assessed by gas chromatography, as for instance described by Krol et al. (2010).

Recently, two studies have proposed ethylene to be a critical component for FLS2 receptor accumulation and flg22-induced ROS production (Boutrot, Segonzac et al. 2010; Mersmann, Bourdais et al. 2010). Additionally, ethylene signaling also seems to be required for callose deposition, a stereotypical long-term response upon MAMP recognition (Clay, Adio et al. 2009).

Transcriptional Changes

Transcriptomic studies revealed that already 30 mins after flg22 treatment, roughly 1000 genes were up regulated in Arabidopsis with approximately 200 genes being down regulated (Zipfel, Robatzek et al. 2004). Subsequent studies also revealed that a similar pattern of gene

regulation was also observed upon elf26 and chitin application, indicating that MAMP signaling converges at an early stage of gene expression (Ramonell, Zhang et al. 2002; Zipfel, Kunze et al. 2006). Amongst the genes induced, there are over 100 of the roughly 600 RLKs present in the Arabidopsis genome, including the PRRs FLS2 and EFR, potentially indicating a positive feedback loop of PTI activation (Shiu, Karlowski et al. 2004; Zipfel, Robatzek et al. 2004). Further MAMP induced genes include pathogenesis-related (PR) genes, transcriptional regulators, as well as kinases and phosphatases (Navarro, Zipfel et al. 2004; Moscatiello, Mariani et al. 2006; van Loon, Rep et al. 2006; Zipfel, Kunze et al. 2006).

Receptor Endocytosis

Quickly after flg22 stimulation, FLS2 undergoes vesicle-mediated endocytosis and disappears from the cell membrane (Robatzek, Chinchilla et al. 2006). Similar observations have also been reported for the XA21 receptor in rice (Bar and Avni 2009; Chen, Gao et al. 2010), leading to the speculation that endocytosis might be widespread in PRR signaling (Beck, Heard et al. 2012). Still, the exact contribution of endocytosis to PTI remains largely unclear.

Stomatal Closure

Stomata are pores on the leaf surface that regulate plant gas exchange and transpiration. As such, they constitute a natural entry site for a significant number of microbes (Sawinski, Mersmann et al. 2013). Sensibly, as a defense response, plants close stomata within minutes after the perception of several MAMPs including flg22, elf18, and chitin (Melotto, Underwood et al. 2006; Cao, Yoshioka et al. 2011). The physiological importance of MAMP-triggered stomatal closure is for instance supported by the fact that fls2 receptor mutants only display a decreased resistance to *Pseudomonas syringae* DC3000 infections when bacteria are sprayed onto the leaf surface instead of being infiltrated into the leaf (Zipfel, Robatzek et al. 2004).

Callose Deposition

In Arabidopsis leaves, the deposition of callose can be made visible from 16 h onwards after MAMP treatment by fixing and staining with aniline blue (Gomez-Gomez, Felix et al. 1999). Despite of being used as a hallmark late response after MAMP detection, the biological foundation of this response as well as its biological relevance in the context of plant defense remain largely unclear (Boller and Felix 2009; Luna, Pastor et al. 2011).

Seedling Growth Inhibition (SGI)

The addition of MAMPs to seedling growth medium can lead to a strong inhibition of seedling growth in a concentration-dependent manner, which is strongly dependent on the PRR-MAMP interaction (e.g. Krol et al. 2010). Whereas the exact molecular details of this arrested seedling growth still remain unclear, a possible explanation would be a shifted resource allocation by the plant from growth to defense in respect to the sensed danger (Walters and Heil 2007; Boller and Felix 2009).

Hormonal Integration of Immune Responses

Downstream of PTI and ETI activation, several plant hormones come into play, further modulating the defense signaling network and potentially spreading the message of imminent danger to yet unaffected tissues (Pieterse, Leon-Reyes et al. 2009). Intriguingly, whereas the initial transcriptome after MAMP detection seems to be similar for the detection of large classes of elicitors (Zipfel, Kunze et al. 2006), the hormonal integration of the defense response can differ quite remarkably depending on the attacker (Glazebrook 2005).

Broadly speaking, pathogens can be divided into such with biotrophic and necrotrophic life styles (Glazebrook 2005). Whereas biotrophic pathogens depend on living cells to derive their nutrients, necrotrophic pathogens kill host cells very early, using lytic enzymes and phytotoxins to feed from dead plant material. However, in frequent cases pathogens gradually switch from biotrophic to necrotrophic life styles and no clear classification is feasible. Therefore, certain pathogens are also referred to as “hemibiotrophic” (Glazebrook 2005).

Given these differences in feeding specification, plants have developed specialized sensing and long-term signaling systems, specifically responding to the invader’s life style. Defense against biotrophic pathogens normally includes the possibility of a local HR and cell death to limit the spread of pathogens by eliminating their nutrient resources (Glazebrook 2005). Additionally, after PTI and ETI induction, the level of the phenolic plant hormone salicylic acid (SA) rises, with Ca^{2+} signaling being an important regulator of SA accumulation (Mishina and Zeier 2007; Du, Ali et al. 2009). After being perceived by a receptor complex containing several NPR (non expressor

of PR) proteins (Fu, Yan et al. 2012), SA signaling acts as an activator of a large set of defense-related genes, which are commonly referred to as pathogenesis-related (PR) genes (Dong 2004; Moore, Loake et al. 2011). Despite of PR genes being a diverse group, several of these genes encode for proteins with anti-microbial activity (van Loon, Rep et al. 2006). Once activated at the site of infection, the SA pathway often leads to a similar response in distal tissue to render yet unaffected tissues alert to an imminent pathogen threat - a phenomenon that is termed as systemic acquired resistance (SAR) (Grant and Lamb 2006; Vlot, Klessig et al. 2008). The highly increased susceptibility of SA accumulation mutants to biotrophic pathogens, such as EDS1 (Enhanced Disease Susceptibility 1) and PAD4 (Phytoalexin Deficient 4) underline the importance of SA signaling in PTI responses against biotrophic and hemibiotrophic pathogens (Feys, Moisan et al. 2001).

In the case of necrotrophic pathogens, the fatty acid derivate jasmonic acid (JA) and the gaseous hormone ethylene are seen as important mediators of downstream signaling (Bari and Jones 2009). In response to pathogen infections and tissue damage, JA levels increase locally (Lorenzo and Solano 2005; Wasternack 2007), and its active derivate JA-Isoleucine (JA-Ile) is perceived by the LRR protein COI1 (Coronatine Insensitive 1) (Feys, Benedetti et al. 1994; Yan, Zhang et al. 2009) to liberate transcription factors for JA-responsive genes in the nucleus (Fonseca, Chico et al. 2009). Amongst these, defensins, proteins with antimicrobial and enzyme inhibitory functions, are regarded as specific markers for JA-dependent defense signaling (Manners, Penninckx et al. 1998; Van der Weerden and Anderson 2012). Similarly to SA and SAR, the activation of JA signaling can lead to a systemic priming of tissue referred to as ISR (Induced Systemic Resistance), which is also induced by wounding and herbivore feeding (Bostock 2005; Schilmiller and Howe 2005). In this context, the increased susceptibility of JA and ethylene signaling and biosynthesis mutants to mostly necrotrophic pathogens underline the importance of JA signaling (Berger 2002; van Loon, Geraats et al. 2006).

Given the differential life style and feeding strategies of biotrophic and necrotrophic pathogens, it is hardly surprising that JA and SA are believed to act antagonistically (Glazebrook 2005).

However, more recent evidence suggests that the interplay between these two and other hormones is more complex and a potentially powerful capacity to finely regulate the immune response to the invader(s) encountered (Pieterse, Leon-Reyes et al. 2009).

Apart from SA, JA, and ethylene, an increasing number of plant hormones, such as auxin (Kazan and Manners 2009), cytokinins (Walters and McRoberts 2006), brassinosteroids (Nakashita, Yasuda et al. 2003), abscisic acid (ABA) (Ton, Flors et al. 2009) and several others have been shown to be involved in plant defense signaling (Bari and Jones 2009). However, given the complexity of the hormonal integration of plant defense processes, a detailed introduction would neither fit the frame of this work, nor is it necessary to understand it. Thus I only refer to a specialized review at this point (Pieterse, Van der Does et al. 2012).

Taking on Bigger Counterparts: Specific Defense Responses against Herbivores

After the recognition of HAMPs and herbivore attack, the immediate plant response is very reminiscent to early PTI events and includes the production of ROS, the induction of ethylene biosynthesis, and the activation of MAP kinases (Maffei, Mithöfer et al. 2007; Wu, Hettenhausen et al. 2007; Wu and Baldwin 2010). Depending on their feeding strategy, insect herbivores can be roughly divided into two categories, namely chewing and sucking herbivores (Schoonhoven, Loon et al. 2005). Here, a recent comparative transcriptional study revealed that early Arabidopsis gene expression after the recognition of the chewing caterpillar *Spodoptera littoralis* is more reminiscent to that upon *Pseudomonas syringae* infection than to that upon the recognition of the sucking herbivore *Myzus persicae* (Bricchi, Bertea et al. 2012).

Induced long-term defenses against herbivores can be classified into two distinct strategies: Direct defense, which targets the attacker itself, and indirect defense, which is aimed at recruiting natural enemies of the attacker (Howe and Jander 2008; Wu and Baldwin 2010). Direct responses to herbivores include the production of toxic secondary metabolites, such as diverse terpenoids and alkaloids, the latter group including some well-known compounds like nicotine and caffeine (Aharoni, Jongsma et al. 2005; Howe and Jander 2008). Moreover, certain

plants produce secondary metabolites that are stored as non-toxic precursors and only released upon herbivore damage. Well-studied examples of such compounds are the glucosinolates in *Brassicaceae* plants (Grubb and Abel 2006). Furthermore, plants can also produce defensive proteins that disrupt the digestive system of herbivores. Well-known examples of such are protease inhibitors (PIs) to inhibit the breakdown of proteins in the insect gut (Green and Ryan 1972; Zavala, Patankar et al. 2004), as well as cysteine proteases, lectins, and chitinases, which directly attack structures involved in the insect digestive system (Peumans and Van Damme 1995; Lawrence and Novak 2006; Mohan, Ma et al. 2006).

As indirect responses, plants can emit volatile compounds to attract predators of herbivores (Kessler and Baldwin 2001). Whereas this has been studied for quite some time for above-ground communication between maize and parasitic wasps (Turlings, Loughrin et al. 1995), there is now evidence that similar modes of below ground communication come into play to protect roots against herbivores (Rasmann, Köllner et al. 2005). Additionally, plants can also use volatiles for inter-plant and even inter-species communications to warn neighboring plants of the imminent herbivore attack (Engelberth, Alborn et al. 2004).

Traditionally, JA is regarded as the master hormone integrating plant defense responses against herbivores as it accumulates upon both, the detection of wounding and that of herbivore OS (Halitschke, Schittko et al. 2001; Glauser, Dubugnon et al. 2009). Moreover, several defensive proteins, such as many PIs were shown to be regulated by JA (Constabel, Bergey et al. 1995; Chen, Wilkerson et al. 2005). Consequently, mutants in JA biosynthesis as well as JA signaling display a strongly compromised resistance against a wide range of both, sucking and chewing herbivores (Howe, McCaig et al. 2004; Verhage, Vlaardingerbroek et al. 2011; Schweizer, Fernández-Calvo et al. 2013).

And Now for Something Completely Different…or not?: Mechanisms Involved in Plant Defense Against Viruses

Although in plants, the detection of and defense against viruses is believed to be mainly achieved by RNA-silencing (Ding and Voinnet 2007; Ruiz-Ferrer and Voinnet 2009; Llave 2010),

several lines of evidence have been compiled by Zvereva and Pooggin (2012) to support a role of PTI in plant defense against viruses.

Firstly, in animals, basal "innate" immunity was shown to be actively involved in defense responses against viruses. For instance, apart from recognizing a wide range of MAMPs, Toll-like receptors (TLRs) also recognize viral RNA and DNA (Song and Lee 2012). As viruses are obligate intracellular pathogens, the TLRs involved in the detection of viral infections are predominately located in the endomembrane system, such as TLR3, TLR7 and TLR8 for viral RNA and TLR9 for viral DNA (Rathinam and Fitzgerald 2011; Jensen and Thomsen 2012).

Additionally, overall, there are certain similarities between immune reactions against microbes and viruses (Soosaar, Burch-Smith et al. 2005), which goes in hand with the observation that several hallmark genes for PTI are induced upon viral infections (Whitham, Quan et al. 2003). Moreover, as for ETI, some NB-LRRs confer resistance against a broad variety of viruses (Soosaar, Burch-Smith et al. 2005; Moffett 2009).

As a last line of evidence connecting viral recognition to PTI in plants, Yang et al (2010) proposed an involvement of BAK1 in antiviral responses by observing an enhanced susceptibility of *bak1-4* mutants to TCV (Turnip Crinkle Virus) infections. However, this claim remains to be investigated since the *bak1-4* mutant used for those experiments displays pleiotropic deficits also in BR signaling (Schwessinger, Roux et al. 2011), and no RLK has so far been brought into connection with the detection of viral infections (Zvereva and Pooggin 2012).

1.3 Endogenous PRR Signaling Systems

Apart from mediating exogenous danger signals, several RLKs have also been shown to perceive endogenous molecules involved in different physiological processes other than defense. For instance, as already outlined before, the RD LRR-RLK BRI1 acts as the receptor for the phytohormone BR, a known mediator of a plethora of plant physiological processes (Wang, Seto et al. 2001; Zhu, Sae-Seaw et al. 2013). Additionally, PRRs have been identified as interaction partners of several small endogenous peptides involved in development and reproduction (Tor, Lotze et al. 2009; Czyzewicz, Yue et al. 2013). A list of all the so-far identified RLK-ligand interaction partners in Arabidopsis is featured in Table 1, indicating their signaling parners and thus underlining the high share of PRRs perceiving endogenous small-peptide ligands. Given this involvement of RLKs in other processes than defense, I would like to provide a short overview of the most important endogenous small-peptide signals identified so far. Still, RLKs are also believed to play an important role in the recognition of endogenous peptidic danger signals (Yamaguchi and Huffaker 2011) as well as in the perception of non-peptidic signaling molecules both in the context of danger and development (Tor, Lotze et al. 2009; Ferrari, Savatin et al. 2013). I will therefore also highlight some of these aspects.

Name	Overall Structure	Receptor domain	Ligand	Origin of Ligand	Reference
FLS2	Non-RD RLK	LRR	Flagellin/ flg22	Bacterial	Chinchilla et al. 2006 Gomez-Gomez et al. 2001
FLS2			CLV3	Endogenous	Lee et al. 2010
EFR	Non-RD RLK	LRR	EF-Tu/elf18	Bacterial	Zipfel et al. 2004
CERK1	RD RLK	LysM	Chitin	Fungal	Miya et al. 2007
CERK1			PGN	Bacterial	Willmann et al. 2011
WAK1	RD RLK	EGF-like	Pectin Fragments	Endogeous	Brutus et al. 2010
PEPR1	RD RLK	LRR	*At*Peps1-8	Enodgenous	Yamaguchi et al. 2006 Bartels et al. 2013
PEPR2	RD RLK	LRR	*At*Pep1 and *At*Pep2	Endogenous	Yamaguchi et al. 2010 Krol et al. 2010
PSKR1 ***PSKR2***	RD RLK	LRR	PSK-α	Endogenous	Matsubayashi et al. 2002
CLV1	RD RLK	LRR	CLV3	Endogenous	Clark et al. 1995 Ogawa et al. 2008
RPK2	RD RLK	LRR	CLV3	Endogenous	Kinoshita et al. 2010

Table 1. An overview of the so-far identified RLK-ligand interaction partners in *Arabidopsis thaliana*. RLKs with endogenous ligands are listed in italics to underline the high share of endogenous RLK-signaling systems. The recognition of CLV3 by FLS2 as reported by Lee et al. (2010) is still contested (see chapter on Clavata/CLE Peptides).

Adapted from Schwessinger and Ronald (2012).

Endogenous Signaling Peptides

Traditionally, plant hormones such as auxin, cytokinins, and ethylene have been assumed to be the main players in cell to cell communication and intercellular signaling processes (Davies 2010). More recently, small signaling peptides have received an increasing attention in this context (Katsir, Davies et al. 2011). A growing number of such small signaling peptides has been discovered in recent years and were shown to elicit a vast array of biological and physiological responses in several plant tissues (Czyzewicz, Yue et al. 2013). This is illustrated in Figure 5, showing the processes and tissues in which some of these small signaling peptides were shown to be involved.

Generally, these peptides are small (5-100 amino acids long) and often the cleavage products of larger post-translationally modified precursor peptides (Czyzewicz, Yue et al. 2013). They have been classified broadly into two major categories, namely post-translationally modified peptides and cysteine-rich peptides (Murphy, Smith et al. 2012). As already outlined, the characterized interaction partners of these small endogenous signaling peptides known so far are RLKs, with several ligand–receptor pairs identified for peptides of the first category (Table 1). However, despite of the structural differences, also cysteine-rich peptides are believed to be mostly perceived by membrane-bound RLKs (Fukuda and Higashiyama 2011). Still, given the partially identified interaction partners and the extensive knowledge available for some post-translationally modified peptides, I would like to focus more on this category of small signaling peptides.

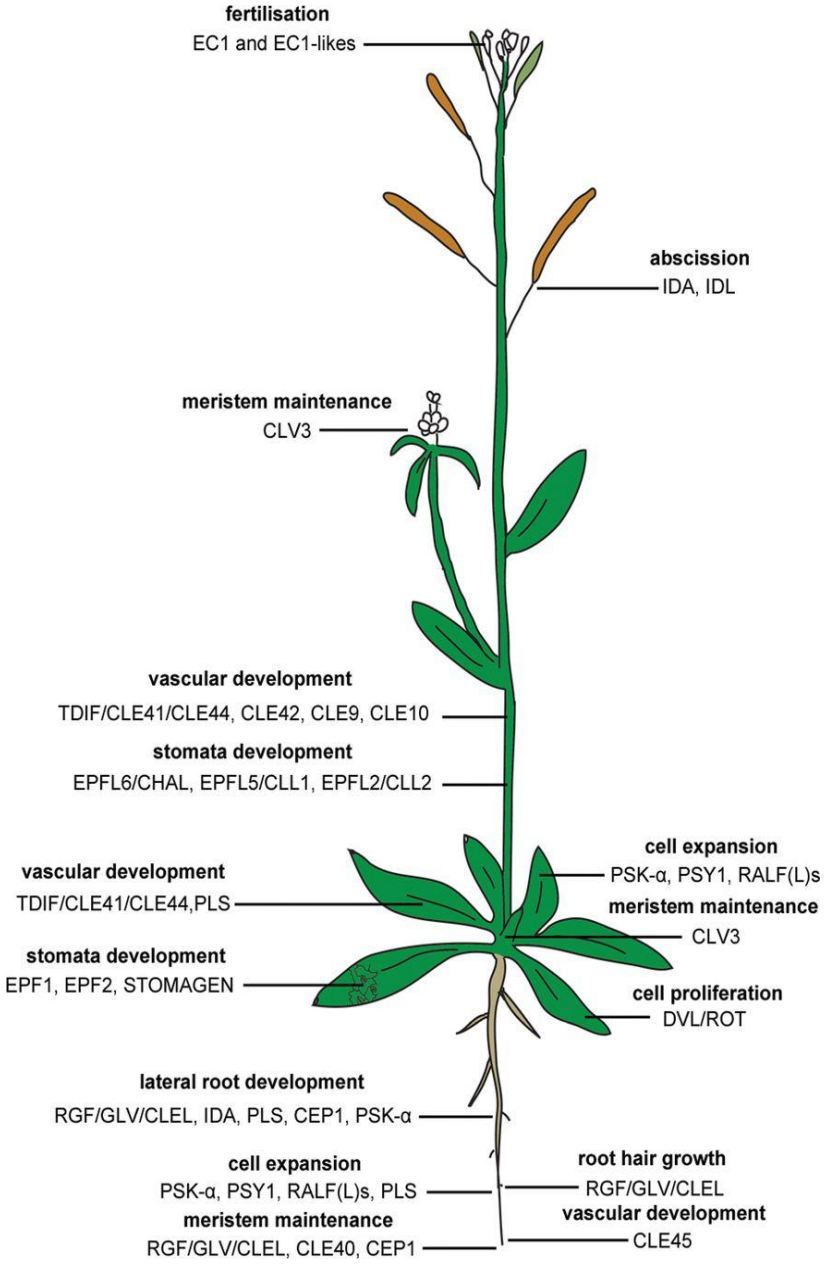

Figure 5. Small endogenous signaling peptides mediate a plethora of plant physiological processes. A visualization provided by Czyzewicz et al. (2013) to underline the spatial involvement of a variety of small endogenous peptides in several plant physiological processes. Not all small peptides featured in this figure are discussed in this introduction. For a full overview, please refer to the original review.

Clavata/CLE Peptides

The CLE/ESR (Clavata Like/Endosperm Surrounding Region) peptides form a family of 32 members in Arabidopsis, which derive from roughly 150 amino acid long precursor proteins with an overall low sequence homology (Wang and Fiers 2010). However, all CLE members share three common features: they code for small proteins of <10 kD, which contain a putative n-terminal secretion signal and a conserved c-terminal CLE-motif (14 AA) (Cock and McCormick 2001; Wang and Fiers 2010). CLE peptides control several plant physiological processes as intercellular signaling molecules and bind to cellular surface located receptors to transmit the signal. CLV3 (Clavata 3), an arabinosylated dodecapeptide, which represents the best characterized CLE peptide in Arabidopsis, is involved in the maintenance of the shoot apical meristem (SAM), but other CLE peptides fulfill a plethora of further tasks in plant development, such as cell fate regulation in the root apical meristem (RAM) (Kondo, Sawa et al. 2006; Betsuyaku, Sawa et al. 2011; Matsubayashi 2011). For CLV3 (Clavata 3), three major receptor kinase complexes are proposed for signaling in the SAM, namely CLV1 (Clavata 1, a LRR-RLK), CLV2 and Coryne (CRN, two RLPs), and the recently identified RPK2 (Receptor-like Protein Kinase 2, a LRR-RLK) (Clark, Running et al. 1995; Kayes and Clark 1998; Kinoshita, Betsuyaku et al. 2010). However, for other tissues and other CLE peptides, different receptors have been proposed as interaction partners (Betsuyaku, Sawa et al. 2011).

Apart from being involved in endogenous developmental processes, CLE peptides have also been shown to be active in environmental responses, such as the interaction with symbiotic nitrogen-fixing bacteria (Mortier, Den Herder et al. 2010). Additionally, CLE-like genes have been found in the genome of parasitic nematodes, which are potentially delivered into the cytoplasm of a host plant cell to alter host cell physiology in the attacker's favor (Wang, Mitchum et al. 2005; Wang, Replogle et al. 2011).

More recently, a role for CLV3 has also been proposed in the context of PTI: Lee et al. (2011) reported the CLV3 peptide to bind to the FLS2 receptor to mediate immunity in the SAM. However, since other research groups could not reproduce these results (Mueller, Chinchilla et

al. 2012; Segonzac, Nimchuk et al. 2012), the results of Lee et al. (2011) are probably an artefact.

Phytosulfokines

Phytosulfokines (PSKs) are disulphated pentapeptides, which were originally discovered to act as growth factors in low-density suspension cultures (Matsubayashi and Sakagami 1996; Matsubayashi, Takagi et al. 1999; Yang, Matsubayashi et al. 1999). In Arabidopsis, PSK-α is derived from roughly 80 amino acid long preproteins, which are encoded by five PSK precursor genes (Yang, Matsubayashi et al. 2001). The mature peptides are then perceived by two receptor proteins, PSKR1 (PSK Receptor 1) and PSKR2, both LRR-RLKs (Matsubayashi, Ogawa et al. 2002). Interaction of mature PSKs with their respective receptors was shown to be important for several developmental processes, such as the promotion of root growth (Kutschmar, Rzewuski et al. 2009) or the control of cell expansion in the hypocotyls (Stührwohldt, Dahlke et al. 2011). Recently, a role of PSKRs in the orchestration of innate immunity signaling has been proposed, most likely as a negative regulator of defense against biotrophic pathogens. This was based on the observation that *pskr1* mutants showed an enhanced resistance against the hemibiotrophic pathogen *Pseudmononas syringae* and generally increased PTI responses to elf18 application (Igarashi, Tsuda et al. 2012) but a decreased resistance against the necrotrophic pathogen *Alternaria brassicola* (Mosher, Seybold et al. 2013).

Other Endogenous Signaling Peptides

In recent years, several other endogenous signaling peptides have been discovered and were shown to be active in a variety of plant physiological processes (Murphy, Smith et al. 2012). Besides of PSKs, CLV3 and CLE/ESR peptides, the category of small post-translationally modified peptides includes PSY1 (Plant Peptide Containing Sulfated Tyrosine1), a peptide involved in cellular proliferation and expansion (Amano, Tsubouchi et al. 2007), RGF1 (Root Growth Factor 1), a peptide involved in root stem cell maintenance (Matsuzaki, Ogawa-Ohnishi et al. 2010), and CEP1 (C-Terminally Encoded Peptide 1), a strong inhibitor of root growth (Ohyama, Ogawa et al. 2008). For all these peptides, the corresponding receptors remain yet to be identified.

Amongst the Cys-rich peptides, stomagen, a regulator of stomatal density (Sugano, Shimada et al. 2009), epidermal patterning factors (EPFs) (Hara, Yokoo et al. 2009), and rapid-alkalinization factors (RALFs), potentially negative regulators of plant growth (Pearce, Moura et al. 2001; Bedinger, Pearce et al. 2010) are examples to be mentioned. Interestingly, the exogenous application of RALFs to plant tissue induces immediate responses reminiscent of PTI, such as medium alkalinization, MAPK activation and growth inhibition. Therefore a role in defense was suggested (Haruta and Constabel 2003). However, since neither MAMP treatment induced the expression of *RALF* genes nor did RALF application induce the expression of defense marker genes, a role in development seems to be more plausible (Haruta and Constabel 2003; Germain, Chevalier et al. 2005; Bedinger, Pearce et al. 2010). Still, RALF signaling again is a remarkable example how apparently similar signaling cascades can lead to potentially different long-term consequences.

Endogenous Signals Involved in Defense

Apart from endogenous peptides involved in developmental processes, an increasing number of endogenous molecules have also been brought into connection with defense signaling. As a general feature, these peptides both elicit a PTI-like response upon exogenous application and the corresponding signaling pathways were shown to be induced by biotic stress. Although these endogenous danger-associated molecular patterns (DAMPs) can be categorized into actively produced (peptidic) signals and passively produced signals originating from damaged structures caused by enemy attack, they are generally thought to be perceived by PRRs with some ligand-receptor pairs already being discovered (Yamaguchi and Huffaker 2011; Ferrari, Savatin et al. 2013).

Systemins

The peptides of the systemin family represent the first identified plant peptides with their activity discovered as early as 1972 (Green and Ryan) and the actual peptides in 1991 (Pearce, Strydom et al. 1991). Systemins are actively produced by the plant upon herbivore and pathogen detection (Pearce, Strydom et al. 1991). Interestingly, systemins share features with both MAMPs and HAMPs as apart from eliciting general PTI-like defense responses, systemins also induce specific anti-herbivore responses, such as the induction of proteinase inhibitor (PI) biosynthesis and the emission of volatile compounds to attract herbivore predators (Pearce, Strydom et al. 1991; Degenhardt, Refi-Hind et al. 2010). Further supporting a role in defense responses against herbivores, systemins were shown to be strong interactors with JA to promote systemic defense responses (Sun, Jiang et al. 2011). Like many other endogenous signaling peptides, systemins are short peptides (18 AAs long) that derive from the C-terminal end of a longer precursor protein (Prosystemin, roughly 200 AAs long) (McGurl, Pearce et al. 1992) via a so far unknown cleavage mechanism. In tomato, the search for the systemin receptor lead to the identification of SR160 (Systemin Receptor 160), a LRR-RLK that turned out to be tomato BRI1 (Scheer and Ryan 2002). However, it is highly doubtful that SR160/BRI1 is indeed the systemin receptor (Boller, 2005). In fact, *sr160* mutants did not respond to brassinolides but were still able to display systemin induced defense responses, indicating that the receptor for systemin is not SR160/BRI1 (Holton, Cano-Delgado et al. 2007). So far, systemin homologues have only been found in the solanoideae plant subfamily (Ryan and Pearce 2003).

Hydroxyproline Rich Systemins

Searching for systemin homologues in tobacco, Pearce et. al (2001) discovered two 18 amino acid long glycopeptides that elicit systemin-like defense responses, as for instance the induction of PI expression. As for systemins, these two 18 AA long peptides, termed *Nt*HypSysI (*Nicotiana tabaccum* Hydroxyproline Rich Systemin I) and *Nt*HypSysII, derive from a longer precursor protein (*Nt*PreProHypSys). However, both HypSys derive from the same precursor protein, with HypSysI being located near the N-terminus and HypSysII near the C-terminus. Moreover, unlike Prosystemin, ProHypSys contains a N-terminal signal sequence for cell wall localization (Pearce, Moura et al. 2001) and furthermore does not share any sequence homology with Prosystemin

(Ryan and Pearce 2003; Narvaez-Vasquez, Orozco-Cardenas et al. 2007). So far, HypSys homologues have been identified only in *Solanaceae* and *Convolvulaceae*, and no receptor yet has been identified (Narvaez-Vasquez, Orozco-Cardenas et al. 2007).

Glycine max Subtilase Peptide (GmSubPep)

The danger signal *Gm*SubPep (*Glycine max* Subtilase Peptide) is in so far a very interesting defense response inducing peptide since similarly to inceptin (see chapter on HAMPs), it derives from a protein with an entirely different primary function (Pearce, Yamaguchi et al. 2010). Such peptides are referred to as cryptic signals and this model is widely established in mammalian systems (Ueki, Someya et al. 2007; Yamaguchi and Huffaker 2011). *Gm*SubPep was identified in *Glycine max* (Soy bean) and is a 12 AA long peptide that derives from an extracellular subtilase. Like many other DAMPs, *Gm*SubPep induces a set of PTI-like responses as well as the expression of genes involved in defense responses against herbivores. However, unlike systemin or HypSys, the expression of the precursor gene is neither induced by defense-related phytohormones, nor by wounding. Moreover, neither the receptor nor the mechanism for the release of *Gm*SubPep from the subtilase precursor protein has yet been identified (Pearce, Yamaguchi et al. 2010). Also, no homologues to *Gm*SubPep have been found in other species so far, but a *Gm*SubPep-like domain was predicted in a subtilase encoding gene in *Medicago truncatula* (Yamaguchi and Huffaker 2011).

GmPep914

The soybean endogenous elicitor peptide *Gm*Pep914 (*Glycine Max* Peptide 914) was originally found by its medium alkalinization activity in suspension cultures. The short (8 AA long) peptide derives from the C-terminus of a 52 AA long precursor peptide, termed *Gm*PROPEP914. *In silico* analyses revealed a homologue named *Gm*Pep890, similarly deriving from the C-terminus of *Gm*Propep890. Both *Gm*Pep914 as well as *Gm*Pep890 are most strongly expressed in roots and their expression can be triggered by MeJA application. Also, the exogenous application of *Gm*Peps induces the expression of several defense marker genes in *Glycine max*. Phylogenetic studies revealed that homologues of *Gm*Peps exist in Fabales and Cucurbitales but not in any other plant families (Yamaguchi, Barona et al. 2011).

Passively Produced Danger Signals

By contrast to the danger signaling peptides described so far, oligogalacturonides (OGs) are oligomers of galacturonosyl residues released from plant cell walls upon degradation (Ferrari, Savatin et al. 2013). This degradation can either be the consequence of pathogen invasion and the use of microbial polygalacturonases (PGs) (Cervone, Hahn et al. 1989) or the activity of endogenous PGs induced by wounding/mechanical damage (Orozco-Cardenas and Ryan 1999). The exogenous application of OGs also leads to a broad spectrum of PTI-like defense responses, such as the production of ROS and the deposition of callose (Galletti, Denoux et al. 2008), the induction of defense genes (Denoux, Galletti et al. 2008), and the activation of MAP Kinase cascades (Galletti, Ferrari et al. 2011). Apart from defense, OGs are also believed to be involved in developmental signaling, potentially antagonising auxins in cell elongation processes where the rigid cell wall has to be amended to allow cellular growth (Ferrari, Savatin et al. 2013). The perception of OGs is mediated through Wall-Associated Kinases (WAKs), a small family of five genes encoding RLKs in Arabidopsis (Verica, Chae et al. 2003). However, given the fact that several WAK receptors exist and the difficulties to produce multiple receptor mutants because of tightly clustered genes (Verica, Chae et al. 2003), it took a cunning approach by Brutus et al. (2010), producing chimeric receptors consisting of either WAK1 ectodomains and EFR kinase domains and vice-versa, to provide evidence that WAKs are the receptors involved in OG signaling.

1.4 The *At*Pep/PEPR System

Similar to many other endogenous peptides involved in defense, the *Arabidopsis thaliana* danger peptides (*At*Peps) have originally been found by their activity to induce medium alkalinization in liquid cell cultures (Huffaker, Pearce et al. 2006). Based on sequence homology, eight *At*Peps (*At*Pep1-8) of a length of 23-29 AAs have been identified, all containing the putative *At*Pep amino acid motif SSGR/KxGxxN (Huffaker, Pearce et al. 2006; Bartels, Lori et al. 2013). However, so far only *At*Pep1 and *At*Pep5 have actually been identified in plant protein extracts with the activity of the other *At*Peps being shown by the application of synthetic peptides (Yamaguchi and Huffaker 2011). As for systemins, *At*Peps derive from the C-terminal end of longer (roughly 100 AAs) signal sequence free precursor peptides (PROPEPs, Figure 6) via a yet unknown cleavage mechanism (Huffaker, Pearce et al. 2006; Yamaguchi and Huffaker 2011). Unlike systemins, homologues to *At*Peps have recently been identified in several dicot and monocot species, including maize, rice, sorghum, soy bean and tomato (Huffaker, Dafoe et al. 2011; Huffaker, Pearce et al. 2013).

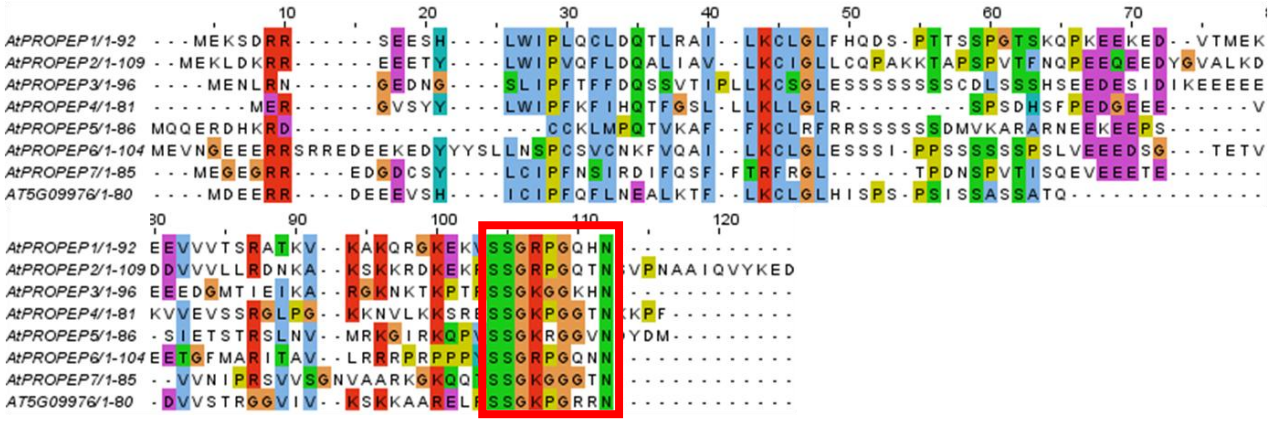

Figure 6. ClustalW alignment of the amino acid sequence of all eight *At*PROPEPs. AT5G09976 encodes for the recently discovered *At*PROPEP8 (Bartels, Lori et al. 2013). The putative SSGR/KxGxxN amino acid motif is framed in red. Coloring is based on the Clustal color scheme.

From Bartels et al. (2013).

In Arabidopsis, the *PROPEP*s1-3 are induced under several conditions of biotic stress, such as microbial infections, the detection of MAMPs such as flg22 and elf18, wounding, MeJA and ethylene application, tightly linking *PROPEP* expression to defense responses (Huffaker, Pearce et al. 2006; Huffaker and Ryan 2007), an observation that was recently shared by Bartels et al. (2013). Moreover, the expression of several *PROPEP*s is induced by *At*Pep application, potentially indicating a positive feedback loop in *At*Pep-signaling (Huffaker and Ryan 2007). Spatially, *PROPEP* basal expression was found in several tissues, namely in the root (*PROPEP*s1-3), leaf vascular tissue (*PROPEP*5), and root-tips (*PROPEP*s4 and 7), suggesting locally different roles in plant physiological processes (Bartels, Lori et al. 2013).

Two Highly Homologous RD-Kinases Act as Pep-Receptors

The perception of *At*Peps is achieved by two highly homologous, membrane-localized LRR-RLKs, termed Pep-Receptor1 (PEPR1) and PEPR2 (Yamaguchi, Pearce et al. 2006; Krol, Mentzel et al. 2010; Yamaguchi, Huffaker et al. 2010). However, whereas PEPR1 is able to recognize all eight *At*Peps, PEPR2 can only detect *At*Pep1 and *At*Pep2 (Bartels, Lori et al. 2013). The expression of both, *PEPR1* and *PEPR2* is induced upon wounding, MAMP treatment, and JA application, although at a potentially much lower level for *PEPR2* (Yamaguchi, Huffaker et al. 2010; Bartels, Lori et al. 2013). Phylogenetically, PEPRs cluster in the subgroup XI of LRR-RLKs, showing a close phylogenetic relationship to several receptors involved in endogenous peptide signaling (Figure 7) (Butenko, Vie et al. 2009).

Despite of being RD-kinases and PEPR1 and PEPR2 were shown to interact with BAK1 upon ligand binding (Postel, Küfner et al. 2010; Schulze, Mentzel et al. 2010) with BAK1 being required for full PTI responses upon *At*Pep application (Roux, Schwessinger et al. 2011). As for PROPEPs, homologues of PEPRs have been identified in several monocot and dicot plants (Bartels, personal communication). Also, similar to *PROPEP*s1-3, the basal expression of both *PEPR*s was observed to be strongest in Arabidopsis root tissue (Bartels, Lori et al. 2013).

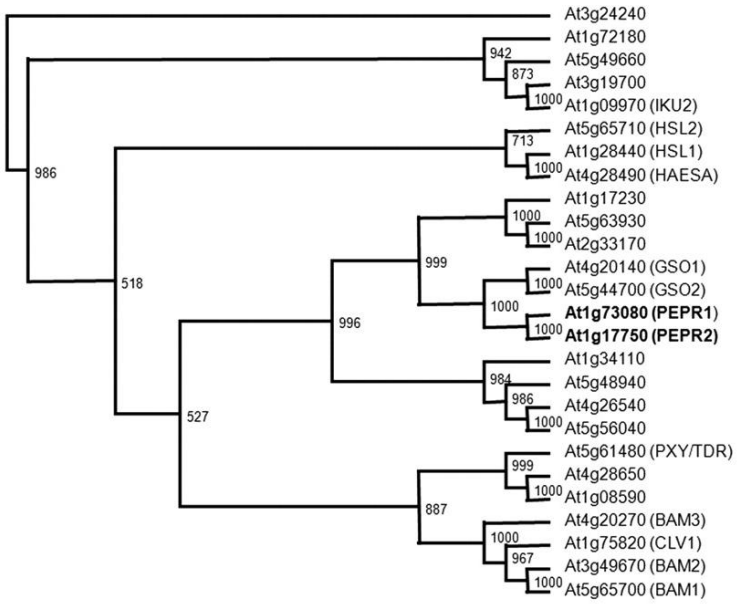

Figure 7. Phylogenetic alignment of LRR-RLK subgroup XI. PEPR1 and PEPR2 are featured in bold, indicating GSO1 and GSO2 as their closest homologues. Phylogenetic analysis was performed using the ClustalW program and visualization using the TreeView program. Names of LRR-RLKs with known biological functions are indicated on the right.

Adapted from Yamaguchi et al. (2010).

Responses Induced by *At*Pep-PEPR Interaction

The application of *At*Peps leads to several PTI-like downstream responses, such as the production of ROS and ethylene, the induction of ion fluxes across the plasma membrane, and the activation of MAP Kinase cascades (Huffaker, Pearce et al. 2006; Huffaker and Ryan 2007; Krol, Mentzel et al. 2010; Yamaguchi, Huffaker et al. 2010). Moreover, gene expression analysis revealed a largely similar set of genes induced compared to MAMP application (Bartels, personal communication) and consequently, the application of *At*Pep1 was shown to confer resistance against *Pseudomonas syringae* (Yamaguchi, Huffaker et al. 2010) whereas the overexpression of *PROPEP1* enhanced the resistance to the root pathogen *Pythium irregulare*. Providing further evidence for an overlap with MAMP signaling, *pepr1 pepr2*, a mutant in both PEPRs and thus fully impaired in *At*Pep perception was reported to be more susceptible to *Pseudomonas syringae* (Ma, Walker et al. 2012; Tintor, Ross et al. 2013) and the necrotrophic pathogen *Botrytis cinerea* (Liu, Wu et al. 2013). However, in contrast to MAMP signaling but similar to systemin or HAMP detection, the application of *At*Peps induces the accumulation of JA and JA-Ile and is also induced by JA (Huffaker and Ryan 2007; Huffaker, Pearce et al. 2013), potentially linking Pep signaling to defense responses against herbivores. Indeed, just recently published studies show that the application of *Zm*Pep3, an *At*Pep homologue in maize, induces

the accumulation of insect-deterring metabolites and enzymes in maize to eventually render maize plants more resistant to the generalist herbivore *Spodoptera exigua* (Huffaker, Pearce et al. 2013).

The Biological Relevance: Models for *At*Pep Function in Plants

The strong induction of PTI by *At*Peps as well as their inducibility by MAMP application led to the hypothesis that the *At*Pep-PEPR system could be regarded as an amplifier of innate immunity, potentially prolonging the signal of an imminent threat after the initial detection of the pathogen and/or serving as a systemic signal to induce resistance in distal parts of the plant (Figure 8A) (Boller and Felix 2009; Yamaguchi and Huffaker 2011). Further evidence supporting this role was provided by the reports that *At*Pep-PEPR signaling is an important positive mediator of ethylene-induced immune responses (Liu, Wu et al. 2013; Tintor, Ross et al. 2013).

Additional evidence was provided by Logemann et al. (2013), who identified several binding sites for WRKY transcription factors in the promoters of *PROPEP2* and *PROPEP3*. Since WRKY transcription factors are known to be induced upon MAMP detection and important transcriptional regulators of PTI, this would indicate a role of *At*Pep-PEPR signaling downstream of the initial PTI response.

Still, other roles for *At*Pep signaling are also proposed. As both PEPRs have an extracellular receptor domain but their ligands lack a secretion signal and therefore are believed to remain at intracellular locations, the *At*Pep-PEPR system was also proposed to be a surveillance system for cellular integrity (Figure 8B). In such a model, *At*Peps would accumulate inside the cell to be released upon cellular damage or lysis and therefore serve as DAMP signals more in the context of damage-associated molecular patterns (Yamaguchi and Huffaker 2011). Since especially herbivore attack or the utilization of lytic enzymes by necrotrophic pathogens would lead to an impaired cellular integrity and JA signaling being required for the integration of responses against these organisms, this would also go in hand with the capacity of *At*Peps to induce JA accumulation. Moreover, a similar role has also been suggested for the systemin peptides

which were shown to be similarly required for a full JA-dependent defense response against attacking herbivores tomato (Sun, Jiang et al. 2011).

Also, both PEPRs cluster in subgroup XI of LRR-RLKs together with receptors so-far solely being described to be involved in plant developmental and reproductive processes with their closest homologues being GSO1 and GSO2 (GASSHO1 and 2, Figure 7) (Yamaguchi, Huffaker et al. 2010). Whereas the ligands for these close homologues still remain elusive, *gso1 gso2* double mutants display several deficiencies in embryo development, linking GSO1 and GSO2 to developmental processes (Tsuwamoto, Fukuoka et al. 2008). Moreover, recent co-expression studies revealed that some *PROPEP*s are actually co-regulated with genes involved in other physiological processes than defense (Bartels, Lori et al. 2013). Still, no developmental phenotype of either *pepr* or *propep* mutants has been discovered yet.

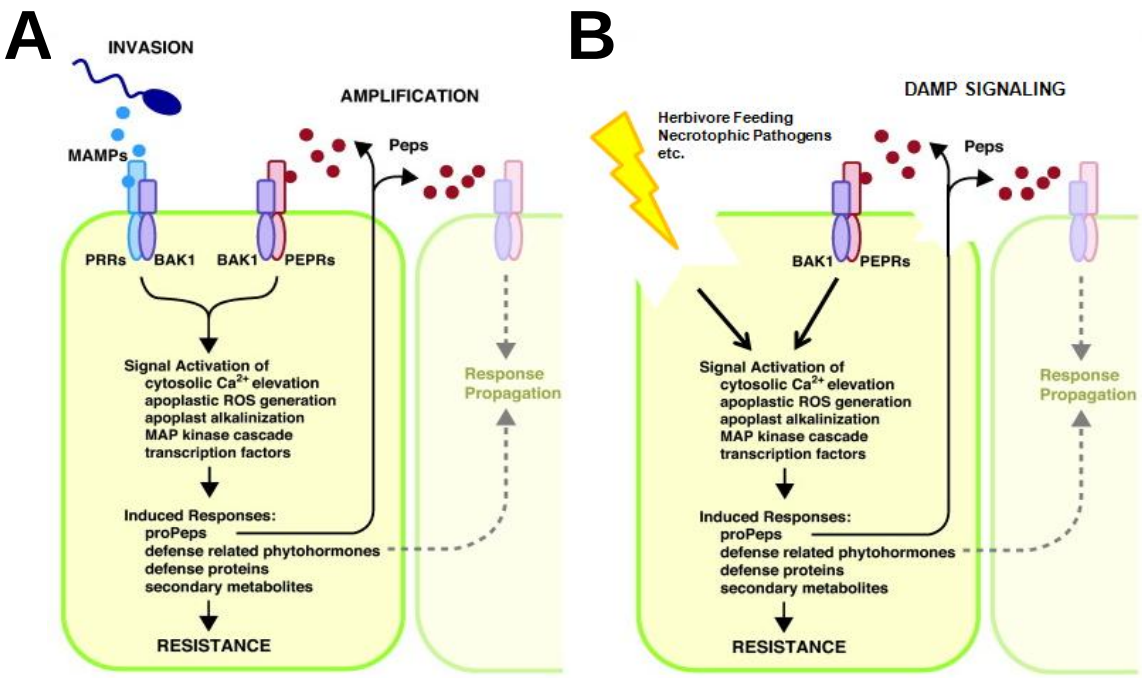

Figure 8. The two major theories for *At*Pep-PEPR system contribution to plant defense so far.

- **A) The amplifier theory:** Outlining a role for the *At*Pep-PEPR system to amplify MAMP-triggred immunity. The release of the *At*Peps is believed to be achieved by a yet unidentified secretion mechanism.
- **B) The DAMP-theory:** This model is based on the potential release of *At*Peps in upon loss of cellular integrity to bind to extracellular receptor domains oft yet unaffected cells to activate defense signaling.

Adapted from Yamaguchi et al. (2011)

1.5 The Aims of this Thesis

At the beginning of this thesis little was known about the mode of action and biological significance of endogenous peptides in plant innate immunity. In contrast, in animal science endogenous peptide signals like cytokines are firmly established to be of crucial importance for efficiently working immune systems (Arai, Lee et al. 1990). In plants, systemins were for many years the only known endogenous peptides with the ability to induce the plants' immune system. But the still elusive systemin receptor greatly impairs complete comprehension of this model system. Rather recently the discovery of the *At*Peps together with both PEPRs in the model plant Arabidopsis opened up a new approach to assess the impact of plant endogenous peptides on plant immunity. Thus, the overall aim of this thesis was to investigate the biological significance of these endogenous danger signaling peptides and their receptors in plant defense. The *At*Pep-PEPR system was regarded to be a particularly useful model since not only the receptor-ligand pair was already known, in addition single mutants for both PEPRs as well as a *pepr1 pepr2* double mutant were already available and characterized (Krol, Mentzel et al. 2010; Yamaguchi, Huffaker et al. 2010).

In the work presented here, the focus was on the *pepr1 pepr2* receptor double mutant that is fully impaired in *At*Pep-mediated signaling. This mutant was used to test current hypotheses like the amplifier and DAMP hypothesis and to determine if the lack of the two receptors indeed alters plant immunity.

In the context of the amplifier theory, as proposed by Boller and Felix (2009), we aimed to investigate the interplay between MAMP and DAMP (namely *At*Pep) signaling. Doing so, we especially addressed the question whether *At*Peps are potential endogenous amplifiers of MAMP-triggered PTI. The results of this work are described in chapters 2 and 3.

To determine a potential biological relevance of the *At*Pep-PEPR system to full-strength defense responses against several forms of biotic stresses, we assessed the resistance of mutants lacking both PEPRs against several invaders. The results of this work are described in the chapters 4 and 5, with studies on a potential relevance of the *At*Pep-PEPR system in the events

of viral infections and herbivore attack. The work described in these chapters was also outlined to investigate the role of the *At*Pep-PEPR system in the previously described DAMP theory. Here, especially herbivore attack would lead to a loss of cellular integrity and thus the release of the presumably intracellular *At*Peps.

2. The Anticipation of Danger: Microbe-Associated Molecular Pattern Perception Enhances *At*Pep-triggered Oxidative Burst

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The work described in this chapter was resulting from a close collaboration between Pascale Flury, Sebastian Bartels, and me. The original intention was to investigate the connection between MAMP and Pep-signaling, and the potential role of *At*Peps as amplifiers of MAMP-induced signaling processes. Here, I contributed to most parts of this work, including the planning of the experimental setup, the execution of most experiments (bioassays, hormone quantification, gene expression analysis), as well as data compilation and manuscript writing.

2.1 Abstract

The endogenous Arabidopsis peptides *At*Peps elicit an innate immune response reminiscent of PTI (pattern-triggered immunity). Detection of various danger signals including microbe-associated molecular patterns (MAMPs) like flg22 leads to elevated transcription of *PROPEPs*, the *At*Pep precursors, and *PEPRs*, the *At*Pep-receptors. It has been hypothesized that *At*Peps are involved in enhancing PTI. Following this idea we analyzed the relationship between MAMP- and *At*Pep-elicited signaling. We found that the perception of MAMPs enhanced a subsequent *At*Pep-triggered production of reactive oxygen species (ROS). Intriguingly, other components of *At*Pep-triggered immunity like Ca^{2+}-influx, MAP kinase phosphorylation, ethylene production and expression of early defense genes as well as ROS-activated genes remained unchanged. In contrast, treatment with MeJA promoted an increase of all analyzed *At*Pep-triggered responses. We positively correlated the intensities of generic *At*Pep-triggered responses with the abundance of the two *At*Pep-receptors by generating constitutively expressing PEPR1 and PEPR2 transgenic lines and by analyzing *pepr1* and *pepr2* mutants.

Further we show that enhanced as well as basal ROS production triggered by *At*Peps is absent in the *rbohD rbohF* double mutant. We present evidence that the enhancement of *At*Pep-triggered ROS is not based on changes in the ROS detoxification machinery and is independent of MAP kinase and Ca^{2+} signaling pathways.

Taken together these results indicate an additional level of regulation beside receptor abundance for the RbohD/F-dependent production of *At*Pep-elicited ROS, which is specifically operated by MAMP-triggered pathways.

2.2 Introduction

The plant immune response is triggered by the recognition of potential danger: Specialized plasma membrane receptors monitor the cellular environment to detect specific danger signals (Boller and Felix, 2009). These danger signals can originate from exogenous sources, such as PAMPs/MAMPs (pathogen/microbe-associated molecular patterns), connected to the presence of potentially harmful microbes, and HAMPs (herbivore-associated molecular patterns), formed

during herbivore feeding. In addition, danger signals can have an endogenous origin; DAMPs (damage-associated molecular patterns) are host molecules modified and/or released to the apoplast by cellular damage (Boller and Felix, 2009).

The *Arabidopsis thaliana* genome encodes seven *PROPEPs*, the precursors of the so called *At*Peps (Huffaker et al., 2006; Huffaker and Ryan, 2007). This family of peptides has the ability to trigger immune responses reminiscent of PTI (pattern-triggered immunity) and were thus characterized as potential DAMPs (Boller and Felix, 2009). *At*Peps are sensed by two pattern-recognition receptors (PRRs) of the receptor-like kinase (RLK) family (PEPR1 and PEPR2), which seem to share structural and functional similarity to the flagellin-receptor FLS2 and the EF-Tu receptor EFR (Yamaguchi et al., 2006; Krol et al., 2010; Yamaguchi et al., 2010). Recently, *At*Pep- as well as flg22- and elf18-triggered responses have been shown to be dependent on the presence of BAK1 and BKK1, indicating convergence of signaling pathways (Chinchilla et al., 2007; Heese et al., 2007; Schulze et al., 2010; Roux et al., 2011).

One of the early responses triggered by MAMPs and DAMPs is the production of apoplastic reactive oxygen species (ROS) by the Arabidopsis NADPH-oxidases RbohD and RbohF (Torres et al., 2006). In recent years multiple functions have been assigned to this so called “oxidative burst”. ROS are supposed to be directly toxic for invading pathogens, thus blocking their further proliferation, but also to act indirectly in defense by crosslinking plant cell wall components (Torres, 2010). Additionally, ROS have been shown to be involved in various intra- and intercellular signaling events. Elevated levels of ROS lead to activation of MAP kinases, ROS-mediated changes of redox-conditions facilitate activation of redox-controlled transcription factors, and ROS-based modifications of lipids can generate signaling molecules like cyclic oxylipins (Torres, 2010). Beside the intracellular signaling activity ROS has also been shown to spread systemically by subsequently inducing ROS production in neighboring cells. In this way, a ROS wave is assumed to spread out from the local area of stress throughout the whole plant body (Miller et al., 2009).

Perception of MAMPs and DAMPs ultimately leads to induced resistance against subsequent microbial infections (Yamaguchi 2010, Zipfel 2004). One aspect of induced resistance is the so called priming effect that is thought to be based on a more sensitive detection system in combination with faster and stronger responses to newly approaching threats (Conrath et al., 2006). This seems to be facilitated by the accumulation of dormant signaling components like MAP kinases and a persistent change in histone modification patterns adjacent to defense-related genes (Beckers et al., 2009; Jaskiewicz et al., 2011).

Since MAMP perception also induces the expression of some *PROPEPs* and both *PEPRs*, it has been hypothesized that these components might enhance PTI (Huffaker and Ryan, 2007).
Here, we show that recognition of the MAMP flg22 massively enhances a subsequent *At*Pep-triggered oxidative burst. This enhancement seems to be exclusive for ROS since all other investigated generic *At*Pep-elicited responses including medium alkalinization, MAP kinase activation, expression of early defense and ROS marker genes and ethylene production, remained unchanged. In contrast, a pretreatment of leaf tissue with methyl jasmonate (MeJA), but not methyl salicylate (MeSA), led to a slight but general enhancement of *At*Pep-triggered responses, probably related to changes in receptor abundance. It has been shown before, and we present further evidence here, that a manipulation of the expression levels of *PEPR* genes affects all the typical *At*Pep-elicited responses. However, the MAMP-triggered enhancement of *At*Pep-ROS is still detectable in *pepr1* or *pepr2* single mutants as well as in transgenic lines constitutively expressing either *PEPR1* or *PEPR2*, and is thus independent of the number of receptors per cell. We further present evidence that the *At*Pep ROS response and its MAMP-dependent enhancement depends on the presence of RbohD and RbohF and that the enhancement is not the result of a reduced ROS detoxification capacity. Thus we propose a second level of regulation for the *At*Pep-elicited oxidative burst which is modified by previous MAMP perception, which might be involved in induced resistance and systemic signaling.

2.3 Results

MAMP Pretreatment Leads to an Enhanced *At*Pep-Triggered Production of ROS

Detection of MAMPs like flg22 or elf18 rapidly induces the expression of *PROPEPs* and *PEPRs* (Zipfel et al., 2004; Huffaker et al., 2006; Zipfel et al., 2006; Denoux et al., 2008). Thus we were wondering about the impact of MAMP perception on the various responses triggered by *At*Peps (Huffaker et al., 2006; Huffaker and Ryan, 2007; Krol et al., 2010; Yamaguchi et al., 2010; Ranf et al., 2011). First we analyzed ROS production in response to *At*Pep1, which is rather low in leaf discs punched from adult leaves (Krol et al., 2010). Indeed, we found a strong increase of ROS production upon *At*Pep1 perception after pretreatment with flg22 (Figure 1). This observed increase in ROS was positively correlated to the flg22 concentration used for the pretreatment (Figure 1A) and independent of the MAMP used although it was less pronounced with elf18 than with flg22 (Figure 1B and Supplementary Figure1). Control treatments of flg22- or elf18-pretreated leaf discs did not induce ROS production (Figure 1B). Surprisingly, the pretreatment of leaf discs with flg22 did not enhance the elf18-triggered ROS and vice versa (Figure 1B and Figure 6C). The final ROS production was independent of the presence of the pretreatment solution since a washing step between pretreatment and final treatment did not change the detectable ROS pattern (Figure 1D). Lack of the flg22 receptor *fls2* or the elf18 receptor *efr* impaired the enhancement of *At*Pep1-triggered ROS by flg22 or elf18 pretreatment, respectively (Figure 1E). The ROS enhancing effect of a flg22-pretreatment could be observed already at 8 h after punching and pretreatment but it was most robust after 16 h (Figure 1C) thus we used this time point for further analyses.

Taken together we observed a strong and robust enhancement of the ROS production triggered by *At*Pep1 when leaf discs were pretreated for at least 8 h with MAMPs like flg22 and elf18. This enhancement is based on the perception of these MAMPs by their receptors but it is independent of the presence of the pretreatment solution at the time when ROS is triggered by *At*Pep1.

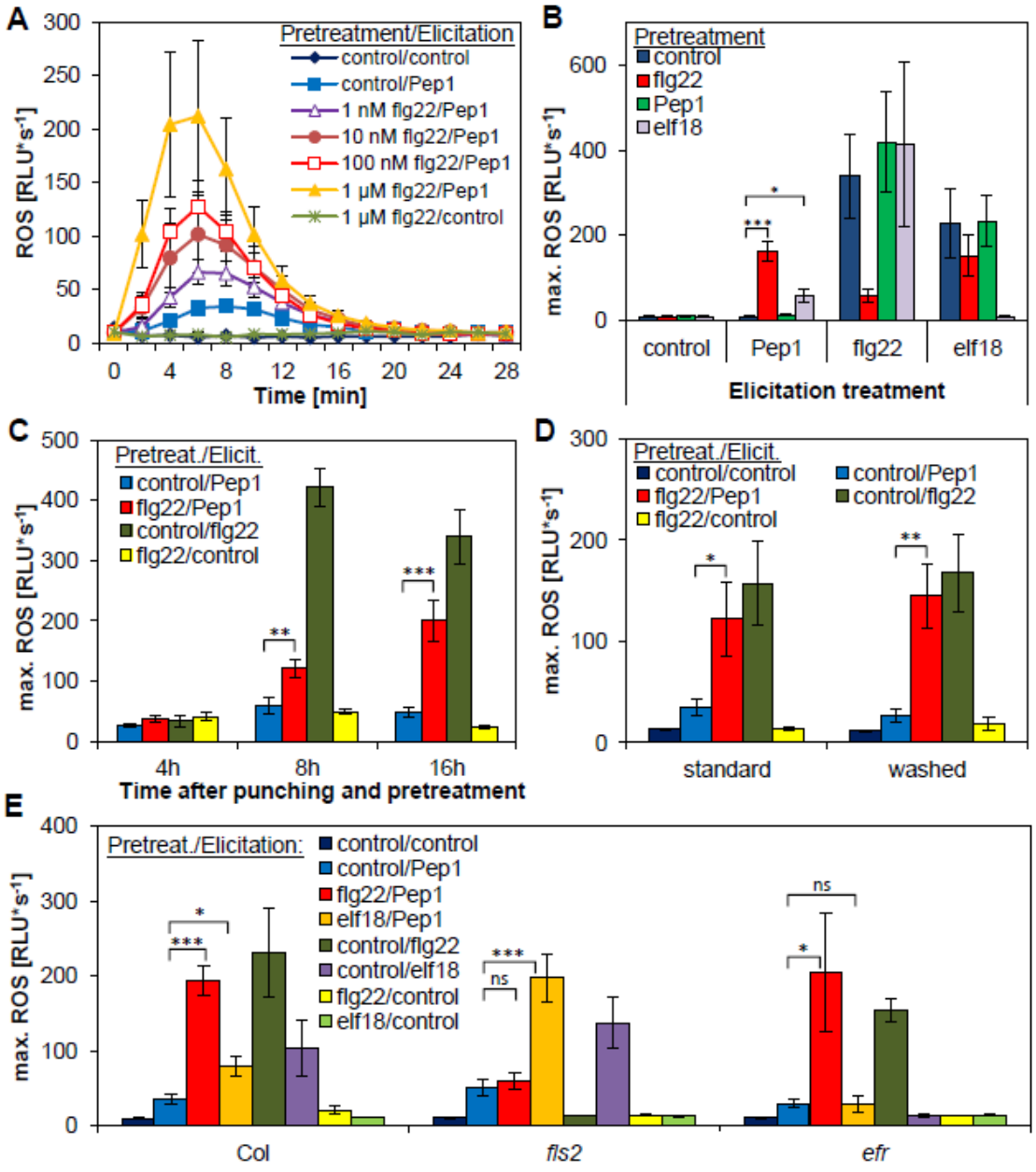


Figure 1. Elevated *At*Pep1-triggered ROS production after pretreatment with flg22.
A) Leaf discs were pretreated with indicated flg22 concentrations or without any peptide (control) for 16 h, and then treated with 1 µM *At*Pep1 or without any peptide (control). Graphs represent mean values of ROS production in at least 8 replicates. Error bars show standard error of the mean. **B and E)** Leaf discs of Col-0 plants **(B)** and *fls2* and *efr* mutants **(E)** were pretreated with the indicated elicitors (1 µM) or without any peptide (control) for 16 h, and then treated with 1 µM of the indicated elicitor or without any peptide (control). **C)** Like **(B)** but the pretreatment was performed for the indicated period of time. **D)** "Standard" was performed like **(B)** but in case of "washed", leaf discs were washed before the final treatment. Columns represent averages of the peak values of ROS production of at least 6 biological replicates, bars indicate standard error of the mean. Asterisks represent t-test results (* = p<0.05; ** = p<0.01; *** = p<0.001; ns = not significant). RLU= relative light units.

Flg22 Perception Does not Enhance other *At*Pep1-Triggered Responses

The spectrum of responses elicited by *At*Pep1 perception is reminiscent of the one triggered by flg22 (Boller and Felix, 2009; Krol et al., 2010; Yamaguchi et al., 2010). Thus we further analyzed a selection of early and late responses to investigate if the MAMP-mediated enhancement of *At*Pep1-triggered responses is a global phenomenon or specific for ROS.

One of the first cellular responses to MAMP or *At*Pep detection is the alkalinization of the surrounding medium (Huffaker et al., 2006). We used liquid cell cultures, either pretreated with 1 µM flg22 or a control solution, and elicited the alkalinization response 16 h after pretreatment. As shown in Figure 2A, despite a clear response to the addition of *At*Pep1 we did not detect any difference between the flg22-pretreated and the control-pretreated cell cultures.

Influx of extracellular Ca^{2+}-ions into the cytosol is another quick response to *At*Pep-perception (Ranf et al., 2011). To detect changes in cytosolic Ca^{2+} concentrations we made use of the aequorin luminescence-based Ca^{2+} detection method that has been used for leaf discs previously (Krol et al., 2010). In this assay, too, we could not detect an enhancement of the *At*Pep1-triggered Ca^{2+} influx in the flg22-pretreated leaf discs compared to the control (Figure 2B).

Next we assessed the phosphorylation kinetics and intensities of the stress activated MAP kinases MPK3 and MPK6. As displayed in Figure 2C we did not detect a stronger MPK3 or MPK6 phosphorylation in flg22-pretreated leaf discs after a 5 min treatment with *At*Pep1. Similarly we did not detect a more rapid phosphorylation of MPK3 and MPK6 after addition of *At*Pep1 in flg22-pretreated leaf discs compared to the control-pretreated ones (Figure 2D).

MPK3 has been connected to basal pathogen resistance whereas MPK6 plays a role in elicitor-induced resistance (Galletti, Ferrari et al. 2011). Thus we also tested whether mutants lacking either MPK3 or MPK6 are compromised for the enhancing effect of flg22 on the subsequent *At*Pep-triggered ROS production. Figure 3 shows that the enhancement of the *At*Pep1-triggered

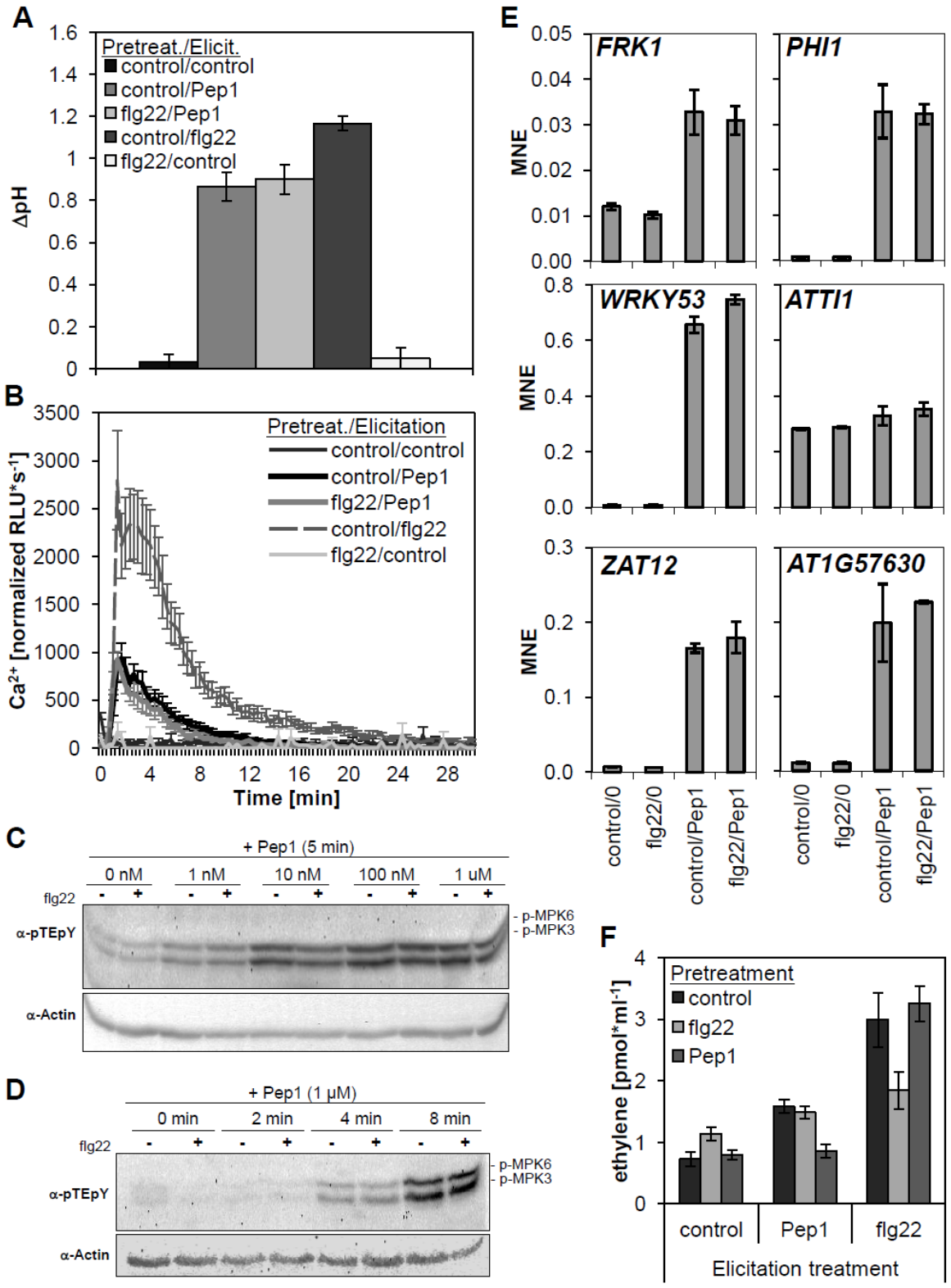
ROS by flg22-pretreatment in *mpk3* and *mpk6* mutant plants was comparable to wild type plants.

We then studied the effect of the flg22-pretreatment on the subsequent induction of defense-related genes in response to *At*Pep1. We selected a set of genes covering distinct signaling pathways: *FRK1* is induced via MAPK-mediated signaling whereas *PHI1* transcription is activated by CDPKs, i.e. calcium-dependent protein kinases (Boudsocq et al., 2010). *WRKY53* has been shown to change its transcription profile due to stress-mediated long term modifications of the methylation pattern of the adjacent histone (Jaskiewicz et al., 2011). *Zat12* is known to be ROS responsive and has been used to monitor the rapid spread of ROS in the plant after local wounding (Miller et al., 2009). Finally we picked *ATTI1* as well as *At1g57630* which have been identified to respond to a variety of treatments that trigger the production of ROS (singlet oxygen, superoxide or hydrogen peroxide) in diverse subcellular compartments (Gadjev et al., 2006).

Our data show that the pretreatment with flg22, despite of its strong induction of e.g. *FRK1* in the short term, had no effect on the expression of these genes after 16 h (Figure 2E, compare control/0 and flg22/0). A subsequent treatment with *At*Pep1 strongly stimulated expression of all genes investigated within 30 min, except *ATTI1*, but this stimulation was independent of the pretreatment (Figure 2E, compare control/Pep1 and flg22/Pep1).

Finally, we measured the release of ethylene in control, flg22 and *At*Pep1-pretreated leaf strips. The pretreatments had little effect on the release of ethylene 16 h later in the control treated samples (Figure 2F, Elicitation: control). When stimulated with flg22, the leaf discs that had not been pretreated responded in the same way as leaf discs pretreated with *At*Pep1. As before, the flg22-pretreated leaf discs responded much less to a second stimulation by flg22 (Figure 2F, Elicitation: flg22). The *At*Pep1-triggered ethylene response was lower than that elicited by flg22; nevertheless, it was clear that it could not be enhanced by flg22-pretreatment (Figure 2F,

Elicitation: *At*Pep1). Again, as before, the *At*Pep1-pretreated leaf strips did not respond to a second stimulation by *At*Pep1.


A
ΔpH
Pretreat./Elicit.
control/control
control/Pep1
flg22/Pep1
control/flg22
flg22/control
B
Ca2+ [normalized RLU*s-1]
Pretreat./Elicitation
control/control
control/Pep1
flg22/Pep1
control/flg22
flg22/control
Time [min]
C
+ Pep1 (5 min)
0 nM
1 nM
10 nM
100 nM
1 uM
flg22
α-pTEpY
α-Actin
p-MPK6
p-MPK3
D
+ Pep1 (1 µM)
0 min
2 min
4 min
8 min
flg22
α-pTEpY
α-Actin
p-MPK6
p-MPK3
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FRK1
PHI1
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ATTI1
ZAT12
AT1G57630
MNE
control/0
flg22/0
control/Pep1
flg22/Pep1
F
ethylene [pmol*ml-1]
Pretreatment
control
flg22
Pep1
control
Pep1
flg22
Elicitation treatment

Figure 2. flg22 pretreatment does not enhance other *At*Pep1-triggered responses.
A) Medium alkalinization assay. Cell cultures were either pretreated with 1 µM flg22 or without any peptide (control) for 16 h, and then treated with 1 µM of the indicated elicitor or a mock-treatment (control). Bars represent mean pH-shift values of 5 biological replicates, error bars indicate standard error. **B) Measurement of cytosolic calcium concentrations.** Leaf discs were pretreated with either 1 µM flg22 or without any peptide (control) for 16 h and then treated with the indicated elicitor (1 µM) or a mock-treatment (control). Graphs represent normalized mean values of 12 biological replicates and error bars show standard error of the mean. **C, D) MAPK phosphorylation.** Leaf discs were pretreated with either 1 µM flg22 (+) or without any peptide (-) for 16 h and then in C) treated for 5 min with the indicated concentrations of *At*Pep1 or a mock-treatment (0 nM), and in D) treated with 1 µM *At*Pep1 for the indicated period of time. MAPK phosphorylation was detected by immunoblotting using an anti-phospo-p44/42-MAP kinase antibody detecting the pTE-pY motif of MPK6 and MPK3. The immunoblot was reprobed with anti-Actin antibody to determine equal loading. **E) Induction of marker gene transcription.** Leaf discs were pretreated with either 1 µM flg22 or without any peptide (control) for 16 h and then directly flash frozen (0) or treated with 1 µM *At*Pep1 for 30 min before freezing (Pep1). Transcript levels of indicated genes were first normalized to the reference gene *UBQ10* (MNE) before calculating the mean of three biological replicates. Error bars indicate standard error of the mean. **F) Ethylene production.** Leaf strips of Col-0 plants were pretreated with either 1 µM flg22 or 1 µM *At*Pep1 or without any peptide (control) for 16 h and then treated (elicitation treatment) with either 1 µM flg22 or 1 µM *At*Pep1 or without any peptide (control) for 4 h. Columns represent averages of detected ethylene values of 6 biological replicates. Error bars indicate standard error of the mean. MNE = mean normalized expression.

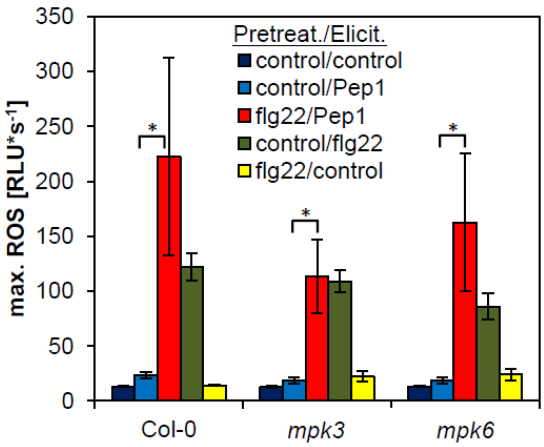


Figure 3. Lack of MPK3 or MPK6 does not impair flg22-mediated elevation of *At*Pep1-triggered ROS production.
A) Leaf discs of Col-0, *mpk3* and *mpk6* mutant plants were pretreated with either 1 µM flg22 or without any peptide (control) for 16 h and then treated with either 1 µM *At*Pep1 or 1 µM flg22 or without any peptide (control) as indicated. Columns represent averages of the peak values of ROS production of 12 biological replicates. Error bars indicate standard error of the mean. Asterisks represent t-test results (* = $p<0.05$). RLU= relative light units.

Lack of RbohD and RbohF Impairs *At*Pep1-Triggered ROS Production

Since ROS production seems to be the only flg22-enhanced *At*Pep-elicited response we analyzed this in more detail. The NADPH oxidases RbohD and RbohF are the main producers of apoplastic ROS in response to elicitors and the presence of pathogens (Torres et al., 2002). However, also cell wall peroxidases and polyamine oxidases can be sources of ROS (Bolwell et al., 2002; Yoda et al., 2009). Thus we determined the ROS-response of *rbohD rbohF* double mutant plants after treatment with *At*Pep1. As shown in Figure 4A, ROS could be detected neither in the flg22-pretreated leaf discs nor in the control-pretreated discs. To exclude a general insensitivity of the *rbohD rbohF* double mutant towards *At*Pep1 we additionally measured the release of ethylene. Since the mutant plants were similar to wild type in this regard (Figure 4B) we concluded that the initial and the flg22-enhanced ROS production upon *At*Pep1 perception is mediated by the enzymatic activities of RbohD and RbohF.

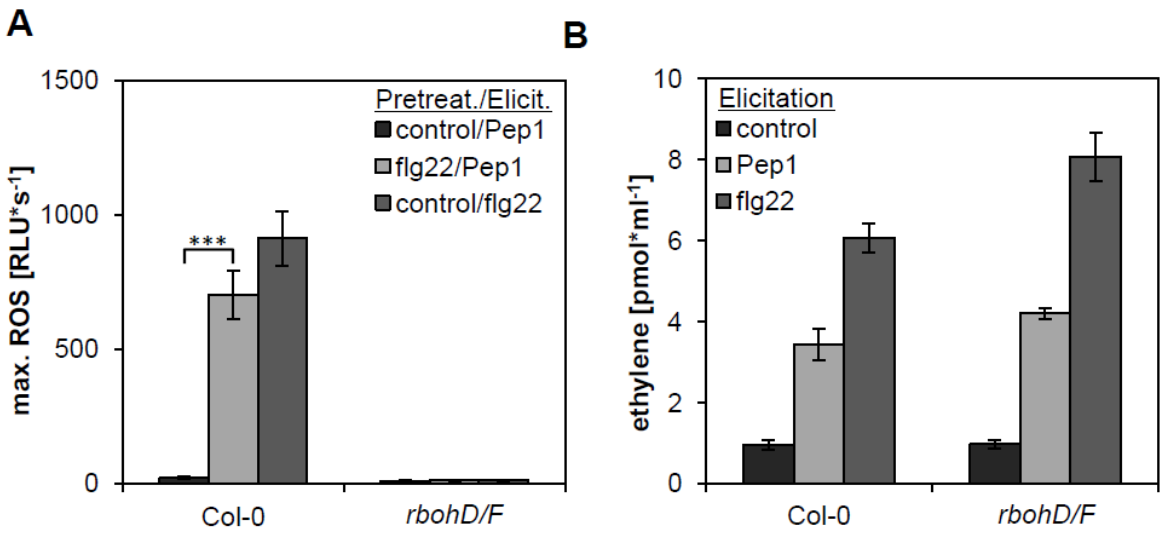


Figure 4. Lack of functional RbohD and RbohF blocks *At*Pep1-triggered ROS, with and without pretreatment, but does not impair the production of ethylene. A) ROS production. Leaf discs of Col-0 and *rbohD rbohF* double knock-out mutants were either pretreated with 1 µM flg22 or without any peptide (control) for 16 h and then treated with either 1 µM *At*Pep1 or 1 µM flg22 as indicated. Columns represent averages of the peak values of ROS production of 12 biological replicates. Error bars indicate standard error of the mean. RLU= relative light units. **B)** Ethylene production. Leaf strips were incubated in water for 16 h and then treated with either 1 µM *At*Pep1, 1 µM flg22 or without any peptide (control). Ethylene production was measured after 4 h of incubation. Error bars indicate standard error of the mean of 6 biological replicates. Asterisks represent t-test results (*** = $p<0.001$).

MAMP-induced Enhancement of *At*Pep-Triggered ROS is Mostly Independent of the Abundance of the Two PEPRs and the Applied *At*Pep

The two *At*Pep-receptors, PEPR1 and PEPR2 differ in their specificity. PEPR1 is able to detect all known *At*Peps whereas PEPR2 mainly binds *At*Pep1 and *At*Pep2 (Yamaguchi et al., 2010). What is the role of the two receptors in the observed phenomena? To answer this question, we first assessed the enhanced ROS production upon *At*Pep1 treatment in flg22-pretreated *pepr1* and *pepr2* single mutants. Despite the known reduced intensity of *At*Pep1-triggered responses, both single mutants showed a stronger *At*Pep1-ROS response when pretreated with flg22 (Figure 5A). Likewise, in wild type plants, all of the *At*Pep peptides stimulated ROS production after a pretreatment with flg22 in a similar way as *At*Pep1 (Figure 5B).

In order to examine the possible role of receptor abundance in the enhanced ROS production, we generated transgenic Arabidopsis plants constitutively expressing either *PEPR1* or *PEPR2* in the *pepr1 pepr2* double mutant background (Supplementary Figure 2). These plants showed a much stronger ethylene response compared to wild type plants when treated with *At*Pep1, suggesting that the higher levels of PEPR1 or PEPR2 caused an enhancement of *At*Pep-triggered responses (Figures 5C and 5D). Interestingly, most of the transgenic plants overexpressing *PEPRs* displayed a more pronounced oxidative burst even in the absence of a pretreatment with flg22 (Figures 5E and 5F). However, in all these plants, the *At*Pep1-triggered ROS was still enhanced when leaf discs were pretreated with flg22. Since the *PEPR* transcription was driven by the constitutive CaMV35S promoter in the *pepr1 pepr2* double mutant background we assume that *PEPR* transcription is not further induced upon flg22 pretreatment thus the flg22-mediated enhancement of *At*Pep1-triggered ROS is independent of induced *PEPR* transcription. Moreover, additional *At*Pep-triggered responses beside ROS are enhanced in the constitutive *PEPR* expressing plants (Figures 5C, D and Figure 7E) indicating that an elevated receptor abundance enhances all *At*Pep-triggered responses. Thus a change in PEPR abundance cannot be the cause for the observed enhanced *At*Pep-triggered ROS after flg22 pretreatment.

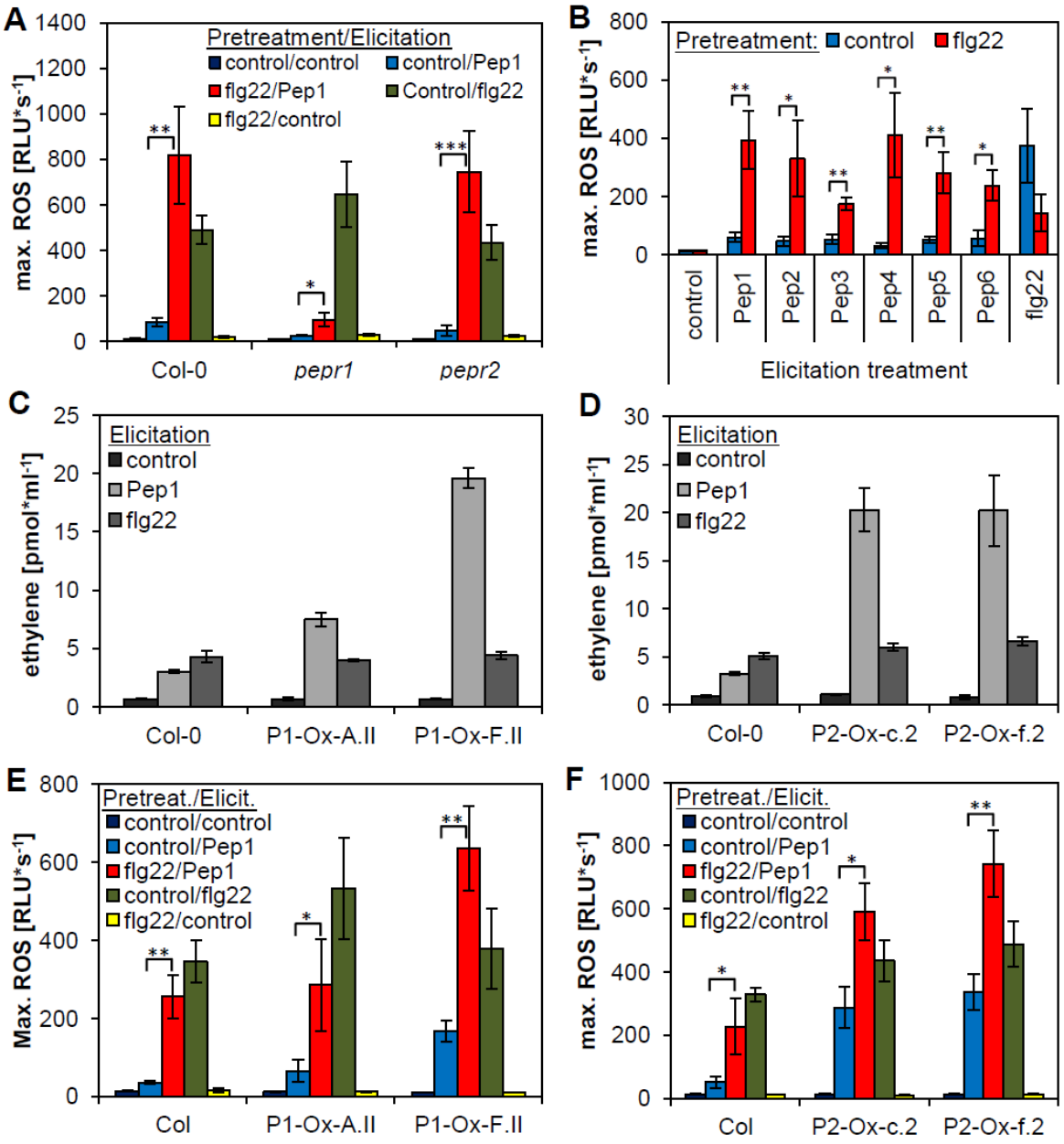


Figure 5. Flg22-dependent enhancement of *At*Pep-triggered ROS is independent of the abundance of PEPR1 or PEPR2 and the added *At*Pep peptide. A,B,E,F) ROS production. Leaf discs of the indicated genotypes **(A,E,F)** or Col-0 **(B)** were either pretreated with 1 µM flg22 or without any peptide (control) for 16 h and then treated with 1 µM of the indicated peptide or without any peptide (control). Columns represent averages of the peak values of ROS production of 12 biological replicates. Error bars indicate standard error of the mean. RLU= relative light units. **C,D) Ethylene production.** Leaf discs of Col-0 and two independent transgenic lines expressing either 35S::PEPR1::YFP (P1-Ox-A.II and F.II) or 35S::PEPR2::YFP (P2-Ox-c.2 and f.2) were floated on water for 16 h, then treated with the indicated elicitors and incubated for 4 h before measurement. Columns represent the mean of 6 independent replicates and error bars indicate the standard error of the mean. Asterisks represent t-test results (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$).

Enhancement of *At*Pep1-Triggered ROS is not Based on an Altered ROS Detoxification Machinery Elicited by the Flg22-Pretreatment

To assess the possibility that the increased *At*Pep-ROS could be a consequence of the depletion of ROS detoxifying compounds due to previous bursts, we assessed the initial flg22 treatment for ROS. Intriguingly, after directly applying flg22 to freshly cut leaf tissue, we could not detect any increased production of ROS with our luminol-based assay. This might indicate that the initial flg22 treatment directly after wounding the leaf tissue does not induce apoplastic ROS production (Figure 6A, left). However, when flg22 is added to plant tissue after the usual 16 h lag phase it induces a strong ROS production (Figure 6A, right). Notably the flg22-induced ROS burst is already saturated at around 100 nM, whereas the *At*Pep-triggered ROS in pretreated samples can still be enhanced by further increasing the concentration of flg22 up to 1 µM for pretreatment (Figures 6B and 1A). Additionally, including a waiting period of 8 h before adding the pretreatment solution, which will enable a detectable flg22-elicited ROS burst (Figure 1C), did not change the enhancement of the ROS production triggered by subsequent *At*Pep1 treatment (Figure 6B). Finally, comparing *At*Pep1-triggered ROS and elf18-triggered ROS in flg22-pretreated samples shows that when the flg22 pretreatment strongly increases the *At*Pep1-triggered ROS it has only a very small impact on the ROS production elicited by elf18 (Figure 6B).

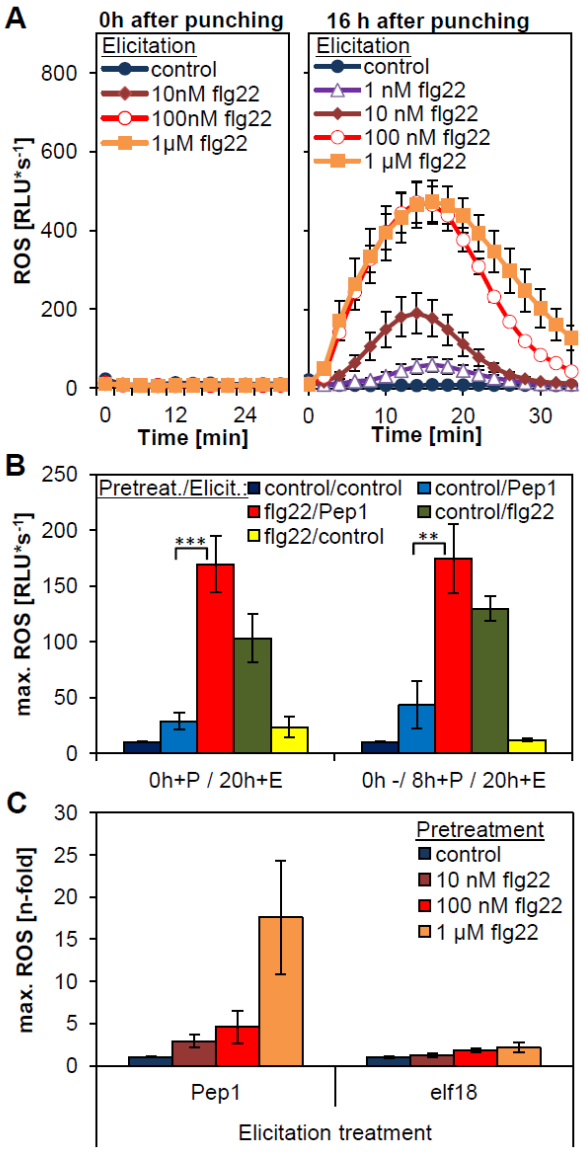


Figure 6. Enhanced *At*Pep-ROS is independent of previous ROS production A) Leaf discs were treated with the indicated concentrations of flg22 either directly after punching (left) or after the standard 16 h incubation time floating on water (right). Graphs display averages of 12 replicates. Error bars indicate standard error of the mean. **B)** Indicated pretreatment (1 µM flg22 or without any peptide (control)) was performed either directly after punching (0h+P/20h+E) or at 8 h after punching (0h-/8h+P/20h+E). 20 h after punching all leaf discs were treated with indicated elicitors or without any peptide (control). Columns represent averages of the peak values of ROS production of 8 biological replicates. Error bars indicate standard error of the mean. RLU= relative light units. **C)** Relative effect of pretreatments on ROS production. Leaf discs were pretreated as indicated for 16 h and then treated with either 1 µM *At*Pep1 or 1 µM elf18 (elicitation). Columns represent relative averages of the peak values of ROS production normalized to the respective control treatment. Error bars indicate relative standard error of the mean of 12 biological replicates. Asterisks represent t-test results (** = p<0.01; *** = p<0.001).

JA Seems to Enhance All *At*Pep-triggered Responses

Beside MAMPs, other molecules have been reported to induce the expression of *PROPEPs* and *PEPRs* (Huffaker et al., 2006; Huffaker and Ryan, 2007; Yamaguchi et al., 2010). Among these, MeJA was especially effective (Yamaguchi et al., 2010). Thus we wondered whether a pretreatment with MeJA or MeSA would enhance ROS production in a similar way as the flg22-pretreatment.

Indeed, pretreatment with MeJA (but not with MeSA) induced a slight enhancement of subsequent *At*Pep1-triggered ROS production (Figure 7A). Accordingly, the JA synthesis mutant *aos* as well as the JA insensitive mutant *coi1-1*, but not the SA synthesis mutant *sid2*, exhibited a reduced ROS release upon *At*Pep1 treatment (Figure 7C). Neither MeJA nor MeSA itself triggered a detectable ROS production (Figure 7A). Thus we hypothesized that the flg22 pretreatment might constitutively elevate endogenous JA and JA-Ile levels, which in turn specifically enhance *At*Pep-triggered ROS production. JA measurements revealed 4-fold increased levels of JA and JA-Ile levels in flg22-pretreated samples compared to control pretreated samples (Supplementary Figure 3). Therefore, we next assessed the specificity of JA on *At*Pep-triggered responses, but in contrast to a pretreatment with flg22, pretreatment with MeJA led to a strong increase of *At*Pep1-triggered ethylene production (Figure 7B). Consistently, we observed a reduction of the *At*Pep1-induced ethylene production in the *aos* and *coi1-1* mutant as well as in the *opr3* mutant, another JA synthesis mutant, but not in the *sid2* mutant (Figure 7D and Supplementary Figure 4). In addition, the inhibition of seedling growth by *At*Pep1 is significantly reduced in the *aos* as well as *coi1-1* mutants whereas it is strongly enhanced in transgenic plants constitutively expressing *PEPR1* or *PEPR2* (Figure 7E).

JA levels have been shown to rapidly increase upon wounding (Glauser, Dubugnon et al. 2009). To exclude the possibility that a rapid wave of JA triggered by the final *At*Pep-treatment somehow modulates the ROS production we measured JA and JA-Ile levels in control- and flg22-pretreated samples directly after addition of *At*Pep1. We found that independent of the pretreatment JA and JA-Ile levels did not change within the first 10 min after *At*Pep1-treatment (Supplementary Figure 3). Additional measurements with later time points revealed that

*At*Pep1 triggers a transient and comparably weak rise in JA and JA-Ile levels around 1 h after treatment (Supplementary Figure 5).

Together these results indicate that, in contrast to the specific effect of flg22, JA enhances all *At*Pep-elicited responses.

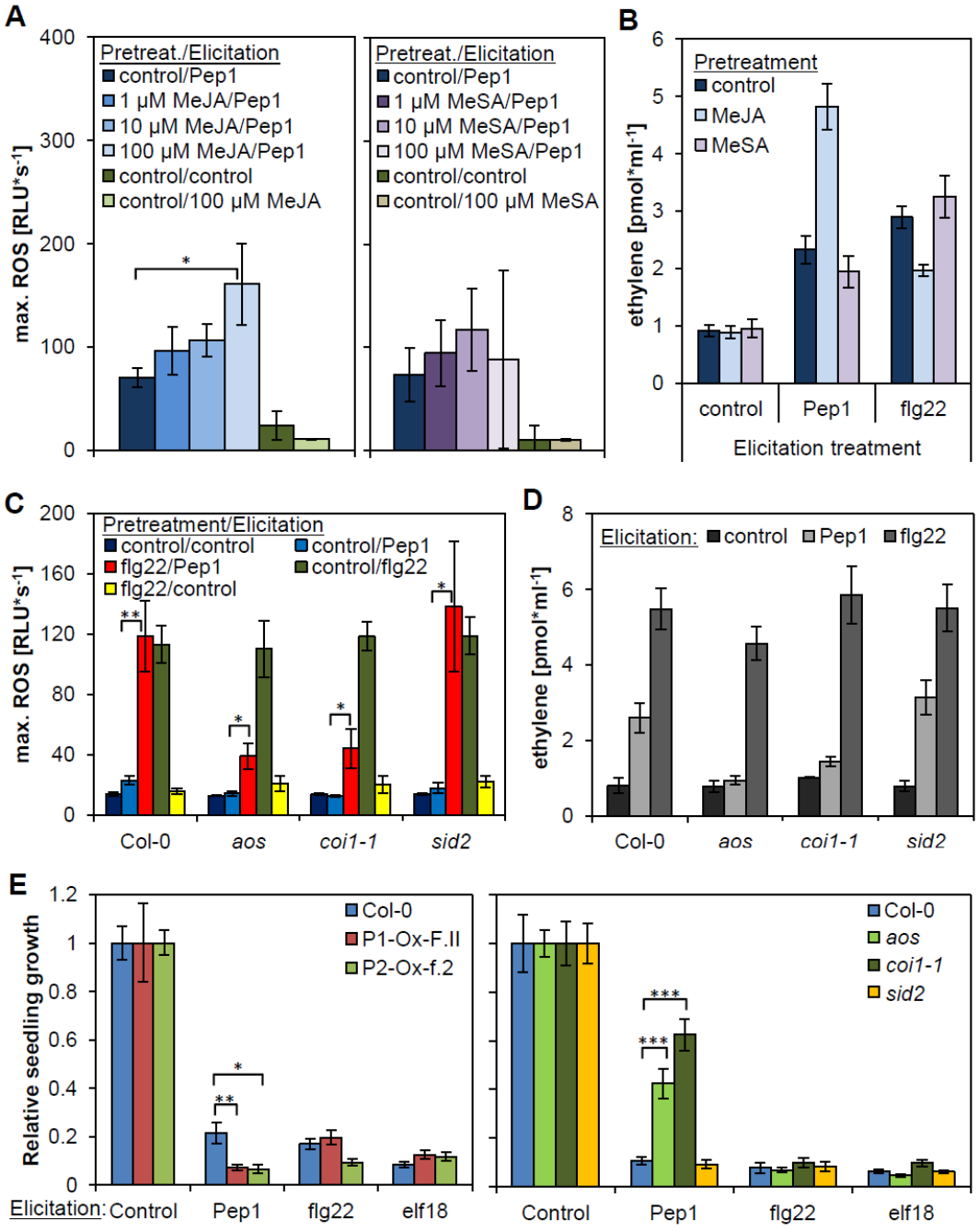


Figure 7. JA regulates *At*Pep-triggered responses in general. A) ROS production in Col-0. Leaf discs were either pretreated with indicated concentrations of MeJA (left) or MeSA (right) or without any hormone (control) for 16 h, and then either treated with 1 µM *At*Pep1, 100 µM MeJA (left), 100 µM MeSA (right) or mock (control) as indicated. Columns represent averages of the peak values of ROS production of 12 biological replicates and error bars indicate standard error of the mean. **B) Ethylene production in Col-0.** Leaf strips were pretreated with the indicated concentrations of either MeJA or MeSA, or mock (control) for 16 h and then treated with either 1 µM of *At*Pep1 or flg22, or mock (control) as indicated (elicitation) and incubated for 4 h before measurement. Columns represent the mean of 6 independent replicates and error bars indicate the standard error of the mean. **C) ROS production in *aos, coi1-1* and *sid2*.** Leaf discs of Col-0, *aos, coi1-1* and *sid2* mutant plants were pretreated with either 1 µM flg22 or without any peptide (control) for 16 h, and then treated with or without 1 µM of elicitor as indicated. Columns represent averages of the peak values of ROS production of 12 biological replicates and error bars indicate standard error of the mean. RLU = relative light units. **D) Ethylene production in *aos*, *coi1-1* and *sid2*.** Leaf strips of Col-0, *aos*, *coi1-1* and *sid2* mutant plants were incubated in water for 16 h and then treated with either 1 µM of *At*Pep1 or flg22, or without any peptide (control) and incubated for 4 h before measurement. Columns represent the mean of 6 independent replicates and error bars indicate the standard error of the mean. **E) Seedling growth inhibition.** 5 day old seedlings of the indicated genotypes were treated for 10 d with 1 µM of the indicated elicitor or without any peptide. Columns represent the mean weight of 12 seedlings out of 6 biological replicates. Error bars indicate standard error of the mean. Asterisks represent t-test results (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$).

2.4 Discussion

MAMPs such as flg22 are characteristic of whole classes of microbes, which might or might not be pathogenic for a specific plant. The perception of MAMPs triggers a defense response, which comprises both early and late responses and ultimately may stop the invasion of a microbe (Boller and Felix, 2009). Similarly, the Arabidopsis endogenous peptides *At*Peps elicit a defense response and mediate an increased resistance to *Pseudomonas syringae* pv. tomato DC3000 in a similar way as flg22 (Huffaker and Ryan, 2007; Yamaguchi et al., 2010). It has been hypothesized that *At*Peps might act as an amplifier of PTI since *PROPEP* as well as *PEPR* expression is induced upon flg22 perception (Zipfel et al., 2004; Huffaker and Ryan, 2007). However, since neither the release of *At*Peps or their precursors PROPEPs has been uncovered nor an impact of *pepr1 pepr2* knock-out on plant defense has been reported yet, the biological function of PROPEPs and PEPRs remains elusive.

Here, we show that a previous stimulation by MAMPs such as flg22 or elf18 strongly increased the apoplastic ROS production upon subsequent *At*Pep perception. This effect was specific to ROS since no other *At*Pep-response investigated was altered by MAMP-pretreatment. Also, it was highly specific for *At*Peps since the subsequent perception of MAMPs did not lead to an increased ROS production. A similarly specific enhancement of ROS production has been reported for grapevine cell suspensions which were pretreated with beta-aminobutyric acid (BABA) and subsequently elicited with oligogalacturonides (OGs) (Dubreuil-Maurizi et al., 2010). In that case the about 2-fold enhanced ROS production seemed to be connected to a slight increase in *RbohD* expression. But the impact of BABA on ROS triggered by other elicitors had not been tested. However, an enhanced RbohD expression suggests a general increase in the capacity to produce ROS rather than an OG-specific enhancement of ROS production.

Our results show that induced ROS production in response to flg22 or *At*Peps depends on functional RbohD/F. Since MAMP-triggered ROS production did not change in MAMP-pretreated samples we ruled out the possibility that an increase in RbohD/F abundance is responsible for the observed specific enhancement of *At*Pep-triggered ROS. Due to the reported induction of *PEPR* expression upon flg22 perception (Zipfel et al., 2004) we

hypothesized that an increase in PEPR abundance might be an explanation for this effect. However, analysis of plants constitutively expressing *PEPR1* or *PEPR2* in the *pepr1 pepr2* double mutant background showed that the flg22-mediated enhancement of *At*Pep-ROS was still present. Moreover, these plants also showed an enhanced basal ROS-production as well as a strong increase of ethylene release upon *At*Pep treatment indicating a global enhancement of *At*Pep-triggered responses when PEPR-levels increase.

MeJA has also been reported to induce *PEPR* expression (Yamaguchi et al., 2010). Consistently, mutants impaired in JA synthesis or detection showed reduced *At*Pep-triggered responses whereas pretreatment with MeJA led to elevated *At*Pep-triggered responses mimicking plants constitutively expressing *PEPRs*. Pretreatment with flg22 caused an elevation of JA and JA-Ile levels which could in principle promote the enhanced *At*Pep-triggered ROS production. On the other hand, flg22-pretreatment had no effect on other *At*Pep1-triggered responses except ROS. Moreover, the JA insensitive mutant *coi1-1* showed, despite overall lower *At*Pep-triggered responses, a flg22-mediated enhancement of *At*Pep1-triggered ROS. Thus, we conclude that JA levels modulate *At*Pep-triggered responses most likely by regulating PEPR expression. In contrast, the enhancement of *At*Pep-triggered ROS by flg22 is largely independent of JA synthesis and perception.

Next we hypothesized that the initial production of ROS triggered by flg22 or elf18 is mediating the enhancement of subsequent *At*Pep-triggered ROS by inhibiting the ROS degrading capacity. However, several lines of evidence contradict this hypothesis: First, depletion or modification of the ROS quenching capacity by a flg22-triggered oxidative burst should also affect the detectable ROS triggered by other elicitors like elf18. But the elf18-triggered ROS production essentially did not change in flg22-pretreated samples. Second, flg22 did not induce a detectable oxidative burst when added to freshly harvested leaf discs. The wounding response seems to suppress a flg22-mediated activation of RbohD/F and thus no apoplastic ROS is present to alter the ROS-degrading capacity. Finally, we found that increasing concentrations of flg22 positively correlated with the enhancement of *At*Pep-triggered ROS production, reaching

1 µM without signs of saturation. In contrast, flg22-triggered ROS production reached saturation as early as 100 nM flg22 concentration. Hence, we conclude that the MAMP-mediated enhancement of *At*Pep ROS production is not connected to a putative modification of the ROS quenching capacity.

Thus we wondered if our observations can be explained by a posttranslational regulation of RbohD and RbohF. It has been shown that *At*RbohD is synergistically activated by Ca^{2+} and direct phosphorylation (Nuhse et al., 2007; Ogasawara et al., 2008). Additionally, it was reported that Arabidopsis plants mutated for several *CDPK*s showed decreased oxidative burst upon flg22, suggesting a role for CDPKs in regulating ROS production (Boudsocq et al., 2010). In contrast, silencing of the stress-linked MAPKs SIPK and WIPK in *N. benthamiana* did not impair MAMP-elicited ROS (Segonzac et al., 2011). However, neither Ca^{2+} influx nor MAPK phosphorylation kinetics or CDPK- and MAPK-dependent gene expression seemed to be enhanced in MAMP-pretreated samples, indicating that *At*Pep-triggered activation of RbohD and RbohF is not achieved via these pathways but might be linked to the PEPRs via different signaling routes. Another possibility would be a persistent modification of Rboh proteins rendering them much more sensitive to subsequent activating signals. In this case even the comparably weaker *At*Pep-triggered Ca^{2+} influx might be sufficient to facilitate full RbohD and RbohF activation and respective ROS release. However, since this modification would be triggered by flg22 perception which we usually did 16 h before the secondary treatment, this mechanism would require a very slow turnover of the RbohD and RbohF to keep the modification. Moreover, a flg22-mediated elevated sensitivity of RbohD/F to activating signals should again lead to enhanced ROS-production triggered by elf18 which we did not detect.

Taken together we found a MAMP-triggered specific enhancement of *At*Pep-elicited ROS production that i) depends on functional *RbohD* and *RbohF*, ii) is not solely based on an induced expression of *PEPR1* and *PEPR2*, iii) appears to be independent of potential changes in the ROS detoxification machinery, iv) appears to be independent of Ca^{2+} and MAPK-mediated signaling pathways, and v) is clearly distinct from the global enhancement of *At*Pep-triggered responses

mediated by JA (Figure 8). We thus propose a new layer of regulation for the RbohD/F-dependent oxidative burst which connects signaling pathways triggered by exogenous and endogenous danger signals. Intriguingly, Gad Miller and colleagues showed that damage (wounding) led to a rapid cell-to-cell spreading of ROS throughout the whole plant which was dependent on RbohD (Miller et al., 2009). But they failed to induce this signal just by H_2O_2 treatment alone indicating that additional compounds are needed for this signaling cascade to be initiated. *At*Peps are endogenous elicitors supposed to be released upon damage or danger. Here we showed that previous MAMP-perception greatly enhances *At*Pep-triggered ROS production. Thus we hypothesize that *At*Peps might take part in this cell-to-cell signaling process for two reasons. First, microbes try to prevent the production of ROS by injecting effectors to block respective signaling pathways (Göhre, Spallek et al. 2008; Gimenez-Ibanez, Hann et al. 2009). A release of *At*Peps would thus make the spread of the ROS-wave more robust. Second, the characteristics of the triggered oxidative burst might encode crucial information (Mittler et al., 2011). Thus *At*Peps might modulate the transduced information to distinguish between situations of potential danger and situations of actual danger. More research is needed to analyze this intriguing connection.

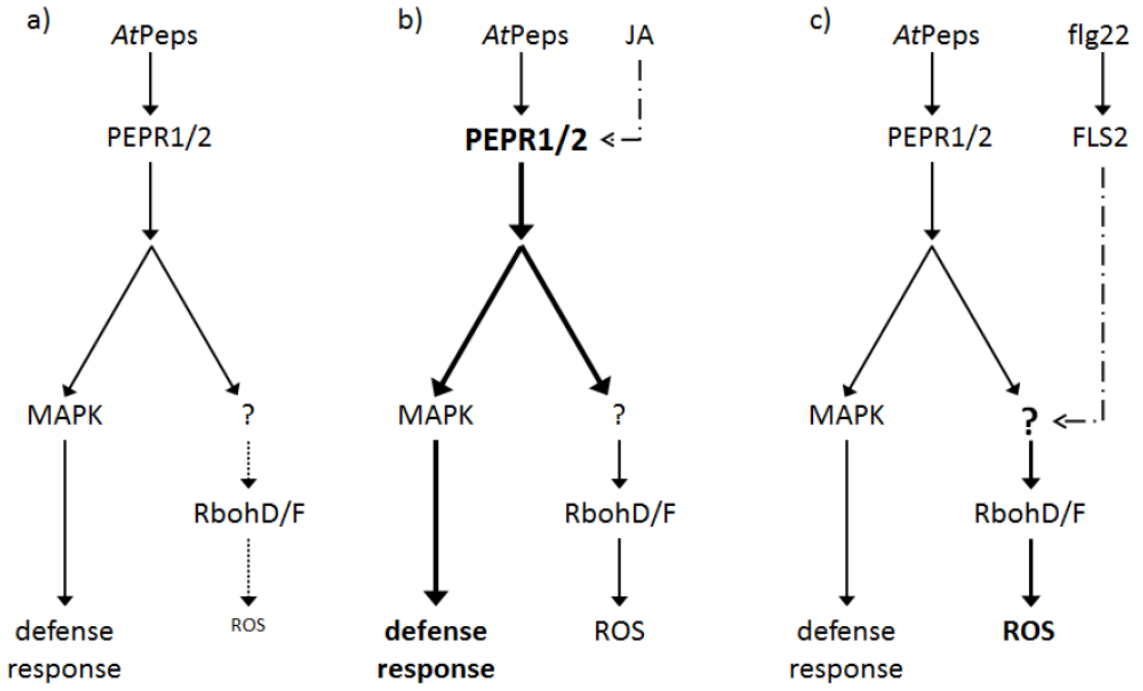
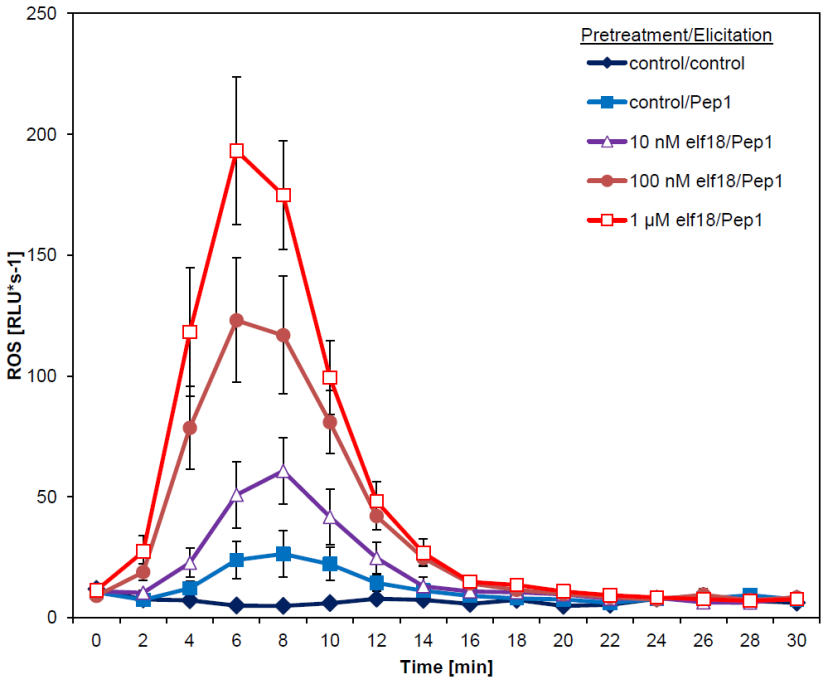
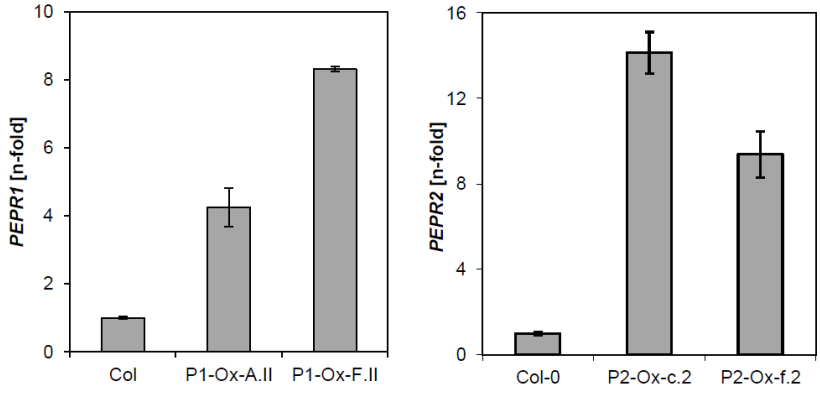


Figure 8. Simplified model on the effect of JA and flg22 on *At*Pep-triggered responses. a) In untreated wild type plants *At*Peps elicit a moderate defense response compared to MAMP-triggered responses (Krol et al., 2010; Ranf et al., 2011). A so far unknown PEPR-specific pathway (?) leading to RbohD/F activation is only weakly expressed therefore *At*Peps trigger only low amounts of apoplastic ROS. **b)** JA positively regulates PEPR transcription (Yamaguchi et al., 2010). Increased levels of PEPRs trigger stronger responses upon *At*Pep-perception. **c)** Previous perception of flg22 specifically triggers expression of the PEPR-specific ROS pathway. Thus, subsequent treatments with *At*Peps lead to highly induced ROS production but other *At*Pep-triggered responses remain unchanged.

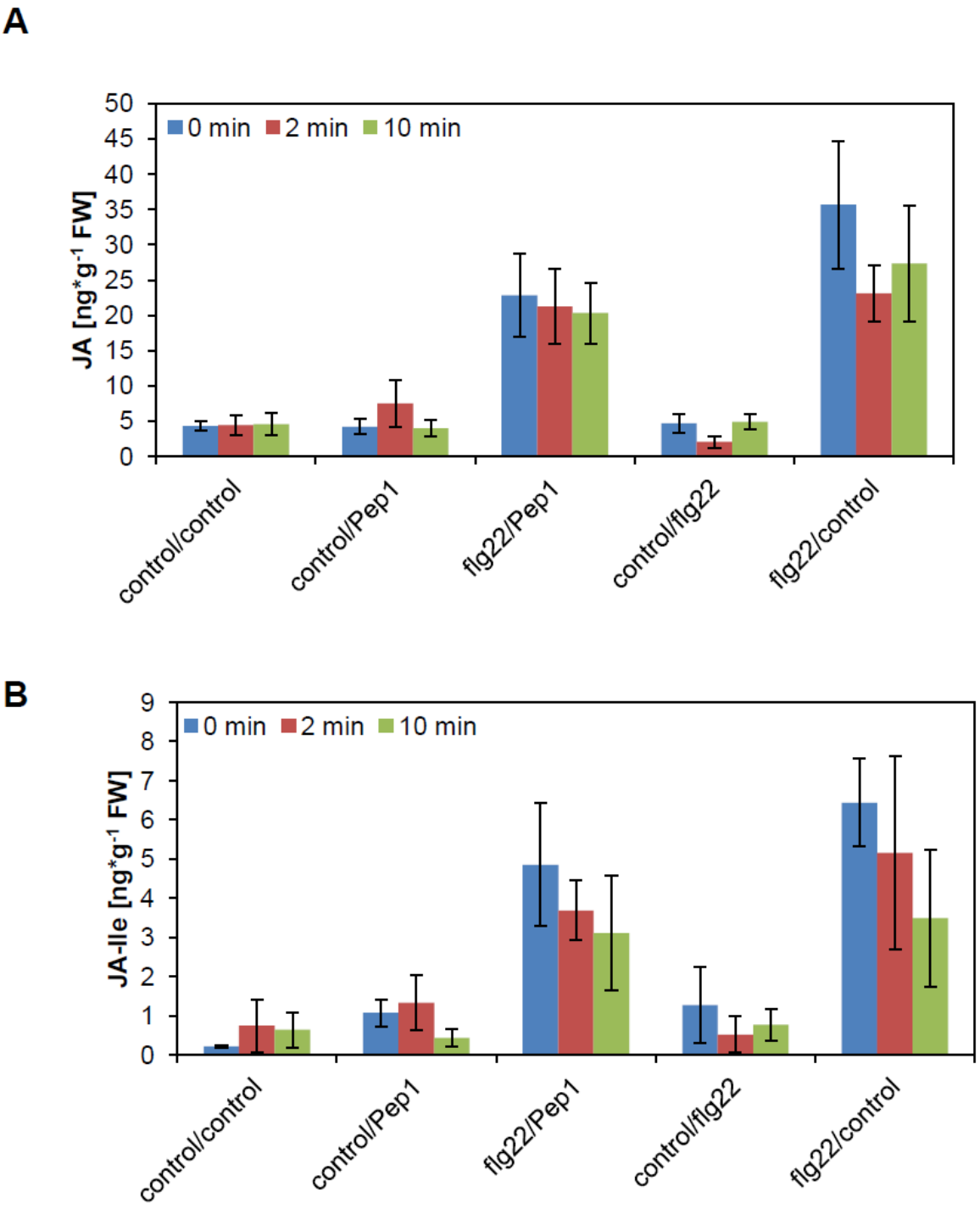
2.5 Supplementary Figures



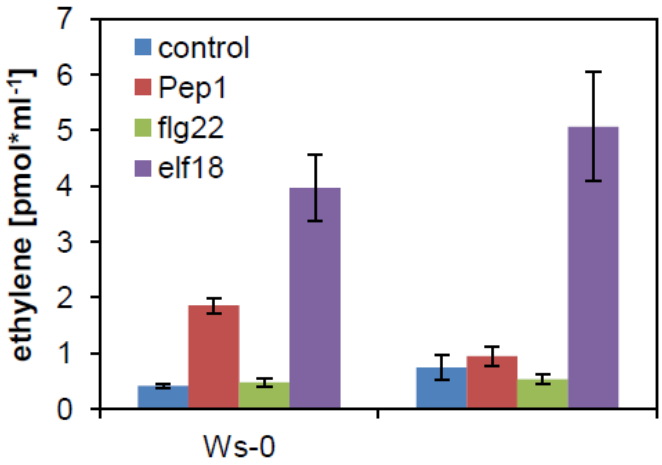
Supplementary Figure 1. Elevated *At*Pep1-triggered ROS production after pretreatment with elf18. Leaf discs were pretreated with indicated elf18 concentrations or without any peptide (control) for 16 h, and then treated with 1 µM *At*Pep1. Graphs represent mean values of ROS production in at least 8 replicates. Error bars show standard error of the mean.



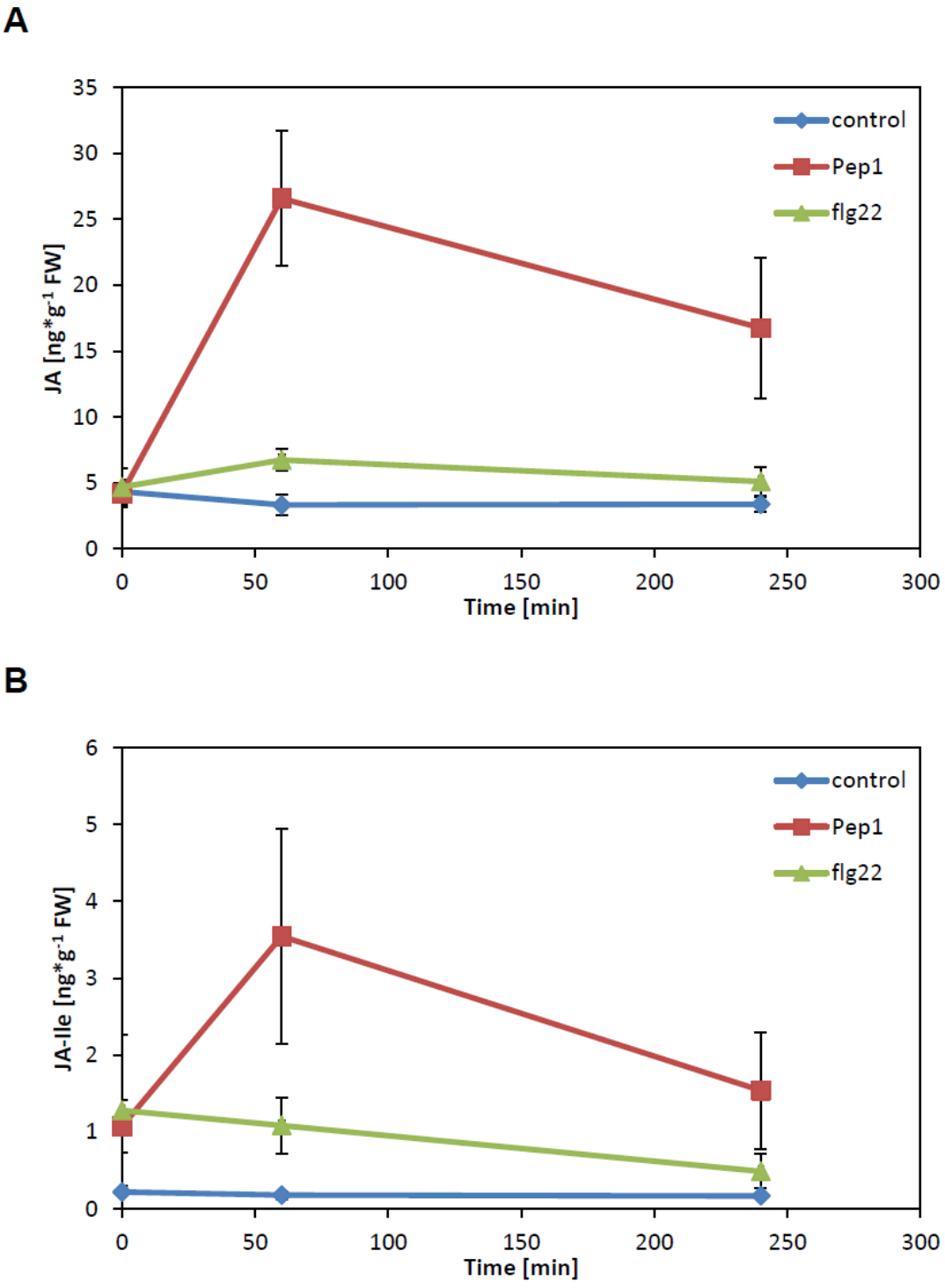
Supplementary Figure 2. Expression of *PEPR1* and *PEPR2* in the generated transgenic lines. Relative transcript levels of *PEPR1* (left) and *PEPR2* (right) in Col-0 and the transgenic lines transformed with CaMV35S::PEPR1::YFP (lines A.II and F.II) and CaMV35S::PEPR2::YFP (lines c.2 and f.2) constructs. Columns represent the mean of two biological replicates relative to Col-0. Error bars indicate relative standard error of the mean.



Supplementary Figure 3. Increased JA levels in flg22-pretreated samples do not change upon *At*Pep1-treatment. Leaf discs of Col-0 plants were incubated in 1 µM flg22 or without any peptide (control) for 16 h, then treated with either 1 µM of *At*Pep1, flg22, or without any peptide (control) and incubated for the indicated time period. A shows JA levels whereas B shows JA-Ile levels. Columns represent the mean of 4 independent replicates and error bars indicate the standard error of the mean.



Supplementary Figure 4. The *opr3* mutant shows reduced responsiveness to *At*Pep1-treatment. Leaf strips of Ws-0 and *opr3* mutant plants were incubated in water for 16 h and then treated with either 1 µM of *At*Pep1, flg22, elf18, or without any peptide (control) and incubated for 4 h before measurement. Columns represent the mean of 6 independent replicates and error bars indicate the standard error of the mean.



Supplementary Figure 5. Treatment with *At*Pep1 triggers a slight increase of JA and JA-Ile levels. Leaf discs of Col-0 plants were incubated in water for 16 h, then treated with either 1 µM of *At*Pep1, flg22, or without any peptide (control) and incubated for the indicated time period. A shows JA levels whereas B shows JA-Ile levels. Graphs represent the mean of 4 independent replicates and error bars indicate the standard error of the mean.

2.6 Material and Methods

Plant Material

The Arabidopsis plants used in this study were grown as one plant per pot at 21° C and an 8 h photoperiod for 4-5 weeks. All mutants used in this study are in the Col-0 ecotype except for *opr3*, which is in the Ws background. The T-DNA insertion lines SALK_059281 (*pepr1*) and SALK_098161 (*pepr2*) were supplied by the Nottingham Arabidopsis Stock Centre (Nottingham, United Kingdom). The *mpk3-1* (SALK_151594) and *mpk6-2* (SALK_073907) mutants were kindly provided by Roman Ulm (University of Geneva), *sid2* was kindly provided by Jean-Pierre Métraux (University of Fribourg), the rbohD/F double mutant by Miguel Angel Torres (University of Madrid), the *aos* and *coi1-1* mutants by Edward Farmer (University of Lausanne) and the *opr3* mutant by Jürgen Zeier (University of Düsseldorf).

The Arabidopsis cell culture was maintained and used for experiments 4–8 days after subculture as described (Felix et al., 1999).

Generation of Transgenic Arabidopsis Lines

The *PEPR1* and *PEPR2* coding regions in pDONR/Zeo were obtained from the Arabidopsis Biological Resource Center (ABRC) based on the work of Gou *et al.*, (2010). Gateway-based cloning was then used to insert PEPR1 and PEPR2 into the binary destination vector pEarley101 (Earley et al., 2006). Arabidopsis plants were transformed by *Agrobacterium tumefaciens* using the floral dip method (Clough and Bent, 1998).

Peptides

Peptides of flg22 (QRLSTGSRINSAKDDAAGLQIA), *At*Pep1 (ATKVKAKQRGKEKVSSGRPGQHN), *At*Pep2 (DNKAKSKKRDKEKPSSGRPGQTNSVPNAAIQVYKED), *At*Pep3 (EIKARGKNKTKPTPSSGKGGKHN), *At*Pep4 (GLPGKKNVLKKSRESSGKPGGTNKKPF), *At*Pep5 (SLNVMRKGIRKQPVSSGKRGGVNDYDM), *At*Pep6 (ITAVLRRRPRPPPYSSGRPGQNN), and elf18 (Ac-SKEKFERTKPHVNVGTIG) obtained from EZBiolabs were dissolved in a solution containing 1 mg/mL bovine serum albumin and 0.1 M NaCl.

Hormone Treatments

Analogously to the peptide treatments, MeSa and MeJa (both Sigma-Aldrich) were diluted in DMSO (Sigma) to 10 mM. This stock solution was then diluted in water or the respective assay

solutions to final concentrations between 1 µM and 100 µM. Additional DMSO was added to maintain equal amounts of DMSO in each dilution. As a negative control, similar amounts of DMSO were used.

Analysis of Plant Hormone Levels

Several leaf discs (90 mg fresh weight) were cut and floated for 16 h in darkness on 1 ml water with 1 µM flg22 or without any peptide (control). Leaf tissue samples were flash-frozen in liquid nitrogen and stored at -80°C until hormone level quantification. Hormone extraction and analysis was then performed as described in Glauser (2013).

Measurement of ROS Generation

Reactive oxygen species released by leaf tissue was assayed by H_2O_2-dependent luminescence of luminol. Leaf discs of 5 mm diameter were cut and floated overnight in darkness in 96-well plates (LIA White, Greiner Bio-One) on 0.1 ml water with or without elicitor peptides or hormones. For elicitation and ROS detection horseradish peroxidase (Sigma) and luminol (Sigma) was added to a final concentration of 10 µg/ml and 100 µM, respectively. Luminescence was measured directly after addition of elicitor peptides or hormones in a plate reader (MicroLumat LB96P, Berthold Technologies) for 30 min.

Alkalinization Assay

For medium alkalinization, aliquots of cell suspensions were assessed 5 days after subculturing. Pretreatment with flg22 or a negative control was performed 16 h before a second elicitor treatment and the pH was measured before and 20 min after the second treatment, using glass pH electrodes.

Cytoplasmic Calcium Measurements

Leaf discs of *A. thaliana* Col-0 expressing apoaequorin were placed into 96-well microplates containing a solution supplemented with 5 µM coelenterazine (Synchem, Felsberg/Altenburg, Germany) and either 1 µM flg22 or without any peptide. The leaf discs were left under complete darkness for 16 h for reconstitution. Data were acquired using a 96-well microplate luminometer (MicroLumat LB96P, Berthold Technologies). The substances were supplied to the wells via a computer-controlled dispensing system. Each experiment ended up with a discharge by adding 1 M CaCl in 10 % ethanol. The relative luminescence was determined from the ratio

of the actual luminescence per second and the total luminescence that was emitted from the probe.

Measurement of Ethylene Production

For measurement of ethylene accumulation leaf material was cut into strips of 10 mm^2 in the evening. 3 leaf strips were placed together in a 6 ml glass vial containing 0.2 ml of ddH_2O with or without elicitor peptides or hormones. Vials with leaf strips were incubated over night in the dark. After 16 h elicitor peptide was added to the desired final concentration and vials were closed with rubber septa. After 4 h of incubation on a shaker at room temperature, ethylene accumulating in the free air space was measured by gas chromatography (GC-14A Shimadzu).

MAPK Phosphorylation

Leaf discs were cut in the evening and left over night (16 h), floating on ddH_2O supplied with or without elicitor peptide. In the morning the elicitor peptide of the secondary treatment was added. Leaf tissue (40 mg per sample) was shock frozen and ground to fine powder before addition of 80 µl extraction buffer (0.35 M Tris-HCl pH 6.8, 30 % (v/v) glycerol, 10 % SDS, 0.6 M DTT, 0.012 % (w/v) bromphenol blue). Total cellular proteins (10 µg) were separated by electrophoresis in 12 % SDS-polyacrylamide gel and electrophoretically transferred to a polyvinylidene fluoride membrane according to the manufacturer's instructions (Bio-Rad). We used polyclonal primary antibodies against phospho-p44/42 MAP kinase (Cell Signaling Technologies) and actin (Sigma-Aldrich), with alkaline phosphatase-conjugated anti-rabbit and anti-mouse immunoglobulins (Sigma-Aldrich) as secondary antibodies, as required. Signal detection was performed using CDPstar (Roche).

Quantitative RT-PCR

Arabidopsis total RNA was extracted using the NucleoSpin RNA plant extraction kit (Macherey-Nagel) and treated with rDNase according to the manufacturer's specifications. Per PCR reaction, cDNA was synthesized from 10 ng of RNA with oligo(dT) primers using the AMV reverse transcriptase according to the manufacturer's instructions (Promega). Quantitative RT-PCR was performed in a 96-well format using a GeneAmp 7500 Sequence Detection System (Applied Biosystems). On the basis of the obtained C_T values, normalized expression to the reference gene *UBQ10* (AT4G05320) was calculated using the qGene protocol (Muller et al.,

2002). The gene-specific primers used were as follows: *UBQ10* (AT4G05320) with UBQ_fw (5'-GGCCTTGTATAATCCCTGATGAATAAG) and UBQ_rv (5'-AAAGAGATAACAGGAACGGAAACATAG), *FRK1* (AT2G19190) with FRK1_fw (5'-TGCAGCGCAAGGACTAGAG) and FRK1_rv (5'-ATCTTCGCTTGGAGCTTCTC), *PHI1* (AT1G35140) with PHI1_fw (5'-TTGGTTTAGACGGGATGGTG) and PHI1_rv (5'- ACTCCAGTACAAGCCGATCC), *WRKY53* (AT4G23810) with WRKY53_fw (5'-TCACTTTTTCTGACCACTTTGG) and WRKY53_rv (5'-AAGGAAGAGATATGTTAAGTTGGG), *ATTI1* (AT2G43510) with ATTI1_fw (5'-GTTGTCTTTTTCATCTTCTTCTTAGTC) and ATTI1_rv (5'-GCACAAAAGCCGAAACCAACATC), *ZAT12* (AT5G59820) with Zat12_fw (5'-TGACTACGTTGAAGAAATCTAGCAG) and Zat12_rv (5'-GTTCTTCCAAGCTCCAACTTGAG), and AT1G57630 with 57630_fw (5'-GGAAGGCCTTCAAAGAAACTTGTC) and 57630_rv (5'-GAACACGAACCAGTTGCTTGAATG), *PEPR1* (AT1G73080) with PEPR1_qRT_fw (5'-GATTCCTATTGAGATATGGAAGAG) and PEPR1_qRT_rv (5'-CCTCTTCTAAGCTGCTGTTCAC), *PEPR2* (AT1G17750) with PEPR2_qRT_fw (5'-ACCAATAATTCACCGCGACATC) and PEPR2_qRT_rv (5'-CGCATTTTCTGGTGCAATGTAC).

Growth Inhibition Assays

Surface sterilized seeds were sown on plates containing MS salts medium (Duchefa), 1% sucrose, and 0.8% agar. 5 days after germination and growth under continuous light seedlings were transferred to liquid MS medium supplied with the elicitors indicated (two seedlings per 500 µl of medium in 24-well plates). The effect of treatment with different peptides on seedling growth was analyzed after 10 days by weighing fresh weight.

3. Several MAMPs, Including Chitin Fragments, Enhance *At*Pep-triggered Oxidative Burst Independently of Wounding

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This chapter was published by invitation of Plant Signaling & Behavior and mainly represents additional results that have not been published in the manuscript described in the previous chapter. Here, my contribution included the collection of most experimental data as well as the compilation and writing of the first draft manuscript.

The first line of plant defense against pathogens is based on the recognition of conserved microbe-associated molecular patterns (MAMPs) by specific membrane-bound receptors leading to the activation of pattern-triggered immunity (PTI) (Jones and Dangl 2006; Boller and Felix 2009). Recently, a family of seven endogenous peptides in *Arabidopsis*, named *At*Peps, was shown to activate PTI-like responses when binding their respective receptors PEPR1 and PEPR2 (Huffaker, Pearce et al. 2006; Yamaguchi, Pearce et al. 2006; Krol, Mentzel et al. 2010; Yamaguchi, Huffaker et al. 2010). Therefore, and since the expression of the PEPRs as well as the *At*Pep-precursor proteins PROPEPs is induced under conditions of biotic stress, *At*Peps are believed to act as an additional endogenous defense system, amplifying the defense response locally and/or spreading this response to distal, non-infected tissue (Ryan, Huffaker et al. 2007; Boller and Felix 2009; Boller and He 2009). Moreover, recent studies suggest an interaction of *At*Pep-signaling with the defense hormone ethylene to maintain PTI responses after the initial MAMP recognition (Liu, Wu et al. 2013; Tintor, Ross et al. 2013). Further underlining the role of *At*Peps as amplifiers of defense, we recently showed that one particular *At*Pep-triggered defense response, the production of reactive oxygen species, is greatly enhanced upon previous MAMP recognition (Flury, Klauser et al. 2013). This increased ROS production seems to be Pep-specific, since no similar effect was observed for MAMPs, where previous elicitor recognition did not lead to an increased production of ROS. Remarkably, this MAMP-triggered enhancement of subsequent responses to *At*Peps was exclusive for ROS and did not affect other responses connected to PTI, such as MAPK activation, ethylene production or defense gene expression.

To analyze this MAMP-mediated enhancement of *At*Pep-triggered ROS production in greater detail we established a more sensitive ROS detection system based on a luminol derivative (L-012, Wako Chemicals). As shown in Figure 1**,** also with the new derivative of luminol we detected the strong enhancement of *At*Pep-triggered ROS by previous treatment with flg22, the active epitope of bacterial flagellin.

To investigate if the observed ROS enhancement might be restricted to bacterial MAMPs we then took this more sensitive ROS detection system and used a fungal elicitor molecule, chitin, which strongly differs from flg22 in its chemical composition and the plants perception system used to detect it. Whereas flg22 is a proteinaceous MAMP composed of 22 amino acids, chitin is an oligomer of N-acetyl-D-glucosamine subunits. Moreover, in contrast to FLS2, the receptor for flg22, which utilizes leucine-rich repeats (LRRs) to detect the peptide, the *Arabidopsis* chitin receptor CERK1 contains three lysine motif (LysM) domains for chitin perception (Liu, Liu et al. 2012). In addition, CERK1 does not require interaction with the co-receptor BAK1 for full activity, whereas FLS2 does (Schulze, Mentzel et al. 2010). Finally, also the PTI signature elicited by chitin is slightly different from the one triggered by flg22 (Felix, Baureithel et al. 1998). For example, chitin elicitation generally induces a much weaker and less transient ROS production in comparison to the ROS production triggered by flg22 (Figure 1) (Li, Zhao-Hui et al. 2009; Flury, Klauser et al. 2013).

Leaf disks only treated with chitin (Figure 1, control/chitin) showed a very weak ROS response at roughly a third of the intensity compared with *At*Pep1-treated leaf disks but still significantly higher than the control treatment (Figure 1, control/control). However, despite of a much weaker ROS-inducing capacity than flg22, chitin pretreatment strongly enhanced a subsequent *At*Pep-triggered ROS production (Figure 1, control/Pep1 vs. chitin/Pep1). Thus, we can conclude that the *At*Pep-ROS enhancing effect is neither specific for bacterial MAMPs, since it is also triggered by fungal MAMPs, nor specific for BAK1-associated MAMP perception systems, indicating that it might be a more general phenomenon.

Since we always measured the ROS response in cut leaf disks, we could never fully exclude the potential necessity of wounding to enable this effect. Previously, alternative setups using unwounded seedlings did not produce ROS levels detectable with the standard luminol-based detection system. Here, with the new assay based on the more sensitive luminol derivative L-012 we were able to detect the comparably low levels of ROS produced by seedlings upon *At*Pep1 or flg22 treatment (Figure 2A).

Using the same setup (except for the luminol) as described in Flury et al. (2013), we observed that 5-d-old seedlings, pretreated with flg22 for 16 h, clearly showed an increased *At*Pep-triggered ROS response compared with seedlings pretreated with a control solution (Figure 2B). Therefore, we can now exclude the possibility that wounding is a factor in the MAMP-induced enhancement of the *At*Pep-ROS response. Moreover, our data shows that already young seedlings are capable to react to MAMPs with a ROS burst, although in our assay the maximal ROS production measured in relative light units (RLUs) of a leaf disk treated with flg22 reached 25,000 RLUs (Figure 1) whereas a 5-d-old seedling produced upon the same treatment a maximum of only 2,500 RLUs (Figure 2A). The latter response was not detectable with the previous luminol-based ROS detection system.

Since plants are believed to have numerous endogenous elicitor molecules apart from *At*Peps (Boller and Felix 2009), it will be intriguing to find out whether this enhanced ROS production upon MAMP perception is restricted to *At*Peps or whether the ROS production triggered by other endogenous elicitors can be similarly enhanced.

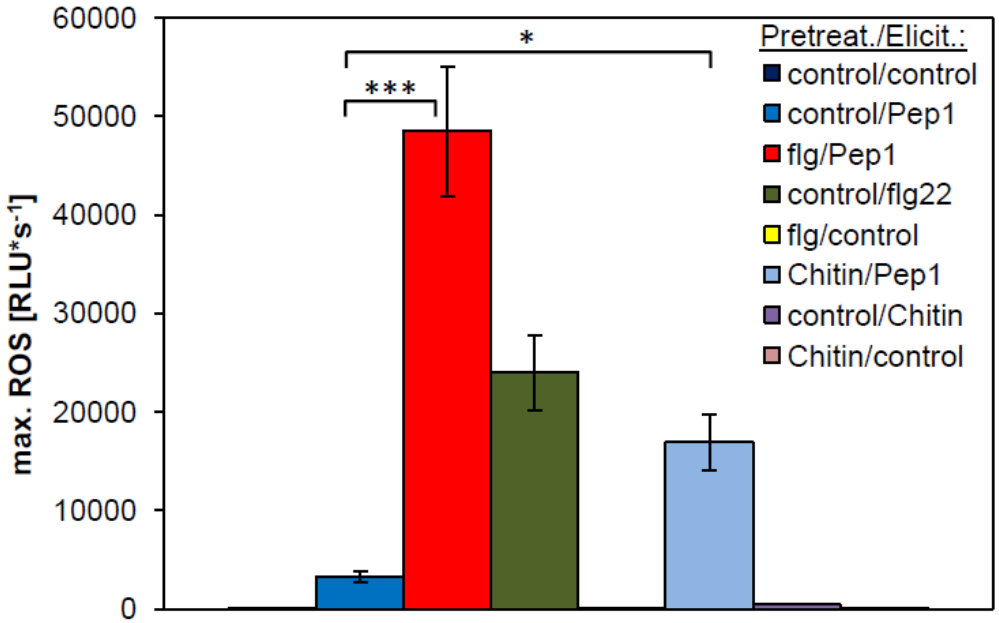

Figure 1. Chitin pretreatment strongly enhances the *At*Pep-triggered ROS response in *Arabidopsis* leaf disks. *Arabidopsis* leaf disks were pretreated, as described previously (Flury, Klauser et al. 2013) with either 1 µM of flg22, chitin or a control solution for 16 h and then treated with 1 µM of the indicated elicitor or without any peptide (control). Columns represent averages of the peak values of ROS production of eight biological replicates detected with the new luminol derivative L-012. Error bars depict 1 SE of the mean. Asterisks represent t-test results (*, $p < 0.05$; **, $p < 0.01$). RLU = relative light units.

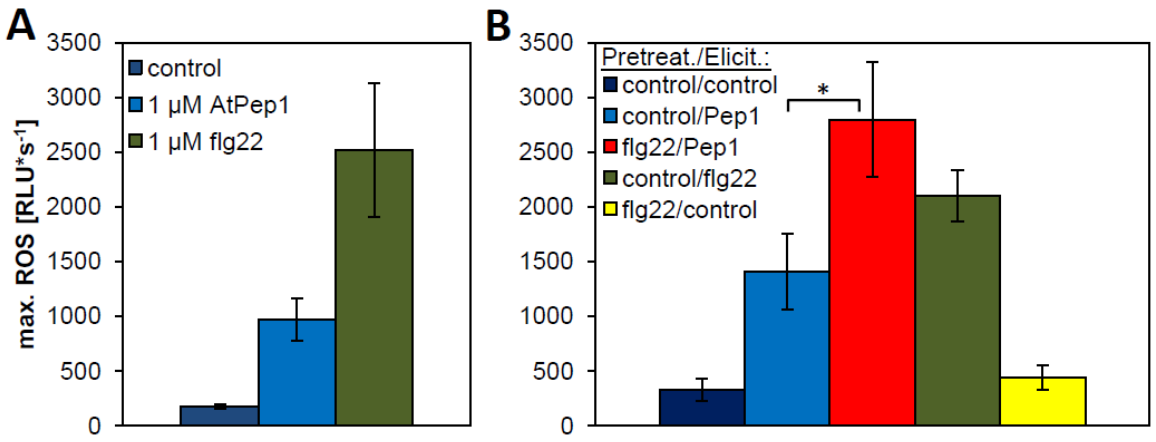

Figure 2. Elicitor-triggered and MAMP-enhanced *At*Pep-triggered ROS produced by *Arabidopsis* seedlings. (**A**) ROS response of 5-d-old *Arabidopsis* seedlings exposed to 1 µM of the respective elicitor. Elicitor treatment and ROS quantification was performed directly after harvesting the seedlings from 0.5-strength MS plates. (**B**) Seedlings display an enhanced *At*Pep-triggered ROS after flg22 pretreatment. Seedlings were pretreated with either 1 µM of flg22 or a control solution for 16 h and then treated with 1 µM of the indicated elicitor or without any peptide (control). (**A and B**) Columns represent averages of the peak values of ROS production of eight biological replicates detected with the new luminol derivative L-012. Error bars depict 1 SE of the mean. Asterisks represent t-test results (*, $p < 0.05$). RLU = relative light units.

4. The Endogenous *Arabidopsis thaliana At*Pep-PEPR Danger Detection System is Induced by Herbivore Oral Secretions to Mediate Defense Responses

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This paper represents one of the several attempts to investigate a potential phenotype of the *pepr1 pepr2* receptor mutant in the context of defense responses against biotic stress. Following up initial leads on a potential involvement of the *At*Pep-PEPR system in herbivore deterrence, I identified collaboration partners at Neuchatel University – specialists in plant-insect interactions - to further pursue herbivore feeding assays and plant hormone quantification. Apart from that, I contributed most experimental work described in this chapter myself, including bioassays, hormone measurements, and GUS expression analysis. Furthermore, I compiled the first draft for the manuscript described in this chapter and outlined both project and experimental setups for this work.

4.1 Abstract

In *Arabidopsis thaliana*, a family of eight endogenous elicitor peptides, referred to as *At*Peps1-8, triggers a PTI-like response upon binding their respective receptors PEPR1 and PEPR2. Since both PEPRs as well as some of the genes encoding the *At*Pep precursor proteins PROPEPs are induced upon diverse conditions of biotic stress, they are regarded as amplifiers of the plant's innate immune system. Indeed, they have been shown to mediate resistance against several microbial pathogens. However, the *At*Pep family also shares certain structural and functional homologies with the systemin peptide family, which is involved in defense responses against herbivores in solanaceous plants. For instance, *At*Peps and systemins both derive from larger precursor proteins, are assumed to be localized in the cytosol, and are known to induce and to be induced by the phytohormone JA. Therefore, a role of *At*Pep-signaling in the context of defense responses against herbivory has long been postulated. Here, using GUS-reporter lines, we show that *PEPR* as well as some *PROPEP* promoters are strongly activated by herbivore feeding. Importantly, promoter activation relied on the detection of herbivore oral secretions rather than on mechanical wounding and JA biosynthesis triggered by oral secretions was reduced in the *At*Pep-insensitive *pepr1 pepr2* double mutant. In accordance with an anti-herbivore function, we show that *pepr1 pepr2* double mutant plants exhibit reduced resistance to feeding *Spodoptera littoralis* larvae, indicating that the endogenous *At*Pep-PEPR danger detection system plays an important role not only in microbial but also in defense against herbivores.

4.2 Introduction

Plants use sophisticated perception and signaling systems to detect biotic dangers, such as microbial pathogens or feeding herbivores, and to subsequently induce an efficient defense response against these threats (Boller and Felix 2009). In the case of microbial pathogens, several membrane-bound pattern recognition receptors (PRRs) have been characterized that specifically detect conserved microbial structures, referred to as microbe-associated molecular patterns (MAMPs) (Boller and Felix 2009). Amongst these, flagellin-sensing 2 (FLS2), the receptor for a conserved domain of bacterial flagellin (flg22) (Felix, Duran et al. 1999; Bauer, Gomez-Gomez et al. 2001), as well as EFR, the receptor for elf18 (a conserved domain in the bacterial elongation factor TU) (Kunze, Zipfel et al. 2004), have been widely studied and well characterized (Boller and Felix 2009; Schwessinger and Ronald 2012).

After the initial recognition of MAMPs by PRRs, plants induce a set of both, immediate and long term responses that are collectively referred to as pattern-triggered immunity (PTI) (Boller and Felix 2009), eventually leading to an enhanced resistance against the invading pathogen (Zipfel, Robatzek et al. 2004; Zipfel, Kunze et al. 2006). Several of these physiological responses, such as for instance the production of reactive oxygen species (ROS) in the apoplast (Torres, Jones et al. 2006), the activation of ethylene biosynthesis (Spanu, Grosskopf et al. 1994), the change in ion fluxes across cell membranes (Boller 1995), and the phosphorylation of MAP kinases (Nühse, Peck et al. 2000) have been thoroughly investigated and are used as hallmarks of PTI to assess plant immunity in detail (Boller and Felix 2009).

In the case of herbivorous insects, plants rely on similar immediate immune responses to induce defense signaling and eventually herbivore deterrence. The initial recognition of herbivore attack is believed to be achieved by both, the detection of mechanical damage (Mithofer, Wanner et al. 2005) and/or the detection of elicitor compounds in insect oral secretions (Alborn, Turlings et al. 1997; Turlings, Alborn et al. 2000), the latter potentially being mediated by a similar set of membrane-bound receptors as for MAMP recognition (Schmelz, Engelberth et al. 2009). Also similar to PTI, the immediate defense responses upon herbivore detection include the production of ROS, the induction of ethylene biosynthesis, and the

activation of MAP kinases (Maffei, Mithöfer et al. 2007; Wu, Hettenhausen et al. 2007; Wu and Baldwin 2010).

Additionally to mechanisms for the detection of exogenous danger, plants also rely on endogenous signaling molecules that elicit defense responses similar to PTI (Boller and Felix 2009). Whereas some of these so-called danger-associated molecular patterns (DAMPs), such as cell wall fragments and cutin monomers, are derived from the degradation of the plant cell wall caused by invading pathogens (Darvill and Albersheim 1984; Fauth, Schweizer et al. 1998; D'Ovidio, Mattei et al. 2004), others like the peptides of the systemin family in solanaceous plants are actively produced by the plant upon herbivore and pathogen detection (Pearce, Strydom et al. 1991). Apart from eliciting general PTI-like defense responses, systemins have also been shown to induce specific anti-herbivore responses, such as the biosynthesis of proteinase inhibitor (PI) and the emission of volatile compounds to attract herbivore predators (Pearce, Strydom et al. 1991; Degenhardt, Refi-Hind et al. 2010; Sun, Jiang et al. 2011).

More recently, a peptide family similar to systemins has been discovered in *Arabidopsis thaliana*, referred to as *At*Peps (Huffaker and Ryan 2007). Like systemins, *At*Peps are small peptides (23-29 amino acids long) derived from the C-terminal ends of larger precursor proteins, the PROPEPs (Huffaker, Pearce et al. 2006; Huffaker and Ryan 2007). Whereas the receptor(s) for systemins are still unknown or under dispute (Holton, Harrison et al. 2008; Lanfermeijer, Staal et al. 2008; Malinowski, Higgins et al. 2009), *At*Peps have been shown to be perceived by two membrane-based receptors referred to as PEP-Receptor 1 (PEPR1) and PEPR2 (Yamaguchi, Pearce et al. 2006; Krol, Mentzel et al. 2010; Yamaguchi, Huffaker et al. 2010). Upon *At*Pep perception, both PEPRs trigger PTI-like defense responses reminiscent of the ones elicited by MAMP or systemin detection.

Given this similarity between MAMP and *At*Peps-induced responses and the fact that PROPEP/*At*Pep expression is induced upon biotic stress, *At*Peps are thought to either amplify or prolong local PTI responses. In addition they might also be involved in spreading the signal of

danger from the damaged or infected area to distal, not yet infected parts of the plant (Boller and Felix 2009; Yamaguchi and Huffaker 2011). Several modes of PTI amplification by *At*Peps have recently been proposed, either by interacting with the ethylene signaling pathway to maintain PTI (Liu, Wu et al. 2013; Tintor, Ross et al. 2013) or by amplifying the ROS response upon previous MAMP detection (Flury, Klauser et al. 2013; Klauser, Flury et al. 2013).
Supporting the amplifier theory, the exogenous application of *At*Peps has been shown to enhance immunity against the hemibiotrophic pathogens *Pseudomonas syringae* (Huffaker and Ryan 2007; Yamaguchi, Huffaker et al. 2010) and the necrotrophic pathogen *Botrytis cinerea* (Liu, Wu et al. 2013). Moreover, the application of *Zm*Pep, an *At*Pep homologue in *Zea mays* has been shown to induce resistance against *Cochliobolis heterostrophus* and *Colletotrichum graminicola* (Huffaker, Dafoe et al. 2011). However, despite of the apparent similarities to systemin, it was only very recently that the exogenous application of *Zm*Peps has been shown to induce herbivore defense signaling, including plant volatile emissions, insect deterrent metabolites and the production of herbivory defense related phytohormones, rendering treated maize plants more resistant to the generalist herbivore *Spodoptera exigua* (Huffaker, Pearce et al. 2013).

Still, since only the exogenous application of Peps has so far been shown to induce an increased resistance against herbivore feeding, the mechanisms of Pep signaling upon herbivore recognition have remained elusive. Here, using prom*PROPEP* and prom*PEPR* reporter lines driving a β-glucuronidase (GUS) reporter gene, we investigated the expression patterns of both, PEPRs as well as PROPEPs upon feeding by caterpillars of the noctuid moth *Spodoptera littoralis*. Additionally, we linked our observations to specific events of herbivory detection, like the actual wounding caused by chewing insects or the abundance of elicitors in herbivore oral secretions (Turlings, McCall et al. 1993; Alborn, Turlings et al. 1997).

To assess the potential role of *At*Pep signaling in defense responses against herbivory, we also assessed standard PTI defense responses as well as the accumulation of JA upon the application of herbivore Oral Secretions (OS) in both, mutant plants fully insensitive to *At*Pep application

(*pepr1 pepr2*) and Col-0 wild-type plants (Krol, Mentzel et al. 2010; Yamaguchi, Huffaker et al. 2010). Finally, herbivore feeding assays using the generalist caterpillar *Spodoptera littoralis* were performed to investigate a potential direct connection between *At*Pep-signaling and herbivore performance.

4.3 Results

Using transgenic plant lines expressing a β-glucuronidase (GUS) reporter gene under the control of the promoter regions of the respective *PROPEP* and *PEPR* genes as described by Bartels et al. (2013) we investigated their spatial and temporal expression under several conditions of biotic stress.

Intriguingly, whereas the promoters of *PEPR1* and *PEPR2* did not produce detectable GUS staining in untreated leaves, herbivore feeding strongly activated these promoters (Figure 1). The promoter of *PEPR1* produced a stronger GUS staining compared to the promoter of *PEPR2*. This increased activity of the PEPR promoters is located directly around areas of herbivore attack but does not extend to unharmed parts of the leaves or the plant. A similar expression pattern was observed for GUS reporter lines under the control of the promoter of *PROPEP3*, but in contrast to the *PEPR*s the induced GUS staining was not limited to the actual feeding sites, but also spread into the leaf veins (Supplementary Figure 1). For other *PROPEP*s, no obvious induction of expression was observed upon herbivore feeding (Supplementary Figure 1).

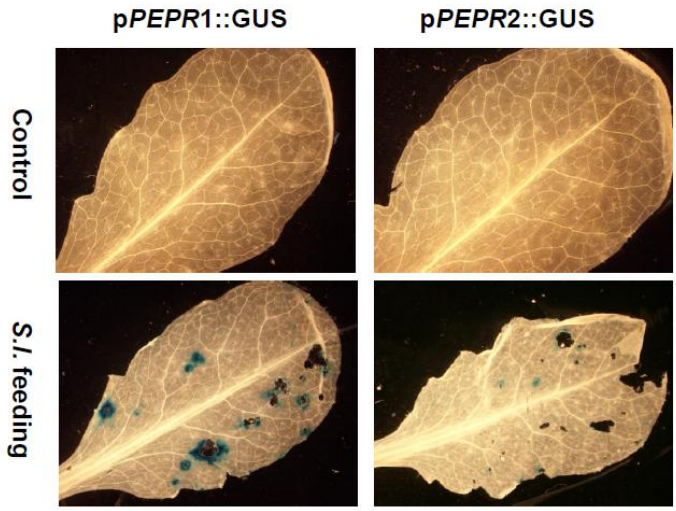


Figure 1. *Spodoptera* feeding strongly induces the promoters of *PEPR1* and *PEPR2*. Transgenic Arabidopsis plants expressing p*PEPR*::GUS reporter constructs were exposed to feeding *Spodoptera littoralis* (*S.l.*) larvae for 12 h. Thereafter, they were detached from the plant, fixed, and stained. For each construct, two independent lines were assessed with very similar results.

To further dissect this strong activation of *PEPR* and *PROPEP3* promoters, we tested whether this induced expression of *PEPR1* and *PEPR2* specifically relied on either the detection of wounding or the recognition of elicitor molecules in herbivore oral secretions. Indeed, in the case of the promoters of *PEPR1* and *PEPR2* wounding did not induce a strong reporter gene expression, whereas the application of *Spodoptera littoralis* oral secretions onto the upper leaf surface led to a strong activation of the *PEPR1* and *PEPR2* promoters right in the area of application (Figure 2). For *pPROPEP3* reporter lines, a similar induction of GUS expression upon the application of OS was observed (Supplementary Figure 1). However, in this case, wounding alone also seemed to induce a strong expression of *PROPEP3*, particularly in the leaf veins in the wounded area. As for herbivore feeding, no strong induction of GUS in other *pPROPEP*::GUS reporter lines was observed upon wounding or OS application (Supplementary Figure 1). Additionally, no activation of the two PEPR promoters was observed in the two reporter lines upon treatment with the MAMPs flg22 and elf18. In contrast, application of flg22 onto the upper leaf surface lead to a strong activation of the *PROPEP3* promoter (Supplementary Figure 2).

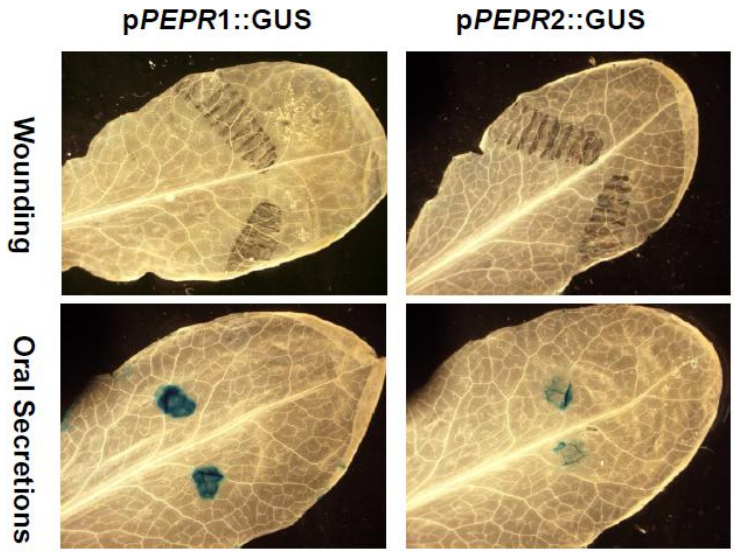


Figure 2. *Spodoptera* oral secretions are sufficient to activate PEPR promoters. Leaves of transgenic Arabidopsis plants expressing p*PEPR*::GUS reporter constructs were either wounded by forceps or were treated with 1 µl of *Spodoptera littoralis* oral secretions. After 12 h, they were detached from the plant, fixed, and stained. For each construct, two independent lines were assessed with similar results.

We compared the effect of herbivore OS on seedling growth between wild-type Col-0 plants and the double mutant (*pepr1 pepr2*), which lacks both Pep receptors and is fully blind to all *At*Peps (Krol, Mentzel et al. 2010; Yamaguchi, Huffaker et al. 2010). As shown in Figure 3, the application of herbivore OS led to a relatively weak seedling growth inhibition in the *pepr1 pepr2* mutant plants, whereas wild-type Col-0 plants were significantly stronger inhibited by the same treatment (Figure 3A). Inhibition of seedling growth was even stronger by flg22 and *At*Pep1 in wild-type plants, but as expected, the double mutant was completely insensitive to *At*Pep1 while it was inhibited by flg22 to the same degree as wild-type plants. Remarkably, this reduced seedling growth inhibition in *pepr1 pepr2* seedlings was particularly obvious in roots, where the application of OS led to an almost total inhibition of root growth in Col-0 wild-type plants, but not in *pepr1 pepr2* mutant plants (Supplementary Figure 3).

However, both the *pepr1 pepr2* mutant plants and the Col-0 wild-type plants responded similarly to a stimulation with OS with regard to ethylene production; producing as much stress ethylene as when the plants were stimulated by flg22 (Figure 3B). As expected, only the Col-0 plants, but not the *pepr1 pepr2* mutant plants showed enhanced ethylene production in response to *At*Pep1 (Figure 3B & Supplementary Figure 4). Noteworthy, ethylene production is regarded to be a much more immediate physiological response to danger signals (Boller and Felix 2009).

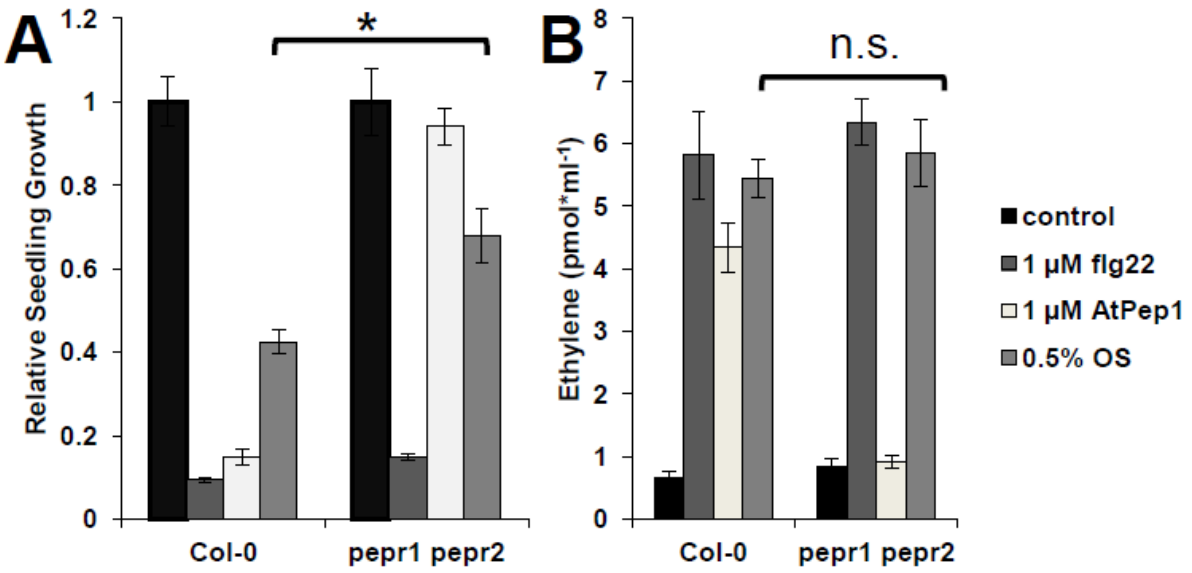


Figure 3. Long-term OS induced defense responses are dependent on *At*Pep signaling.
A: Arabidopsis seedlings of the lines Col-0 (wild-type) and the *pepr1 pepr2* double mutant were grown under sterile conditions for five days and then transferred into MS medium either containing 1 µM of the respective elicitors, 0.5% (v/v) OS, or no elicitor (control). Seedling growth was then assessed 10 d after transfer by weighing the fresh weight of the seedlings. Error bars show +/- 1 SE of six independent replicates.
B: Leaf disks of Arabidopsis Col-0 and *pepr1 pepr2* plants were treated with 1 µM of the respective elicitors, 0.5% (v/v) OS or without any elicitor (control) and ethylene production was assessed 5 h after the application of the eliciting compounds. Error bars show +/- 1 SE of eight independent replicates, asterisks indicate significant differences between genotypes (t-test, $p < 0.05$).

As JA is an important hormone involved in herbivory and wounding responses, we compared JA levels between *pepr1 pepr2* and Col-0 wild type plants after the application of OS. Interestingly, in Col-0 wild-type plants, both the levels of JA and JA-Ile increased by a factor of five within four hours, while it increased significantly less, only by a factor of about two in *pepr1 pepr2* mutant plants (Figure 4). For wound-induced JA biosynthesis however, no differential induction between the *pepr1 pepr2* and Col-0 wild type plants was observed (Supplementary Figure 5).

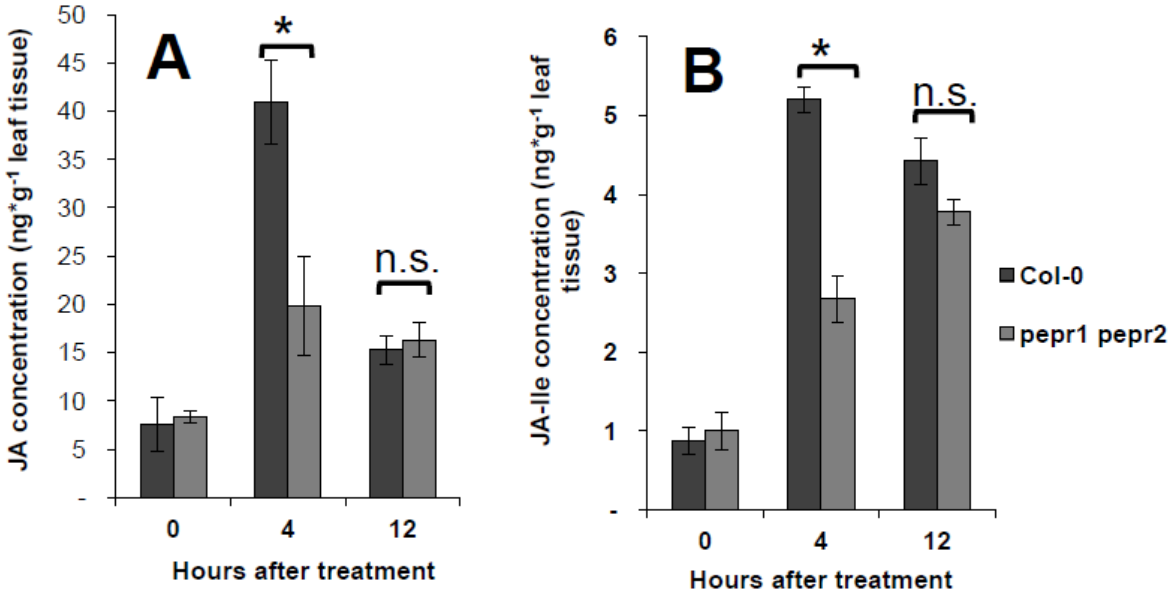


Figure 4. The detection of herbivore oral secretions induces JA biosynthesis in an *At*Pep-dependent manner. Plant leaves were treated by pipetting 1 µl of *Spodoptera littoralis* OS onto the upper leaf surface. After the time indicated, leafs were detached from the plant, flash frozen in liquid nitrogen and the levels of JA (**A**) and JA-Ile (**B**) were analyzed. Error bars show +/- 1 SE of eight independent replicates, asterisks indicate significant differences between genotypes (t-test, $p < 0.05$).

Finally, we compared the performance of the generalist herbivore *Spodoptera littoralis* feeding on *pepr1 pepr2* double mutant plants to Col-0 wild-type plants to assess the contribution of the signaling started by the PEPR activation to resistance against herbivory (Figure 5). Larvae that were left feeding on the plants ten days grew significantly larger on the *pepr1 pepr2* mutants than on the Col-0 wild type plants.

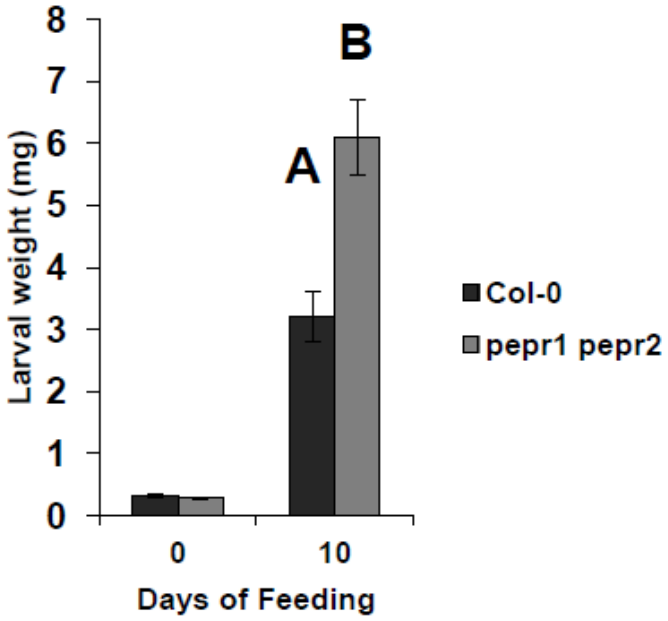


Figure 5. Plants impaired in *At*Pep-signaling display an increased susceptibility to herbivore feeding. Arabidopsis Col-0 wild-type and *pepr1 pepr2* mutant plants were exposed to feeding *Spodoptera littoralis* larvae for 10 days. Error bars indicate +/- 1 SE of caterpillar weight per larvae with 6 individual plants (10 larvae per plant) assessed. Letters indicate a significant difference using a one-way ANOVA (after arcsine transformation).

Taken together, these results show that upon the perception of *Spodoptera littoralis* OS, Arabidopsis plants exhibit a PTI-like response and activate the *At*Pep-PEPR system. Indeed, when this system is impaired the response to herbivore OS is significantly reduced an culminates in a considerably weakened resistance to herbivore attack.

4.4 Discussion

Recently, it was shown that the exogenous application of *Zm*Pep3, an *At*Pep ortholog in maize, induced defense responses against herbivore feeding, such as the induction of JA signaling, the production of herbivore-deterrent secondary metabolites and the induction of plant volatiles that attract herbivore predators (Huffaker, Pearce et al. 2013). These findings suggest a role for Pep-signaling in plant responses against herbivores, but the specific role of the Pep-PEPR system in the context of plant defense against herbivores still needed full confirmation.

Hereby, we show that the promoters of both *PEPRs* and *PROPEP3* are induced upon the recognition of herbivore OS, resulting in specific defense signaling in a JA-dependent manner and eventually rendering the plant more resistant to leaf-chewing herbivores. Moreover, by using the *pepr1 pepr2* double mutant plants, which are fully impaired in the recognition of *At*Peps, we provide evidence that endogenous *At*Pep-signaling is crucial for an efficient defense response against herbivore feeding in *Arabidopsis thaliana.*

Originally, it was shown that the expression of both *PEPR*s and some *At*Peps is induced upon wounding (Huffaker and Ryan 2007; Yamaguchi, Huffaker et al. 2010). Using p*PEPR*::GUS reporter lines, we surprisingly did not detect a strong GUS staining in mechanically wounded leaf tissue. However, we observed that feeding insects as well as the application of *Spodoptera littoralis* OS alone caused a very strong local induction of *PEPR* promoter activity. This activation was stronger for the *PEPR1* promoter than for the promoter of *PEPR2*, thereby supporting our assumption that PEPR1 is the more important Pep-receptor since already the basal *PEPR1* expression generally is higher than that of *PEPR2*, and PEPR1 detects all AtPeps, whereas PEPR2 only detects *At*Pep1 and *At*Pep2 (Krol, Mentzel et al. 2010; Bartels, Lori et al. 2013). We also observed a strong activation of the Promoter of *PROPEP3*, both in the case of herbivore feeding and the application of herbivore OS. No such induction was observed for other p*PROPEP*::GUS constructs. Recent studies have shown that particularly the expression of the *PROPEP2* and *PROPEP3* genes is induced in the context of defense signaling, with their promoters providing binding sites for WRKY transcription factors known to mediate PTI responses (Bartels, Lori et al. 2013; Logemann, Birkenbihl et al. 2013). In contrast, the expression of e.g. *PROPEP*5 has been

suggested to rather be co-regulated with non-defense related physiological events such as reproduction (Bartels, Lori et al. 2013). This goes in hand with our observations that none of the remaining p*PROPEP*::GUS reporter lines were inducible by the application of herbivore OS, nor by direct herbivore feeding.

Active elicitors from oral secretions are known to induce plant responses in a JA dependent manner (Turlings, McCall et al. 1993; Alborn, Turlings et al. 1997; Turlings, Alborn et al. 2000). Since two recent studies showed that the exogenous application of *At*Peps induces the accumulation of both JA and JA-Isoleucine (Flury, Klauser et al. 2013; Huffaker, Pearce et al. 2013), we further investigated this interplay between JA- and Pep-signaling. After the application of herbivore OS to *pepr1 pepr2* plants we observed decreased peak levels of JA and JA-Ile accumulation compared to Col-0 wild-type plants. However, upon wounding alone, JA-level accumulation in *pepr1 pepr2* plants increased to similar levels as those in Col-0 wild-type plants. Upon wounding, JA and JA-Ile levels are known to be rapidly but very transiently induced (Glauser, Dubugnon et al. 2009), whereas in the case of the application of OS, this induction seems to be much weaker but also less transient. Hence, *At*Pep-signaling might play a role in the specific recognition of herbivory and with the help of elicitor molecules in herbivore oral secretions. This hypothesis is supported by our observations that the *pepr1 pepr2* mutants exhibited impaired long-term PTI defense responses upon the application of herbivore OS, reflected also in a decreased seedling growth arrest. As a general phenomenon upon the detection of MAMPs and DAMPs, Arabidopsis seedlings reduce their growth rate, which is believed to go in hand with a reallocation of resources towards the induction of defense responses (Boller and Felix 2009), thereby rendering the plant more resistant to the imminent attack at the cost of a reduced growth. However, early PTI responses, such as the production of the stress hormone ethylene, remained similar between *pepr1 pepr2* and Col-0 wild type plants, possibly indicating no involvement of *At*Pep-PEPR-signaling in the direct recognition of elicitors in herbivore OS.

Underlining this potential involvement of *At*Pep-PEPR-signaling in activation and/or maintenance of defense responses, caterpillars of the generalist herbivore *Spodoptera littoralis* displayed a considerably enhanced growth on *pepr1 pepr2* plants compared to Col-0 wild-type plants.

In recent years, several lines of evidence supporting a role of the *At*Pep-system in defense responses against herbivores have been provided. Yang et al. (2011) proposed a role of BAK1 (bri1-associated receptor kinase) in the recognition of herbivore OS and the subsequent JA-dependent activation of defense responses against herbivores in *Nicotiana attenuata*. Upon ligand binding, PEPRs are known to form a heteromeric complex with BAK1, which is required for full defense response inducing activity of PEPRs as well as for many other PRRs (Chinchilla, Zipfel et al. 2007; Chinchilla, Shan et al. 2009; Schulze, Mentzel et al. 2010). Therefore, it is tempting to speculate that the involvement of BAK1 in antiherbivore responses might be connected to Pep-signaling (Yang, Hettenhausen et al. 2011). However, since BAK1 is believed to dimerize with many yet unknown PRRs, it could also be involved in other steps of herbivore feeding recognition and antiherbivore defense signaling, as for instance the direct recognition of active elicitors in herbivore OS.

Interestingly, *At*Peps can induce JA accumulation and JA itself can induce the expression of both *PEPR*s as well as some *PROPEP*s (Flury, Klauser et al. 2013; Huffaker, Pearce et al. 2013). Here, the temporalsequence of *At*Pep and JA signaling upon natural herbivore attack is of great interest. Whereas the JA inducing properties of *At*Pep-PEPR-signaling underline their potential role as amplifiers of JA-signaling and thus defense responses against herbivores, no prolonged increase of JA accumulation was observed in Col-0 wild type plants compared to the *pepr1 pepr2* mutants upon the recognition of herbivore OS. Yet, JA peak levels upon the application of herbivore OS were decreased in the *pepr1 pepr2* mutants.

Spatially, the role of *At*Pep-PEPR-signaling also needs further investigation. Given the fact that *PEPR* promoters are activated only around the site of herbivory, one would assume *At*Pep-

PEPR-signaling to take place very locally to induce the production of feeding deterrent metabolites. However, *At*Pep-PEPR-signaling could also lead to a systemic signal, rendering distal parts of the plant more resistant to the imminent attack.

In summary, we propose that *At*Pep-PEPR-signaling mediates defense responses upon the detection of herbivore OS, either by amplifying the local immune response at the site of feeding and/or potentially inducing JA and other systemic signaling components. Since the expression of some *PROPEP*s as well as both *PEPR*s, is also known to be induced by JA, this amplification of defense responses could also be following a positive feedback mechanism between Pep- and JA signaling (Figure 6). Following this up, it would be interesting to shed light on which signaling events precede the activation of *At*Pep-PEPR-signaling and which signals are induced by *At*Pep-dependent signaling. For this, the p*PEPR*::GUS and p*PROPEP*::GUS reporter lines described here, as well our bioassays to assess the plant defense response upon the detection of herbivore OS could prove powerful tools to dissect the molecular mechanisms of defense against herbivores.

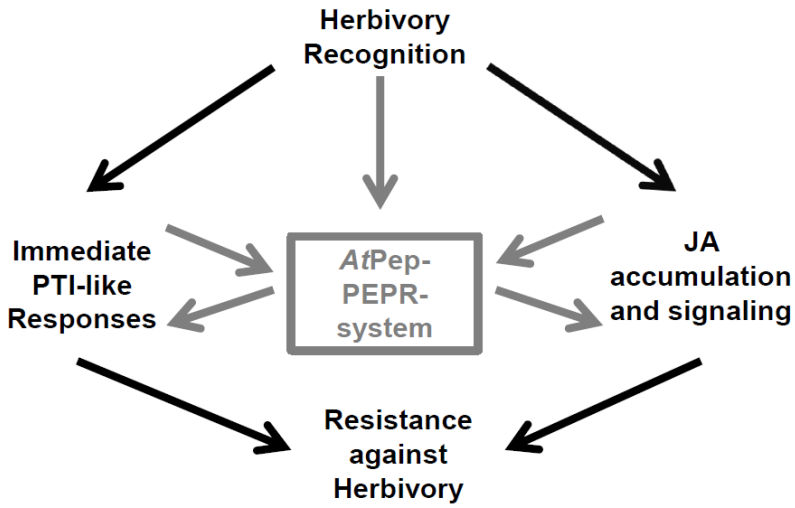
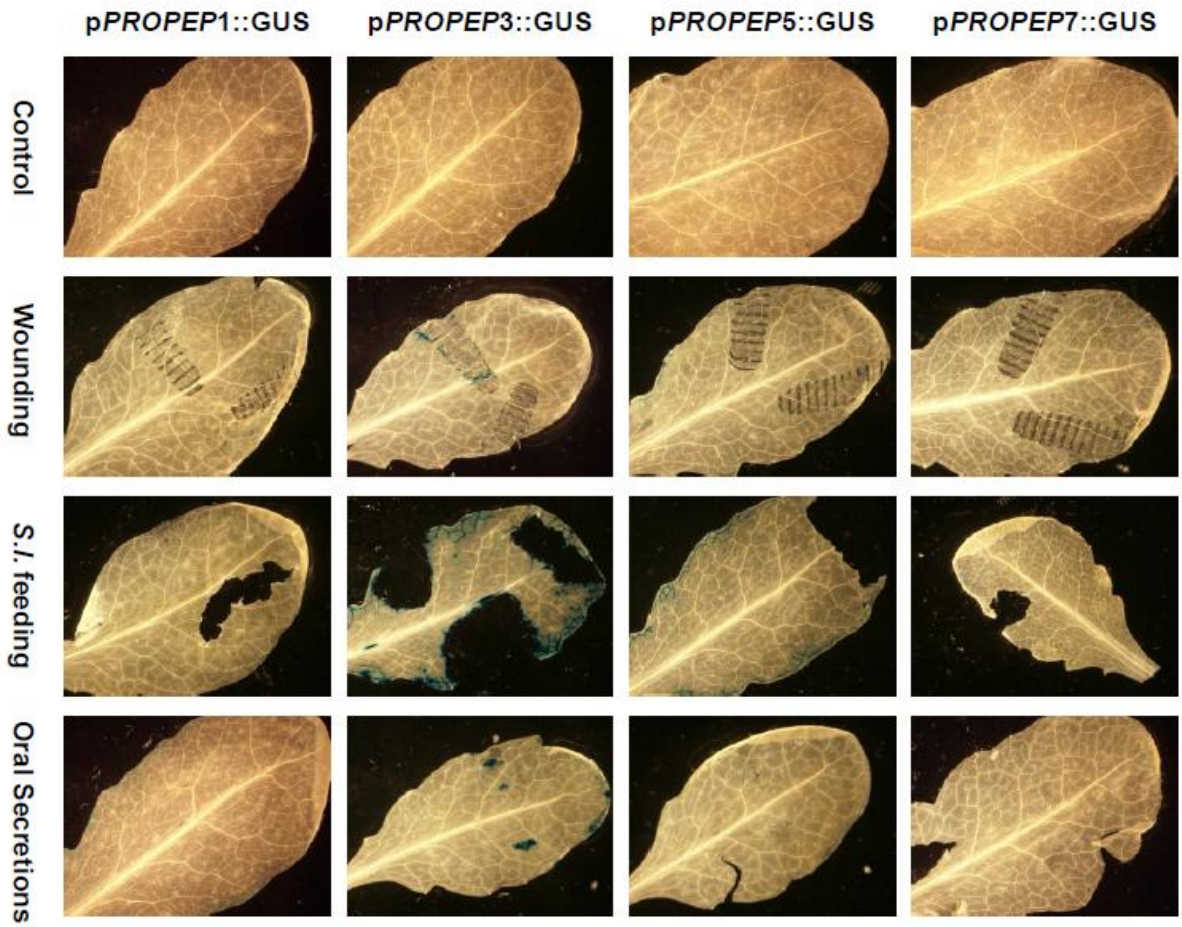
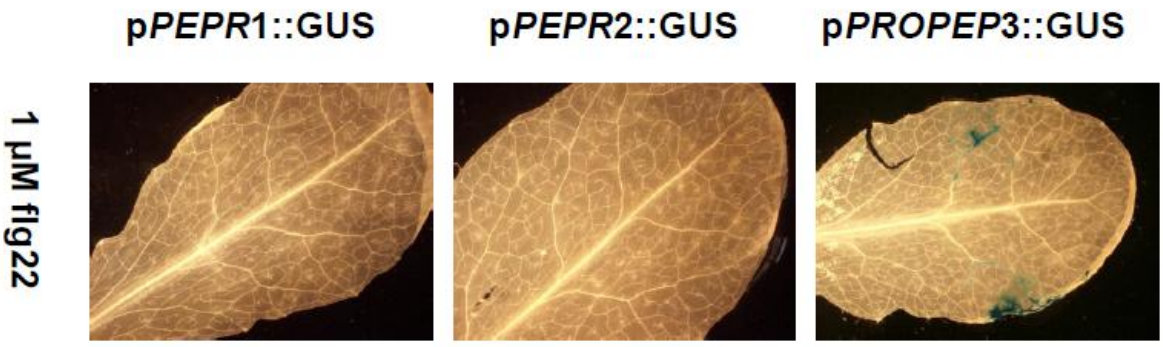


Figure 6. *At*Pep-signaling is required for full-strength in defense responses against herbivory in *Arabidopsis thaliana*. Upon herbivory recognition, both, immediate PTI-like responses (ethylene accumulation, ROS production, Ca^{2+}-signaling) as well as JA accumulation are induced. Additionally, the recognition of elicitors in OS of feeding herbivores also induces the accumulation of *PEPR* and *PROPEP3* transcripts. The *At*Pep-PEPR-system triggers PTI-like responses and eventually leads to the accumulation of the phytohormone JA. Moreover, JA signaling and PTI responses can themselves induce *PEPR* and *PROPEP* expression, possibly establishing a positive feedback loop for enhanced defense signaling (grey arrows). Thus, *At*Pep-PEPR-signaling is required for full-strength defense responses in *Arabidopsis thaliana* against the generalist herbivore *Spodoptera littoralis*.

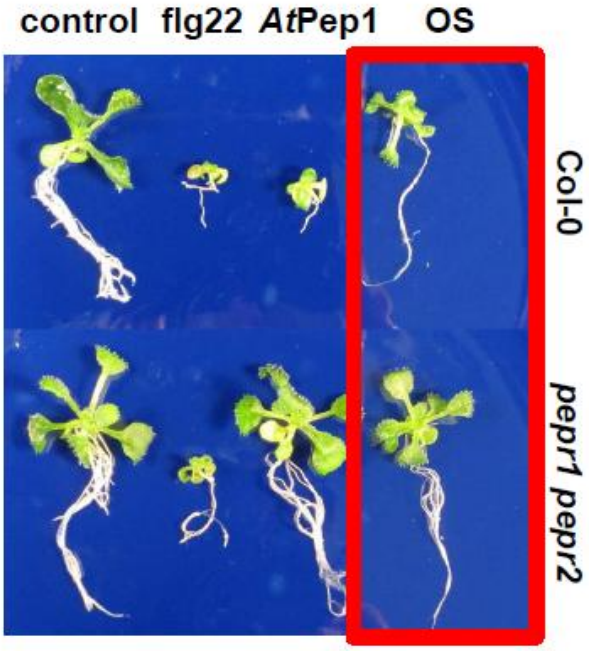
4.5 Supplementary Figures



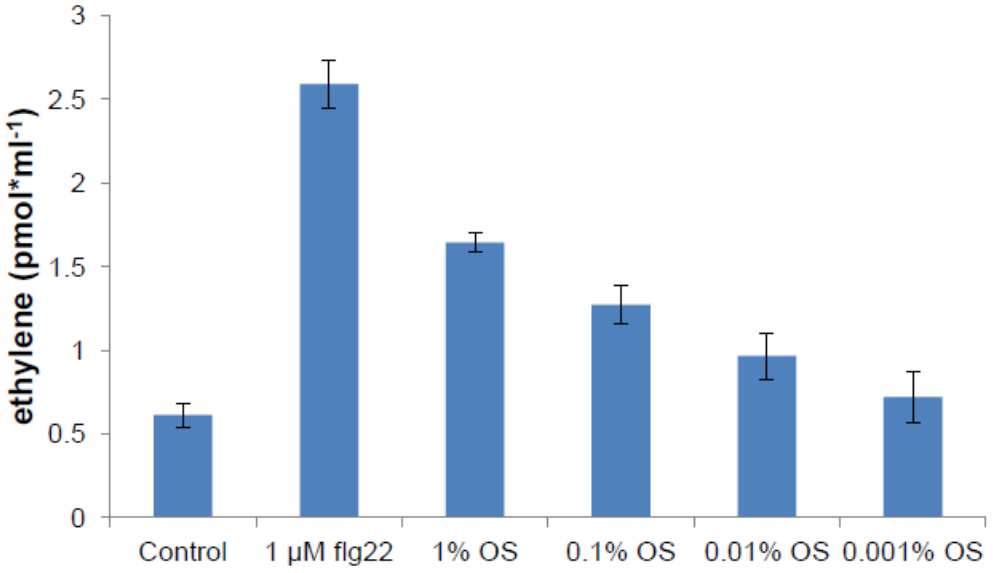
Supplementary Figure 1. p*PROPEP::GUS* reporter lines reveal a distinct pattern upon herbivore detection. Leaves of transgenic Arabidopsis plants expressing the indicated GUS reporter constructs were either wounded by forceps, exposed to feeding *Spodoptera* littoralis larvae or were treated with 1 µl of *Spodoptera littoralis* oral secretions. After 12 h, they were detached from the plant, fixed, and stained. For each construct, two independent lines were assessed with similar results.



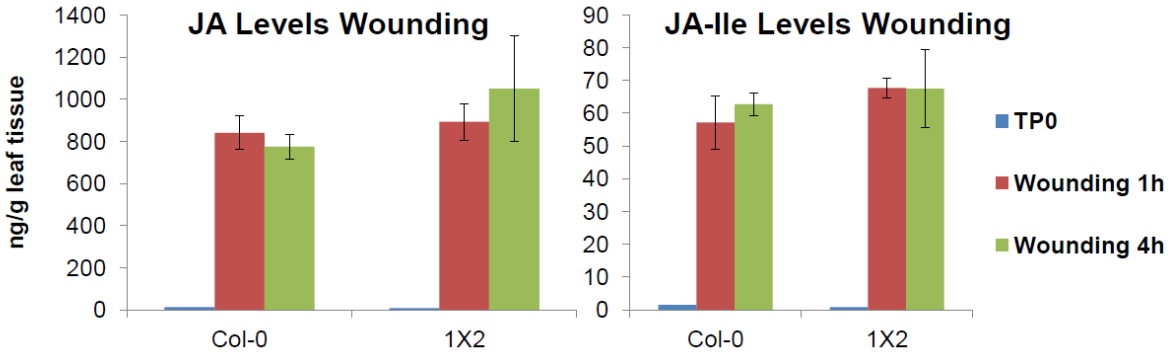
Supplementary Figure 2. The application of flg22 activates the promoter of *PROPEP*3 but not the ones of *PEPR1* and *PEPR2*. Leaves of transgenic Arabidopsis plants expressing the indicated GUS reporter constructs were treated 10 µl of 1 µM flg22, pipette onto the upper leaf surface. After 12 h, they were detached from the plant, fixed, and stained. For each construct, two independent lines were assessed with similar results.



Supplementary Figure 3. *Spodoptera littoralis* OS particularly reduce root growth in *Arabidopsis thaliana* seedlings. Arabidopsis seedlings of the lines Col-0 (wild-type) and the *pepr1 pepr2* double mutant were grown under sterile conditions for five days and then transferred into MS medium either containing 1 µM of the respective elicitors, 0.5% (v/v) OS, or no elicitor (control).



Supplementary Figure 4. Herbivore Oral secretions induce a dose-dependent ethylene response. Leaf disks of Arabidopsis Col-0 and *pepr1 pepr2* plants were treated with the respective concentration (v/v) of OS or without any elicitor (control) and ethylene production was assessed 5 h after the application of the eliciting compounds. Error bars show +/- 1 SE of eight independent replicates.



Supplementary Figure 5. Wounding induces JA biosynthesis independently of *At*Pep-signaling. Plant leaves were treated by forceps wounding. After the time indicated, leafs were detached from the plant, flash frozen in liquid nitrogen and the levels of JA and JA-Ile were analyzed. Error bars show +/- 1 SE of eight independent replicates.

4.6 Material and Methods

Plant Material

Arabidopsis plants of the indicated phenotypes were grown individually in small pots at 21° C and a 10 h photoperiod for 4-5 weeks. T-DNA insertion mutants for the *pepr1 pepr2* mutants are in a Col-0 background and were obtained from Birgit Kemmerling (University of Tübingen). The p*PEPR*::GUS and p*PROPEP*::GUS reporter lines used are described in Bartels et al. (2013).

Elicitor Peptides and Insect Oral Secretions

Peptides of flg22 (QRLSTGSRINSAKDDAAGLQIA) and *At*Pep1 (ATKVKAKQRGKEKVSSGRPGQHN) obtained from EZBiolabs were dissolved in a solution containing 1 mg/mL bovine serum albumin and 0.1 M NaCl.

Oral secretions of *Spodoptera littoralis* larvae (obtained from Syngenta Crop Protection, Switzerland) were obtained by gently pushing the forehead region of third and fourth instar larvae as described by Turlings (1993). Until usage, oral secretions were stored at -20°C.

GUS Staining

Plant leaves were either crunched using forceps, exposed to feeding *Spodoptera littoralis* larvae, or treated with 1 µl of the indicated elicitor (1 µM) or 1 µl of *Spodoptera littoralis* oral secretions (both by applying on the upper leaf surface) for 24 h. Subsequently, the tissue was fixed in ice-cold 90 % acetone for 20 min, washed with water and then placed in GUS staining buffer (1 mM 5-bromo-4-chloro-3-indolyl β-d-glucuronidase (Gold BioTechnology, St. Louis, Missouri, USA), 100 mM sodium phosphate (pH 7.5), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA, and 0.1% (v/v) Triton X-100) at 37 °C for 12h. Plant tissue was cleared with 70% (v/v) ethanol and photographed using an Olympus SZX12 binocular in combination with an Olympus DP72 camera and the CellSens imaging software (Olympus America, Pennsylvania, USA).

Plant Hormone Analysis

Several leaf discs (90 mg fresh weight) were cut from leaves treated by either exposing them to feeding *Spodoptera littoralis* larvae, application of 1 µl of insect oral secretions on the upper leaf surface, or supplying 1 µl of the respective elicitor (1 µM) to the upper leaf surface. Leaf tissue samples were then flash-frozen in liquid nitrogen and stored at -80°C until hormone level

quantification. Hormone extraction and analysis was performed as described by Glauser et al. (2013).

Herbivore Feeding Assays

Adult plants in the vegetative stage were separately exposed to *Spodoptera littoralis* first instar larvae (10 per plant) for 10 days. Larvae were then counted to assess survivorship and weighed. Weight of the larvae was measured at the beginning and at the end of the experiment for each plant to determine mass gained. A total of 15 plants of each of the two Arabidopsis lines tested (Col-0 and *pepr1 pepr2*) were used.

Growth Inhibition Assays

Five days after germination, sterile seedlings were transferred to liquid MS medium either containing peptides at 1 µM final concentration or OS at a concentration of 0.5 % (v/v). The MS medium was filter sterilized after the addition of the respective elicitors (one seedling per 500 µl of medium in 24-well plates). The effect of treatment with different elicitors on seedling growth of different plant genotypes was analyzed after 10 days by weighing the fresh weight.

Measurement of Ethylene Production

For measurement of ethylene accumulation, three leaf disks of 4-5 week old plants were harvested using a 5 mm cork borer and placed into a 6 ml glass vial containing 0.5 ml of ddH2O, placed back into the growth chamber and left overnight (~ 16 h). Elicitor peptides (1 µM final concentration) and oral secretions (0.5 % v/v final concentration) were added and vials were closed with air-tight rubber septa. After 4 h of incubation at room temperature, ethylene accumulating in the free air space was measured by gas chromatography (GC-14A Shimadzu).

5. The Immunity Regulator BAK1 Contributes to Resistance against diverse RNA Viruses

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The work described in this chapter compiles two initially independent approaches. The first approach constituted a collaboration between Camilla Kørner and me to investigate a potential contribution of the *At*Pep-PEPR system to antiviral defenses, the second by Dagmar Hann to follow up her initial observations on a potential involvement of BAK1 in defense responses against viruses. Here, I contributed to the design of the project and experimental layout, the performance of several experiments that led to the published results (microarray database analysis, gene expression analysis, bioassays, pathotests) as well as in the writing process of the eventual manuscript.

5.1 Abstract

The plant's innate immune system detects potential biotic threats through recognition of microbe-associated molecular patterns (MAMPs), or danger-associated molecular patterns (DAMPs), by pattern recognition receptors (PRRs). A central regulator of pattern-triggered immunity (PTI) is the BRI1 associated kinase 1 (BAK1) which undergoes complex formation with PRRs upon ligand binding. Although viral patterns inducing PTI are well known from animal systems, nothing similar has been reported for plants. Antiviral defense in plants is rather thought to be mediated by posttranscriptional gene silencing of viral RNA, or through effector-triggered immunity, i.e. recognition of virus-specific "effectors" by resistance proteins. Nevertheless, infection by compatible viruses can also lead to the induction of defense gene expression, indicating that plants may also recognize viruses through PTI. Here we show that PTI, or at least the presence of the regulator BAK1, is important for antiviral defense of Arabidopsis plants. Arabidopsis *bak1* mutants show increased susceptibility to three different RNA viruses during compatible interactions. Furthermore, crude viral extracts, but not purified virions, induce several PTI marker responses in a BAK1-dependent manner. Overall, we conclude that BAK1-dependent PTI contributes to antiviral resistance in plants.

5.2 Introduction

Both plants and animals perceive microbe-associated molecular patterns (MAMPs) as a first line of defense against potential microbial pathogens (Janeway and Medzhitov 2002; Zipfel and Felix 2005; Boller and Felix 2009). Such MAMPs are broadly conserved molecular microbial structures essential for the microbial life style, and absent from the host. Upon MAMP perception by pattern recognition receptors (PRRs), a signaling cascade is initiated, which ultimately leads to PTI, i.e. "pattern-triggered immunity" (Janeway and Medzhitov 2002; Boller and Felix 2009).

PTI is well studied in animals and active against a wide range of different pathogens, including viruses. The best characterized PRRs in animals are Toll-like receptors (TLRs), which recognize a wide range of MAMPs, including viral RNA and DNA (Song and Lee 2012). In order to permit efficient detection of extracellular and intracellular microbes, animal TLRs are either localized to the plasma membrane or associated with the endomembrane system (Jensen and Thomsen 2012). As viruses are obligate intracellular pathogens, they are predominately recognized by intracellular receptors, such as TLR3, TLR7 and TLR8 for viral RNA and TLR9 for viral DNA (Rathinam and Fitzgerald 2011; Jensen and Thomsen 2012).

In plants, no similar mechanisms have been described thus far. Antiviral defense strategies in plants thought to be mediated by recognition and subsequent degradation of viral genomic RNA or replication intermediates by the host post-transcriptional gene silencing machinery, leading to the generation of viral small interfering RNAs (vsiRNA) (Ruiz-Ferrer and Voinnet 2009). The production of vsiRNAs leads to sequence-specific degradation or translational inhibition of viral target RNAs (Ding 2010). However, whether or not virus-specific molecules are directly recognized by innate immune receptors in plants such as the case in animal systems is still unknown.

In plants, the molecular mechanisms underlying PTI share a number of features known from MAMP perception systems in animals (Boller and Felix 2009). Today, several MAMPs from fungi, oomycetes and bacteria, as well as a number of plant PRRs involved in their perception

are known. However, there are no equivalent MAMPs derived from viruses. All currently known PRRs of plants appear to be plasma membrane proteins with an extracellular receptor domain and either an intracellular kinase domain (receptor-like kinases, RLK) or without a structured intracellular domain (receptor-like proteins, RLP). Interestingly, a number of these RLKs and RLPs have been shown or are believed to interact with the LRR-RLK BAK1 (for BRI1-associated kinase 1) upon ligand binding (Chinchilla, Zipfel et al. 2007; Heese, Hann et al. 2007; Schulze, Mentzel et al. 2010). This interaction likely leads to cross phosphorylation between the two kinases and subsequent activation of downstream signaling (Schulze, Mentzel et al. 2010; Schwessinger, Roux et al. 2011). As BAK1 interacts with several PRRs, it is regarded a general regulator of plant immunity (Chinchilla, Zipfel et al. 2007; Heese, Hann et al. 2007; Chinchilla, Shan et al. 2009).

Plants also have surveillance systems to monitor cell integrity in a mechanistically similar manner to PTI. Wounding of Arabidopsis plants, for example, is believed to induce the production of *At*PEPs, small peptides that are relatively poorly conserved amongst higher plants and derived from longer peptides called *ProPEPs* (Wasternack, Stenzel et al. 2006; Huffaker and Ryan 2007; Krol, Mentzel et al. 2010; Yamaguchi and Huffaker 2011). Similarly to many MAMPs, *At*Peps are perceived by PRRs, namely Pep-Receptor 1 (PEPR1) and PEPR2 (Krol, Mentzel et al. 2010). Interestingly, PEPR1 and PEPR2 also interact with BAK1 upon *At*Pep recognition, which leads to the induction of a stereotypical defense response reminiscent of PTI (Boller and Felix 2009; Krol, Mentzel et al. 2010). Thus, BAK1 is a central player in different aspects of immunity, including classical PTI as well as DAMP signaling and thus constitutes an ideal molecular tool for the identification of novel components of immunity.

Given the similarities between plant and animal innate immune systems, we hypothesized that plant viruses also induce PTI by yet unknown mechanisms. This hypothesis is supported by the induction of gene expression typically associated with PTI and salicylic acid (SA) signaling during compatible viral infections (Whitham, Quan et al. 2003; Love, Yun et al. 2005; Carr, Lewsey et al. 2010; Hanssen, van Esse et al. 2011). To test this hypothesis we infected a range of PTI

signaling mutants with three different RNA viruses and found that plants mutated for the common regulator of PTI, BAK1, exhibited increased susceptibility to virus infection. This suggested that viral elicitors, or DAMPs produced in response to the virus, were recognized in a BAK1-dependent manner and that specific recognition of these molecular patterns contributed to resistance against these viruses. Consistently, we found that extracts of infected plants induced PTI responses in Arabidopsis, such as MAPK activation, increased production of the plant hormone ethylene or root growth inhibition in a BAK1-dependent manner when applied to healthy plants. In conclusion, we show that BAK1 is involved in antiviral defenses in plants and we provide evidence that virus encoded MAMPs or virus-induced DAMPs elicit classical PTI responses.

5.3 Results

Analysis of publicly available microarray data (https://www.genevestigator.com/gv/; (Whitham, Quan et al. 2003; Love, Yun et al. 2005; Carr, Lewsey et al. 2010; Hanssen, van Esse et al. 2011)) and previously published microarrays analysis of cDNA derived from tissue of Arabidopsis plants infected with the tobamovirus *Oilseed rape mosaic virus* (ORMV) (Hu, Hollunder et al. 2011) revealed that genes related to innate immunity exhibited modulated expression during compatible virus infections, and that these gene expression changes increased over time (Figure 1, Supplementary Figure 1). Interestingly, also *PEPR1* and *ProPEP2* were induced. Hence, we speculated that compatible virus infection could also interfere with DAMP signaling. To test whether DAMP-signaling was indeed induced upon virus infection, we infected Arabidopsis wildtype (WT) Col-0 plants with purified virions of three different RNA viruses, namely the tobamoviruses *Tobacco mosaic virus* (TMV) strain U1 and ORMV, and the tombusvirus *Turnip crinkle virus* (TCV). TCV and ORMV both cause strong symptoms in Arabidopsis plants, while TMV causes only very mild symptoms in Arabidopsis (Dardick, Golem et al. 2000). The transcripts of the precursor proteins *AtproPEP2* and *AtproPEP3*, which are known to be induced by wounding, biotic stress and MAMP signaling (Huffaker and Ryan 2007), were strongly up regulated by all three RNA viruses in systemic infected Arabidopsis leaf tissue compared to mock treated tissue (Figure 2A). To define the potential involvement of PEP signaling in anti-

viral defenses more closely, we also analyzed the expression of both *PEPR1* and *PEPR2*. Infection with TMV which causes only mild symptoms in Arabidopsis, significantly induced *PEPR1* and *PEPR2* at 21 DPI, while the slight enhancement observed for TCV and ORMV infections was statistically not significant (Figures 2B and 2C). Overall, these results indicate that components of the danger signaling pathway can be induced by RNA virus infections.

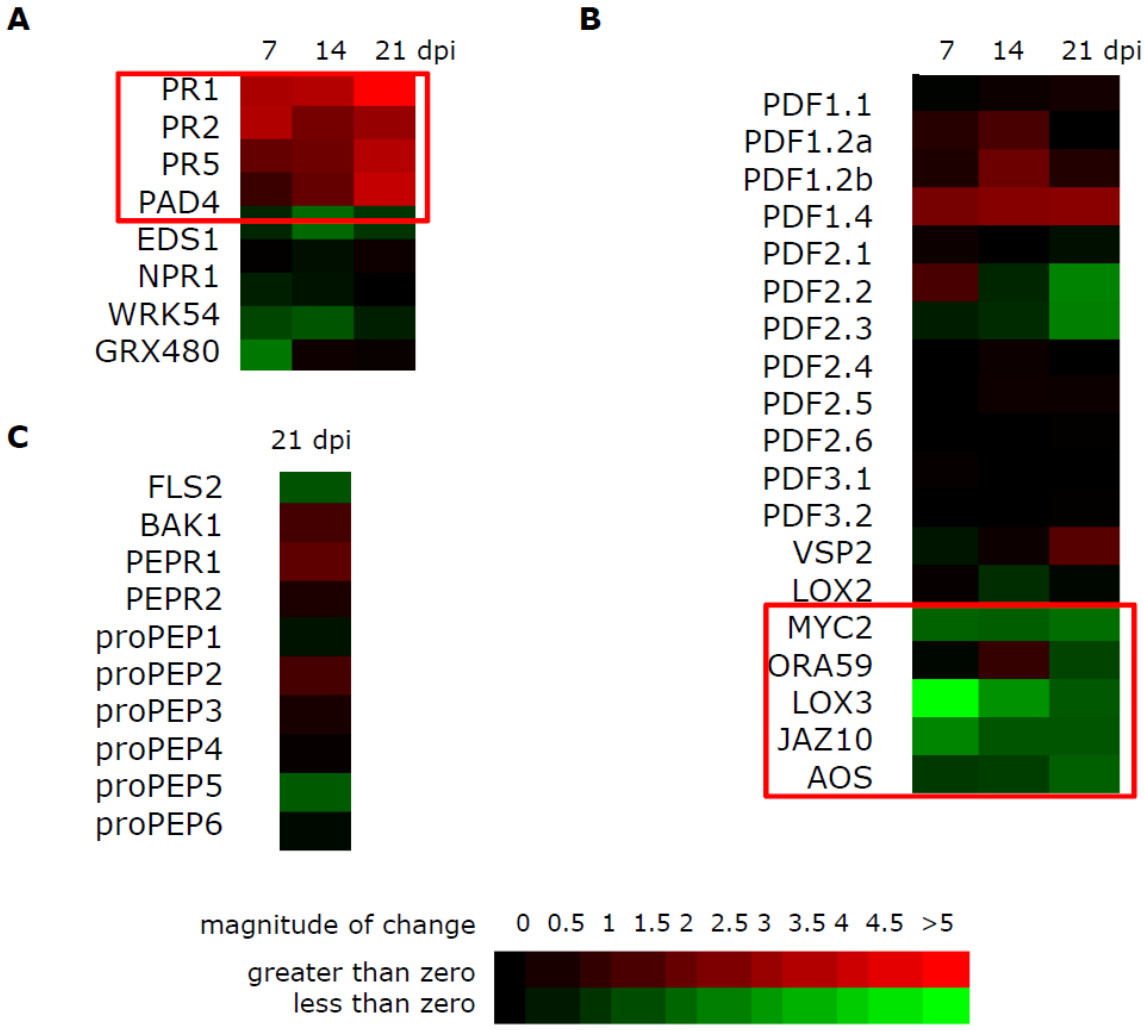


Figure 1. Expression of SA- JA- and PTI-pathway genes upon infection with the compatible virus ORMV. Whole plants infected with ORMV were harvested at 7, 14, 21 dpi. Total RNA was isolated and used for microarray analysis (Hu et al. 2011). Heat maps show $\log_2$ expression levels of genes involved in defense response via SA **(A),** JA**(B)** or PTI **(C)**. Strongly up- or downregulated genes are shown in red squares.

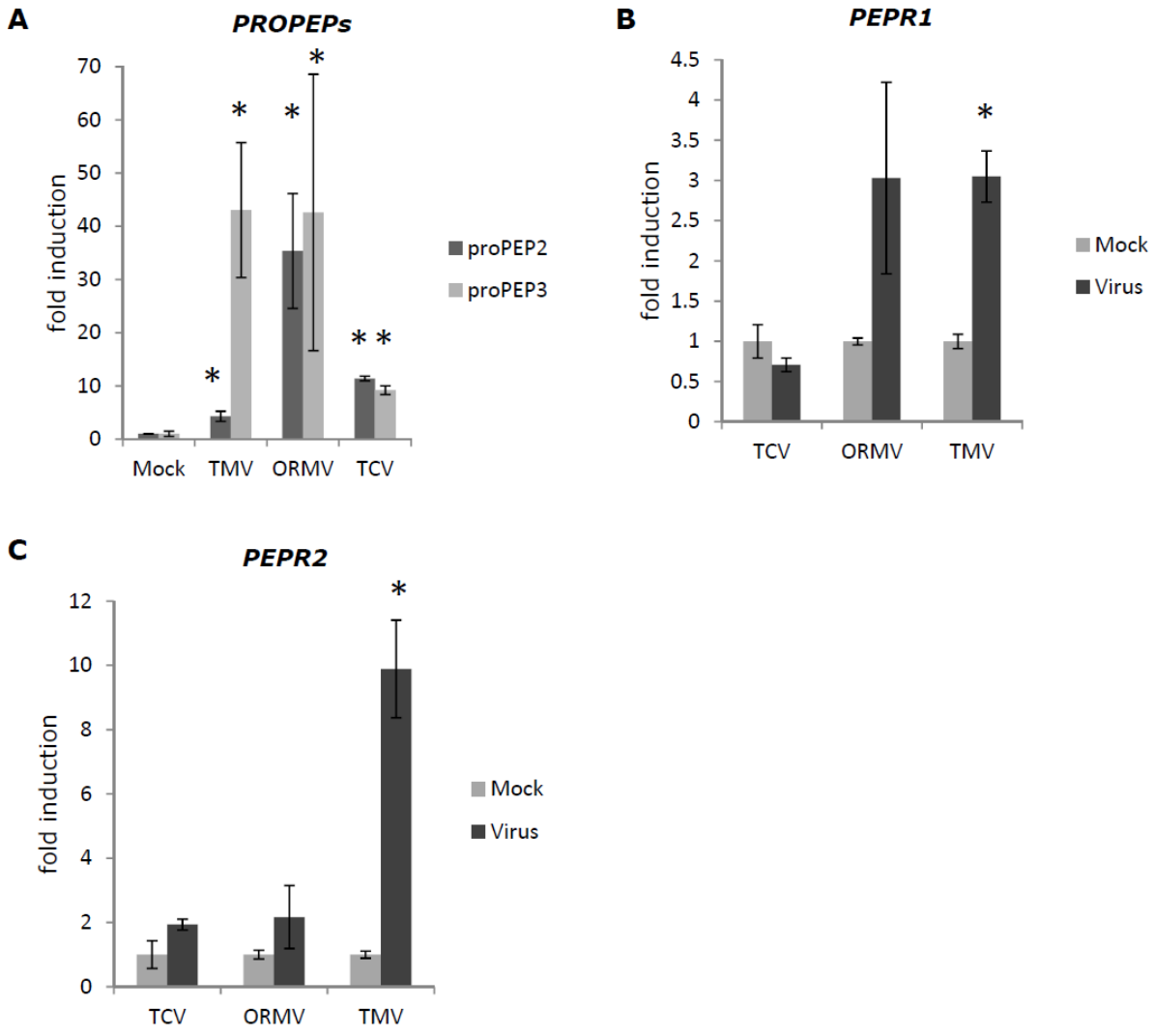


Figure 2. Components of PRR-triggered immunity are upregulated during viral infections. A) *PROPEP2* and *PROPEP3* are upregulated during infections with the tobamoviruses TMV and ORMV as well as the tombusvirus TCV. **B)** *PEPR1* is significantly upregulated ($p < 0.05$, student's t-Test) by TMV, but not by ORMV or TCV. **C)** *PEPR2* is significantly upregulated ($p < 0.05$, student's t-Test) by TMV, but not by ORMV or TCV. Significantly different results ($p < 0.05$, student's t-Test) are indicated by an asterisk and the error bars represent the standard error of the mean (sem -/+). All cDNAs were generated from tissue of Arabidopsis Col-0 plants infected with the respective virus for 21 days and the expression was normalized to the housekeeping gene ubiquitin. These experiments were repeated twice with similar results.

To investigate a potential role of *At*PEP signaling in anti-viral defenses we infected WT, the *pepr1/2* double mutant as well as mutants of BAK1, a common regulator of PTI responses and interactor of PEPR1/2 upon ligand binding, with TCV sap (Supplementary Figure 4A). We chose two different BAK1 mutants, namely the knockout mutant *bak1-4* (Chinchilla et al., 2007; Heese et al., 2007) and the single amino acid mutant *bak1-5* (Chinchilla, Zipfel et al. 2007; Heese, Hann et al. 2007; Schwessinger, Roux et al. 2011), which is impaired in PTI signaling but functions normally in BR signaling. We also included the flg22-specific MAMP receptor mutant *fls2* as a negative control, since an involvement of FLS2 in antiviral defenses seems unlikely (Gomez-Gomez and Boller 2000). At 21 DPI, TCV infection causes growth retardation and altered leaf morphology with rounder and slightly crinkled leaves in Col-0 WT, while the petiole length remains largely unaltered (Figure 3A). TCV infection of *pepr1/2* and *fls2* mutants produced similar symptoms as observed in the Col-0 wild type control plants (Supplementary Figure 2B). Interestingly, disease symptoms such as growth retardation and altered leaf morphology were strongly enhanced in the *bak1-4* and *bak1-5* mutants upon TCV infection, suggesting that *BAK1*-dependent DAMP and/or MAMP signaling may be involved in antiviral defense. Consistent with the assumption that disease symptoms would represent a measure for susceptibility, we observed a correlation between disease symptoms and virus accumulation. There was a tendency towards increased accumulation of viral coat protein in *bak1* mutants (Figure 3B), and a clearly increased accumulation of viral RNA in *bak1* mutants, compared to Col-0 WT, *pepr1/2* and *fls2* (Figure 3C). Together, these results demonstrate that *bak1* mutants are hypersusceptible to TCV, while *pepr1/2* or *fls2* mutants exhibit similar susceptibility to TCV as WT plants. This suggests that AtPEP signaling is not sufficient to suppress or attenuate viral infection.

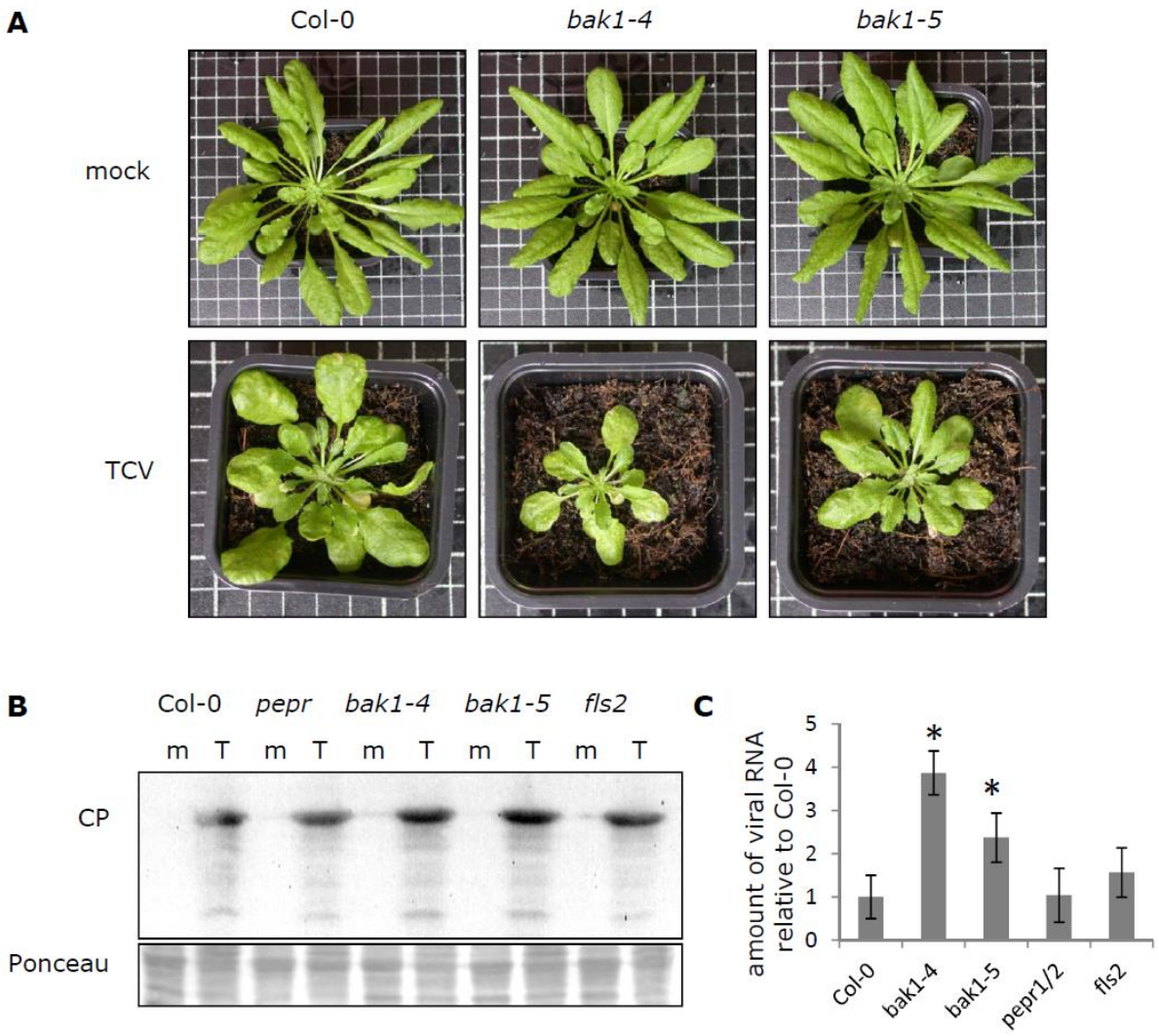


Figure 3: Characterization of TCV infections at 21 dpi in Col-0 and *bak1* mutant Arabidopsis lines on symptom and viral accumulation level. A) Col-0, the *bak1-4* knockout and the *bak1-5* point mutation mutants were infected with TCV crude extract and symptoms were scored three weeks post inoculation. The pictures present the average phenotype observed in the different lines. **B)** Expression level of the TCV coat protein detected by anti-CP antibodies (DSMZ) on Western blots loaded with leaf tissue extracts derived from the mock (m) and TCV (T) infected lines shown above and in and supplementary figure (SF) 2B. **C)** qRT-PCR of TCV CP accumulation in the infected lines shown in A and SF 2B normalized to the housekeeping gene ubiquitin. The bars represent the mean of three biological repeats with the sem (+/-) indicated by the error bars. Significant results ($p < 0.05$, student's t-Test) are marked by an asterisk. Each experiment was independently repeated at least twice and the results shown here are representative for each repeat.

To investigate whether the increased susceptibility of *bak1* mutants was specific for TCV or whether *bak1* mutants were generally more susceptible to virus infection, we analyzed virus-induced symptoms and virus accumulation of the tobamovirus ORMV, which causes disease in Arabidopsis plants as indicated by strong viral replication and symptom development. Infection of WT plants with ORMV led to growth retardation, severely curled and serrated leaves with shortened petioles and compact rosette formation. These symptoms developed similarly in wild type as well as in *fls2* and *pepr1/2* mutants (Supplementary Figure 3), but were again enhanced in *bak1-4* and *bak1-5* mutants (Figure 4A). Enhanced symptom development was paralleled by increased virus accumulation in *bak1* mutants compared to the wild type; we found a tendency towards higher levels of ORMV coat protein (Figure 4B) and a two-fold increase in ORMV RNA in *bak1* mutant plants compared to WT, *fls2* and *pepr1/2* mutants (Figure 4C). Taken together, the *bak1* mutants were not only more susceptible for TCV but also for ORMV infection.

Arabidopsis Col-0 plants infected with TCV or ORMV display apparent viral symptoms (Dempsey, Pathirana et al. 1997; Cai, Chen et al. 2009). In contrast, TMV infection of Arabidopsis Col-0 plants causes only mild symptoms, including shortened petioles and an overall more compact rosette (Figure 5A) (Dardick, Golem et al. 2000; Pereda, Ehrenfeld et al. 2000; Serrano, Gonzalez-Cruz et al. 2008). Given the strong symptom formation in WT plants upon ORMV and TCV infections, we included the symptomatically weaker TMV to our assays. Similar to the results obtained with ORMV and TCV, TMV-associated symptoms were also enhanced in *bak1* mutants characterized by an even more compact rosette than in virus infected Col-0 plants and altered leaf morphology upon infection (Figure 5A), while *fls2* and *pepr1/2* mutants displayed similar infection symptoms as the WT control (Supplementary Figure 3). Consistent with the observation obtained by TCV and ORMV infection that stronger disease symptoms correlate with higher virus titers, the TMV coat protein appeared slightly more abundant while the genomic RNA accumulated to significantly higher levels in the *bak1* mutants compared to WT or *fls2* and *pepr1/2* mutants (Figure 5B and C).

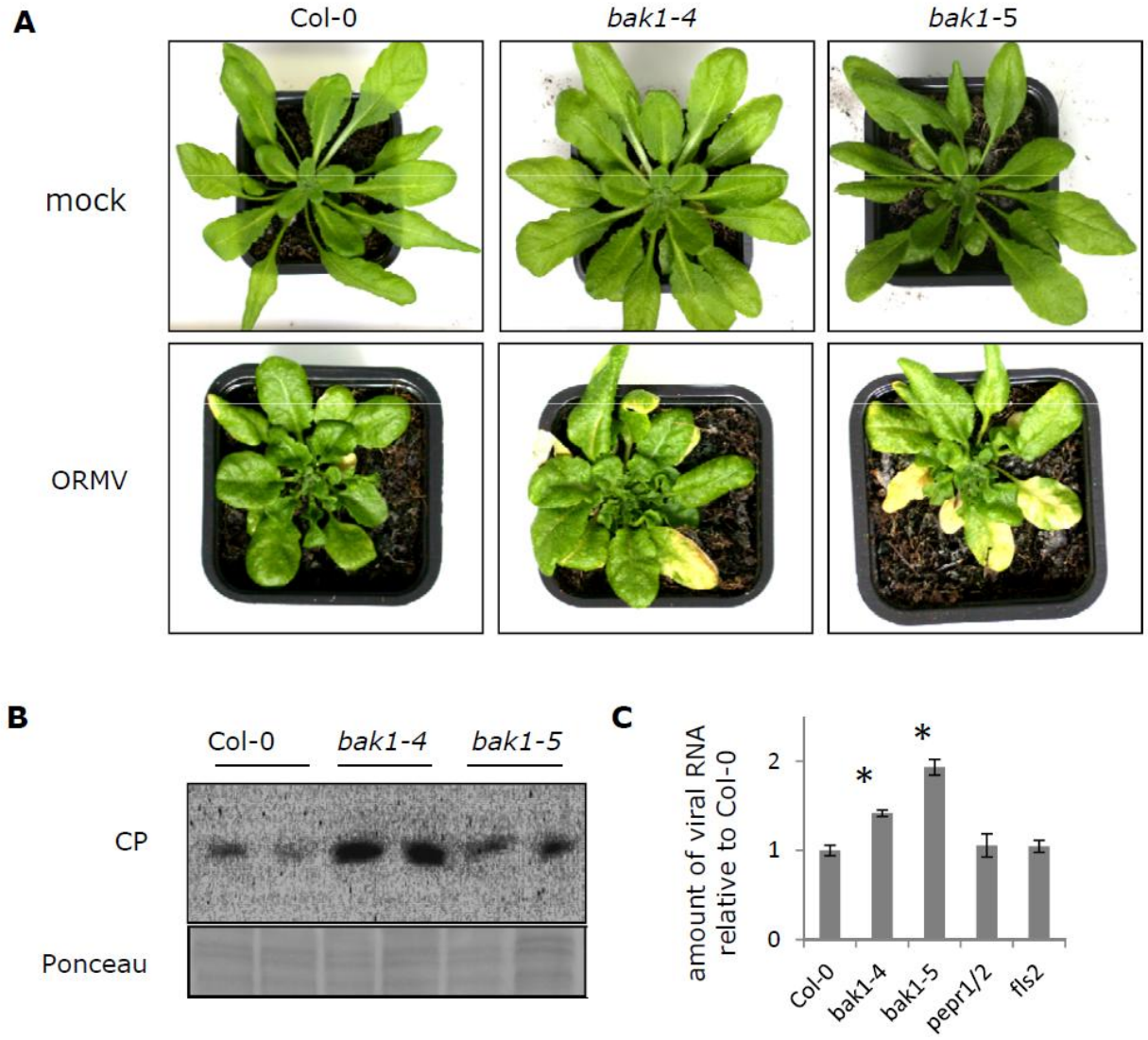


Figure 4. Characterization of ORMV infections at 21 dpi in Col-0 and *bak1* mutant Arabidopsis lines on symptom and viral titer level. A) Col-0, *bak1-4* knockout and *bak1-5* point mutation mutants were infected with ORMV virions and symptoms were scored three weeks post inoculation. The pictures present the average phenotype observed in the different lines. **B)** Accumulation of the ORMV coat protein detected by anti-CP antibodies (DSMZ) on Western blots of leaf tissue extracts from the infected lines shown above and in and SF 4. The samples were loaded as duplicates. **C)** qRT-PCR of ORMV RNA accumulation in the infected lines shown in A and SF 3 normalized to the housekeeping gene ubiquitin. The bars represent the mean of three biological repeats with the sem (+/-) indicated by the error bars. Significant results ($p < 0.05$, student's t-Test) are marked by an asterisk. Each experiment was independently repeated at least twice and the results shown here are representative for each repeat.

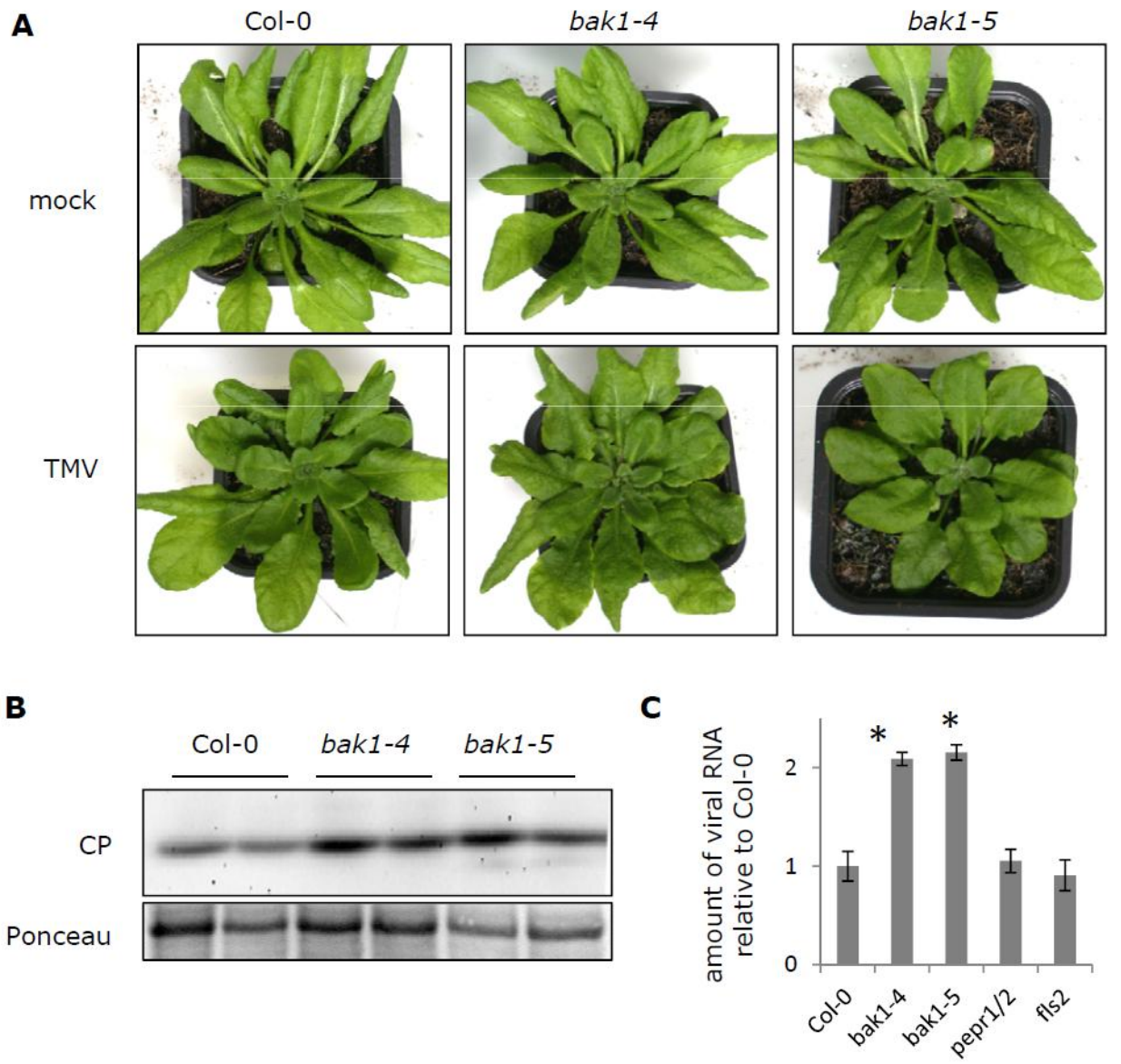


Figure 5. Characterization of TMV infections at 21 dpi in Col-0 and *bak1* mutant Arabidopsis lines on symptom and viral titer level. A) Col-0, *bak1-4* knockout and the *bak1-5* point mutation mutants were infected with TMV virions and symptoms were scored three weeks post inoculation. The pictures present the average phenotype observed in the different lines. **B)** Accumulation of the TMV coat protein detected by anti-CP antibodies (DSMZ) on Western blots of leaf tissue extracts derived from the infected lines shown in A. The samples were loaded as duplicates. **C)** qRT-PCR of TMV RNA accumulation in the infected lines shown in A and SF 3 normalized to the housekeeping gene ubiquitin. The bars represent the mean of three biological repeats with the sem (+/-) indicated by the error bars. Significant results ($p < 0.05$, student's t-Test) are marked by an asterisk. Each experiment was independently repeated at least twice and the results shown here are representative for each repeat.

Taken together, our results suggest that a BAK1-dependent pathway is important in the plants' defenses against viruses. By contrast, although induction of *AtproPEPs* and PEPR suggested that PEP signaling might be involved in antiviral defense, unaffected virus titers and symptoms in *pepr1x2* mutants demonstrate that PEP signaling alone is not sufficient to restrict virus infection in Arabidopsis.

Considering the increased susceptibility of *bak1* mutants to RNA viruses, we wondered whether extracts of virus infected leaf tissues contain MAMPs/DAMPs that induce PTI responses in plants. To address this question we prepared crude extracts of TCV-infected or mock-treated *N. benthamiana* leaves (Supplementary Figure 4A) and tested whether these extracts were able to induce typical PTI responses such as MAPK activation, ethylene production and seedling growth inhibition in Arabidopsis. In plant innate immunity PRR activation often leads to the downstream phosphorylation of mitogen-activated protein kinases (MAPKs) which occurs within minutes of MAMP/DAMP treatment (Nühse, Peck et al. 2000). To monitor MAPK activation upon crude extract application, we infiltrated highly diluted extracts of mock-infected and TCV-infected *N. benthamiana* plants into Arabidopsis leaves for 15 minutes. Proteins were extracted and subjected to Western blot analysis with a commercially available antibody detecting the dually phosphorylated MAPKs. Very little activated MAPK was detected when the leaf tissue was treated with 5% v/v "mock extract". In contrast, high amounts of activated MAPKs were detected when the leaf tissue was treated with 5% v/v "TCV extract" (Figure 6A).

Besides early responses such as MAPK activation, MAMPs and DAMPs also induce the production of phytohormones. During PTI the production of ethylene has been particularly well studied and offers a reliable and sensitive method to detect MAMP/DAMP activity (Zipfel, Robatzek et al. 2004; Chinchilla, Bauer et al. 2006; Chinchilla, Zipfel et al. 2007). Therefore, we also analyzed whether highly diluted "TCV extracts" were able to induce ethylene production in Arabidopsis. As a negative control, we started by evaluating to which extend the “mock” extract is capable of inducing ethylene responses in Arabidopsis leaf strips. When the mock extract was

applied at 5% volume per volume, we could not observe a significant increase in ethylene, although we repeatedly observed a tendency towards higher ethylene production upon this treatment (Figure S4C). However, in contrast to mock extracts TCV extracts repeatedly induced a significant response. The ethylene peak caused by TCV extract was comparable to the response to crude crab shell chitin, which represents another well known MAMP. Compared to flg22, both chitin as well as TCV extract induced a much weaker response, although chitin was applied at a high concentration (100 µg/ml) (Figure 6B, Supplementary Figure 4C). This difference in activity is not surprising considering that flg22 is a highly purified synthetic peptide applied at very high concentrations (1 µM), while the TCV extract represents a complex mixture in which the active compound might only be present in minute amounts. The eliciting molecule may be of proteinaceous nature as no ethylene response was observed when the crude extracts where pre-treated with proteinase K (Supplementary Figure 4B). We compared the ethylene-inducing activity of "mock extracts", "TCV crude extracts" and "purified TCV" to that of the known MAMPs, flg22 and chitin (Supplementary Figure 4C). When the "mock extract" was applied at 5% volume per volume, there was no significant increase in ethylene. In contrast, "TCV crude extract", applied at the same concentration, caused a marked and significant increase in ethylene production, in a similar range as chitin, albeit to a lesser degree than flg22 (Figure S4C).

To evaluate the BAK1-dependency of the ethylene response, we also investigated the potential of crude viral extracts to induce ethylene in *bak1-4*, *bak1-5*, *fls2*, *pepr1/2* double mutant plants. The diluted "TCV extract" induced strong ethylene production in the *fls2* and *pepr1/2* mutants like in wild type plants, but only a small ethylene production in the *bak1-4* mutant and none at all in the *bak1-5* mutant (Figure 6B). These results indicate that one or more components of these extracts induce(s) ethylene production in a BAK1-dependent, but *PEPR1/2*-independent manner.

Based on these experiments it is not clear whether the eliciting activity comes from the plant and therefore constitutes a DAMP or is a component of the virus. To address this question we

prepared crude as well as highly purified extracts of TCV-infected or mock-treated control *N. benthamiana* leaves (Figure S4A) and compared their activities in a third bioassay to look at the activity of elicitors of the PTI response, namely the seedling growth inhibition assay (Gomez-Gomez, Felix et al. 1999; Wang, Komurasaki et al. 2010). We applied crude as well as highly purified extracts of TCV-infected (5% v/v) or mock-treated control (5% v/v) *N. benthamiana* leaves to one week old seedlings of WT, *pepr1/2*, *fls2*, *bak1-4* and *bak1-5* and continued to grow these seedlings under continuous light for ten days. While the overall seedling growth was only slightly affected by crude viral extracts, the roots of WT, *fls2* and *pepr1/2* showed severe growth inhibition (Figure 6C). Compared to the root growth inhibition observed by saturating concentrations of flg22, the inhibition was less pronounced, but still statistically significant (Supplementary Figure 4D). Whether or not the seedlings became infected by TCV due to the long incubations with TCV sap extract has not been investigated. Conversely, the *bak1-4* and *bak1-5* mutants did not display any significant root growth inhibition suggesting that a *BAK1*-dependent signaling pathway is activated by the crude extract. Interestingly, highly purified extracts of TCV did not induce significant root growth inhibition (Figure 6C) or ethylene production (Figure S4C). This suggests that the elicitor of root growth inhibition and potentially PTI is not derived from intact TCV virions but is co-extracted from plant tissue or an intermediate product of TCV. Taken together these results show that a compound(s) in the crude extract from TCV infected *N.benthamiana* elicit(s) several PTI-responses; this activity is *BAK1*-dependent, and the elicitor perceived is not the virion itself.

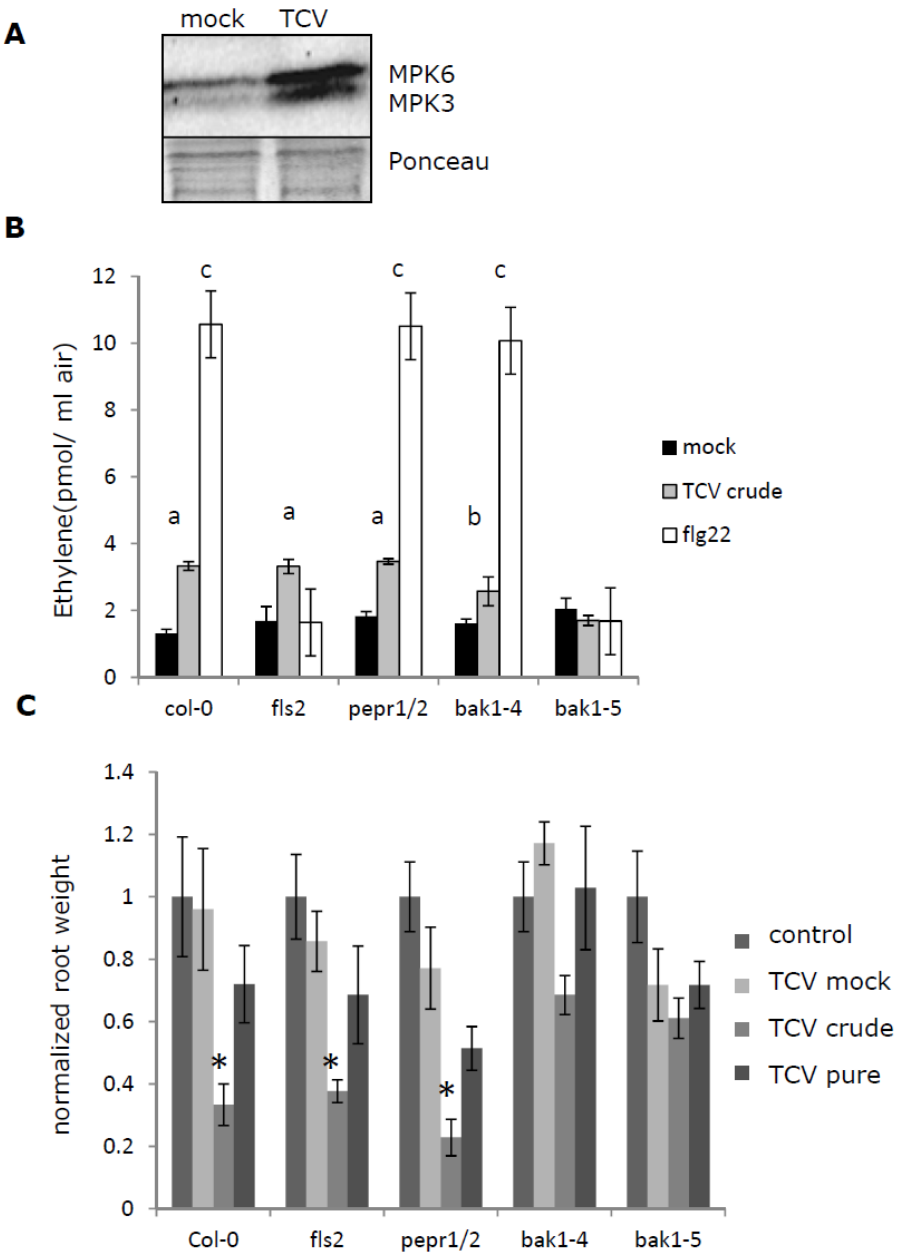


Figure 6: Compound(s) within crude extracts of TCV infected *N. benthamiana* leaves induce immune responses in Arabidopsis in a *BAK1*-dependent manner. A) Four weeks old Arabidopsis leaves (n =3) were infiltrated with crude extracts from mock-infected or TCV-infected *N. benthamiana* leaves. After 15 minutes, the tissue was harvested, extracted and subjected as a watery dilution (5%v/v) to Western blot analysis using anti-pERK1-2 monoclonal antibodies. **B)** Leaf strips of four week old Arabidopsis plants (n = 4) of Col-0 WT, *pepr1/pepr2* double mutant, *fls2*, *bak1-4* knockout and the *bak1-5* point mutation mutants were treated with "TCV extract" (5% v/v), "mock extract" (5% v/v) or a positive flg22 control (1mM) and ethylene accumulation was measured four hours after treatment. Significant differences ($p < 0.05$, one-way ANOVA) are marked by different letters. **C)** One week old Arabidopsis seedlings of Col-0, *pepr1/pepr2* double mutant, *fls2*, *bak1-4* knockout and *bak1-5* point mutation mutants (n = 6) were untreated (control) or treated with crude extracts of TCV-infected leaves (5%v/v) or with purified virions (5% v/v) in sterile multiplates containing liquid MS medium and the root weight was measured ten days post treatment. n=8. Significant differences ($p < 0.05$, student t-test) are marked by an asterisk. These experiments were repeated at least twice with two different sets of extracts and similar results were obtained.

5.4 Discussion

Innate immunity constitutes the first line of defense against biotic threats and protects eukaryotes from a wide range of potential pathogens. The perception of MAMPs by PRRs activates a range of fast and efficient defense responses which collectively lead to PTI in both, animals and plants (Boller and Felix 2009). Plants also sense danger by monitoring cell integrity. Is cell integrity disturbed, plant endogenous DAMPs are either actively or passively released and recognized by PRRs at the plasma membrane, leading to PTI-like defense responses (Huffaker and Ryan 2007; Boller and Felix 2009; Krol, Mentzel et al. 2010). Interestingly, compatible virus-host interactions are associated with upregulation of PTI marker genes, indicating that the presence of the viral pathogen is sensed by the host by a yet unknown mechanism (Whitham, Quan et al. 2003; Love, Yun et al. 2005).

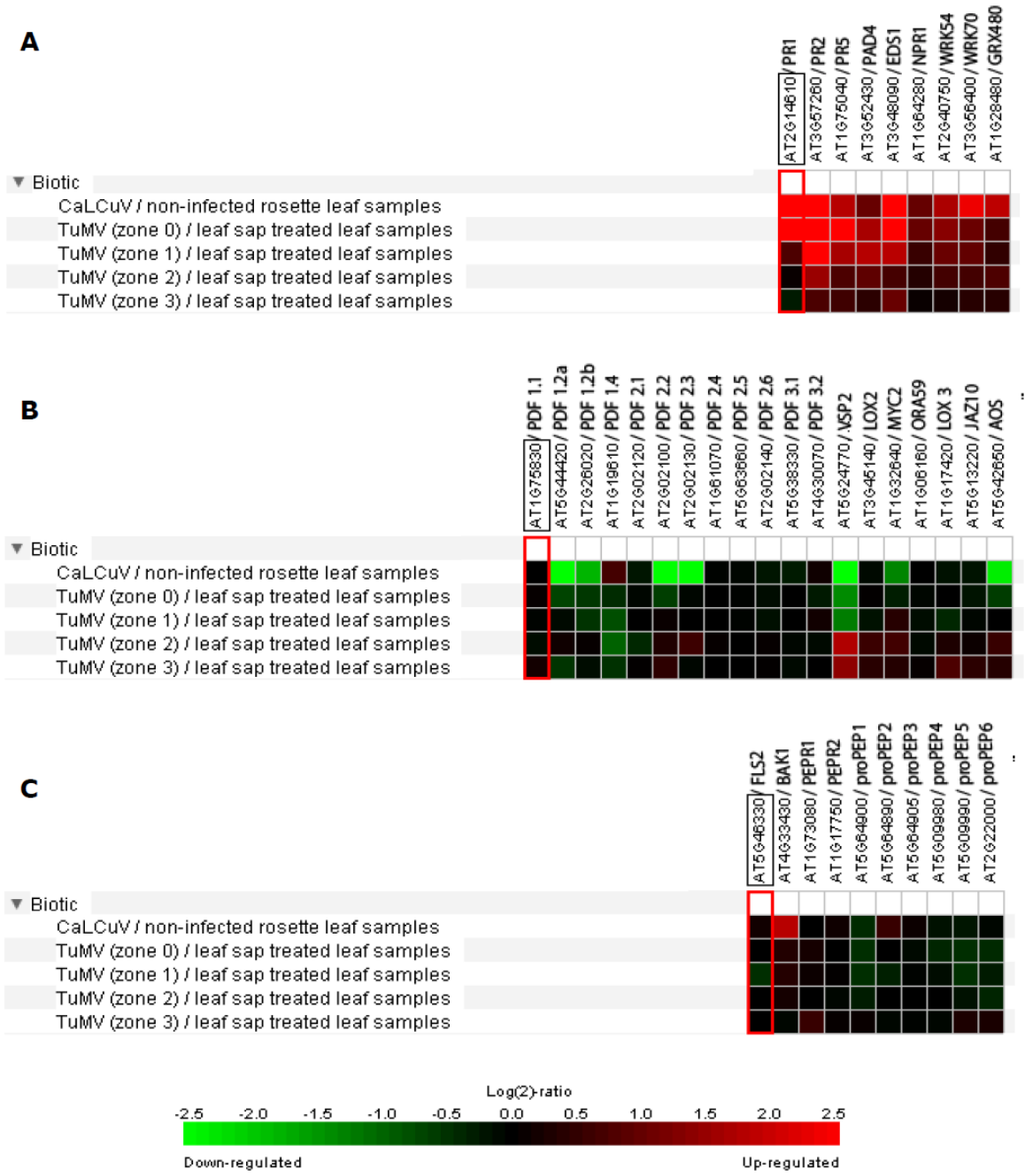
Here we present first evidence that viruses are either directly or indirectly recognized by the PTI surveillance system in a BAK1-dependent manner. As a first indication, we observed that mutants in the central PTI regulator *BAK1* are more susceptible to three different RNA viruses, namely ORMV, TMV and TCV. However, BAK1 is not only important for regulation of innate immunity, but also involved in cell death control and brassinosteroid (BR) signaling, a phytohormone important for plant growth (Li, Wen et al. 2002; Wang, Kota et al. 2008). To exclude the possibility that the increased susceptibility of *bak1-4* mutants (knockout mutant) to RNA viruses results from an impairment in BR signaling, we also included the *bak1-5* mutant in our analysis, which is strongly impaired in PTI but not affected in BR responses (Schwessinger, Roux et al. 2011). This is in contrast to the *bak1-4* mutant, which is impaired in both signaling pathways (Li, Wen et al. 2002; Chinchilla, Zipfel et al. 2007; Heese, Hann et al. 2007). In all our pathogen assays, the *bak1-4* and *bak1-5* mutants were similarly affected, suggesting that BR signaling does not contribute to viral replication and symptom development. Interestingly, a viral nuclear shuttle protein (NSP) of the *Tomato yellow spot begomovirus* (ToYSV) interacted with the kinase domain of tomato BAK1 in yeast-two hybrid assays, suggesting that it might be an important virulence target for plant viruses (Sakamoto, Deguchi et al. 2012). In addition, this result suggests that BAK1 not only plays an important role in antiviral defense to RNA viruses but also to DNA viruses, because ToYSV is a single-stranded DNA virus. Thus, *BAK1* seems to

contribute to antiviral defenses due to its role as a central regulator of immunity and independent of its ability to enhance BR signaling. However, since BAK1 appears to be involved in many RLK-mediated pathways, the increased symptom development in *bak1* mutants could still be indirect and caused by effects on convergent but unrelated pathways. For example, the MP of TMV and ORMV interacts with CDC48, and CDC48 interacts with SERK1, which may activate defense with BAK1 as coreceptor (Aker, Hesselink et al. 2007; Niehl, Amari et al. 2012; Niehl, Amari et al. 2012). Thus defenses could be triggered in infected cells through changes at the ER which induce ER stress, the induction of CDC48 and MP extraction from the ER.

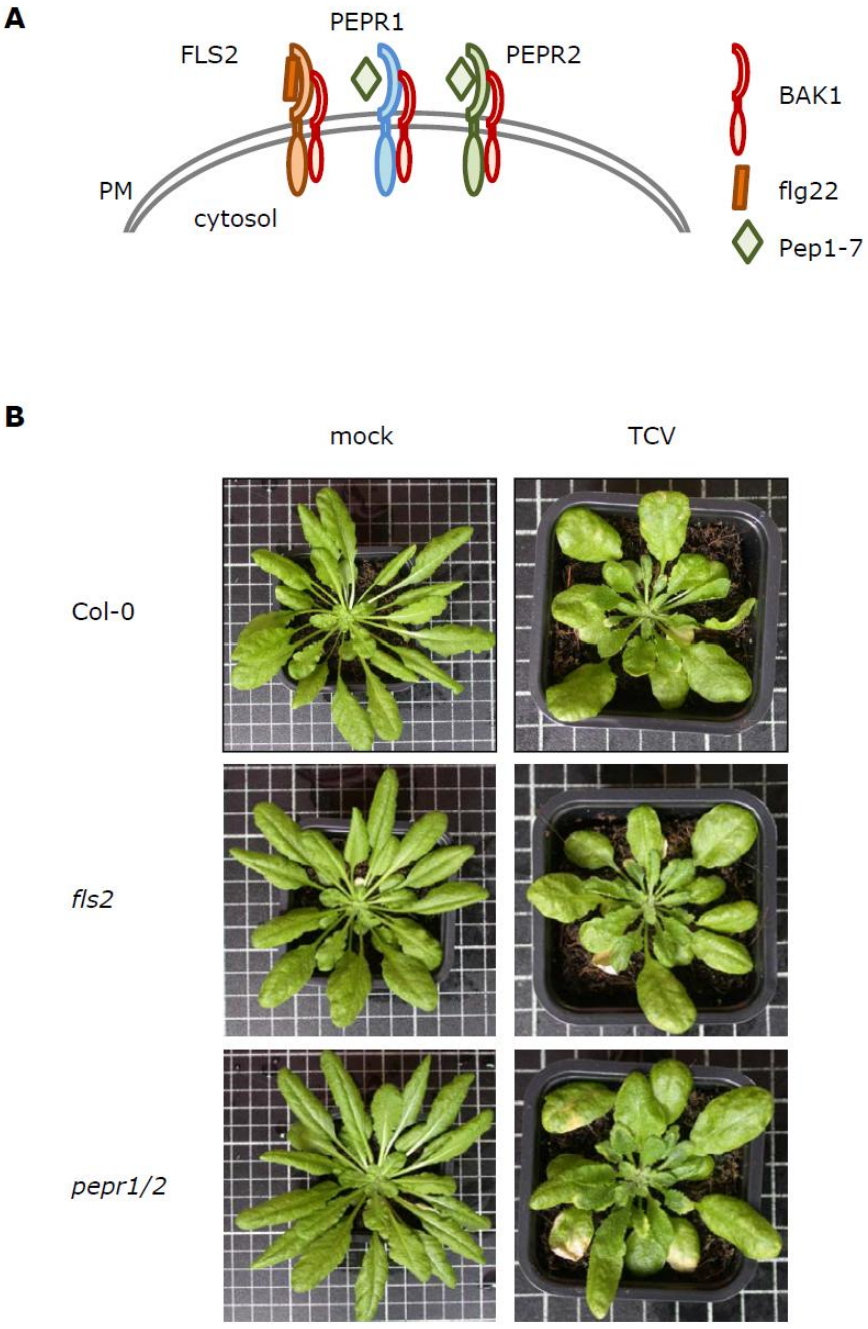
Our results did not reveal differences in susceptibility of *pepr1/2* mutants to virus infection, although the receptors as well as *AtPep2* and *AtPep3* are upregulated during viral infections. This could be consequence of functional redundancy among different DAMP perception systems or indicating that the virus-induced PTI response is *At*PEP independent. Still, the involvement of BAK1 in antiviral immunity suggests that either a viral MAMP or a virus-induced DAMP may be recognized by an unknown BAK1-interacting receptor. To test this, we obtained crude and highly purified TCV extracts from infected *N. benthamiana* plants and used these for standard PTI assays in Arabidopsis. Crude but not highly purified TCV extracts induced a number of PTI marker responses including ethylene production, MAPK activation and seedling growth inhibition. Based on these observations, we conclude that TCV-infected tissue contains an elicitor-active compound sufficient for extracellular PTI induction and that intact TCV virions alone are not perceived as a MAMP in Arabidopsis Col-0. This is in line with a previous study where it has been shown that TMV coat protein is extracellularly perceived and induces ROS production in epidermal peels of tobacco plants shortly after application (Allan, Lapidot et al. 2001). Consistent with the infection assays, ethylene production and seedling growth inhibition upon crude extract application were *BAK1*-dependent. The requirement of BAK1 for signaling further suggests that the elicitor is perceived at the plasma membrane. Overall, these results support the hypothesis that PTI-responses are induced by PRR-mediated recognition of MAMPs/DAMPs present in the crude extracts.

Since viruses are strictly intracellular one explanation for the observed PTI induction is that TCV infections lead to the production of a plant derived DAMP. In support of this hypothesis, only crude extracts resulted in PTI responses while highly purified virions did not. However, the crude TCV extract also contains virus encoded proteins, viral RNA or sRNA in their free form. By contrast, purified virion preparations only contain the assembled coat protein, and the viral genome packaged inside the particles. Thus, it is equally well possible that viral proteins expressed during infection, viral RNA, or viral sRNA induce PTI responses. However, a conserved recognition mechanism between Arabidopsis and *N. benthamiana* is likely since the active compound is recognized in Arabidopsis despite of being obtained from infected tissue of *N. benthamiana*. Overall we suggest a model in which virus infections continuously release a DAMP and or MAMP into the apoplast where the eliciting activity is then recognized by a yet unknown BAK1-dependent receptor. Further research will be needed to identify the antiviral MAMP/DAMP receptor. Our finding that BAK1 is involved in antiviral innate immunity against several RNA viruses makes BAK1 a powerful tool in the search for the virus MAMP/DAMP and its receptor.

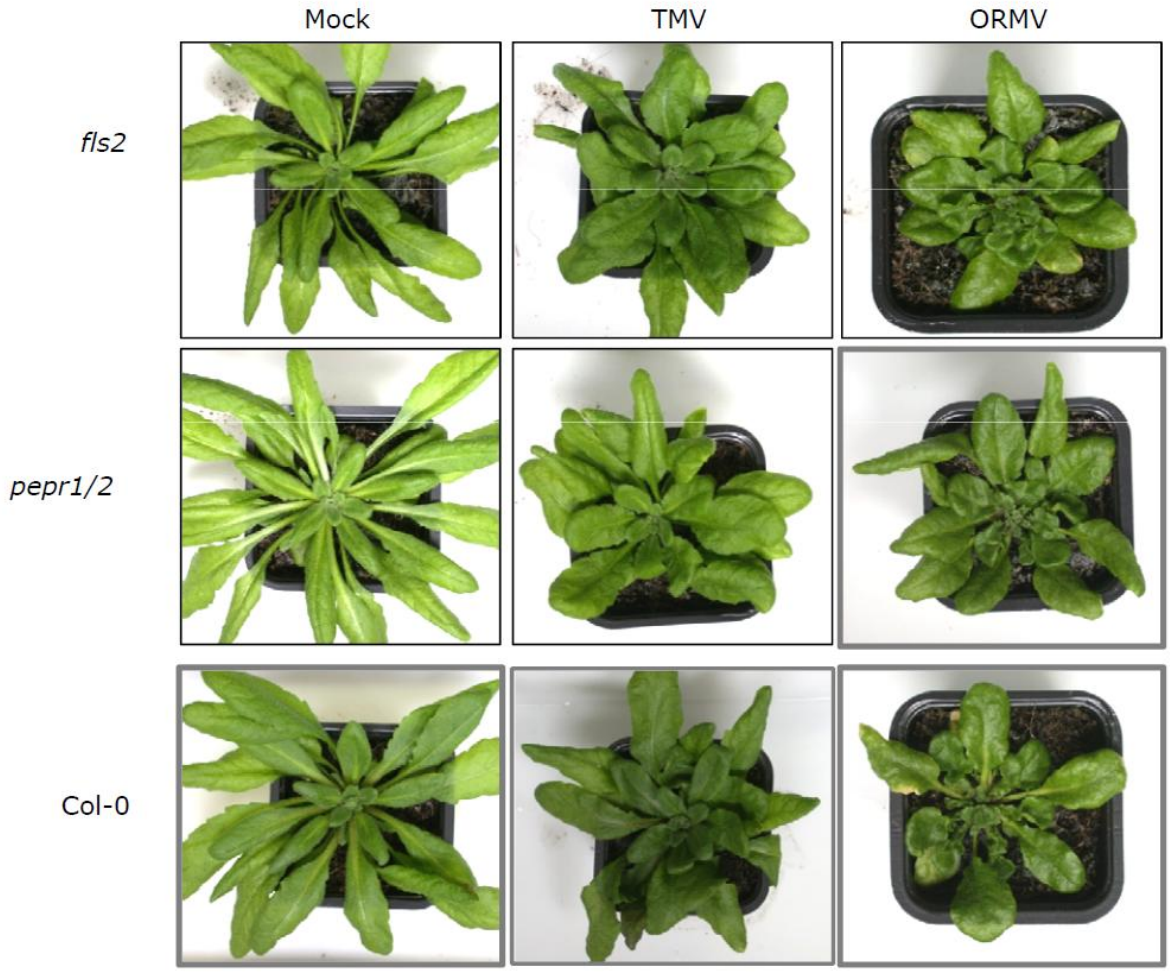
5.5 Supplementary Figures



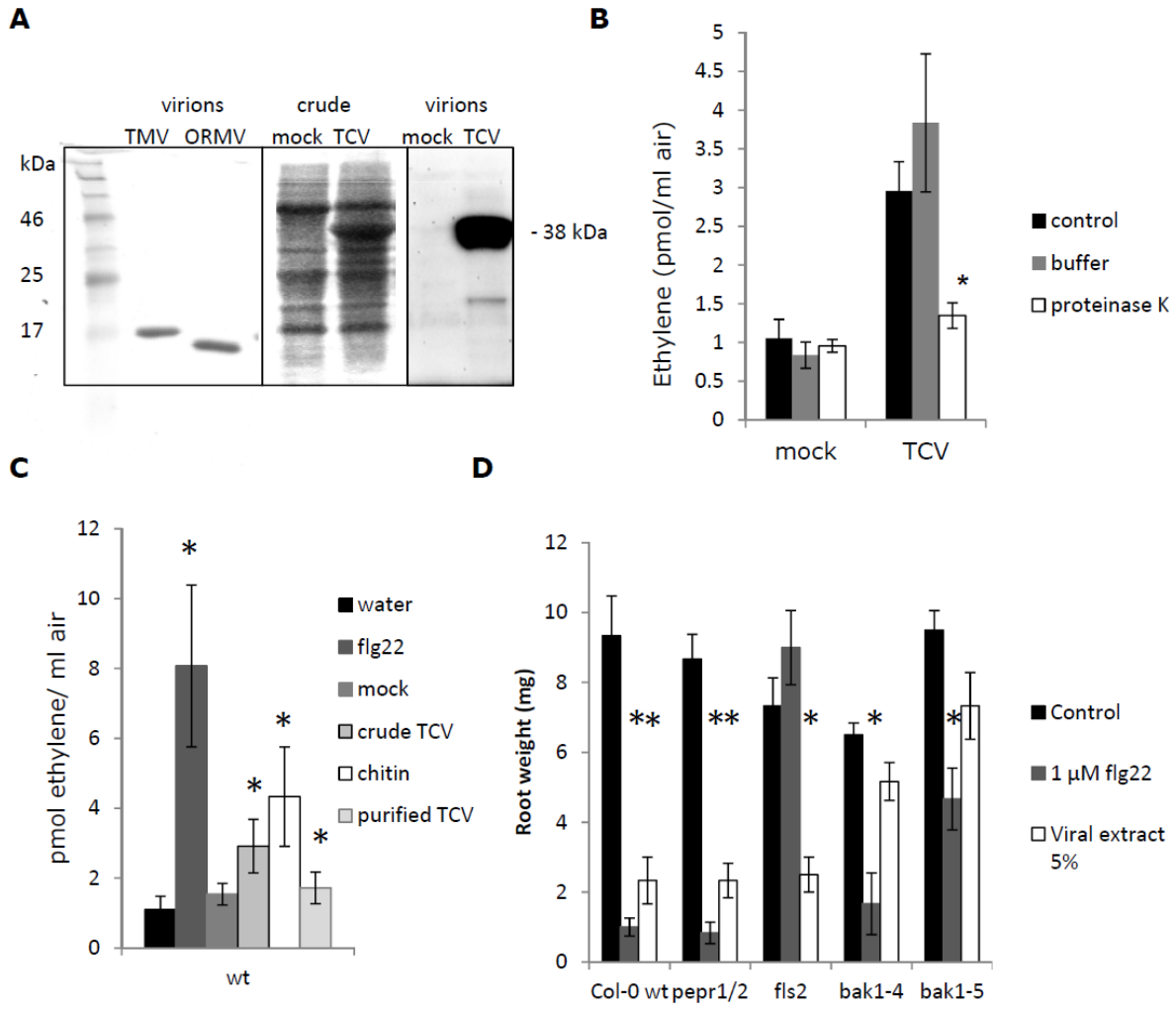
Supplementary Figure 1. Expression of SA-. JA- and PTI-pathway genes upon viral infection. Using array data from plants infected with viral pathogens (CalCuV and TuMV) deposited in the genevestigator database the expression levels of genes involved in defense response via SA **(A)**, JA **(B)** or PTI **(C)** were retrieved.



Supplementary Figure 2. A) Schematic representation of MAMP and DAMP perception systems. **B)** Symptoms of TCV of various Arabidopsis lines at 21 dpi. Col-0, the *fls2* and *pepr1/2* knockout lines were infected with "TCV crude extract" from *N. benthamiana*. The pictures present the average phenotype observed in the different lines. The experiment was repeated three times with similar results. The pictures shown here are derived from the same infection experiment as in Figure 2A.



Supplementary Figure 3. Symptomatic characterization of 21 day old ORMV and TMV infections of Col-0, *fls2* and *pepr1/pepr2* mutant lines. The *fls2* and *pepr1/2* knockout lines were infected with TMV or ORMV virions and symptoms were scored three weeks post inoculation. The pictures present the average phenotype observed in the different lines and this experiment was repeated three times with similar results. The pictures shown here are derived from the same infection as in Figure 4 and 5.



Supplementary Figure 4. Purity of virion preparations and TCV crude extracts. A) Approximately 1 µg of TMV or ORMV virions were separated on 15% SDS-PAGE gels followed by Coomassie staining. Only the ~17 kDa coat proteins were detectable in the virion preparations. Likewise, 10 ml of highly purified TCV virions resulted in bands corresponding to the size of viral coat protein (38 kDa). In contrast the crude extract shows a highly complex mixture containing various plant proteins. **B)** Leaf strips of four week old Arabidopsis plants were treated with mock or TCV crude extract diluted in water, the proteinase K buffer or proteinase K buffer plus proteinase K and ethylene content was measured four hours after treatment. Significant differences to the mock control($p < 0.05$, student t-test) are marked by an asterisk. These experiments were repeated at least twice with similar results. **C)** Determination of the baseline observed with mock extract treatments in ethylene. For this leaf strips of four week old Arabidopsis plants (n = 4) of Col-0 wt were treated with water, "TCV extract" (5% v/v), "mock extract" (5% v/v) or a positive flg22 control (1mM) and ethylene accumulation was measured four hours after treatment. Significant differences ($p < 0.05$, students t-test) are marked by asterisks. **D)** One week old Arabidopsis seedlings of Col-0, *pepr1/pepr2* double mutant, *fls2*, *bak1-4* knockout and *bak1-5* point mutation mutants (n = 6) were treated with mock (control) or with crude extracts of TCV-infected leaves (5%v/v) in sterile multiplates containing liquid MS medium and the root weight was measured ten days post treatment. A flg22 treatment (1 mM) was included as a positive control. Significant differences to the negative control ($p < 0.05$, student t-test) are marked by an asterisk.

5.6 Material & Methods

Plants, Growth Conditions, and Virus Inoculation

Arabidopsis thaliana plants were grown in growth chambers (Sanyo, Japan) at 21°C with 8h/16h light/dark cycle. To initiate virus infection, two leaves of 4 weeks old plants were rub-inoculated either with sap from healthy or TCV-infected plants or with 150ng purified TMV or ORMV virion particles per leaf.

TCV Crude Extracts

N. benthamiana plants were infected with TCV by infiltration of Agrobacterium GV3101 carrying pBIN19-TCV or an empty vector construct. Two to three days post infiltration non infiltrated systemic tissue was harvested and frozen in liquid nitrogen. The tissue was ground and extracted in 1/10 (w/v) PBS-Tween (0.5%) overnight on a rotation wheel at 4°C. The extracts were centrifuged three times at 4000 g for 15 minutes to remove cellular debris and analyzed by SDS-page and Coomassie staining. Prior to PTI assays the extract was diluted 1/20 in distilled water. Extracts were produced at least five times independently and each time analyzed for PTI induction by ethylene measurements. The mock extract derived from empty vector infiltrated plants was treated exactly the same way as extracts derived from virus infected plants.

Purified TCV Virions

Highly purified TCV virions were prepared according to the protocol of Leberman (Songkram, Ohta et al. 2010).

TMV and ORMV Virion Preparation

TMV- or ORMV-infected leaves were frozen and ground in liquid nitrogen. For each gram tissue powder one ml buffer A (0.5M NaP pH7; 0.1% 2-ME) and one vol of butanol/chloroform 50/50 was added and mixed by shaking (1-2 min). The mixture was centrifuged for 10 min at RT and 14000 rpm. The upper aqueous phase was removed and centrifuged at 15000 rpm for 15 min. Following addition of 1/10 vol of 40% PEG8000 to the upper aqueous phase, incubation for10 minutes on ice, and centrifugation for10 min at 14000 rpm, supernatant was discarded and the pellet resuspended in 0.2 vol 10 mM Na_2HPO_4 pH 7. The sample was again centrifuged for 10 min at 5000 g and the supernatant transferred into a fresh 1.5 ml tube. Sodium chloride was

added to a total of 1% and PEG to a total of 4% followed by centrifugation at 5000 g for 10 min and the removal of the supernatant. The final pellet was resuspended in 10 mM Na_2HPO_4 pH 7 and the concentration measured photometrically, whereby an OD_{260} = 3 equals 1 mg/ml of virion particles.

Quantitative RT-PCR

Arabidopsis total RNA was extracted using the NucleoSpin RNA plant extraction kit (Macherey-Nagel, Germany) and treated with rDNase according to the manufacturer's specifications. cDNA was synthesized from 500ng of RNA with random primers using the AMV reverse transcriptase system according to the manufacturer's instructions (Promega, USA). Quantitative RT-PCR was performed in a 96-well format using a Light Cycler 480 machine (Roche Applied Science, Switzerland). On the basis of the obtained C_T values, normalized expression to the reference gene *UBQ10* (AT4G05320) was calculated using the qGene protocol (Job, Wakamiya et al. 2010). The gene-specific primers used were as follows: *UBQ10* (AT4G05320) with UBQ_fw (5'-GGCCTTGTATAATCCCTGATGAATAAG) and UBQ_rv (5'-AAAGAGATAACAGGAACGGAAACATAG), *PEPR1* (AT1G73080) with PEPR1_fw (5'-CAACAACAATGTGGAGGATA) and PEPR1_rv (5'-AACGAGATTACCGAACTGAA), *PEPR2* (AT1G17750) with PEPR2_fw (5'-AAGAAGATGGCTTAATGCTG) and PEPR2_rv (5'-GAGTTGTGCCAGTAACAGTG), *ProPEP2* (AT5G64890) with ProPEP2_fw (5'-TCACCAAACTATTGGATTTCAA) and ProPEP2_rv (5'-GACTCAATTGACTTCTTAATC), *ProPEP3* (AT5G64905) with ProPEP3_fw (5'-CAACGATGGAGAATCTCAGA) and ProPEP3_rv (5'-CTAATTGTGTTTGCCTCCTTT), *BAK1* (AT4G33430) with BAK1_fw (5'-GACCTTGGGAATGCAAATCTATC) and BAK1_rv (5'-AAAACTGATTGGAGTGAAAAGTGAAA). For the quantification of viral RNA, the following Primer combinations were used: TMV with TMV_fw (5'-GACCTGACAAAAATGGAGAAGATCT) and TMV_rev (5'-GAAAGCGGACAGAAACCCGCTG), ORMV with ORMV_fw (5'-AGGTGGGGTAACAGTGAGCGTGA) and ORMV_rev (5'-GCTTTCGCTTGGCATCCGCG), TCV with TCV_fw (5'-GTCGATTTCGGCAAACTCAT) and TCV_rev (5'-GCTGGTTGAGCCAGTTCTGT).

MAP Kinase Activation Assays

Arabidopsis leaves of four to six week old plants were infiltrated with crude extract from mock-treated or TCV-infected *N.benthamiana* plants (5%v/v in water) for 15 min. Leaf discs (50 mg) were then frozen in liquid nitrogen and proteins were extracted in 100 µl extraction buffer (50 mM Tris-HCl pH 7.5, 150 nM NaCl, protease inhibitor cocktail (Sigma-Aldrich, USA) for 30 min at 4°C. Subsequently 100 µl Lämmli loading buffer (2x) were added to each sample. Samples were subjected to immunoblot analysis using the anti-p42/44-phospho-ERK antibody (Cell Signaling, USA). Blots were developed using CDP-star technology (NEB, USA).

Ethylene Production

Ethylene production was measured as described previously (Felix, Duran et al. 1999). For the induction of ethylene by crude viral extracts, the extracts were added to the water in which the leaf strips were floating at 5% (v/v).

Seedling Growth Inhibition Assay

5 days after germination, sterile seedlings were exposed to liquid MS medium supplied with viral extracts of 5% (v/v) final concentration or to a control treatment with buffer containing 1 µM flg22 (one seedling per 500 µl of medium in 24-well plates). The effect of the treatment on seedling growth was analyzed after 10 days by weighing the fresh dry weight of the roots.

6. General Discussion

Since the beginning of this thesis, several reports have shed light on the function of the Pep-PEPR system in plant defense. The *pepr1 pepr2* receptor mutant has been shown display an enhanced susceptibility against the biotrophic bacterial pathogen *Pseudomonas syringae* (Ma, Walker et al. 2012; Tintor, Ross et al. 2013) and the necrotrophic ascomycete *Botrytis cinerea* (Liu, Wu et al. 2013). Moreover, homologues of *At*Peps have been found across all sequenced genomes of higher plants and the exogenous application *Zm*Peps, the *At*Pep homologues in maize, has subsequently been shown to enhance defense responses against the fungal pathogens *Cochliobolis heterostrophus* and *Colletotrichum graminicola,* as well as the generalist herbivore *Spodoptera exigua* (Huffaker, Dafoe et al. 2011; Huffaker, Pearce et al. 2013).

Apart from that, *At*Peps have also been shown to be enhancers of ethylene-induced defense responses (Liu, Wu et al. 2013; Tintor, Ross et al. 2013) and to be necessary for the maintenance of intracellular calcium balances, claimed to be required for full-strength PTI responses upon MAMP detection (Qi, Verma et al. 2010; Ma, Walker et al. 2012). Moreover, promoter studies have identified several binding sites for WRKY transcription factors, known transcriptional modulators of plant defense (Pandey and Somssich 2009), in the promoters of *PROPEP2* and *PROPEP3* - an additional observation strongly linking the expression of these two *PROPEP*s to PTI signaling events (Logemann, Birkenbihl et al. 2013). Together with the work presented here, the *At*Pep-PEPR system has thus been shown to be involved in a variety of defense responses against several invaders, strongly differing in structure and pathogenesis.

6.1 The *At*Pep-PEPR System is Induced upon Several Conditions of Biotic Stress

Several events lead to the expression of components of the *At*Pep-PEPR system. For instance, it has been shown that the application of MAMPs, the phytohormone derivatives MeSA, MeJA, and Ethephon, as well as wounding led to an increased transcript accumulation of both *PEPR*s as well as certain *PROPEP*s (Huffaker, Pearce et al. 2006; Huffaker & Ryan 2007; Yamaguchi,

Huffaker et al. 2010). We now additionally observed that the expression of several members of the *At*Pep-PEPR system was induced upon herbivore attack and viral infections (Chapter 4, Figures 1 and 2; Chapter 5, Figure 2). Therefore, the *At*Pep-PEPR system was shown to be induced upon the recognition of several structural classes of biotic invaders, namely microbes, viruses and herbivorous insects. However, this induced expression seems to happen at different stages of biotic interaction, as it was observed 1 h after the application of MAMPs like flg22 and elf18 (Yamaguchi, Huffaker et al. 2010), 12 h after the application of herbivore OS (Chapter 4, Figure 2), and 21 DPI in the case of viral infections (Chapter 5, Figure 2). These temporal differences in the expression of the *At*Pep-PEPR system could thus indicate potentially different roles of this system in the deterrence of specific invaders. However, it remains astonishing to think that the *At*Pep-PEPR system could be a universal element integrating defense responses against these vastly different categories of biotic invaders.

6.2 JA is a Prerequisite for Full-Strength *At*Pep Signaling

Our observations indicate that Pep-triggered PTI responses are strongly impaired in mutants lacking a functional JA pathway, therefore linking a fully functional *At*Pep-PEPR signaling system to JA signaling (Chapter 2, Figure 7). This is particularly intriguing since similar observations were also claimed for the interaction of the *At*Pep-PEPR system with SA (Huffaker, Pearce et al. 2006). Especially in the context of the antagonistic fashion of JA and SA signaling in the integration of defense responses against necrotrophic/herbivorous and biotrophic invaders (Glazebrook 2005), this induction of the *At*Pep-PEPR system by both pathways seems surprising. Indeed, in contrast to Huffaker et al. (2006), we could not see any reduction of Pep-triggered PTI responses in mutants impaired in SA signaling (Chapter 2, Figure 7). Also, the application of MeSA to plant tissue did not increase Pep-triggered PTI responses, as it was observed in case of MeJA application (Chapter 2, Figure 7).

Moreover, we could show that the application of *At*Peps also induces JA levels, an observation later shared with Huffaker et al. (2013). Also here, we did not observe any accumulation of SA upon the application of *At*Peps (Chapter 2, Figure S5, SA data not included). Given this induction of the *At*Pep-PEPR system by JA as well as the induction of JA accumulation by *At*Pep

application, it is of course tempting to speculate that these two systems act in a positive feedback loop to induce and maintain defense responses.

6.3 *At*Pep and MAMP Signaling are Tightly Linked

As already outlined, the expression of several components of the *At*Pep-PEPR system was shown to be induced upon the recognition of MAMPs, HAMPs, as well as upon viral infections. Intriguingly, both the recognition of MAMPs as well as HAMPs leads to the induction of immediate PTI responses (Boller and Felix 2009; Maffei, Mithöfer et al. 2007; Wu and Baldwin 2010). Similarly, we have now shown that the application of extracts of virus infected plants also triggers hallmark PTI responses, such as the production of ethylene, the activation of MAPK cascades, and seedling growth inhibition (Chapter 5, Figure 6). Additionally, the *At*Pep-PEPR system itself was also shown to trigger PTI-like responses (Huffaker, Pearce et al. 2006; Krol, Mentzel et al. 2010, Bartels, Lori et al. 2013), therefore potentially backing up the original PTI response or propagating this response to distal tissues, independently of the biotic invader.

One specific *At*Pep-triggered PTI response, namely the production of ROS, can be strongly induced by the previous detection of MAMPs, whereas other Pep-triggered PTI responses remain largely unchanged (Chapter 2, Figure 2). Despite of the fact that the expression of both PEPRs were shown to induced by MAMP application (Yamaguchi, Huffaker et al. 2010), this ROS boost was shown to be independent of the amount of PEPR receptors (Chapter 2, Figure 5). Therefore, apart from a transcriptional regulation of the *At*Pep-PEPR system upon MAMP detection, an additional level of induction seems to be in place. Still, the exact role of this induced ROS response can only be speculated, with ROS being brought into connection of both local resistance as well as systemic signaling (Apel and Hirt 2004; Lamb and Dixon 1997; Miller, Schlauch et al. 2009). Also, the exact mechanism leading to this increased ROS response remains to be investigated since neither a transcriptional control nor a saturation of the ROS detoxification machinery seem plausible causes.

6.4 The *At*Pep-PEPR System is Required for Full-Strength Defense Responses Against a Variety of Invaders

JA signaling was shown to be strongly involved in defense responses against herbivores (Howe and Jander 2008) with JA levels accumulating in the event of herbivore attack (Halitschke, Schlittko et al. 2001). Further supporting the positive feedback mechanism between *At*Pep and JA signaling, we could show that the JA accumulation upon herbivore OS application is reduced in *pepr1 pepr2* mutants (Chapter 4, Figure 4). Moreover, *pepr1 pepr2* mutants also displayed a strongly increased susceptibility to the generalist herbivore *Spodoptera littoralis* (Chapter 4, Figure 5). Therefore, we suggest a role of the *At*Pep-PEPR system, strongly and positively interacting with the JA pathway, in the context of herbivore deterrence. This was also supported by recent observations of other groups, indicating a role of the Pep signaling in defense responses against the generalist herbivore *Spodoptera exigua* (Huffaker, Pearce et al. 2013). Moreover, JA was also shown to be required for full-strength defense responses against necrotrophic pathogens (Glazebrook 2005). Here, the recent report of the *pepr1 pepr2* mutant being more susceptible to the necrotrophic ascomycete *Botrytis cinerea* could further support this role (Liu, Wu et al. 2013).

However, apart from herbivores and necrotrophic pathogens, there were also claims that the *pepr1 pepr2* mutants were displaying an increased susceptibility to the hemibiotrophic pathogen *Pseudomonas syringae* (Tintor, Ross et al. 2013; Ma, Walker et al. 2012). As SA was shown to be a major hormonal player in these interactions, this would also go in line with the claims of SA also positively interacting with the *At*Pep-PEPR system. However, in our laboratory, we could not confirm the observation of the *pepr1 pepr2* mutant being more susceptible to *Pseudomonas syringae* (Flury 2011). Thus, it remains to be debated in how far the involvement of the *At*Pep-PEPR system in potentially contradicting defense responses and hormonal signaling pathways can be supported by the evidence provided. Also, given the complexity of the hormonal integration of plant defense responses, this rather simplistic model of JA and SA antagonism might need to be reconsidered under certain circumstances.

Despite of the *At*Pep-PEPR system being induced upon virus infections, our pathotests did not reveal an increased susceptibility of *pepr1 pepr2* mutants to the RNA viruses ORMV, TMV, and TCV (Chapter 5, Figures 3 to 5). Instead, plants lacking a functional co-receptor BAK1 displayed a strongly increased susceptibility to all three viruses, potentially indicating the involvement of other BAK1-dependent RLKs in virus detection. However, given the fact that potentially several DAMP or "VAMP (Virus Associated Molecular Pattern)" signals could play a role in the recognition of virus infections, this functional redundancy could mask a potential beneficial role of the *At*Pep-PEPR system in antiviral defense. Also, in our assays, viral infections were performed by wounding leaf tissue with virus coated quartz particles, whereas in nature, a common way of virus infections is mediated by aphid feeding (Pirone and Blanc 1996). Considering that as for other herbivores, aphid feeding locally induces the activation of PEPR and certain *At*Pep promoters (Gaylord Désurmont, personal communication), the *At*Pep-PEPR system could play a distinct role in the prevention of these infection mechanisms – a role that we want to further explore in a new collaboration.

To summarize, I would like to propose a model where the *At*Pep-PEPR system is activated upon several forms of biotic stress. To propagate defense responses, the *At*Pep-PEPR system strongly and positively interacts with PTI and JA signaling, both in a potentially positive feedback mechanism (Figure 1). This positive contribution of the *At*Pep-PEPR system to both JA and PTI-triggered immune response then leads to an increased resistance to several microbial and herbivore invaders. One particular PTI response, namely the production of ROS, is strongly induced by previous MAMP recognition, potentially leading to a systemic signaling cascade or direct local resistance (dashed lines). However, the exact role of this increased ROS response further remains to be investigated.

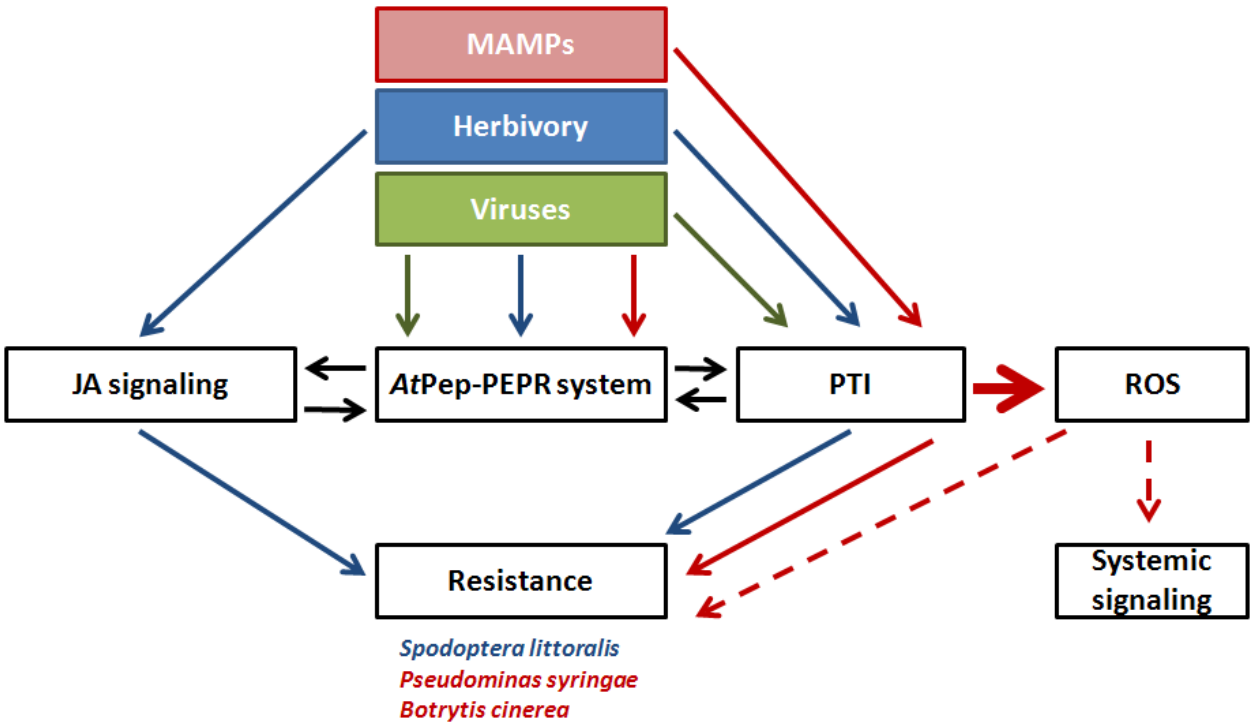


Figure 1: The integration of the *At*Pep-PEPR system in the orchestration of defense responses against biotic invaders. An intact *At*Pep-PEPR signaling system was shown to be required for full resistance against the microbial pathogens *Pseudomonas syringae* and *Botrytis cinerea* as well as the generalist herbivore *Spodoptera littoralis*. Furthermore the *At*Pep-PEPR system was shown interact with both JA signaling as well as PTI in a potentially positive feedback mechanism, facilitating defense responses against the above mentioned invaders. As one particular PTI response, *At*Pep-triggered ROS can be greatly induced by previous MAMP perception. However, the exact role of this ROS response remains to be investigated, potentially being involved in either local defense or systemic signaling.

6.5 Which Model to Choose – Theoretical Integrations of Practical Observations

As outlined in the introduction, two major theories have been proposed for the involvement of the *At*Pep-PEPR system in plant defense responses. Both of these theories, namely the amplifier theory and the DAMP theory, had data, initially, supporting their cause as well as certain constraints.

Addressing the data described in this work in the context of these two theories, several lines of evidence arise that can rather be used for supporting either theory depending on circumstances (Figure 2). On the one hand, the importance of a functional *At*Pep-PEPR system for full defense responses against herbivores can be interpreted to support the DAMP theory as feeding herbivores cause cellular damage that subsequently might release *At*Peps to bind their extracellular receptors. Additionally, the strong positive interaction of the *At*Pep-PEPR system with JA further supports a role of *At*Peps as DAMPs. JA is known to be the main hormonal integrator of defense responses against herbivores and necrotrophic pathogens (Glazebrook 2005; Howe and Jander 2008) and these two categories of enemies normally both cause cellular damage, either by feeding (herbivores) or the secretion of lytic enzymes (necrotrophs). Consequently, a role of the *At*Pep-PEPR system in defense against these two categories has been provided recently (Huffaker, Pearce et al. 2013; Liu, Wu et al. 2013).

On the other hand, the induction of *At*Pep-triggered ROS upon previous MAMP detection also provides evidence for the *At*Pep-PEPR system to be linked with the amplifier theory. Also, the reports that *pepr1 pepr2* mutants display an enhanced susceptibility to the hemibiotrophic pathogen *Pseudomonas syringae* (Ma, Walker et al. 2012; Tintor, Ross et al. 2013) and the identification of WRKY binding sites in the promoters of *PROPEP2* and *PROPEP3* (Logemann, Birkenbihl et al. 2013) provide further evidence to support a role of the *At*Pep-PEPR system as amplifiers of PTI.

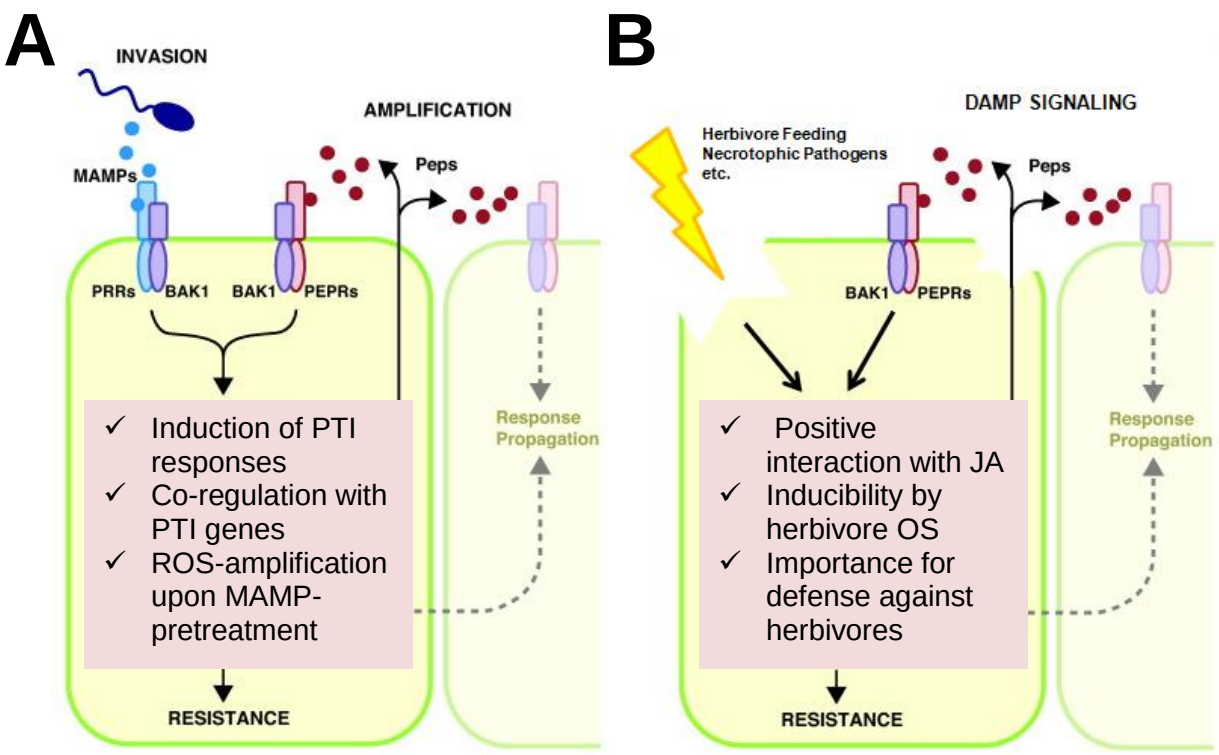


Figure 2. Compiled evidence supporting the major theories of the biological function of the *At*Pep-PEPR system.

A) Recent evidence obtained to support a role of the *At*Pep-PEPR system as amplifiers of MAMP signaling include the increased *At*Pep-triggered ROS response upon MAMP detection, the co-regulation of several components of the *At*Pep-PEPR system with major PTI genes (Logemann, Birkenbihl et al. 2013) as well as the induction of general PTI responses upon *At*Pep application.

B) Evidence supporting a role as "Damage associated molecular patterns" includes the strong interaction of the *At*Pep-PEPR system with the plant hormone JA, the induction of the expression of both *PEPR* as well as *PROPEP3* upon the recognition of herbivore OS as well as the decreased resistance of the *pepr1 pepr2* mutant against the generalist herbivore *Spodoptera littoralis*.

With the results of this and other recent works, we thus cannot provide conclusive evidence on which model to be preferred as most observations can rather be used in favor of one theory and not for ruling out the other. However, we would like to underline the fact that these theories do not necessarily have to be interpreted to be contradicting each other, but could also work in a synergistic manner or could work in separately depending on the attacking organism(s).

6.6 Outlook

Despite of the plethora of evidence compiled in recent years, several questions remain to be addressed to more deeply understand the role of *At*Pep-PEPR signaling and its impact on plant defense (Figure 2). Furthermore, this evidence could also be used to further link the *At*Pep-PEPR system to the proposed models.

- Firstly, it still remains elusive whether full-length PROPEPs do also have the ability to bind PEPRs and therefore induce defense responses. Also, to date, it is not known how the potential cleavage from PROPEPs to *At*Peps actually is achieved.

- Secondly, the mechanism on how the presumably intracellular PROPEPs/Peps reach the extracellular receptor domains of the PEPRs also needs to be addressed. So far, the main hypothesis is that upon biotic stress, the PROPEPs/Peps accumulate in the cytoplasm to then be released from damaged cells upon wounding, lysis or during HR. However, other ways on how Peps could leave the cytoplasm should also be considered. For instance, given the fact that Peps are generally very small peptides, they could be cleaved in the cytoplasm to then be released by a yet unidentified unconventional secretion mechanism.

- Thirdly, as for many other small cryptic peptides in danger signaling systems, a function besides of defense could also be considered since the expression of most *PROPEP*s is not co-regulated with other defense genes but merely with developmental processes (Bartels, Lori et al. 2013). This potential function would however likely be independent of PEPR recognition since so far, no developmental phenotype of the *pepr1 pepr2* mutant has been observed.

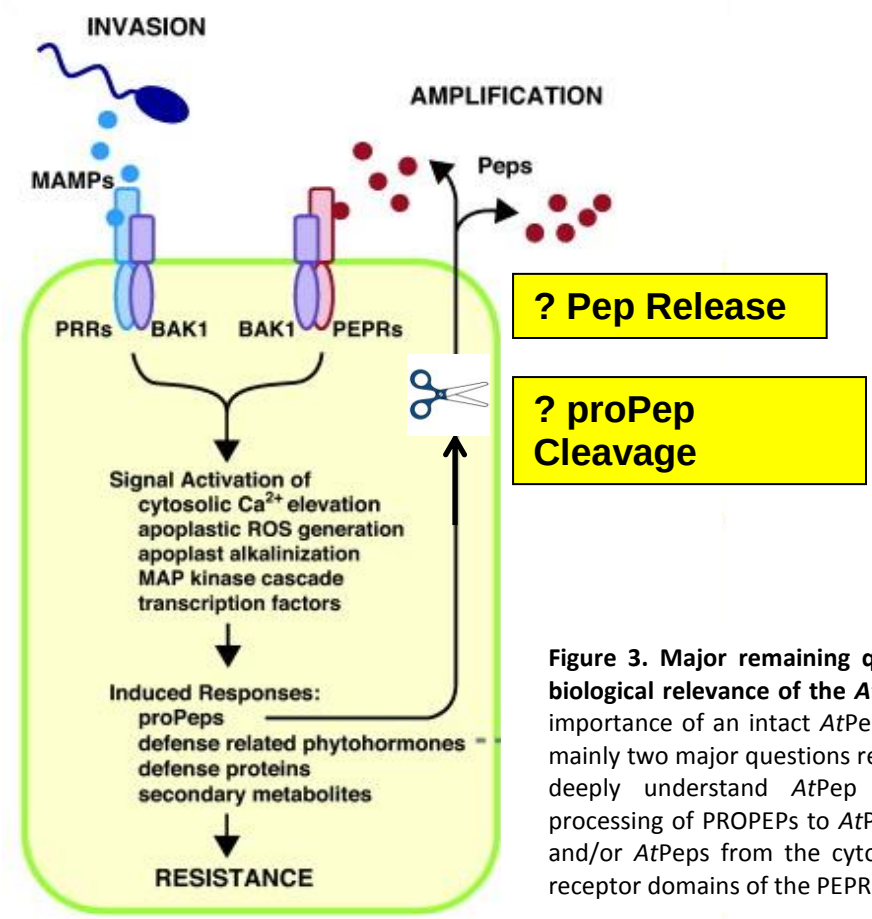


Figure 3. Major remaining questions to further dissect the biological relevance of the *At*Pep-PEPR system. Whereas the importance of an intact *At*Pep-PEPR system has been shown, mainly two major questions remain to be investigated to more deeply understand *At*Pep signaling. These include the processing of PROPEPs to *At*Peps and the release of PROPEPs and/or *At*Peps from the cytoplasm to bind the extracellular receptor domains of the PEPRs.

Despite of these remaining questions, the *At*Pep-PEPR system constitutes the first peptidic DAMP system where both, receptors and elicitors have fully been discovered. Recent work by several groups including ourselves assessing this DAMP system have revealed its importance in PTI responses as well as in the resistance against a plethora of enemies, greatly differing in structure and pathogenesis. Therefore, we underlined the pivotal role of DAMP signaling as an additional layer in plant defense responses against a variety of invaders and essential for the plant's well-being under unfavorable conditions.

Appendix: Looking BAK again: Is BAK1 Involved in Defense Responses against Herbivores?

Introduction

In recent years, the membrane-bound receptor-like kinase (RLK) BAK1 (BRI1-Associated Kinase) has been shown to interact with several other RLKs upon ligand-binding to facilitate downstream signaling responses (Chinchilla, Zipfel et al. 2007). Originally discovered to be interacting with BRI1 (Brassinosteroid-Insensitive 1), the receptor for the plant hormone brassinosteroid (BR) (Wang, Seto et al. 2001), BAK1 has subsequently been shown to be an interaction partner of FLS2 (Flagellin-Sensing2) and EFR (Elongation-Factor TU Receptor), two receptors for microbial elicitors (Chinchilla, Zipfel et al. 2007). Also, PEPR1 (Pep-Receptor 1) and PEPR2 (Pep-Receptor 2), receptors for endogenous danger signaling peptides, have been shown to interact with BAK1 upon ligand binding (Schulze, Mentzel et al. 2010). In these interactions, BAK1 acts as a positive regulator of downstream defense responses since *bak1* null mutants display a decreased amplitude of responses induced (Chinchilla, Zipfel et al. 2007; Chinchilla, Shan et al. 2009; Schulze, Mentzel et al. 2010; Roux, Schwessinger et al. 2011). Moreover, BAK1 has recently been proposed to be required for full strength defense responses upon the recognition of yet unknown viral elicitors, as a) *bak1* mutants displayed decreased PTI (pattern-triggered immunity) responses, such as MAPK phosphorylation, ethylene production, and seedling growth inhibition upon treatment with crude extracts of TCV infected *Nicotiana benthamiana* plants, and b) *bak1* mutants were shown to be more susceptible to the infection of three RNA viruses, such as TCV (Turnip Crinkle Virus), ORMV (Oilseed Rape Mosaic Virus) and TMV (Tobacco Mosaic Virus) (Kørner, Klauser et al. 2013).

In the field of plant-insect interactions, Yang and colleagues (2011) observed an accumulation of *BAK1* transcripts in *Nicotiana attenuata* leaves shortly after the onset of herbivore feeding (2011). Investigating *BAK1* silenced *Nicotiana attenuata* plants, they additionally observed a much decreased accumulation of the plant defense hormone Jasmonic Acid (JA), its active derivate Jasmonoyl Isoleucine (JA-Ile) as well as a decreased expression of several marker genes

involved in defense responses against herbivores when challenged with herbivores or herbivore oral secretions (OS) (Yang, Hettenhausen et al. 2011). These results suggested that BAK1 was not only to be involved in the recognition of several microbial elicitors, but may be a positive mediator in the recognition of yet to be identified herbivore-derived elicitors (Yang, Hettenhausen et al. 2011).

However, given the fact that BAK1 not only is a facilitator of defense responses, but also of hormonal signaling (Roux, Schwessinger et al. 2011), silencing of *BAK1* expression could lead to pleiotropic phenotypes, especially in the context of brassinosteroid signalling (Kørner, Klauser et al. 2013). Therefore, in the current study we decided to use a *bak1* mutant that was shown to be specifically impaired in PTI signaling (Roux, Schwessinger et al. 2011) to investigate two PTI-like responses shown to be induced by yet unknown elicitors in herbivore oral secretions (Klauser, Desurmont et al. 2013). Intriguingly, the results obtained suggest a positive role for BAK1 in the induction of immune responses upon the recognition of herbivore elicitors. Although the corresponding receptors remain elusive so far, our results indicate a conserved role of BAK1 in the detection of several biotic threats, including microbe-, virus-, and now herbivore-derived elicitors.

Results and Discussion

The recognition of elicitors in herbivore OS was shown to induce several PTI-like responses (Maffei, Mithöfer et al. 2007; Wu, Hettenhausen et al. 2007; Wu and Baldwin 2010). Here, we compared the amplitude of two of these PTI-like responses, namely seedling growth inhibition (SGI) and the production of the phytohormone ethylene, upon the application of *Spodoptera littoralis* OS in two different *bak1* mutants. Whereas the *bak1-4* mutant is impaired in both, brassinosteroid and defense signaling, the *bak1-5* mutant was shown to be specifically impaired in defense responses only and displays intact BR responses (Heese, Hann et al. 2007; Roux, Schwessinger et al. 2011). Intriguingly, upon the application of *Spodoptera littoralis* OS to the growth medium, *bak1-4* and *bak1-5* both displayed a reduced seedling growth inhibition compared to Col-0 wild type plants, though to a lesser extent for *bak1-4* (Figure 1). Similarly the growth inhibition caused by the microbial elicitor flg22 did cause a decreased response in both

bak1 mutants. This is in accordance previously published results (Heese, Hann et al. 2007; Roux, Schwessinger et al. 2011).

Similar observations were made for the production of the phytohormone ethylene, a quick physiological response upon elicitor recognition (Boller and Felix 2009). However, in this assay, only the *bak1-5* mutant displayed a significantly decreased ethylene production upon both, the application of flg22 as well as the application of herbivore OS. The decrease in flg22-induced ethylene production was again less pronounced in the *bak1-4* mutant and not significant in the case of OS-induced ethylene biosynthesis.

Altogether, these results suggest a role for BAK1 as a facilitator in the recognition of specific elicitors in herbivore OS independently of BR signaling. Still, neither the elicitors nor the corresponding receptors in this interaction have been identified yet. Since membrane-bound receptors have been proposed for several herbivore elicitors identified (Schmelz, Engelberth et al. 2009), it is of course tempting to speculate that BAK1 might have a similar function in both, the detection of microbial and herbivore-derived elicitors. Therefore, it will be interesting to confirm this potentially positive role of BAK1 in the initiation of defense responses against herbivores with more assays, such as MAP kinase phosphorylation or reporter gene expression. Moreover, in case this positive role can be confirmed, BAK1 might prove to be a powerful tool to detect both, the receptors and ligands involved in the induction of defense responses against herbivores.

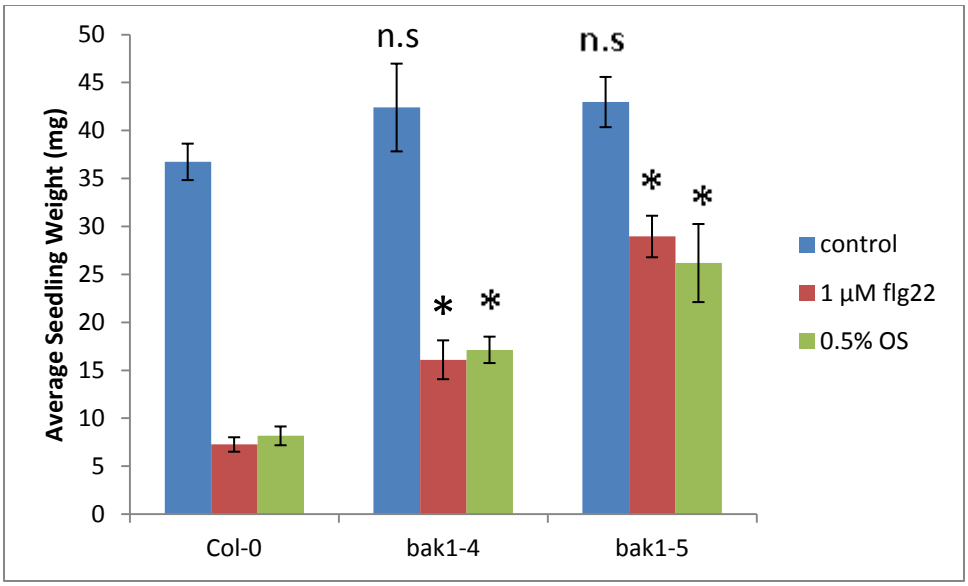


Figure 1. The BAK1 null mutants *bak1-4* and *bak1-5* display a reduced seedling growth inhibition to herbivore oral secretions applied. Arabidopsis seedlings of the lines Col-0 (wild-type) and *bak1-4* and *bak1-5* mutants were grown under sterile conditions for five days and then transferred into MS medium either containing 1 µM flg22, 0.5% (v/v) OS, or no elicitor (control). Error bars show +/- 1 SE of six independent replicates, asterisks indicate significant differences between mutant genotypes and Col-0 wild-type plants (t-test, $p < 0.05$). Similar results were obtained in two independent assays.

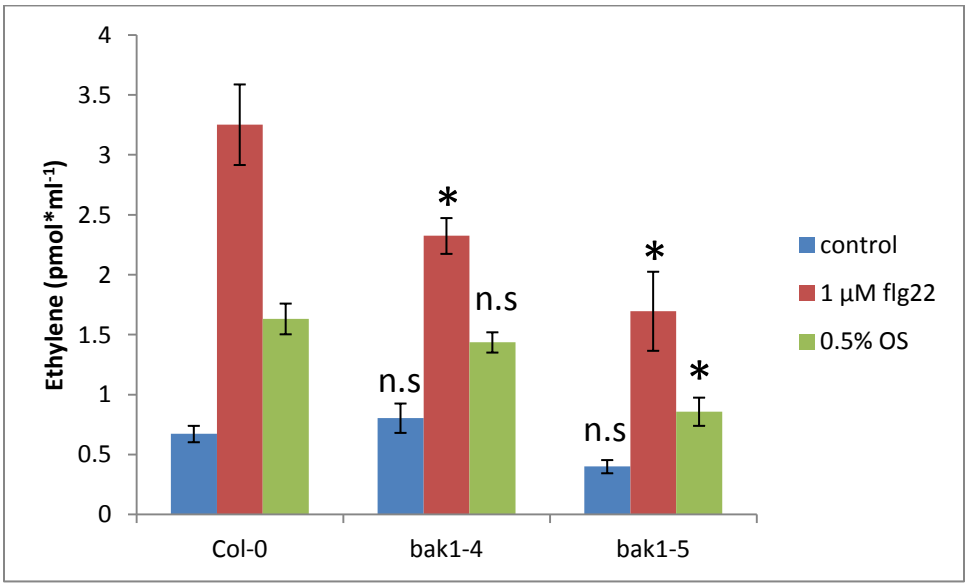


Figure 2. The *bak1-5* mutant displays a reduced ethylene response to herbivore oral secretions applied. Leaf disks of Arabidopsis Col-0 and *bak1-4* and *bak1-5* mutants were treated with 1 µM flg22, 0.5% (v/v) OS or without any elicitor (control) and ethylene production was assessed 5 h after the application of the eliciting compounds. Error bars show +/- 1 SE of six independent replicates, asterisks indicate significant differences between mutant genotypes and Col-0 wild-type plants (t-test, $p < 0.05$). Similar results were obtained in two independent assays.

List of Abbreviations

AA	Amino Acid
ABA	Abscisic Acid
ACC	1-Aminocyclopropane-1-carboxylic acid
ACS	ACC-Synthase
A.t. / At	*Arabidopsis thaliana*
BAK1	BRI1 associated Kinase 1
BKK1	BAK1-like 1
BR	Brassinosteroid
BRI1	Brassionosteroid Insensitive 1
Bp	Basepair
BSA	Bovine Serum Albumin
CalCuV	Cabbage Leaf Curl Virus
CaMV	Cauliflower Mosaic Virus
cDNA	Complementing DNA
CDPK	Calcium-Dependent Protein Kinases
CEP1	C-Terminally Encoded Peptide
CERK1	Chitin Elicitor Receptor Kinase 1
CLE	CLAVATA3/ENDOSPERM SURROUNDING REGION
CLV1	CLAVATA1
CLV2	CLAVATA2
CLV3p	CLAVATA3 Peptide
COI1	Coronatine Insensitive 1
Col-0	*Columbia-0* Ecotype
CP	Coat Protein
D	Aspartate
DAMP	Damage-Associated Molecular Pattern
dd	Double-distilled
DMSO	Dimethyl Sulfoxide
Dpi	Day(s) post inoculation
DTT	Dithiothreitol
EDS1	Enhanced Disease Susceptibility 1
EDTA	Ethylendiamintetraacetatic acid
EFR	Elongation Factor TU Receptor
EF-Tu	Elongation Factor Thermo Unstable
elf18/26	18/26 amino acids peptide of the N-terminus of EF-Tu
EPF	Epidermal Patterning Factor
ER	Endoplasmic Reticulum
ESR	Endosperm Surrounding Region
EST	Expressed Sequence Tag
ET	Ethylene
ETI	Effector-Triggered Immunity
ETS	Effector-Triggered Susceptibility
flg22	22 amino acids peptide of the N-terminus of flagellin
FLS2	Flagellin Sensing 2
FRK1	Flagellin Responsive Kinase 1

G	Glutamate
GC	Gas Chromatograph
GFP	Green Fluorescent Protein
Gm	*Glycine max*
GSO	GASSHO
GUS	β-Glucuronidase
HAMP	Herbivory-Associated Molecular Pattern
HopU1	Hrp-dependent Outer Protein U1
HR	Hypresensitive Response
HypSys	Hydroxyproline-containing glycopeptides
JA	Jasmonic Acid
JA-Ile	Jasmonoyl-Isoleucine
JAZ	Jasmonate ZIM-Domain
K	Lysine
kDa	kilodaltona
Ler	*Landsberg erecta* ecotype
LOX	Linolene Oxidase
LRR	Leucine Rich Repeat
LysM	Lysin Motif
MAMP	Microbe-Associated Molecular Pattern
MAPK	Mitogen-Activated Protein Kinase
MeJA	Methyl Jasmonate
MeSA	Methyl Salicylate
MP	Movement Protein
MS	Murashige & Skoog Medium
N	Asparagine
NADPH	Nicotinamidadenine Dinucleotidephosphate
NB	Nucleotide Binding
Nb	*Nicotiana benthamiana*
NPR	Non-Expresser of PR
NSP	Nuclear Shuttle Protein
OG	Oligogalacturonides
OPR	Oxophytodienoic Acid Reductase
ORA59	*Octadecanoid-Responsive Arabidopsis 59*
ORMV	Oilseed Rape Mosaic Virus
OS	Oral Secretions
PAD4	Phytoalexin Deficient 4
PAMP	Pathogen-Associated Molecular Pattern
PCR	Polymerase Chain Reaction
PDF	Defensin
PEG	Polyethylene Glycol
Pep	Danger Peptide
Pep914	Danger Peptide 914
PEPR	Pep-Receptor
PGN	Peptidoglycan
PI	Protease Inhibitor
PM	Plasma Membrane
PR	Pathogenesis-Related

PROPEP	Precursor of *At*Pep
PROSYSTEMIN	Precursor of Systemin
PRR	Pattern Recognition Receptor
PSKR	Phytosulfokine Receptor
PTI	Pattern/PAMP-Triggered Immunity
qRT	Quantitative Real Time PCR
R	Arginine
R	Resistance (Gene)
RALF	Rapid Alkalinization Factor
RAM	Root Apical Mersitem
RboH	Respiratory-burst oxidase homologue
RGF	Root Growth Factor
RLK	Receptor-Like Kinase
RLP	Receptor-Like Protein
RLU	Relative Light Unit
ROS	Reactive Oxygen Species
RPK	Receptor-Like Protein Kinase 2
S	Serine
SA	Salicylic Acid
SAM	Shoot Apical Meristem
SAR	Systemic Acquired Resistance
SERK	Somatic Embryogenesis Receptor Kinase
SGI	Seedling Growth Inhibition
SID2	Salicylic Acid Induction Deficient
SIPK	Salicylic Acid Induced Protein Kinase
Sl	*Solanum lycopersicon*
SR160	Systemin Receptor 160
SubPep	Subtilase Peptide
TCV	Turnip Crinkle Virus
TLR	Toll-Like Receptor
TMV	Tobacco Mosaic Virus
ToYSV	Tomato Yellow Spot Virus
TuMV	Turnip Mosaic Virus
UBQ	Ubiquitin
vsiRNA	Viral small interfering RNA
VSP	Vegetative Storage Protein
WAK1	Wall-Associated Kinase 1
WIPK	Wound-Induced Protein Kinase
Ws	*Wassilijewska* ecotype
WT	Wild Type
XA21	Xanthomonas Resistance 21
YFP	Yellow Fluorescent Protein
ZAT	Zinc Transporter of *Arabidopsis thaliana*
Zm	*Zea mays*

References

Abramovitch, R. B., R. Janjusevic, E. Stebbins, G. Martin (2006). "Type III effector AvrPtoB requires intrinsic E3 ubiquitin ligase activity to suppress plant cell death and immunity." Proceedings of the National Academy of Sciences **103**: 2851-2856.

Aharoni, A., M. A. Jongsma, H. Bouwmeester (2005). "Volatile science? Metabolic engineering of terpenoids in plants." Trends in Plant Science **10**: 594-602.

Aker, J., R. Hesselink, R. Engel, R. Karlova, J. Borst, A. Visser, S. De Vries (2007). "In vivo hexamerization and characterization of the Arabidopsis AAA ATPase CDC48A complex using forster resonance energy transfer-fluorescence lifetime imaging microscopy and fluorescence correlation spectroscopy." Plant Physiology **145**: 339-350.

Albert, M. (2013). "Peptides as triggers of plant defence."Journal of Experimental Botany **64**: 5269-5279.

Albert, M., A. K. Jehle, M. Lipschis, K. Mueller, Y. Zeng, G. Felix (2010). "Regulation of cell behaviour by plant receptor kinases: pattern recognition receptors as prototypical models." European Journal of Cell Biology **89**: 200-207.

Alborn, H., T. Turlings, T. Jones, G. Stenhagen, J. Loughrin, J. Tomlinson (1997). "An elicitor of plant volatiles from beet armyworm oral secretion." Science **276**: 945-949.

Albrecht, C., E. Russinova, B. Kemmerling, M. Kwaaitaal, S. De Vries (2008). "Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR KINASE proteins serve brassinosteroid-dependent and-independent signaling pathways." Plant Physiology **148**: 611-619.

Allan, A. C., M. Lapidot, J. Culver, R. Fuhr (2001). "An early tobacco mosaic virus-induced oxidative burst in tobacco indicates extracellular perception of the virus coat protein." Plant Physiology **126**: 97-108.

Amano, Y., H. Tsubouchi, H. Shinohara, M. Ogawa, Y. Matsubayashi (2007). "Tyrosine-sulfated glycopeptide involved in cellular proliferation and expansion in Arabidopsis." Proceedings of the National Academy of Sciences **104**: 18333-18338.

Apel, K. and H. Hirt (2004). "Reactive oxygen species: Metabolism, oxidative stress, and signal transduction." Annual Review of Plant Biology **55**: 373-399.

Arai, K.-i., F. Lee, A. Miyajima, S. Miyatake, N. Arai, T. Yokota (1990). "Cytokines: coordinators of immune and inflammatory responses." Annual Review of Biochemistry **59**: 783-836.

Bar, M. and A. Avni (2009). "EHD2 inhibits ligand-induced endocytosis and signaling of the leucine-rich repeat receptor-like protein LeEix2." Plant Journal **59**: 600-611.

Bari, R. and J. D. Jones (2009). "Role of plant hormones in plant defence responses." Plant Molecular Biology **69**: 473-488.

Bartels, S., M. Lori, M. Mbengue, M. van Verk, D. Klauser, T. Hander, S. Robatzek, T. Boller (2013). "The family of AtPeps and their precursors in Arabidopsis: Differential expression and localization but similar induction of pattern-triggered immune responses." Journal of Experimental Botany **In Press**.

Bauer, Z., L. Gomez-Gomez, T. Boller, G. Felix (2001). "Sensitivity of different ecotypes and mutants of Arabidopsis thaliana toward the bacterial elicitor flagellin correlates with the presence of receptor-binding sites." Journal of Biological Chemistry **276**: 45669-45676.

Beck, M., W. Heard, M. Mbengue, S. Robatzek (2012). "The INs and OUTs of pattern recognition receptors at the cell surface." Current opinion in plant biology **15**: 367-374.

Bedinger, P. A., G. Pearce, P. Covey (2010). "RALFs: Peptide regulators of plant growth." Plant Signaling & Behavior **5**: 1342-1346.

Berger, S. (2002). "Jasmonate-related mutants of Arabidopsis as tools for studying stress signaling." Planta **214**: 497-504.
Betsuyaku, S., S. Sawa, M. Yamada (2011). "The function of the CLE peptides in plant development and plant-microbe interactions." The Arabidopsis Book **9**.
Boller, T. (1995). "Chemoperception of microbial signals in plant cells." Annual Review of Plant Physiology and Plant Molecular Biology **46**: 189-214.
Boller, T. and G. Felix (2009). "A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors." Annual Review of Plant Biology **60**: 379-406.
Boller, T. and S. Y. He (2009). "Innate Immunity in Plants: An Arms Race Between Pattern Recognition Receptors in Plants and Effectors in Microbial Pathogens." Science **324**: 742-744.
Böni, R. (2012). "Characteristics of the AtPep/PEPR system." Master Thesis.
Bostock, R. M. (2005). "Signal crosstalk and induced resistance: Straddling the line between cost and benefit." Annual Review of Phytopathology **43**: 545-580.
Boudsocq, M., M. R. Willmann, M. McCormack, H. Lee, L. Shan, P. He, J. Bush, S. Cheng, J. Sheen (2010). "Differential innate immune signalling via Ca2+ sensor protein kinases." Nature **464**: 418.
Boutrot, F., C. Segonzac, K. Chang, H. Qiao, J. Ecker, C. Zipfel, J. Rathjen (2010). "Direct transcriptional control of the Arabidopsis immune receptor FLS2 by the ethylene-dependent transcription factors EIN3 and EIL1." Proceedings of the National Academy of Sciences **107**: 14502-14507.
Bricchi, I., C. M. Bertea, A. Occhipinti, I. Popanov, M. Maffei (2012). "Dynamics of Membrane Potential Variation and Gene Expression Induced by Spodoptera littoralis, Myzus persicae, and Pseudomonas syringae in Arabidopsis." PLoS ONE **7**(10).
Brutus, A., F. Sicilia, A. Macone, F. Cervone, G. de Lorenzo (2010). "A domain swap approach reveals a role of the plant wall-associated kinase 1 (WAK1) as a receptor of oligogalacturonides." Proceedings of the National Academy of Sciences **107**: 9452-9457.
Butenko, M. A., A. K. Vie, T. Brembu, R. Aalen, A. Bones (2009). "Plant peptides in signalling: looking for new partners." Trends in Plant Science **14**: 255-263.
Cai, L., K. Chen, X. Zhang, L. Yan, M. Hou, Z. Xu (2009). "Biological and molecular characterization of a crucifer tobamovirus infecting oilseed rape." Biochemical Genetics **47**: 451-461.
Cao, F. Y., K. Yoshioka, D. Desveaux (2011). "The roles of ABA in plant–pathogen interactions." Journal of Plant Research **124**: 489-499.
Carr, J. P., M. G. Lewsey, P. Palukaitis (2010). "Signaling in Induced Resistance." Advances in Virus Research **76**: 57-121.
Cervone, F., M. G. Hahn, G. de Lorenzo, A. Darvill, P. Albersheim (1989). "Host-pathogen interactions XXXIII. A plant protein converts a fungal pathogenesis factor into an elicitor of plant defense responses." Plant Physiology **90**: 542-548.
Chen, F., M.-J. Gao, Y. Miao, Y. Yuan, M. Wang, Q. Li, B. Mao, L. Jiang, Z. He (2010). "Plasma membrane localization and potential endocytosis of constitutively expressed XA21 proteins in transgenic rice." Molecular Plant **3**: 917-926.
Chen, H., C. G. Wilkerson, J. Kuchar, B. Phinney, G. Howe (2005). "Jasmonate-inducible plant enzymes degrade essential amino acids in the herbivore midgut." Proceedings of the National Academy of Sciences **102**: 19237-19242.
Chinchilla, D., Z. Bauer, M. Regenass, T. Boller, G. Felix (2006). "The Arabidopsis receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception." Plant Cell **18**: 465-476.
Chinchilla, D., L. Shan, P. He, S. de Vries, B. Kemmerling (2009). "One for all: the receptor-associated kinase BAK1." Trends in Plant Science **14**: 535-541.

Chinchilla, D., C. Zipfel, S. Robatzek, B. Kemmerling, T. Nuernberger, J. D. Jones, G. Felix, T. Boller (2007). "A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence." Nature **448**: 497-500.

Clark, S. E., M. P. Running, E. Meyerowitz (1995). "CLAVATA3 is a specific regulator of shoot and floral meristem development affecting the same processes as CLAVATA1." Development **121**: 2057-2067.

Clay, N. K., A. M. Adio, C. Denoux, G. Jander, F. Ausubel (2009). "Glucosinolate Metabolites Required for an Arabidopsis Innate Immune Response." Science **323**: 95-101.

Cock, J. M. and S. McCormick (2001). "A Large Family of Genes That Share Homology withCLAVATA3." Plant Physiology **126**: 939-942.

Conrath, U., C. M. Pieterse, B. Mauch-Mani (2002). "Priming in plant–pathogen interactions." Trends in Plant Science **7**: 210-216.

Constabel, C. P., D. R. Bergey, C. Ryan (1995). "Systemin activates synthesis of wound-inducible tomato leaf polyphenol oxidase via the octadecanoid defense signaling pathway." Proceedings of the National Academy of Sciences **92**: 407-411.

Cunnac, S., M. Lindeberg, A. Collmer (2009). "*Pseudomonas syringae* type III secretion system effectors: repertoires in search of functions." Current Opinion in Microbiology **12**: 53-60.

Czyzewicz, N., K. Yue, T. Beeckman, I. de Smet (2013). "Message in a bottle: small signalling peptide outputs during growth and development." Journal of Experimental Botany: ert283.

D'Ovidio, R., B. Mattei, S. Roberti, D. Bellincampi (2004). "Polygalacturonases, polygalacturonase-inhibiting proteins and pectic oligomers in plant-pathogen interactions." Biochimica Et Biophysica Acta **1696**: 237-244.

Da Cunha, L., M.-V. Sreerekha, D. Mackey (2007). "Defense suppression by virulence effectors of bacterial phytopathogens." Current Opinion in Plant Biology **10**: 349-357.

Dangl, J. L., D. M. Horvath, B. Staskawicz (2013). "Pivoting the Plant Immune System from Dissection to Deployment." Science **341**: 746-751.

Dangl, J. L. and J. D. Jones (2001). "Plant pathogens and integrated defence responses to infection." Nature **41**: 826-833.

Dardick, C. and P. Ronald (2006). "Plant and animal pathogen recognition receptors signal through non-RD kinases." PLoS Pathogens **2**: 14-28.

Dardick, C. D., S. Golem, J. Culver (2000). "Susceptibility and symptom development in Arabidopsis thaliana to Tobacco mosaic virus is influenced by virus cell-to-cell movement." MPMI **13**: 1139-1144.

Darvill, A. G. and P. Albersheim (1984). "Phytoalexins and their elicitors - a defense against microbial infections in plants." Annual Review of Plant Physiology and Plant Molecular Biology **35**: 243-275.

Davies, P. J. (2010). The plant hormones: their nature, occurrence, and functions. Plant Hormones, Springer**:** 1-15.

Degenhardt, D. C., S. Refi-Hind, J. Stratmann, D. Lincoln (2010). "Systemin and jasmonic acid regulate constitutive and herbivore-induced systemic volatile emissions in tomato." Phytochemistry **71**: 2024-2037.

Dempsey, D. M. A., M. S. Pathirana, K. Wobbe, D. Klessig (1997). "Identification of an Arabidopsis locus required for resistance to turnip crinkle virus." Plant Journal **11**: 301-311.

Denoux, C., R. Galletti, N. Mammarella, S. Gopalan, D. Werck, G. de Lorenzo, S. Ferrari, F. Ausubel, j. Dewdney (2008). "Activation of defense response pathways by OGs and Flg22 elicitors in Arabidopsis seedlings." Molecular Plant **1**: 423-445.

Ding, S.-W. and O. Voinnet (2007). "Antiviral immunity directed by small RNAs." Cell **130**: 413-426.

Ding, S. W. (2010). "RNA-based antiviral immunity." Nature Reviews Immunology **10**: 632-644.

Dodds, P. N. and J. P. Rathjen (2010). "Plant immunity: towards an integrated view of plant–pathogen interactions." Nature Reviews Genetics **11**: 539-548.
Dong, C., R. J. Davis, R. Flavell (2002). "MAP kinases in the immune response." Annual Review of Immunology **20**: 55-72.
Dong, X. N. (2004). "NPR1, all things considered." Current Opinion in Plant Biology **7**: 547-552.
Doss, R. P., J. E. Oliver, W. Proebsting, S. Potter, S. Kuy, S. Clement, T. Williamson, J. Carney, D. de Vilbiss (2000). "Bruchins: insect-derived plant regulators that stimulate neoplasm formation." Proceedings of the National Academy of Sciences **97**: 6218-6223.
Du, L., G. S. Ali, K. Simons, J. Hon, T. Yang, A. Reddy, B. Poovaiah (2009). "Ca2+/calmodulin regulates salicylic-acid-mediated plant immunity." Nature **457**: 1154-1158.
Engelberth, J., H. T. Alborn, E. Schmelz, J. Tomlinson (2004). "Airborne signals prime plants against insect herbivore attack." Proceedings of the National Academy of Sciences **101**: 1781-1785.
Fauth, M., P. Schweizer, A. Buchala, C. Markstadter, M. Riederer, T. Kato, H. Kauss (1998). "Cutin monomers and surface wax constituents elicit H2O2 in conditioned cucumber hypocotyl segments and enhance the activity of other H2O2 elicitors." Plant Physiology **117**: 1373-1380.
Felix, G., K. Baureithel, T. Boller (1998). "Desensitization of the perception system for chitin fragments in tomato cells." Plant Physiology **117**: 643-650.
Felix, G., J. D. Duran, S. Volko, T. Boller (1999). "Plants have a sensitive perception system for the most conserved domain of bacterial flagellin." Plant Journal **18**: 265-276.
Ferrari, S., D. V. Savatin, F. Sicilia, G. Gramegna, F. Cervone, G. de Lorenzo (2013). "Oligogalacturonides: plant damage-associated molecular patterns and regulators of growth and development." Frontiers in Plant Science **4**.
Feys, B. J., C. E. Benedetti, C. Penfold, J. Turner (1994). "Arabidopsis mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen." Plant Cell **6**: 751-759.
Feys, B. J., L. J. Moisan, M. Newman, J. Parker (2001). "Direct interaction between the Arabidopsis disease resistance signaling proteins, EDS1 and PAD4." The EMBO Journal **20**: 5400-5411.
Flor, H. H. (1971). "Current status of the gene-for-gene concept." Annual Review of Phytopathology **9**: 275-296.
Flury, P. (2011). "The Role of AtPeps in Local and Systemic Resistance." Master Thesis.
Flury, P., D. Klauser, B. Schulze, T. Boller, S. Bartels (2013). "The anticipation of danger: MAMP perception enhances *At*Pep-triggered oxidative burst." Plant Physiology.
Fonseca, S., J. M. Chico, R. Solano (2009). "The jasmonate pathway: the ligand, the receptor and the core signalling module." Current opinion in Plant Biology **12**: 539-547.
Fu, Z. Q., M. Guo, B. Jeong, F. Tian, T. Elthon, R. Cerny, D. Staiger, J. Alfano (2007). "A type III effector ADP-ribosylates RNA-binding proteins and quells plant immunity." Nature **447**: 284-288.
Fu, Z. Q., S. Yan, A. Saleh, W. Wang, J. Ruble, W. Oka, R. Mohan, S. Spoel, Y. Tada, N. Zheng (2012). "NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants." Nature **486**: 228-232.
Fukuda, H. and T. Higashiyama (2011). "Diverse functions of plant peptides: entering a new phase." Plant and Cell Physiology **52**: 1-4.
Galletti, R., C. Denoux, S. Gambetta, J. Dewdney, F. Ausubel, G. de Lorenzo, S. Ferrari (2008). "The AtrbohD-mediated oxidative burst elicited by oligogalacturonides in Arabidopsis is dispensable for the activation of defense responses effective against Botrytis cinerea." Plant Physiology **148**: 1695-1706.
Galletti, R., S. Ferrari, G. de Lorenzo (2011). "Arabidopsis MPK3 and MPK6 Play Different Roles in Basal and Oligogalacturonide- or Flagellin-Induced Resistance against *Botrytis cinerea*." Plant Physiology **15**: 804-814.

Germain, H., E. Chevalier, S. Caron, D. Matton (2005). "Characterization of five RALF-like genes from Solanum chacoense provides support for a developmental role in plants." Planta **220**: 447-454.
Gimenez-Ibanez, S., D. R. Hann, V. Ntoukakis, E. Peptutschnig, V. Lipka, J. Rathjen (2009). "AvrPtoB Targets the LysM Receptor Kinase CERK1 to Promote Bacterial Virulence on Plants." Current Biology **19**: 423-429.
Glauser, G., L. Dubugnon, S. Mousavi, S. Rudaz, J.L. Wolfender, E. E. Farmer (2009). "Velocity Estimates for Signal Propagation Leading to Systemic Jasmonic Acid Accumulation in Wounded Arabidopsis." Journal of Biological Chemistry **284**: 34506-34513.
Glauser, G., A. Vallat, D. Balmer (2013). Hormone Profiling. Arabidopsis Protocols. Sanchez-Serrano and Salinas. NY, USA, Humana Press.
Glazebrook, J. (2005). "Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens." Annual Review of Phytopathology **43**: 205-227.
Göhre, V., T. Spallek, H. Haweker, S. Mersmann, T. Mentzel, T. Boller, M. De Torres, J. Mansfield, S. Robatzek (2008). "Plant Pattern-Recognition Receptor FLS2 Is Directed for Degradation by the Bacterial Ubiquitin Ligase AvrPtoB." Current Biology **18**: 1824-1832.
Gomez-Gomez, L. and T. Boller (2000). "FLS2: An LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis." Molecular Cell **5**: 1003-1011.
Gomez-Gomez, L. and T. Boller (2002). "Flagellin perception: a paradigm for innate immunity." Trends in Plant Science **7**: 251-256.
Gomez-Gomez, L., G. Felix, T. Boller (1999). "A single locus determines sensitivity to bacterial flagellin in Arabidopsis thaliana." Plant Journal **18**: 277-284.
Grant, M. and C. Lamb (2006). "Systemic immunity." Current Opinion in Plant Biology **9**: 414-420.
Green, T. and C. Ryan (1972). "Wound-induced proteinase inhibitor in plant leaves: a possible defense mechanism against insects." Science **175**: 776-777.
Greenberg, J. T. and N. Yao (2004). "The role and regulation of programmed cell death in plant-pathogen interactions." Cellular Microbiology **6**: 201-211.
Grubb, C. D. and S. Abel (2006). "Glucosinolate metabolism and its control." Trends in Plant Science **11**: 89-100.
Guttman, D. S., B. A. Vinatzer, S. Sarkar, M. Ranall, G. Kettler, J. Greenberg (2002). "A functional screen for the type III (Hrp) secretome of the plant pathogen Pseudomonas syringae." Science **295**: 1722-1726.
Halitschke, R., U. Schittko, G. Pohnert, W. Bohland, I. Baldwin (2001). "Molecular Interactions between the Specialist HerbivoreManduca sexta (Lepidoptera, Sphingidae) and Its Natural Host Nicotiana attenuata. III. Fatty Acid-Amino Acid Conjugates in Herbivore Oral Secretions Are Necessary and Sufficient for Herbivore-Specific Plant Responses." Plant Physiology **125**: 711-717.
Hanssen, I. M., H. P. van Esse, A. Ballester, S. Hogewoning, N. Parra, A. Paeleman, B. Lievens, A. Bovy, B. Thoma (2011). "Differential tomato transcriptomic responses induced by pepino mosaic virus isolates with differential aggressiveness." Plant Physiology **156**: 301-318.
Hara, K., T. Yokoo, R. Kajita, T. Onishi, S. Yahara, K. Peterson, K. Torii, T. Kamkimoto (2009). "Epidermal cell density is autoregulated via a secretory peptide, EPIDERMAL PATTERNING FACTOR 2 in Arabidopsis leaves." Plant and Cell Physiology **50**: 1019-1031.
Haruta, M. and C. P. Constabel (2003). "Rapid alkalinization factors in poplar cell cultures. Peptide isolation, cDNA cloning, and differential expression in leaves and methyl jasmonate-treated cells." Plant Physiology **131**: 814-823.
Hecht, V., J. P. Vielle-Calzada, M. Hartog, E. Schmidt, K. Boutilier, U. Grossniklaus, S. De Vries (2001). "The Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture." Plant Physiology **127**: 803-816.

Heese, A., D. R. Hann, S. Gimenez-Ibanez, A. Jones, K. He, J. Li, J. Schroeder, S. Peck, J. Rathjen (2007). "The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants." Proceedings of the National Academy of Sciences **104**: 12217-12222.

Holton, N., A. Cano-Delgado, K. Harrison, T. Montoya, J. Chory, G. Bishop (2007). "Tomato BRASSINOSTEROID INSENSITIVE1 is required for systemin-induced root elongation in Solanum pimpinellifolium but is not essential for wound signaling." Plant Cell **19**: 1709-1717.

Holton, N., K. Harrison, T. Yokota, G. Bishop (2008). "Tomato BRI1 and systemin wound signalling." Plant Signaling & Behavior **3**: 54-55.

Howe, G. A. and G. Jander (2008). "Plant immunity to insect herbivores." Annual Review of Plant Biology **59**: 41-66.

Howe, G. A., B. C. McCaig, T. Schilmiller, H. Chen, L. Lei (2004). "The jasmonate-COI1 signaling pathway plays a dual role in the regulation of stress responses and seed development in tomato." Plant Biology **2004**: 23.

Hu, Q., J. Hollunder, A. Niehl, C. Koerner, D. Gereige, D. Windels, A. Arnold, M. Kuiper, F. Vazquez, M. Pooggin, M. Heinlein (2011). "Specific impact of tobamovirus infection on the Arabidopsis small RNA profile." PLoS One **6**: e19549.

Huffaker, A., N. J. Dafoe, E. Schmelz (2011). "ZmPep1, an Ortholog of Arabidopsis Elicitor Peptide 1, Regulates Maize Innate Immunity and Enhances Disease Resistance." Plant Physiology **155**: 1325-1338.

Huffaker, A., G. Pearce, C. Ryan (2006). "An endogenous peptide signal in Arabidopsis activates components of the innate immune response." Proceedings of the National Academy of Sciences **103**: 10098-10103.

Huffaker, A., G. Pearce, N. Veyrat, M. Erb, T. Turlings, R. Sartor, Z. Shen, S. Briggs, M. Vaughan, H. Alborn, P. Teal, E. Schmelz (2013). "Plant elicitor peptides are conserved signals regulating direct and indirect antiherbivore defense." Proceedings of the National Academy of Sciences **110**: 5707-5712.

Huffaker, A. and C. A. Ryan (2007). "Endogenous peptide defense signals in Arabidopsis differentially amplify signaling for the innate immune response." Proceedings of the National Academy of Sciences **104**: 10732-10736.

Igarashi, D., K. Tsuda, F. Katagiri (2012). "The peptide growth factor, phytosulfokine, attenuates pattern-triggered immunity." Plant Journal **71**: 194-204.

Janeway, C. A., Jr. and R. Medzhitov (2002). "Innate immune recognition." Annual Review Immunology **20**: 197-216.

Jehle, A. K., M. Lipschis, M. Albert, V. Fallazadeh-Mamaghani, U. Fürst, K. Mueller, G. Felix (2013). "The Receptor-Like Protein ReMAX of Arabidopsis Detects the Microbe-Associated Molecular Pattern eMax from Xanthomonas." Plant Cell **25**: 2330-2340.

Jensen, S. and A. R. Thomsen (2012). "Sensing of RNA viruses: a review of innate immune receptors involved in recognizing RNA virus invasion." Journal of Virology **86**: 2900-2910.

Jones, J. D. G. and J. L. Dangl (2006). "The plant immune system." Nature **444**: 323-329.

Katsir, L., K. A. Davies, D. Bergmann, K. Laux (2011). "Peptide signaling in plant development." Current Biology **21**: 356-364.

Kayes, J. M. and S. E. Clark (1998). "CLAVATA2, a regulator of meristem and organ development in Arabidopsis." Development **125**: 3843-3851.

Kazan, K. and J. M. Manners (2009). "Linking development to defense: auxin in plant–pathogen interactions." Trends in Plant Science **14**: 373-382.

Kemmerling, B., T. Halter, S. Mazzotta, S. Mosher, T. Nürnberger (2011). "A genome-wide survey for Arabidopsis leucine-rich repeat receptor kinases implicated in plant immunity." Frontiers in Plant Science **2**.

Kessler, A. and I. T. Baldwin (2001). "Defensive function of herbivore-induced plant volatile emissions in nature." Science **291**: 2141-2144.

Kinoshita, A., S. Betsuyaku, Y. Osakabe, S. Mizuno, S. Nagawa, Y. Stah, R. Simon, K. Yamaguchi, H. Fukuda, S. Sawa (2010). "RPK2 is an essential receptor-like kinase that transmits the CLV3 signal in Arabidopsis." Development **137**: 3911-3920.

Klauser, D., G. Desurmont, G. Glauser, A. Vallat, P. Flury, T. Boller, T. Turlings, S. Bartels et al. (2013). "The Arabidopsis AtPep-PEPR danger detection system is induced by Spodoptera littoralis oral secretions to mediate defense responses against herbivores." Plant Journal **Submitted**.

Klauser, D., P. Flury, T. Boller, S. Bartels (2013). "Several MAMPs, including chitin fragments, enhance AtPep-triggered oxidative burst independently of wounding." Plant Signaling & Behavior **8**: e25346.

Kobayashi, M., I. Ohura, K. Kawakita, N. Yokota, M. Fujiwara, K. Shinamoto, N. Doke, H. Yoshioka (2007). "Calcium-dependent protein kinases regulate the production of reactive oxygen species by potato NADPH oxidase." Plant Cell **19**: 1065-1080.

Kondo, T., S. Sawa, A. Kinoshita, S. Mizuno, T. Kakimoto, H. Fukuda, Y. Sakagami (2006). "A plant peptide encoded by CLV3 identified by in situ MALDI-TOF MS analysis." Science **313**: 845-848.

Kørner, C. , D. Klauser, A. Niehl, A. Dominguez-Ferreras, D. Chinchilla, T. Boller, M. Heinlein, D. R. Hann (2013). "The immunity regulator BAK1 contributes to resistance against diverse RNA viruses." MPMI.

Krol, E., T. Mentzel, D. Chinchilla, T. Boller, G. Felix, B. Kemmerling, S. Postel, M. Arents, E. Jeworutzki, K. Al-Rasheid, D. Becker, R. Hedrich (2010). "Perception of the Arabidopsis danger signal peptide 1 involves the pattern recognition receptor *At*PEPR1 and its close homologue *At*PEPR2." Journal of Biological Chemistry **285**: 13471-13479.

Kunze, G., C. Zipfel, S. Robatzek, K. Niehaus, T. Boller, G. Felix (2004). "The N terminus of bacterial elongation factor Tu elicits innate immunity in Arabidopsis plants." Plant Cell **16**: 3496-3507.

Kutschmar, A., G. Rzewuski, N. Stuhrwoldt, G. Beemster, D. Inze, M. Sauter (2009). "PSK-α promotes root growth in Arabidopsis." New Phytologist **181**: 820-831.

Lacombe, S., A. Rougon-Cardoso, E. Sherwood, N. Peters, D. Dahlbeck, P. van Esse, M. Smoker, G. Rallapalli, B. Thomma, B. Staskawicz, J.D. Jones, C. Zipfel (2010). "Interfamily transfer of a plant pattern-recognition receptor confers broad-spectrum bacterial resistance." Nature Biotechnology **28**: 365-U394.

Lamb, C. and R. A. Dixon (1997). "The oxidative burst in plant disease resistance." Annual Review of Plant Physiology and Plant Molecular Biology **48**: 251-275.

Lanfermeijer, F. C., M. Staal, R. Malinowski, J. Stratman, J. Elzenga (2008). "Micro-electrode flux estimation confirms that the *Solanum pimpinellifolium cu3* mutant still responds to systemin." Plant Physiology **146**: 129-139.

Lawrence, S. D. and N. G. Novak (2006). "Expression of poplar chitinase in tomato leads to inhibition of development in Colorado potato beetle." Biotechnology Letters **28**: 593-599.

Lecourieux, D., R. Raneva, A. Pugin (2006). "Calcium in plant defence-signalling pathways." New Phytologist **171**: 249-269.

Lee, H., O.-K. Chah, J. Sheen (2011). "Stem-cell-triggered immunity through CLV3p-FLS2 signalling." Nature **473**: 376-379.

Lehti-Shiu, M. D., C. Zou, K.Hanada, S. Shiu (2009). "Evolutionary history and stress regulation of plant receptor-like kinase/pelle genes." Plant Physiology **150**: 12-26.

Li, J., J. Q. Wen, K. Lease, J. Doke, F. Tax, J. Walker (2002). "BAK1, an Arabidopsis LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling." Cell **110**: 213-222.

Li, J., C. Zhao-Hui, M. Batoux, V. Nekrasov, M. Roux, D. Chinchilla, C. Zipfel, J.D. Jones (2009). "Specific ER quality control components required for biogenesis of the plant innate immune receptor EFR." Proceedings of the National Academy of Sciences **106**: 15973-15978.

Liu, T., Z. Liu, C. Song, Y. Hu, Z. Han, J. She, F. Fang, J. Wang, C. Jin, J. Chang, J.M. Zhou, J. Chai (2012). "Chitin-induced dimerization activates a plant immune receptor." Science **336**: 1160-1164.

Liu, Z., Y. Wu, F. Yang, Y. Zhang, S. Chen, O. Xie, X. Tian, J.M Zhou (2013). "BIK1 interacts with PEPRs to mediate ethylene-induced immunity." Proceedings of the National Academy of Sciences.

Llave, C. (2010). "Virus-derived small interfering RNAs at the core of plant–virus interactions." Trends in Plant Science **15**: 701-707.

Logemann, E., R. P. Birkenbihl, V. Rawat, K. Schneeberger, E. Schmelzer, I. Somssich (2013). "Functional dissection of the PROPEP2 and PROPEP3 promoters reveals the importance of WRKY factors in mediating microbe-associated molecular pattern-induced expression." New Phytologist.

Lorenzo, O. and R. Solano (2005). "Molecular players regulating the jasmonate signalling network." Current Opinion in Plant Biology **8**: 532-540.

Love, A. J., B. W. Yun, V. Laval, G,. Loake, J. Milner (2005). "Cauliflower mosaic virus, a compatible pathogen of Arabidopsis, engages three distinct defense-signaling pathways and activates rapid systemic generation of reactive oxygen species." Plant Physiology **139**: 935-948.

Luna, E., V. Pastor, J. Robert, V. Flors, B. Mauch-Mani, J. Ton (2011). "Callose deposition: a multifaceted plant defense response." MPMI **24**: 183-193.

Ma, Y., R. K. Walker, Y. Zhao, G. Berkowitz (2012). "Linking ligand perception by PEPR pattern recognition receptors to cytosolic Ca2+ elevation and downstream immune signaling in plants." Proceedings of the National Academy of Sciences **109**: 19852-19857.

Maffei, M. E., A. Mithöfer, W. Boland (2007). "Insects feeding on plants: Rapid signals and responses preceding the induction of phytochemical release." Phytochemistry **68**: 2946-2959.

Maischak, H., P. A. Grigoriev, H. Vogel, W. Boland, A. Mithofer (2007). "Oral secretions from herbivorous lepidopteran larvae exhibit ion channel-forming activities." FEBS letters **581**: 898-904.

Malinowski, R., R. Higgins, Y. Luo, L. Piper, A. Nazir, V. Bajwa, S. Clouse, P. Thompson, J. Stratman (2009). "The tomato brassinosteroid receptor BRI1 increases binding of systemin to tobacco plasma membranes, but is not involved in systemin signaling." Plant Molecular Biology **70**: 603-616.

Manners, J. M., I. A. Penninckx, K. Vermaere, R. Brown, A. Morgan, D. Maclean, M. Curtis, B. Cammue, W. Broekaert (1998). "The promoter of the plant defensin gene PDF1. 2 from Arabidopsis is systemically activated by fungal pathogens and responds to methyl jasmonate but not to salicylic acid." Plant Molecular Biology **38**: 1071-1080.

Matsubayashi, Y. (2011). "Small post-translationally modified Peptide signals in Arabidopsis." The Arabidopsis Book **9**.

Matsubayashi, Y., M. Ogawa, M. Akiko, Y. Sakagami (2002). "An LRR receptor kinase involved in perception of a peptide plant hormone, phytosulfokine." Science **296**: 1470-1472.

Matsubayashi, Y. and Y. Sakagami (1996). "Phytosulfokine, sulfated peptides that induce the proliferation of single mesophyll cells of Asparagus officinalis L." Proceedings of the National Academy of Sciences **93**: 7623-7627.

Matsubayashi, Y., L. Takagi, N. Omura, M. Akiko, Y. Sakagami (1999). "The endogenous sulfated pentapeptide phytosulfokine-α stimulates tracheary element differentiation of isolated mesophyll cells of Zinnia." Plant Physiology **120**: 1043-1048.

Matsuzaki, Y., M. Ogawa-Ohnishi, A. Mori, Y. Matsubayashi (2010). "Secreted peptide signals required for maintenance of root stem cell niche in Arabidopsis." Science **329**: 1065-1067.

McGurl, B., G. Pearce, M. Orozco-Cardenas, C. Ryan (1992). "Structure, expression, and antisense inhibition of the systemin precursor gene." Science **255**: 1570-1573.

Medzhitov, R. and C. Janeway Jr (2000). "Innate immunity." The New England Journal of Medicine **343**: 338-344.

Melotto, M., W. Underwood, J. Koczan, K. Nomura, S.Y He (2006). "Plant stomata function in innate immunity against bacterial invasion." Cell **126**: 969-980.

Mersmann, S., G. Bourdais, S. Rietz, S. Robatzek (2010). "Ethylene signaling regulates accumulation of the FLS2 receptor and is required for the oxidative burst contributing to plant immunity." Plant Physiology **154**: 391-400.

Miller, G., K. Schlauch, R. Tam, D. Cortes, M. Torres, V. Shulaev, J. Dangl, R. Mittler (2009). "The plant NADPH oxidase RBOHD mediates rapid systemic signaling in response to diverse stimuli." Science Signaling **2**: 1-10.

Mishina, T. E. and J. Zeier (2007). "Pathogen-associated molecular pattern recognition rather than development of tissue necrosis contributes to bacterial induction of systemic acquired resistance in Arabidopsis." Plant Journal **50**: 500-513.

Mithöfer, A. and W. Boland (2008). "Recognition of herbivory-associated molecular patterns." Plant Physiology **146**: 825-831.

Mithofer, A., G. Wanner, W. Boland (2005). "Effects of feeding Spodoptera littoralis on lima bean leaves. II. Continuous mechanical wounding resembling insect feeding is sufficient to elicit herbivory-related volatile emission." Plant Physiology **137**: 1160-1168.

Mittler, R., S. Vanderauwera, N. Suzuki, G. Miller, V. Tognetti, K. Vandepoele, M. Gollery, V. Shulaev, F. Van Breusegem(2011). "ROS signaling: the new wave?" Trends in Plant Science **16**: 300-309.

Miya, A., P. Albert, T. Shinya, Y. Desaki, K. Ichimura, K. Shirasu, Y. Narusaka, N. Kawakami, H. Kaku, N. Shibuya (2007). "CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis." Proceedings of the National Academy of Sciences **104**: 19613-19618.

Moffett, P. (2009). "Mechanisms of Recognition in Dominant *R* Gene Mediated Resistance." Advances in Virus Research **75**: 1-229.

Mohan, S., P. Ma, T. Pechan, E. Bassford, W. Williams, D. Luthe (2006). "Degradation of the *S. frugiperda* peritrophic matrix by an inducible maize cysteine protease." Journal of Insect Physiology **52**: 21-28.

Monaghan, J. and C. Zipfel (2012). "Plant pattern recognition receptor complexes at the plasma membrane." Current Opinion in Plant Biology **15**: 349-357.

Moore, J. W., G. J. Loake, S. Spoel (2011). "Transcription dynamics in plant immunity." Plant Cell **23**: 2809-2820.

Morillo, S. A. and F. E. Tax (2006). "Functional analysis of receptor-like kinases in monocots and dicots." Current Opinion in Plant Biology **9**: 460-469.

Mortier, V., G. Den Herder, R. Whitford, W. Na de Welde, S. Rombauts, k. D'Haeseleer, M. Holsters, S. Goormachtig (2010). "CLE peptides control Medicago truncatula nodulation locally and systemically." Plant Physiology **153**: 222-237.

Moscatiello, R., P. Mariani, D. Sanders, F. Mathuis (2006). "Transcriptional analysis of calcium-dependent and calcium-independent signalling pathways induced by oligogalacturonides." Journal of Experimental Botany **57**: 2847-2865.

Mosher, S., H. Seybold, P. Rodriguez, M. Stahl, K. Davies, S. Dayaratne, S. Morillo, M. Wierzba, B. Favery, H. Keller (2013). "The tyrosine-sulfated peptide receptors PSKR1 and PSY1R modify the immunity of Arabidopsis to biotrophic and necrotrophic pathogens in an antagonistic manner." Plant Journal **73**: 469-482.

Mueller, K., D. Chinchilla, M. Albert, A. Jehle, H. Kalbacher, T. Boller, G. Felix (2012). "Contamination risks in work with synthetic peptides: flg22 as an example of a pirate in commercial peptide preparations." Plant Cell **24**: 3193-3197.

Murphy, E., S. Smith, I. de Smet (2012). "Small signaling peptides in Arabidopsis development: how cells communicate over a short distance." Plant Cell **24**: 3198-3217.

Nakashita, H., M. Yasuda, T. Nitta, T. Asami, S. Fujioka, Y. Arai, K. Sekimata, S. Takatsuto, I, Yamaguchi, S. Yoshida (2003). "Brassinosteroid functions in a broad range of disease resistance in tobacco and rice." Plant Journal **33**: 887-898.

Narvaez-Vasquez, J., M. L. Orozco-Cardenas, C. Ryan (2007). "Systemic wound signaling in tomato leaves is cooperatively regulated by systemin and hydroxyproline-rich glycopeptide signals." Plant Molecular Biology **65**: 711-718.

Navarro, L., C. Zipfel, O. Rowland, I. Keller, S. Robatzek, T. Boller, J.D. Jones (2004). "The transcriptional innate immune response to flg22. interplay and overlap with Avr gene-dependent defense responses and bacterial pathogenesis." Plant Physiology **135**: 1113-1128.

Niehl, A., K. Amari, D. Gereige, K. Brandner, Y. Mely, M. Heinlein(2012). "Control of Tobacco mosaic virus movement protein fate by CELL-DIVISION-CYCLE protein48." Plant Physiology **160**: 2093-2108.

Niehl, A., K. Amari, M. Heinlein (2012). "CDC48 function during TMV infection: Regulation of virus movement and replication by degradation?" Plant Signaling & Behavior **8**.

Nühse, T. S., S. C. Peck, H. Hirt, T. Boller (2000). "Microbial elicitors induce activation and dual phosphorylation of the Arabidopsis thaliana MAPK 6." Journal of Biological Chemistry **275**: 7521-7526.

Ohyama, K., M. Ogawa, Y. Matsubayashi (2008). "Identification of a biologically active, small, secreted peptide in Arabidopsis by in silico gene screening, followed by LC-MS-based structure analysis." Plant Journal **55**: 152-160.

Orozco-Cardenas, M. and C. A. Ryan (1999). "Hydrogen peroxide is generated systemically in plant leaves by wounding and systemin via the octadecanoid pathway." Proceedings of the National Academy of Sciences **96**: 6553-6557.

Pandey, S. P. and I. E. Somssich (2009). "The role of WRKY transcription factors in plant immunity." Plant Physiology **150**: 1648-1655.

Panstruga, R. and P. N. Dodds (2009). "Terrific protein traffic: the mystery of effector protein delivery by filamentous plant pathogens." Science **324**: 748.

Pearce, G., D. S. Moura, J. Stratman, C. Ryan (2001). "Production of multiple plant hormones from a single polyprotein precursor." Nature **411**: 817-820.

Pearce, G., D. S. Moura, J. Stratman, C. Ryan (2001). "RALF, a 5-kDa ubiquitous polypeptide in plants, arrests root growth and development." Proceedings of the National Academy of Sciences **98**: 12843-12847.

Pearce, G., D. Strydom, S. Johnson, C. Ryan (1991). "A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins." Science **253**: 895-897.

Pearce, G., Y. Yamaguchi, G. Barona, C. Ryan (2010). "A subtilisin-like protein from soybean contains an embedded, cryptic signal that activates defense-related genes." Proceedings of the National Academy of Sciences **107**: 14921-14925.

Pereda, S., N. Ehrenfeld, C. Medina, J. Delgado, P. Arce-Johnson (2000). "Comparative analysis of TMV-Cg and TMV-U1 detection methods in infected *Arabidopsis thaliana*." Journal of Virological Methods **90**: 135-142.

Peumans, W. J. and E. Van Damme (1995). "Lectins as plant defense proteins." Plant Physiology **109**: 347.

Pieterse, C. M., A. Leon-Reyes, S. van Endt, S. van Wees (2009). "Networking by small-molecule hormones in plant immunity." Nature Chemical Biology **5**: 308-316.

Pieterse, C.M., D. van der Does, C. Zamioundis, A. Leon-Reyes, S. van Wees (2012). "Hormonal modulation of plant immunity." Annual Review of Cell and Developmental Biology **28**: 489-521

Pirone, T. P. and S. Blanc (1996). "Helper-dependent vector transmission of plant viruses." Annual Review of Phytopathology **34**: 227-247.

Postel, S., I. Küfner, C. Beuter, S. Mazzotta, A. Schwedt, A. Borlotti, T. Halter, B. Kemmerling, T. Nurnberger (2010). "The multifunctional leucine-rich repeat receptor kinase BAK1 is implicated in Arabidopsis development and immunity." European Journal of Cell Biology **89**: 169-174.

Qi, Z., R. Verma, C. Gehring, Y. Yamaguchi, Y. Zhao, C. Ryan, G. Berkowitz (2010). "Ca^{2+} signaling by plant *Arabidopsis thaliana* Pep peptides depends on *At*PepR1, a receptor with guanylyl cyclase activity, and cGMP-activated Ca^{2+} channels." Proceedings of the National Academy of Sciences **107**: 21193-21198.

Ramonell, K. M., B. Zhang, R. Ewing, Y. Chen, D. Xu, G. Stacey, S. Somerville (2002). "Microarray analysis of chitin elicitation in Arabidopsis thaliana." Molecular Plant Pathology **3**: 301-311.

Rasmann, S., T. G. Köllner, J. Degenhardt, I. Hiltpold, S. Toepfer, U. Kuhlmann, J. Gershenzon, T. Turlings (2005). "Recruitment of entomopathogenic nematodes by insect-damaged maize roots." Nature **434**: 732-737.

Rathinam, V. A. and K. A. Fitzgerald (2011). "Cytosolic surveillance and antiviral immunity." Current Opinion in Virology **1**: 455-462.

Robatzek, S., D. Chinchilla, T. Boller (2006). "Ligand-induced endocytosis of the pattern recognition receptor FLS2 in Arabidopsis." Genes & Development **20**: 537-542.

Roux, M., B. Schwessinger, C. Albrecht, D. Chinchilla, A. Jones, N. Holton, F. Malovsky, M. Tor, S. De Vries, C. Zipfel (2011). "The Arabidopsis leucine-rich repeat receptor-like kinases BAK1/SERK3 and BKK1/SERK4 are required for innate immunity to hemibiotrophic and biotrophic pathogens." Plant Cell **23**: 2440-2455.

Ruiz-Ferrer, V. and O. Voinnet (2009). "Roles of plant small RNAs in biotic stress responses." Annual Review of Plant Biology **60**: 485-510.

Ryan, C. A., A. Huffaker, Y. Yamaguchi (2007). "New insights into innate immunity in Arabidopsis." Cellular Microbiology **9**: 1902-1908.

Ryan, C. A. and G. Pearce (1998). "Systemin: A polypeptide signal for plant defensive genes." Annual Review of Cell and Developmental Biology **14**: 1-17.

Ryan, C. A. and G. Pearce (2003). "Systemins: A functionally defined family of peptide signal that regulate defensive genes in Solanaceae species." Proceedings of the National Academy of Sciences: 14577-14580.

Sakamoto, T., M. Deguchi, O. Brustolini, A. Santos, F. Silva, E. Fontes (2012). "The tomato RLK superfamily: phylogeny and functional predictions about the role of the LRRII-RLK subfamily in antiviral defense." BMC Plant Biol **12**: 229.

Sawinski, K., S. Mersmann, S. Robatzek, M. Boehmer (2013). "Guarding the Green: Pathways to Stomatal Immunity." MPMI **26**: 626-632.

Scheer, J. M. and C. A. Ryan (2002). "The systemin receptor SR160 from Lycopersicon peruvianum is a member of the LRR receptor kinase family." Proceedings of the National Academy of Sciences **99**: 9585-9590.

Schilmiller, A. L. and G. A. Howe (2005). "Systemic signaling in the wound response." Current Opinion in Plant Biology : 369-377.

Schmelz, E. A., M. J. Carroll, S. LeClere, S. Phipps, J. Meredith, P. Chourey, H. Alborn, P. Teal (2006). "Fragments of ATP synthase mediate plant perception of insect attack." Proceedings of the National Academy of Sciences **103**: 8894-8899.

Schmelz, E. A., J. Engelberth, H. Alborn, J. Tomlinson, P. Teal (2009). "Phytohormone-based activity mapping of insect herbivore-produced elicitors." Proceedings of the National Academy of Sciences **106**: 653-657.

Schoonhoven, L. M., J. van Loon, M. Dicke (2005). Insect-Plant Biology, Oxford University Press.

Schulze, B., T. Mentzel, A. Jehle, K. Mueller, S. Beeler, T. Boller, G. Felix, D. Chinchilla (2010). "Rapid heteromerization and phosphorylation of ligand-activated plant transmembrane receptors and their associated kinase BAK1." Journal of Biological Chemistry **285**: 9444-9451.

Schweizer, F., P. Fernández-Calvo, M. Zander, M. Diez-Diaz, S. Fonseca, G. Glauser, M. Lewse, J. Ecker, R. Solano, P. Reymond (2013). "Arabidopsis Basic Helix-Loop-Helix Transcription Factors MYC2, MYC3, and MYC4 Regulate Glucosinolate Biosynthesis, Insect Performance, and Feeding Behavior." Plant Cell.

Schwessinger, B. and P. C. Ronald (2012). "Plant innate immunity: perception of conserved microbial signatures." Annual Review of Plant Biology **63**: 451-482.

Schwessinger, B., M. Roux, Y. Kadota, V. Ntoukakis, J. Sklenar, A. Jones, C. Zipfel (2011). "Phosphorylation-dependent differential regulation of plant growth, cell death, and innate immunity by the regulatory receptor-like kinase BAK1." PLoS Genetics **7**: e1002046.

Segonzac, C., Z. L. Nimchuk, M. Beck, P. Tarr, S. Robatzek, E. Meyerowitz, C. Zipfel (2012). "The shoot apical meristem regulatory peptide CLV3 does not activate innate immunity." Plant Cell **24**: 3186-3192.

Serrano, C., J. Gonzalez-Cruz, J. Gonzalez, F. Jaregui, C. Medina, P. Mantilla, J. Matrus, P. Arce-Johnson (2008). "Genetic and histological studies on the delayed systemic movement of *Tobacco mosaic virus* in *Arabidopsis thaliana*." BMC Genetics **9**: 59.

Shan, L., P. He, J. Li, A. Heese, S. Peck, T. Nuernberger, G. Martin, J. Sheen (2008). "Bacterial effectors target the common signaling partner BAK1 to disrupt multiple MAMP receptor-signaling complexes and impede plant immunity." Cell Host & Microbe **4**: 17-27.

Shiu, S.-H. and A. B. Bleecker (2001). "Receptor-like kinases from Arabidopsis form a monophyletic gene family related to animal receptor kinases." Proceedings of the National Academy of Sciences **98**: 10763-10768.

Shiu, S.-H. and A. B. Bleecker (2003). "Expansion of the receptor-like kinase/Pelle gene family and receptor-like proteins in Arabidopsis." Plant Physiology **132**: 530-543.

Shiu, S. H., W. M. Karlowski, R. Pan, Y. Tzeng, K. Mayer, W. Li (2004). "Comparative analysis of the receptor-like kinase family in Arabidopsis and rice." Plant Cell **16**: 1220-1234.

Song, D. H. and J. O. Lee (2012). "Sensing of microbial molecular patterns by Toll-like receptors." Immunol ogy Reviews **250**: 216-229.

Soosaar, J. L., T. M. Burch-Smith, S. Dinesh-Kumar (2005). "Mechanisms of plant resistance to viruses." Nature Reviews Microbiology **3**: 789-798.

Spanu, P., D. G. Grosskopf, G. Felix, T. Boller (1994). "The apparent turnover of 1-aminocyclopropane-1-carboxylate synthase in tomato cells is regulated by protein-phosphorylation and dephosphorylation." Plant Physiology **106**: 529-535.

Stührwohldt, N., R. I. Dahlke, B. Steffens, A. Johnson, M. Sauter (2011). "Phytosulfokine-α controls hypocotyl length and cell expansion in Arabidopsis thaliana through phytosulfokine receptor 1." PLoS One **6**: e21054.

Sugano, S. S., T. Shimada, Y. Imai, K. Okawa, A. Tamai, M. Mori, I. Hara-Nishimura (2009). "Stomagen positively regulates stomatal density in Arabidopsis." Nature **463**: 241-244.

Sun, J. Q., H. L. Jiang, C. Li (2011). "Systemin/Jamsonate-mediated systemic defense signaling in tomato." Molecular Plant: 1-9.

Thordal-Christensen, H. (2003). "Fresh insights into processes of nonhost resistance." Current Opinion in Plant Biology **6**: 351-357.

Tintor, N., A. Ross, K. Kanehara, K. Yamada, L. Fan, B. Kemmerling, T. Nuernberger, K. Tsuda, Y. Saijo (2013). "Layered pattern receptor signaling via ethylene and endogenous elicitor peptides during Arabidopsis immunity to bacterial infection." Proceedings of the National Academy of Sciences.

Ton, J., V. Flors, B. Mauch-Mani (2009). "The multifaceted role of ABA in disease resistance." Trends in Plant Science **14**: 310-317.
Tor, M., M. T. Lotze, N. Holton (2009). "Receptor-mediated signalling in plants: molecular patterns and programmes." Journal of Experimental Botany **60**: 3645-3654.
Torres, M. A., J. Dangl, J. D. Jones (2002). "Arabidopsis gp91phox homologues AtrbohD and AtrbohF are required for accumulation of reactive oxygen intermediates in the plant defense response." Proceedings of the National Academy of Sciences **99**: 517-522.
Torres, M. A., J. D. Jones, J. Dangl (2006). "Reactive oxygen species signaling in response to pathogens." Plant Physiology **141**: 373-378.
Truitt, C. L., H.-X. Wei, P.Pare (2004). "A plasma membrane protein from Zea mays binds with the herbivore elicitor volicitin." Plant Cell **16**: 523-532.
Truman, W., M. T. Zabala, M. Grant (2006). "Type III effectors orchestrate a complex interplay between transcriptional networks to modify basal defence responses during pathogenesis and resistance." Plant Journal **46**: 14-33.
Tsuda, K. and F. Katagiri (2010). "Comparing signaling mechanisms engaged in pattern-triggered and effector-triggered immunity." Current Opinion in Plant Biology **13**: 459-465.
Tsuwamoto, R., H. Fukuoka, Y. Takahata (2008). "GASSHO1 and GASSHO2 encoding a putative leucine-rich repeat transmembrane-type receptor kinase are essential for the normal development of the epidermal surface in Arabidopsis embryos." Plant Journal **54**: 30-42.
Turlings, T., J. H. Loughrin, P. McCall, U. Rose, J. Lewis, J. Tomlinson (1995). "How caterpillar-damaged plants protect themselves by attracting parasitic wasps." Proceedings of the National Academy of Sciences **92**: 4169-4174.
Turlings, T. C., H. T. Alborn, J. Loughrin, J. Tomlinson (2000). "Volicitin, an elicitor of maize volatiles in oral secretion of Spodoptera exigua: isolation and bioactivity." Journal of Chemical Ecology **26**: 189-202.
Turlings, T. C., P. J. McCall, H. Alborn, J. Tomlinson (1993). "An elicitor in caterpillar oral secretions that induces corn seedlings to emit chemical signals attractive to parasitic wasps." Journal of Chemical Ecology **19**: 411-425.
Ueki, N., K. Someya, Y. Matsuo, K. Wakamatsu, H. Mukai (2007). "Cryptides: functional cryptic peptides hidden in protein structures." Peptide Science **88**: 190-198.
Van der Weerden, N. L. and M. A. Anderson (2012). "Plant defensins: common fold, multiple functions." Fungal Biology Reviews.
van Loon, L. C., B. P. Geraats, H. Linthorst (2006). "Ethylene as a modulator of disease resistance in plants." Trends in plant science **11**: 184-191.
van Loon, L. C., M. Rep, C. M. Pieterse (2006). "Significance of inducible defense-related proteins in infected plants." Annual review of phytopathology **44**: 135-162.
Verhage, A., I. Vlaardingerbroek, C. Raaymakers, N. Van Dam, M. Dicke, S. Van Wees, C.M Pieterse (2011). "Rewiring of the jasmonate signaling pathway in Arabidopsis during insect herbivory." Frontiers in plant science **2**.
Verica, J. A., L. Chae, H. Tong, P. Igmire, Z.H He (2003). "Tissue-specific and developmentally regulated expression of a cluster of tandemly arrayed cell wall-associated kinase-like kinase genes in Arabidopsis." Plant Physiology **133**: 1732-1746.
Vlot, A. C., D. F. Klessig, S. Park(2008). "Systemic acquired resistance: the elusive signal(s)." Current Opinion in Plant Biology **11**: 436-442.
Walters, D. and M. Heil (2007). "Costs and trade-offs associated with induced resistance." Physiological and Molecular Plant Pathology **71**: 3-17.
Walters, D. R. and N. McRoberts (2006). "Plants and biotrophs: a pivotal role for cytokinins?" Trends in Plant Science **11**: 581-586.

Wan, J. R., X. C. Zhang, D. Neece, K. Ramonell, S. Clough, S. Kim, M. Stacey, G. Stacey (2008). "A LysM receptor-like kinase plays a critical role in chitin signaling and fungal resistance in Arabidopsis." Plant Cell **20**: 471-481.

Wang, G. D. and M. Fiers (2010). "CLE peptide signaling during plant development." Protoplasma **240**: 33-43.

Wang, J., A. Replogle, R. Hussey, T. Baum, X. Wang, E. Davis, M. Mitchum (2011). "Identification of potential host plant mimics of CLAVATA3/ESR (CLE)-like peptides from the plant-parasitic nematode Heterodera schachtii." Molecular Plant Pathology **12**: 177-186.

Wang, X., U. Kota, K. He, K. Blackburn, J. Li, M. Goshe, S. Huber, S. Clouse (2008). "Sequential transphosphorylation of the BRI1/BAK1 receptor kinase complex impacts early events in brassinosteroid signaling." Developmental Cell **15**: 220-235.

Wang, X., M. G. Mitchum, B. Gao, C. Li, H. Diab, T. Baum, R. Hussey, E. Davis (2005). "A parasitism gene from a plant-parasitic nematode with function similar to CLAVATA3/ESR (CLE) of Arabidopsis thaliana." Molecular Plant Pathology **6**: 187-191.

Wang, Z.-Y., H. Seto, S. Fujioka, S. Yoshida, J. Chory (2001). "BRI1 is a critical component of a plasma-membrane receptor for plant steroids." Nature **410**: 380-383.

Wasternack, C. (2007). "Jasmonates: An update on biosynthesis, signal transduction and action in plant stress response, growth and development." Annals of Botany **100**: 681-697.

Wasternack, C., I. Stenzel, B. Hause, G. Hause, C. Kutter, H. Maucher, J. Neumerkel, I. Feussner, O. Miersch (2006). "The wound response in tomato-role of jasmonic acid." Journal for Plant Physiology **163**: 297-306.

Whitham, S. A., S. Quan, H. Chang, B. Cooper, B. estes, T. Zhu, X. Wang, Y. Hou (2003). "Diverse RNA viruses elicit the expression of common sets of genes in susceptible Arabidopsis thaliana plants." Plant Journal **33**: 271-283.

Willmann, R., H. M. Lajunen, G. Erbs, M.A. Newman, D. Kolb, K. Tsuda, F. Katagiri, J. Fliegmann, J.J. Bono, J. Cullimore (2011). "Arabidopsis lysin-motif proteins LYM1 LYM3 CERK1 mediate bacterial peptidoglycan sensing and immunity to bacterial infection." Proceedings of the National Academy of Sciences **108**: 19824-19829.

Wu, J. and I. T. Baldwin (2010). "New insights into plant responses to the attack from insect herbivores." Annual Review of Genetics **44**: 1-24.

Wu, J. Q., C. Hettenhausen, S. Meldau, I. T. Baldwin (2007). "Herbivory rapidly activates MAPK signaling in attacked and unattacked leaf regions but not between leaves of Nicotiana attenuata." Plant Cell **19**: 1096-1122.

Yamaguchi, Y., G. Barona, C. Ryan, G. Pearce (2011). "GmPep918, an Eight-Amino Acid Peptide Isolated from Soybean Leaves, Activates Defense-Related Genes." Plant Physiology **156**: 932-942.

Yamaguchi, Y. and A. Huffaker (2011). "Endogenous peptide elicitors in higher plants." Current Opinion in Plant Biology **14**: 351-357.

Yamaguchi, Y., A. Huffaker, A. Bryan, F. Tax, C. Ryan (2010). "PEPR2 is a second receptor for the Pep1 and Pep2 peptides and contributes to defense responses in Arabidopsis." Plant Cell **22**: 508-522.

Yamaguchi, Y., G. Pearce, C. Ryan (2006). "The cell surface leucine-rich repeat receptor for AtPep1, an endogenous peptide elicitor in Arabidopsis, is functional in transgenic tobacco cells." Proceedings of the National Academy of Sciences **103**: 10104-10109.

Yan, J. B., C. Zhang, M. Gu, Z. Bai, W. Zhang, T. Qi, Z. Cheng, W. Peng, H. Lou, F. Nan, Z. Wang, D. Xie (2009). "The Arabidopsis CORONATINE INSENSITIVE1 Protein Is a Jasmonate Receptor." Plant Cell **21**: 2220-2236.

Yang, D.-H., C. Hettenhausen, I.T Baldwin, J. Wu (2011). "BAK1 regulates the accumulation of jasmonic acid and the levels of trypsin proteinase inhibitors in Nicotiana attenuata's responses to herbivory." Journal of Experimental Botany **62**: 641-652.

Yang, D.-H., C. Hettenhausen, et al. I.T Baldwin, J. Wu (2011). "The multifaceted function of BAK1/SERK3: Plant immunity to pathogens and responses 1 to insect herbivores." Plant Signaling & Behavior **6**: 1322-1324.

Yang, H., X. Gou, D. Xi, J. Du, H. Lin, J. Li (2010). "BAK1 and BKK1 in Arabidopsis thaliana confer reduced susceptibility to turnip crinkle virus." European Journal of Plant Pathology **127**: 149-156.

Yang, H., Y. Matsubayashi, H. Hanai, Y. Sakagami (1999). "Oryza sativa PSK gene encodes a precursor of phytosulfokine-α, a sulfated peptide growth factor found in plants." Proceedings of the National Academy of Sciences **96**: 13560-13565.

Yang, H., Y. Matsubayashi, K. Nakamura, Y. Sakagami (2001). "Diversity of Arabidopsis genes encoding precursors for phytosulfokine, a peptide growth factor." Plant Physiology **127**: 842-851.

Zavala, J. A., A. G. Patankar, K. Gase, D. Hui, I.T. Baldwin (2004). "Manipulation of endogenous trypsin proteinase inhibitor production in Nicotiana attenuata demonstrates their function as antiherbivore defenses." Plant Physiology **134**: 1181-1190.

Zhu, J.-Y., J. Sae-Seaw, Z.Y. Wang (2013). "Brassinosteroid signalling." Development **140**(8): 1615-1620.

Zipfel, C. (2013). "Combined roles of ethylene and endogenous peptides in regulating plant immunity and growth." Proceedings of the National Academy of Sciences **110**: 5748-5749.

Zipfel, C. and G. Felix (2005). "Plants and animals: a different taste for microbes?" Current Opinion in Plant Biology **8**: 353-360.

Zipfel, C., G. Kunze, D. Chinchilla, A. Caniard, J.D. Jones, T. Boller, G. Felix (2006). "Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation." Cell **125**: 749-760.

Zipfel, C., S. Robatzek, L. Navarro, E. Oakeley, J.D. Jones, G. Felix, T. Boller (2004). "Bacterial disease resistance in Arabidopsis through flagellin perception." Nature **428**: 764-767.

Zvereva, A. and M. Pooggin (2012). "Silencing and innate immunity in plant defense against viral and non-viral pathogens." Viruses **4**: 2578-2597.

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- Flury P*, Klauser D*, Schulze B, Boller T and Bartels S **(2013)** The Anticipation of Danger: MAMP Perception Enhances AtPep-triggered Oxidative Burst. **Plant Physiology**.

- Klauser D, Flury P, Boller T and Bartels S **(2013)** Several MAMPs, including chitin fragments, enhance *At*Pep-triggered oxidative burst independently of wounding. **Plant Signaling and Behaviour.**

- Kørner C*, Klauser D*, Niehl A, Heinlein M, Boller T and Hann D **(2013)** The immunity regulator BAK1 contributes to resistance against diverse RNA viruses. **Molecular Plant-Microbe Interactions.**

- Bartels S, Lori M, Mbengue M, van Verk M, Klauser D, Hander T, Böni R, Robatzek S and Boller T **(2013)** The family of AtPeps and their precursors in Arabidopsis: differential expression and localization but similar induction of pattern-triggered immune responses. **Journal of Experimental Botany**.

- Klauser D, Desurmont G, Glauser G, Vallat A, Flury P, Boller T, Turlings T, Bartels S **(2013)** The Arabidopsis AtPep-PEPR danger detection system is induced by Spodoptera littoralis oral secretions to mediate defense responses against herbivores. **The Plant Journal (submitted).**

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