

Functional Genomic Studies of Novel Players in Innate

Immunity and Classification of Promoter Activation

Patterns of the *AtPROPEP* Genes and their

Corresponding Receptors in the Model Plant

Arabidopsis thaliana

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Dedicated to my Parents

for their love, endless support and sincere encouragement.

Summary

The first layer of innate immunity in plants is initiated through the perception of microbe-associated molecular patterns (MAMPs) or damage-associated molecular patterns (DAMPs) by pattern recognition receptors. MAMP/DAMP perception initiates downstream defense responses, a process which ultimately leads to pattern triggered immunity, as reviewed in the first chapter of this thesis.

In the second chapter of this thesis, based on a deep-sequencing expression profiling approach, a number of hitherto overlooked genes have been identified that are induced in wild type *Arabidopsis* seedlings upon treatment with both the MAMP, flg22, and the DAMP, *AtPep1*. This implies the possible involvement of the corresponding gene products in innate immunity. Four of them, named PP2-B13, ACLP1, SERP1 and GRP89, respectively, were studied in more detail. Homozygous mutant lines for the genes encoding these proteins were obtained and analyzed. The mutants *pp2-b13*, *aclp1*, *serp1* and *grp89* exhibited an increased susceptibility to infection by the virulent pathogen *P. syringae* pv. *tomato* DC3000 and also by its avirulent *hrcC* mutant. Furthermore, it was observed that the *aclp1* mutant was deficient in ethylene production upon flg22 treatment, while the mutants *pp2-b13*, *serp1* and *grp89* were deficient in reactive oxygen species production.

As mentioned, in addition to MAMPs, plants can sense and recognize DAMPs, i.e. endogenous elicitors which activate the immune system in response to biotic and also abiotic stimuli. So far, eight peptides have been described as DAMPs or endogenous danger peptides in *Arabidopsis thaliana*, named *AtPeps1-8*. These peptides are derived from precursor proteins called the *AtPROPEPs*. The leucine-rich-repeat receptor kinases, *AtPEPR1* and *AtPEPR2*, act as the receptors for the *AtPep* peptides. In the third chapter of this thesis, promoter-GUS reporter constructs were used to study the expression pattern of the genes encoding the

*At*PROPEPs as well as the *At*PEPRs under biotic and abiotic stress, including *At*Pep1, flg22, Methyl jasmonate, and NaCl treatments. We found that the genes for the two *At*PEPR receptors were differentially regulated in response to MAMPs (flg22) and DAMPs (*At*PEP1). In addition, we showed that the activation pattern of the genes encoding the eight *At*PROPEPs was totally different, despite the similarity of the members of the Pep family. This allowed us to classify the activity of the *At*PROPEP promoters, based on their differential response to biotic and abiotic stimuli.

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1. An Introduction to Plant Immunity

The plant innate immune system is a sophisticated and highly coordinated multi-layered defense system under the tight regulation and control of many genes to protect the host from infection by potential pathogens (Dangl *et al.*, 2013). Plant defense is built on the combination of two basic strategies: "passive" structural and chemical barriers that are pre-formed and prevent entry and spreading of potential pathogens constitutively, and an "active" innate immunity system, which is based on inducible defense responses (Bigeard *et al.*, 2015; Cecchini *et al.*, 2015; Serrano *et al.*, 2014; Spoel and Dong, 2012). The front lines of the passive defense system against pathogen infections are pre-formed physical barriers or chemical secretion, including the cuticle layer with its waxes, the plant cell wall and pre-formed antimicrobial secondary metabolites. The presence of these pre-formed barriers stop microbes' entries whether these microbes are pathogenic or not. Those microbes which can overcome these passive barriers but cannot overcome the early defense response from the plants are called "Non-host pathogens"(Mishina and Zeier, 2007; Nurnberger, *et al.*, 2005).

Nevertheless, some of the potentially pathogenic microbes are able to overcome these pre-formed physical or chemical barriers. In other words, these passive defensive systems are not enough to totally protect the host against all pathogenic microorganisms. Thus, in the co-evolution of host-microbe interactions, plants have gained a highly effective inducible innate immunity system to protect themselves against potential attack by microbial pathogens (Chisholm *et al.*, 2006; Jones and Dangle, 2006).

The innate immune system can be activated in two conceptually different ways, as PTI ("pattern-triggered immunity") or as ETI ("effector-triggered immunity" (Jones and Dangle, 2006).

PTI is activated a result of recognizing a variety of evolutionarily highly conserved nonspecific elicitors. These are signature components of microbes termed as microbe-associated molecular

patterns (MAMPs). They are perceived by specific plasma membrane localized receptors called pattern recognition receptors (PRRs). Receptor complex activation as the consequence of the MAMP perception is one of the important key aspects of the innate immune system. (Boller and Felix 2009). Initially, Jones and Dangle (2006) called the molecules recognized by the plant "pathogen-associated molecular patterns (PAMPs)". Later, it was realized that this was not the most accurate term, because PAMPs would reflect that they come only from pathogens, while it seems that they come from all kinds of bacteria and fungi (e.g. flagellin from bacteria and chitin from all kinds of fungi). Therefore, PAMPs were renamed into MAMPs which is more fitting to what is really happening (Boller and Felix, 2009).

This triggered defense mechanism is also called the basal defense (Grennan 2006; Dodds and Rathjen, 2010). Over the last decade, there have been tremendous efforts for better understanding of the MAMPs and the cognate plant receptors that recognize them (Boller and Felix 2009; Segonzac and Zipfel, 2011; Zipfel 2014).

PTI is just the first level of defense against potential pathogens, which refers to general defense responses in plants as a consequence of the perception of unspecific signals from microbes by PRR (Nicaise *et al.*, 2009). Apart from PTI, there is another layer of plant innate immunity against invading agents, called effector-triggered immunity, which shows remarkable robustness against pathogens that can overcome PTI (Cui *et al.*, 2015). ETI is a more specific defense response compared to PTI, because it is triggered by specific effectors produced only by a specific pathogen (Jones and Dangle, 2006; Cui *et al.*, 2015).

A variety of gram-negative plant bacteria such as *P. syringae* and also some animal bacterial pathogens have a gene cluster called *hrp* (for "hypersensitive response and pathogenicity"; Hueck, 1998; Cornelis and Van Gijsegem, 2000); These genes encode a type III secretion system (T3SS) or Hrp system that allows bacteria to inject specific proteins, called effectors, into plant (or animal) cells (Alfano and Collmer, 1997); both pathogenic bacteria and symbiotic

bacteria are able to deliver such effector molecules into the host cells. The principal role of these is to neutralize or overcome PTI (Cui *et al.*, 2015; He *et al.*, 2006). However, plants have evolved so-called resistance genes (R genes), the products of which (R proteins) recognize specific effector proteins of a given aggressive pathogen, directly or indirectly. The ETI response is the molecular basis for classic gene-for-gene theory of plant pathology (Boller and He, 2009; Gohre and Robatzek, 2008; Gassmann and Bhattacharjee, 2012; Nicaise *et al.*, 2009; Van der Biezen, and Jones, 1998). The ETI response at the site of the infection is usually more robust than the PTI response and often culminates in programmed cell death, which is called the hypersensitive response (HR) (Boller and He 2009; Jones and Dangl 2006).

1.1 MAMPs are Sensed by Plants

Well-studied MAMPs are flagellin, prokaryotic elongation factor-Tu (EF-Tu), lipopolysaccharide (LPS) of Gram-negative bacteria; glucans and glycoproteins from oomycetes, and chitin of fungal pathogens (Boller and Felix, 2009).

One of the best characterized MAMPs is flagellin (Boller and Felix, 2009; Felix *et al.*, 1999), Flagellin is the building block of flagellum, an important structure for bacterial motility (Zipfel and Felix, 2005). Flagellins from different bacteria have evolutionarily highly conserved N- and C-terminal region but their central regions are hyper-variable (Figure 1-1; Felix *et al.*, 1999). The N- and C-terminal regions are needed for filament architecture and also motility functions, while the hyper-variable region is at the surface of the flagellum (Figure. 1-2; Ramos *et al.*, 2004; Yonekura *et al.*, 2003; Zipfel and Felix, 2005).

Plant recognize a highly conserved stretch of 22 amino acids in the N-terminus of flagellin, called flg22 (Felix *et al.*, 1999; Figure 1-1). Using flg22 as a model, many mechanistic details have been unveiled in understanding the signaling components and events as a consequence of PTI (Block and Alfano, 2011; Segonzac and Zipfel, 2011).



Figure 1-1. Alignment of N-terminal sequences of eubacterial flagellin sequences. Schematic representation of flagellin gene structure with conserved N- and C-terminal sequences and a variable middle part (top). Flg22 is highlighted (Adopted from Felix *et al.*, 1999).

Importantly, MAMP responses are effective in limiting pathogen growth, as it is observed that pre-treatment of Arabidopsis with flg22 (a peptide derived from flagellin) strongly reduces growth of *P. syringae* pv. *tomato* DC3000 (*Pst*DC3000), a virulent pathogen of this model plant (Zipfel *et al.*, 2004).

1.1.1 Receptor-like kinases (RLKs)

Transmembrane proteins with versatile N-terminal extracellular signal domains and a C-terminal intracellular kinase domain are called RLKs. As one of the largest gene families, RLKs encompass 610 members in *Arabidopsis thaliana* (Shiu and Blecker, 2001; Torii, 2004). Some of these RLKs are involved in a wide variety of developmental process, such as CLAVATA1 which regulates meristem and also flower development in response to the endogenous plant peptide clv3, which directly binds to the receptor (Clark *et al.*, 1993; Ogawa *et al.*, 2008). Others, like FLS2 (Boller and Felix, 2009), have a role in defense against bacterial pathogens.

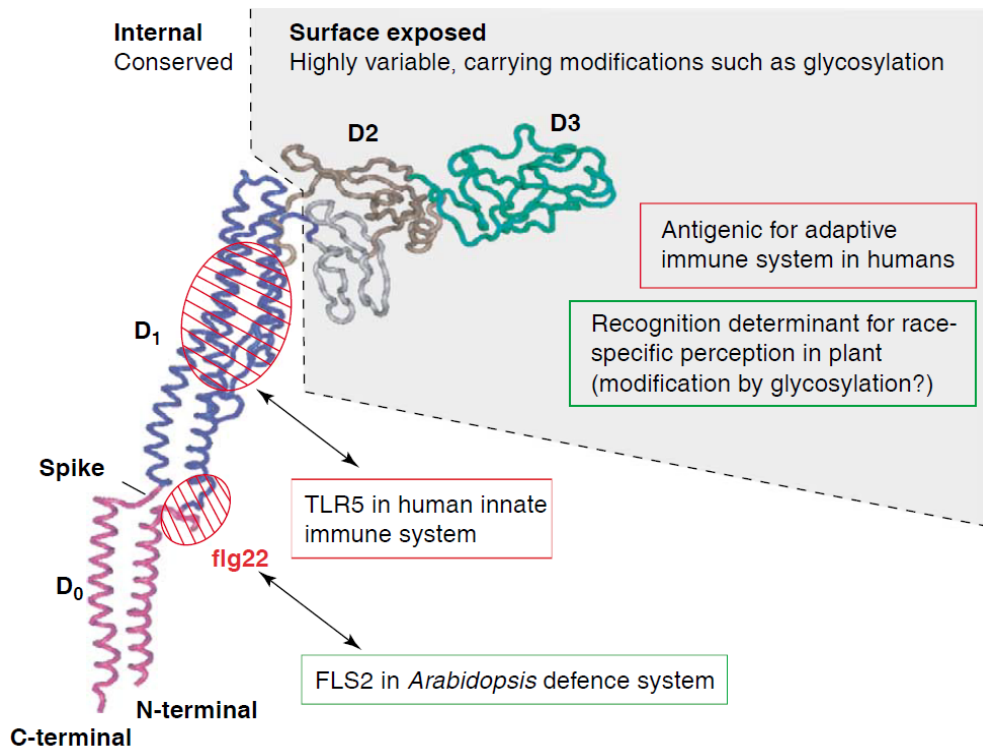


Figure. 1-2. Structure of a flagellin monomer from *Salmonella*. The regions that can be recognized by FLS2 and TLR5, respectively, are shown in red color (Zipfel and Felix, 2005).

In addition, RLKs have roles in hormone perception, the wounding response, and also in symbiosis (Torii, 2012). Many of the RLKs, such as CLV1 and FLS2 (Flagellin-Sensitive 2), contain an extracellular Leucine-rich repeat (LRRs) domain which interact with the stimulus to be recognized (Kobe and Deisenhofer, 1995; Torii, 2004; Zipfel 2014). Remarkably, studies show that Leucine-rich repeat receptor like kinases (LRR-RKs) can act as dimers; some may form a receptor complex with leucine-rich repeat receptor-like proteins (LRR-RPs) that lack a cytoplasmic kinase domain (Torii, 2004; Zipfel, 2014).

The most well-known PRRs (FLS2, EFR, CERK1, which recognize flg22 (of flagellin), the bacterial epitopes elf18 (of EF-Tu) and fungal chitins, respectively) belong to the LRR-RLKs; they are considered as a major component of multiprotein complexes at the plasma membrane, which contain additional transmembrane proteins required for the triggering and specification of immune signaling (Macho and Zipfel, 2014). PRR complexes are under the tight control and regulation by different protein phosphatases, E3 ligases, and also other regulatory elements, demonstrating the complex regulation of these molecular machines (Greeff *et al.*, 2012).

1.1.2 Receptor-like proteins (RLPs)

Transmembrane proteins with extracellular LRRs and a short cytoplasmic tail, which lack an intracellular signaling domain are called RLPs. So far, 57 RLPs were identified in Arabidopsis (Torri *et al.*, 2004). Members of this group of receptors have roles in different developmental process in Arabidopsis including growth, development and defense (Shiu and Bleecker, 2003). Since RLPs lack a signaling, or interaction domain in their intracellular region, they appear to work mostly in conjunction with RLKs (Macho and Zipfel, 2014; Shiu and Bleecker, 2003).

1.1.3 PRRs Perceive Conserved Molecular Signatures to Initiate PTI

Perception of MAMPs by plant cell surface PRRs leads to the activation of downstream defense responses including ion fluxes across the plasma membrane (e.g. increase in Ca^{2+} influx) in 30 seconds to 2 minutes; oxidative burst produced by the NADPH oxidase encoded by *AtrbohD* that is started in 2-3 minutes after flg22 perception and reach to the peak in 10-14 minutes (Bigeard *et al.*, 2015). Reactive oxygen species (ROS) production is needed for deposition of callose at the cell wall (Gomez-Gomez and Boller, 2000; Zhang *et al.*, 2007). Within a few minutes after flg22 perception, signal transduction via mitogen-activated protein kinase cascades is activated (Asai *et al.*, 2002; Felix *et al.*, 1999). Within an hour, stomatal closure is initiated, and also ethylene (ET) biosynthesis through activation of 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS; Liu *et al.*, 2004). Increased accumulation of plant defense salicylic acid (SA) hormone also occurs later, within a few hours, as a consequence of flg22 perception (Colcombet and Hirt, 2008; Dunning *et al.*, 2007; Melotto *et al.*, 2006; Navarro *et al.*, 2004; Nicaise *et al.*, 2009; Tsuda *et al.*, 2008). Furthermore, reprogramming the transcription of many genes was found to occur after around 30 minutes upon flg22 perception (Bigeard *et al.*, 2015; Zipfel *et al.*, 2004).

The best-studied and best-characterized PRRs are the flagellin receptor FLS2 (Gómez-Gómez and Boller, 2000) and EF-Tu receptor EFR from *Arabidopsis* (Zipfel *et al.*, 2004), the chitin receptors CERK1 and CEBiP from *Arabidopsis* and rice, respectively, and Xa21 that recognizes Ax21 (activator of Xa21-mediated immunity) from *Xanthomonas* species and related genera (Monaghan and Zipfel, 2012). FLS2 from the model plant *Arabidopsis* and Xa21 from rice are both RLKs, and share sequence and structural similarities (Figure 1-3; Greeff *et al.* 2012; Lee *et al.*, 2009).

Based on recent biochemical, structural, and genetic studies, the elicitors are perceived by receptors through three major different ways (Macho and Zipfel, 2014). It is including

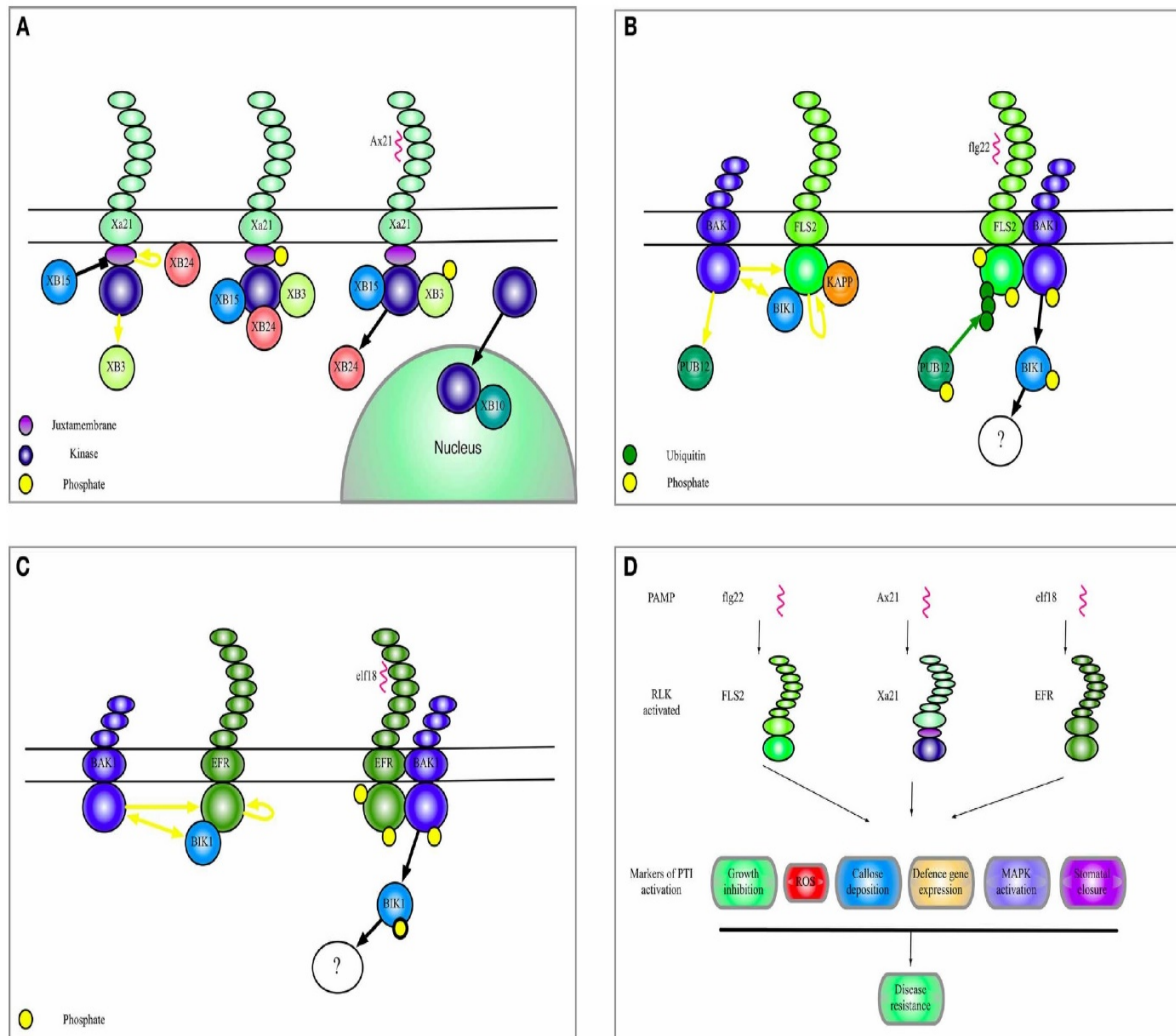


Figure 1-3. Complex formation of Xa21, FLS2, and EFR upon elicitor perception: **A)** A model to illustrate the interaction of co-receptors with Xa21. **B)** The complexes formed by the RLK FLS2 in flg22 perception. **C)** elf18 is perceived by RLK EFR. **D)** The subsequent effect of selected RLK activation. Yellow dots = phosphate groups; yellow arrows = phosphorylation of a substrate protein. Yellow blunt arrows = dephosphorylation of a substrate protein; Green dots and green arrows = ubiquitination; Black arrows = translocation, association, or dissociation (Greeff *et al.*, 2012).

homodimerization (Chitin perception in Arabidopsis results in homodimerization of AtCERK1 and the generation of an active receptor complex); heterodimerization (flagellin perception at the extracellular LRRs of FLS2 initiates the immediate formation of a stable heterodimer with the co-receptor BAK1 (BRI1-associated receptor kinase 1), in Arabidopsis) and heteromultimerization (chitin perception in rice), where a multimeric receptor formed by dimers of OsCEBiP and then OsCERK1, mediates chitin binding (Figure 1-4; Macho and Zipfel, 2014).

1.1.3.1 FLS2 is Responsible for Flagellin Perception

FLS2 is one of the well-studied PRRs that has been identified in nearly all plant species tested so far (Boller and Felix, 2009). It has an extracellular leucine-rich repeat domain, a single membrane-spanning domain and an intracellular serine/threonine kinase domain. The extracellular LRR domain of FLS2 recognizes the presence of bacterial flagellin and activates defense responses (Dunning *et al.*, 2007; Gomez-Gomez *et al.*, 2001). For proper perception of flg22 by FLS2, the co-receptor BAK1 is needed (Chinchilla *et al.*, 2007). BAK1 is a LRR-RLK that has a role in regulating the brassinosteroid receptor BRI1 (Li *et al.*, 2002; Nam *et al.*, 2002), but it is also involved in PRR-dependent signaling to initiate innate immunity (Chinchilla *et al.*, 2007). Remarkably, FLS2 and BAK1 form a complex *in vivo*, in a specific ligand-dependent manner (Chinchilla *et al.*, 2007). Furthermore, it has been observed that after perception of the flg22, heteromerization of FLS2 with BAK1 occurs almost instantaneously, which induces formation of a stable FLS2-BAK1 complex *in vitro* and is independent of kinase activity (Schulze *et al.*, 2010). It is worth noting that phosphorylation of the FLS2-BAK1 complex is associated with the heteromerization process which is a very quick and specific event that occurs in 15 seconds upon flg22 perception (Schulze *et al.*, 2010).

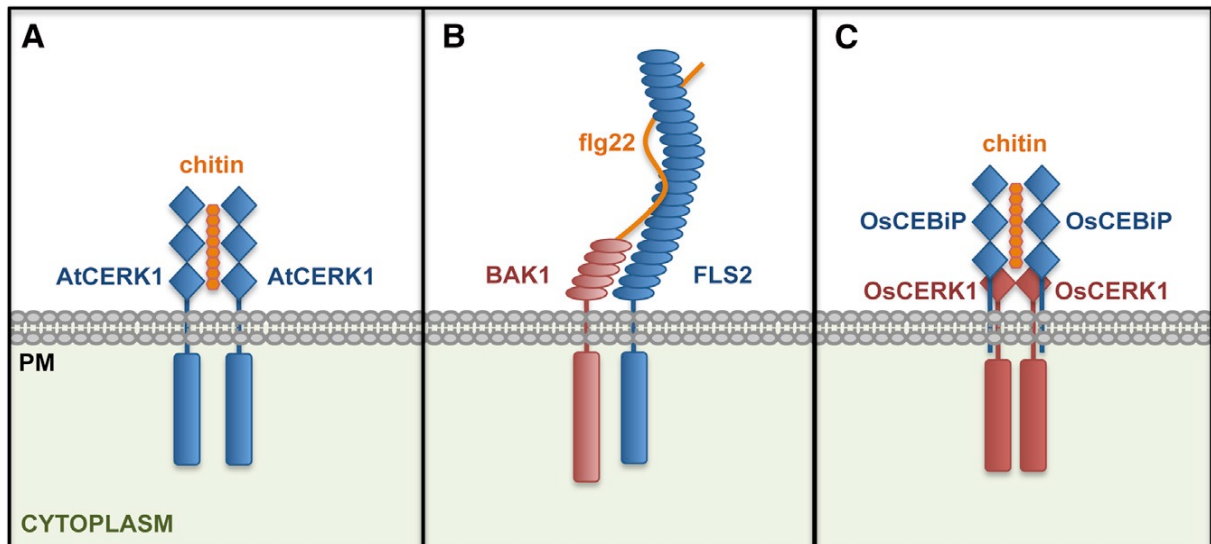


Figure 1-4. Ligand perception mediated by **A)** Heterodimerization (flagellin perception at the extracellular LRRs of FLS2); **B)** Homodimerization (Chitin perception in Arabidopsis); and **C)** Multimerization (Chitin perception in Rice) of Plant PRRs (Macho and Zipfel, 2014).

It has recently been observed that FLS2 interacts with E3 enzymes that polyubiquitinate the receptor after flg22 signaling (Gohre *et al.*, 2008). As it has been described for the mammalian Toll-like receptor 4 (TLR4) and TLR9, FLS2 is subsequently degraded by the proteasome (Chuang and Ulevitch, 2004) and in proteasome-mediated degradation of FLS2, PUB12 and PUB13 are involved (Lu *et al.*, 2011). It has been shown that plant U-Box 12 (PUB12) and PUB13, (both E3 ubiquitin enzymes) are BAK1 phosphorylation targets, and this modification process is needed for its association with FLS2 (Duplan and Rivas, 2014; Greeff *et al.*, 2012).

1.1.3.2 BAK1 Role in Plant Innate Immunity

BAK1 is a member of the somatic embryogenesis receptor kinase (SERK) family including five members named: SERK1, SERK2, BAK1/SERK3, BAK1-like (BKK1)/SERK4, and SERK5 (Dardick and Roland 2006; Shiu and Bleeker, 2003). Although FLS2 interacts with SERK1, SERK2, SERK5, and BKK, its predominant association, upon flg22 stimulation, is with the BAK1 protein. BAK1 was first identified and characterized as an RLK involved in brassinosteroid signaling via the receptor BRI1 (Li *et al.*, 2002). By now, it has confirmed that BAK1 is a common component in many RLK signaling complexes (Shan *et al.*, 2008).

Mutation or deletion of BAK1 leads to a reduction of flg22 and elf18 induced responses (Chinchilla *et al.*, 2007; Heese *et al.*, 2007). Based on these findings, it seems that BAK1 controls signaling initiated by several different Leucine-rich repeat receptor kinases (LRR-RKs) and probably represents a general regulatory adapter protein (Chinchilla *et al.*, 2007; Kemmerling *et al.*, 2007; Schwessinger and Zipfel, 2008). It has also been observed that the expression of BAK1 is up-regulated upon viral infection (Kørner *et al.*, 2013).

1.1.3.3 FLS2 Interacts with BAK1 in flg22 Sensing

As mentioned above, perception of flg22 leads to a close interaction of the FLS2 receptor with members of the SERK family, particularly with BAK1. Recently, the crystal structure of the FLS2-flg22-BAK1 ectodomain complex was determined at 3.06 Å and revealed information on FLS2-flg22-BAK1 interaction at the atomic level (Sun *et al.*, 2013).

This has been facilitated by the fact that the ectodomains of FLS2 and BAK1 are sufficient to form an flg22-induced complex (Sun *et al.*, 2013; Figure 5-1). It was shown that the structure of FLS2 is superhelical and flg22 binds to the concave surface of FLS2 by running across 14 LRRs (LRR3 to LRR16). It was observed that the heterodimerization of FLS2-BAK1 is both flg22 and receptor-mediated where flg22 was sandwiched between FLS2 and BAK1. The C-terminal segment of flg22 binds FLS2 and BAK1.

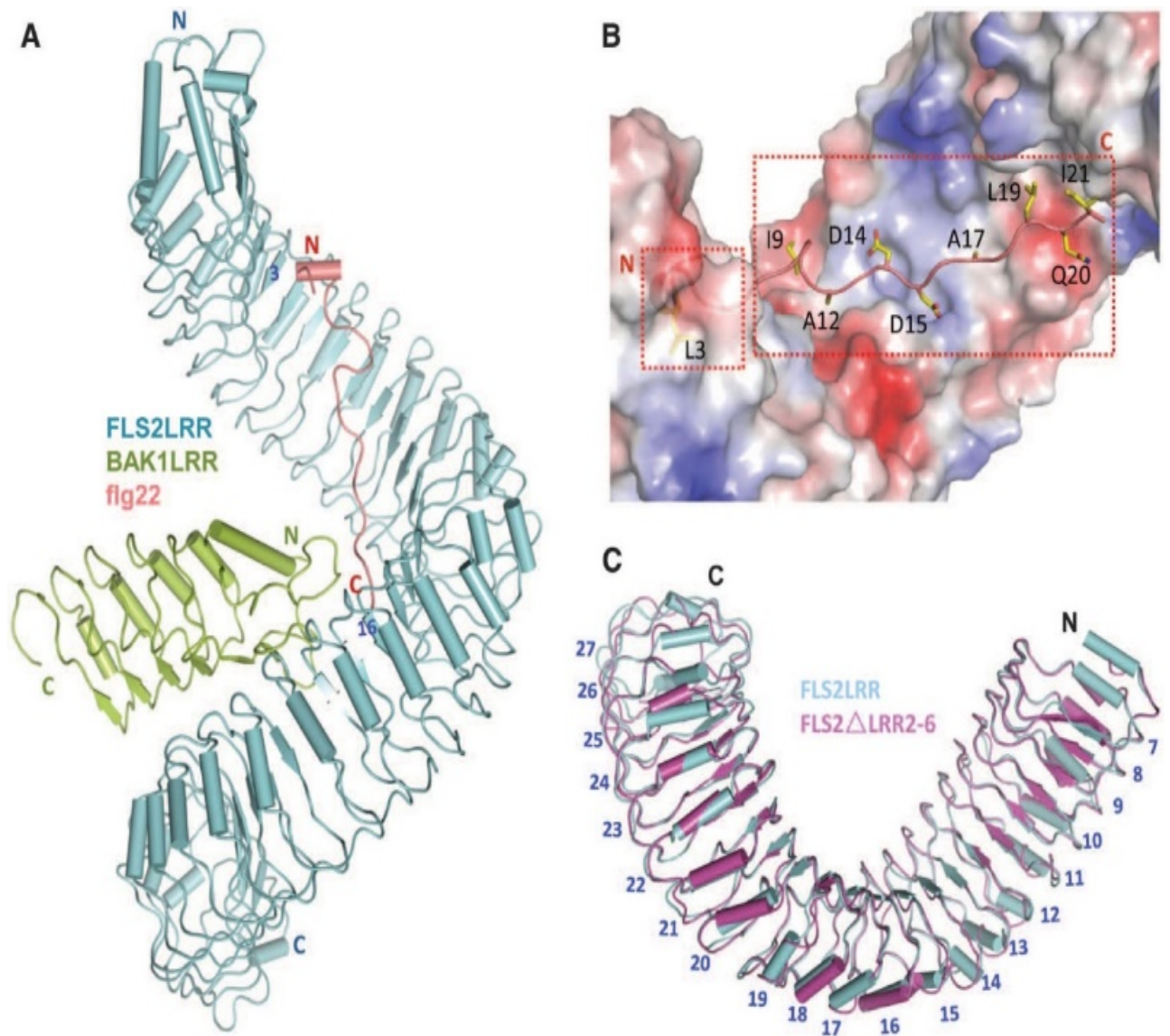


Figure 1-5. Ectodomains mediate the flg22-induced heterodimerization of FLS2 and BAK1. **A)** The structure of FLS2-flg22-BAK1 complex. The positions of LRR3 and LRR16 are indicated by blue numbers. “N” and “C” illustrate the N and C terminus, respectively. **B)** The shallow groove at the inner surface of FLS2LRR solenoid is the region that flg22 binds **C)** Structural comparison of the ligand-bound FLS2LRR with the free FLS2DLRR2-6 (Sun *et al.*, 2013).

The binding region of the Flg22 to the receptor is the shallow groove at the inner surface of the FLS2. Based on the data from crystal structure, the FLS2 recognizes both the C- and N-terminal region of flg22 by conserved and also a non-conserved site, respectively (Figure 1-5; Sun *et al.*, 2013).

1.1.3.4 Botrytis-induced Kinas 1 (BIK1) and its Role in flg22 Signal Transduction

One of the consequences of flg22 binding and FLS2-BAK1 heterodimer formation is the phosphorylation of BIK1, a cytoplasmic protein kinase associated with the activated receptor (Lu *et al.*, 2010). BIK1 is a member of the receptor-like cytoplasmic kinases which plays a significant role in early flagellin signaling from the FLS2/BAK1 receptor complex (Lu *et al.*, 2010). BIK1 phosphorylation leads to additional phosphorylation events in both FLS2 and BAK1 (Lu *et al.*, 2010). Releasing BIK1 activates downstream signaling components. Lu *et al.* (2010) proposed a model of BIK1 in flagellin signaling (Figure 1-6). Briefly, BIK1 is associated with FLS2 and BAK1 in an inactive state in the absence of flg22. In the presence of flg22, FLS2 and BAK1 become phosphorylated, and subsequently, activated BAK1 phosphorylates BIK1 protein and as a result transphosphorylates the FLS2–BAK1 complex; at the next step, the fully active FLS2–BAK1 complex phosphorylates BIK1. Then, active BIK1 is released from the FLS2–BAK1 complex to activate downstream intracellular signaling.

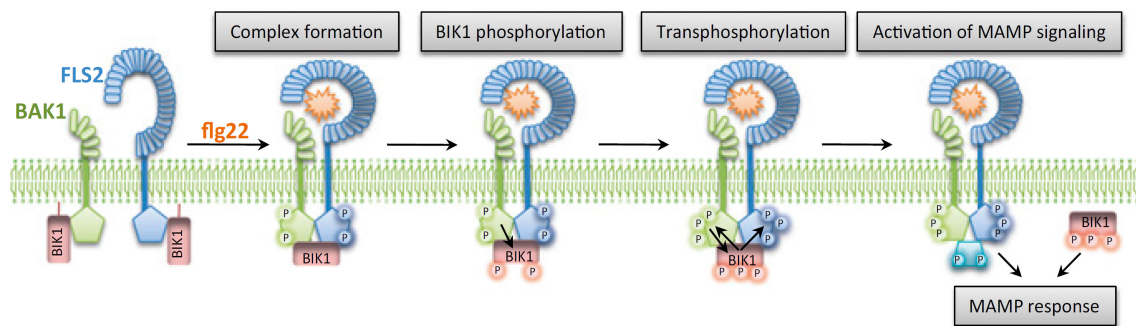


Figure 1-6. A proposed model of BIK1 in flg22 signaling (Lu *et al.*, 2010).

1.2 Endogenous Peptide Signals Can Activate Components of the Innate Immune System in *A. thaliana*

Plants and other multicellular organisms such as mammals possess a sophisticated system to monitor cellular integrity and to detect the presence of damaged cells (Bartels and Boller 2015; Yamaguchi and Huffaker, 2011; Figure 1-7). In plants as well as in mammals, this is based on the recognition of endogenous host derived elicitors, the so-called "Damage-associated molecular patterns" (DAMPs; Boller and Felix, 2009; Yamaguchi and Huffaker, 2011). Perception of DAMPs leads to the induction of similar defense responses as the perception of MAMPs, both in plants and in mammals (Boller and Felix, 2009; Heil and Land, 2014).

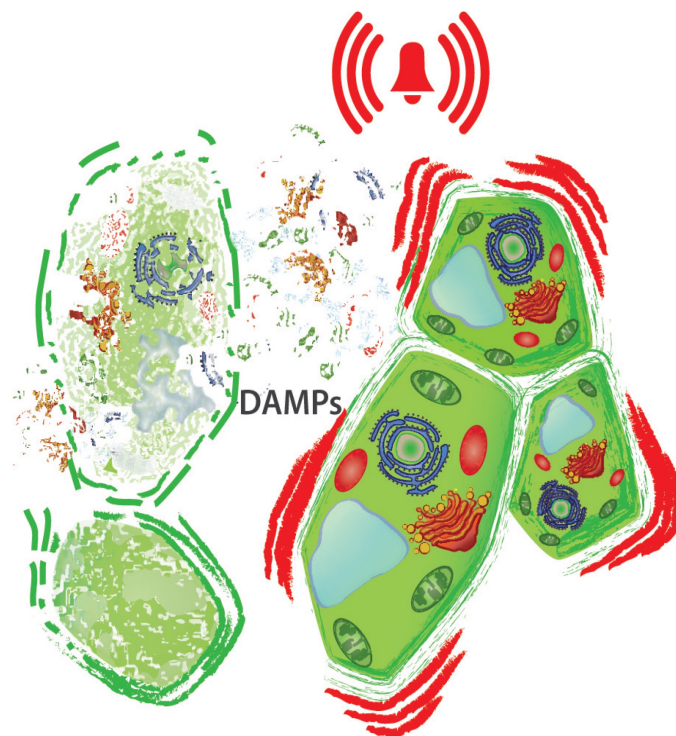


Figure 1-7. Damaged-self recognition. The damaged cells (**left**) releases intracellular molecules to the extracellular space which can serve as damage-associated molecular patterns (DAMPs) that function as a signal preparing the neighboring, intact cells (**right**) for enemy recognition and wound sealing (Heil and Land, 2014).

In 2006, a small peptide, called *AtPep1*, was isolated from an extract of wounded *A. thaliana* leaves, which could activate defense-related genes and also the synthesis of ROS (Huffaker *et*

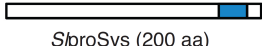

al., 2006). *AtPep1* is a 23-aa peptide from *Arabidopsis* which is derived from a 92-aa precursor proteins encoded within a small gene called *AtPROPEP1*. This gene is inducible by wounding and also methyl jasmonate (MeJA) treatment. In that research, they have also shown that *AtPROPEP1* gene has seven paralogues which are named *AtPROPEP1- AtPROPEP7*. Except *AtPROPEP6* which is located on chromosome 2, all the others are located in chromosome 1. They also have shown that the protein products of *AtPROPEPs* are conserved at the C-terminal region. In that research, they have presented evidence that *AtPROPEPs* have orthologs in other plant species. The discovery of the endogenous peptide signal *AtPep1* in *Arabidopsis* has opened a new field of plant innate immunity research, as reviewed recently (Bartels and Boller, 2015). Since endogenous peptide elicitors similar to the *AtPep* family have been identified in different species across the plant kingdom, it seems that they have been maintained over evolution (Bartels and Boller, 2015). They may play a role in regulating and balancing the immune system to attack by both pathogens and also herbivores (Yamaguchi and Huffaker, 2011). It has been proposed that DAMPs signaling (such as *AtPeps* in *A. thaliana*) intensifies or prolongs the stereotypical defense response triggered by MAMPs (Ross *et al.*, 2014). Thus, it seems that DAMPs are important for the fine-tuning of the defense response (Flury *et al.*, 2013; Logemann, *et al.*, 2013; Moore *et al.*, 2011).

1.2.1 Major Classes of Endogenous Peptide Elicitors in Plants

So far, several classes of plant-derived molecules, which elicit defense responses, have been identified (Yamaguchi and Huffaker, 2011). Endogenous peptide elicitors in plants are classified into different groups based on the structure of their precursor proteins, which include different processing mechanisms to release the active signal (Yamaguchi and Huffaker, 2011).

Yamaguchi and Huffaker (2011), classified these peptides in three major groups (Figure 1-8). The differences between the amino acid sequences of these endogenous peptide signals in different plant families and species indicate the diversity of receptor partners that perceive these elicitors and also show that there is a diversity in processing and also different export mechanisms for activation of these peptide signals in the cell (Yamaguchi and Huffaker, 2011).

(I) Peptides from precursor proteins without an N-terminal secretion signal

	Precursor protein	Peptide sequence	Characterized from
Systemins	 SproSys (200 aa)	AVQSKPPSKRDPPKMQTD S/Sys (18 aa)	Tomato (Pearce <i>et al.</i> , 1991). Pepper (Constabel <i>et al.</i> , 1998). Nightshade (Constabel <i>et al.</i> , 1998). Potato (Constabel <i>et al.</i> , 1998).
Pep	 AtPROPEP1 (92 aa)	ATKVKAKQRGKEKVSSGRPGQHN AtPep1 (23 aa)	Arabidopsis (Huffaker <i>et al.</i> , 2006). Maize (Huffaker <i>et al.</i> , 2011).

(II) Peptides from precursor proteins with an N-terminal secretion signal

HypSys	 NtpreproHypSys (165 aa)	RGANLPOOSOASSOOSKE NtHypSysI (18 aa) NRKPLSOOSOKPADGQRP NtHypSysII (18 aa)	Tobacco (Pearce <i>et al.</i> , 2001). Tomato (Pearce <i>et al.</i> , 2003). Petunia (Pearce <i>et al.</i> , 2007). <i>N. attenuata</i> (Heiling <i>et al.</i> , 2010). Black nightshade (Pearce <i>et al.</i> , 2009). Sweet potato (Chen <i>et al.</i> , 2008).
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(III) Cryptic peptides derived from proteins with separate primary functions

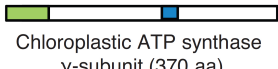
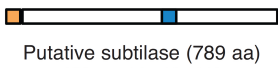
Inceptin	 Chloroplastic ATP synthase γ-subunit (370 aa)	*ICDINGVCVDA Inceptin (11 aa)	Fall armyworm larval oral secretion after ingestion of cowpea (Schmelz <i>et al.</i> , 2007).
SubPep	 Putative subtilase (789 aa) (Glyma18g48580)	NTPPRRAKSRPH GmSubPep (12 aa)	Soybean (Pearce <i>et al.</i> , 2010).

Figure 1-8. The five families of endogenous peptide elicitors in plants. Precursor protein and peptide sequences for each family are illustrated. Blue, orange and green boxes indicate positions of bioactive peptide, signal sequence for secretion and chloroplast localization signal, orderly. HypSys (hydroxyproline-rich systemin) are modified to contain hydroxyprolines (O in red) with pentose attachments, and inceptin contains a pair of cysteines (asterisk) that forms a disulfide bond; reference to each identified peptide elicitors in each individual plant is represented (Adopted from Yamaguchi and Huffaker, 2011).

(I) Peptides from Precursor Proteins Without an N-terminal Secretion Signal

The best example of this group is tomato systemin, which was the first endogenous peptide signal identified in plants (Pearce *et al.*, 1991). Systemin, a peptide with 18 aa residues, induces various defense responses in tomato leaves and cell cultures (reviewed in Boller and Felix, 2009). It is formed from the C-terminal domain of a 200-aa precursor protein (McGurl *et al.*, 1992). It has been claimed that the functional analogs of systemin in mammals are the cytokines; both are peptides or small proteins, both are induced by wounding and pathogens, and both are activating defense responses (Ryan and Pearce, 1998). Despite the tremendous effort to identify the receptor for systemin, finding the receptor was not successful; however, it has been speculated that the systemin receptor in tomato most likely belongs to the class of LRR-RLKs (Heil and Land, 2014). Recent studies showed that systemin is not the only peptide molecule that elicit defense responses; a large class of small peptide molecules which can trigger plant defense has been identified so far (Albert, 2013), which play different roles in the intact tissue (Bartels *et al.*, 2013). As mentioned, apart from systemin, a well-studied family of endogenous peptide elicitors, there are the *At*Peps from *A. thaliana*. They are derived from the family of *At*PROPEPs, which do not have an N-terminal secretion signal (Yamaguchi and Huffaker, 2011).

(II) Peptides from Precursor Proteins with an N-terminal Secretion Signal

In tobacco two 18-aa glycopeptides induces defense responses. These peptides are named NtHypSysI and NtHypSysII. They are hydroxyproline-rich systemins, and both are derived from the same precursor protein Ntprepro-HypSys (Pearce *et al.*, 2001), which carries an N-terminal secretion signal. Orthologs of these peptides also have been identified in other solanaceous plants (Pearce and Ryan, 2003; Pearce *et al.*, 2009).

Recently, through an in-silico approach another group of peptide signals in *A. thaliana* has been identified (Hou *et al.*, 2014), which have precursor proteins with an N-terminal secretion signal. These newly identified elicitor precursors are named the prePIP family. It has been shown that the active part of the protein, which induce immune responses and also pathogen resistance in *A. thaliana*, is the C-terminal conserved regions in prePIP1 and also prePIP2 (Figure 1-9; Hou *et al.*, 2014). PrePIP1 is secreted into the extracellular space and is cleaved at the C-terminus, which then triggers immune responses and subsequently enhances pathogen resistance in *A. thaliana*. Moreover, it has also been observed that PIP1 and Pep1 cooperate to amplify the immune responses triggered by MAMPs (Hou *et al.*, 2014), and based on genetic and biochemical analysis approaches, it has been suggested that the receptor-like kinase 7 (RLK7) can functions as a receptor of PIP1.

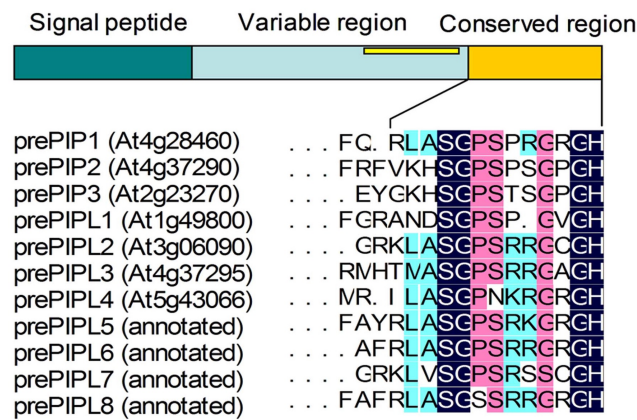


Figure 1-9. Schematic presentation of prePIP homologs in *A. thaliana* (Hou *et al.*, 2014).

(III) Cryptic Peptide Signals Derived from Proteins with Separate Primary Functions

The terms “cryptic peptides” is used to indicate the pool of peptides formed through the proteolytic action of peptidases on precursor proteins. Cryptic peptides may have totally different biological activities that can be discriminated from the function of their precursor proteins (Autelitano *et al.*, 2006; Duckworth *et al.*, 2004). Therefore, recently, biochemical

mechanisms, in which cryptic peptides are generated, have been investigated by many researchers; this opened up a new field in peptide studies (Bellia *et al.*, 2013; Samir and Link, 2011). Some cryptic peptides play an immunoregulatory role in mammalian systems (Ueki *et al.*, 2007). Many of them are produced in the maturation or degradation processes of functional proteins including mitochondrial enzymes and also regulatory proteins (Ueki *et al.*, 2007). Inceptin family peptides are the first cryptic peptides which were discovered to have a role in inducing immunity in plants. Inceptin is a disulfide-bridged peptide which has been isolated from oral secretions of *Spodoptera frugiperda* larvae; it elicits defense responses such as ET production and also leads to an increase in the levels of the phytohormones SA and jasmonic acid in *Vigna unguiculata* (Schmelz *et al.*, 2006; Schmelz *et al.*, 2007). The precursor of inceptin is a plant protein that is eaten by the insect larva. In general, inceptin is regarded as a potent indirect signal which is able to initiate specific plant responses to insect attacks (Yamaguchi and Huffaker, 2011).

Recently, Pearce *et al.*, (2010) identified a 12-aa peptide from soybean which can activate the expression of defense genes upon herbivory attack. Since it is derived from a member of the subtilisin-like protease (subtilase) family, it was named Glycine max Subtilase Peptide (GmSubPep). Perception of the peptide by its corresponding receptor leads to the initiation of defense signaling cascades. It has been also confirmed that the gene encoding GmSubPep was not induced by defense-related phytohormones or wounding and is constitutively expressed in all actively growing tissues (Pearce *et al.*, 2010; Yamaguchi and Huffaker, 2011).

1.2.2 The Family of AtPROPEPs Proteins and their Involvement in Innate Immunity

Despite the discovery of systemin as the first endogenous plant elicitor peptide, long time ago, Arabidopsis plant elicitor peptide1 (*AtPep1*) was the first endogenous peptide signal related to defense responses in Arabidopsis. It originates from a larger precursor protein encoded by the Arabidopsis gene named *AtPROPEP1* (Huffaker *et al.*, 2006; Yamaguchi and Huffaker,

2011). So far, eight AtPROPRPs have been identified as precursors of AtPeps in *A. thaliana* (AtPep1 to AtPep8; Bartels *et al.*, 2013; Huffaker *et al.*, 2006).

The length of AtPeps are 23-29-aa, and based on sequence homology comparison, all of them have a highly conserved amino acid motif SSGR/KxGxxN (Figure 1-10; Bartels *et al.*, 2013).

AtPep1 is derived from the C-terminus of a 92-aa precursor protein AtPROPEP1, and it has recently been shown that AtPROPEP1 is localized at the tonoplast (Bartels, *et al.*, 2013).

AtPep1 peptide comprises 23 amino acids, and the C-terminal region of this small protein

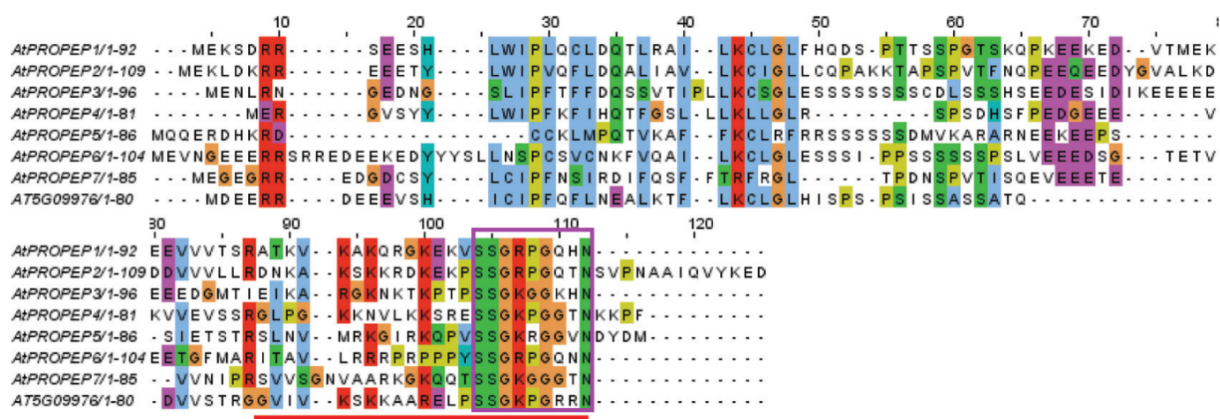


Figure 1-10. Amino acid alignment of the eight *Arabidopsis* AtPROPEPs. The red bar at the bottom highlights the 23-29-aa that are active to induce the defense response. The pink box shows the highly conserved motif among Arabidopsis AtPROPEPs (Adopted from Bartels *et al.*, 2013).

specifically binds to two receptors which are called AtPEPR1 (Pep-Receptor1) and AtPEPR2 (Pep-Receptor2) that subsequently activate downstream signaling cascades (Yamaguchi *et al.* 2006; Yamaguchi *et al.*, 2010). Although the activation of defense responses has been shown for all eight synthetic AtPeps, only AtPep1 and AtPep5 have been isolated from plant protein extracts (Bartels *et al.*, 2013; Yamaguchi and Huffaker 2011). It has been observed that the expression of *AtPROPEP1* gene is induced upon flg22 treatment, AtPep1 itself, wounding, ET, and also MeJA (Huffaker *et al.*, 2006). As shown in Figure 1-8, eight AtPROPEPs have been identified in Arabidopsis, so far (Bartels *et al.*, 2013); it still remains unclear whether these AtPROPEPs are redundant or if they have specialized roles and functions (Bartels and Boller, 2015).

Specific changes in gene expression play a crucial role in plant immunity, and expression studies indicate that transcript levels of numerous genes are changed concurrently upon pathogen perception (Eulgem, 2005), including leucine Zipper domain (bZIP), Myeloblastosis (MYB), Ethylene Responsive Factor (ERF) and *WRKY* gene families (Moore *et al.*, 2011). Interestingly, studies have shown that *AtPep* treatments of Arabidopsis plants induce the transcription of their own precursor genes and also defense genes (Bartels and Boller, 2015). Recently Logemann *et al.*, (2013) showed that *WRKY* transcription factors (TFs) are the major regulators of MAMP-induced *AtPROPEP2* and also *AtPROPEP3* gene expression.

These observations indicate that there is a positive feedback in the signaling pathways to generate additional processed peptides to up-regulate downstream defense responses (Ryan *et al.*, 2007).

Recently, a homolog of *AtPep1* called *ZmPep1* was identified in maize, and it was shown that it can regulate maize disease resistance responses (Huffaker *et al.*, 2011). Moreover, it was observed that *ZmPep3* regulates responses against attacks by herbivores and, expression of the *ZmPROPEP3* gene is rapidly induced by *Spodoptera exigua* oral secretions (Huffaker *et al.*, 2011).

This elicitor is so active that, in the concentration at 5 pM, it can stimulate the production of jasmonic acid, ET, and increased expression of genes encoding proteins associated with defense against herbivores.

These observations demonstrate that Peps are conserved signals even between dicots and monocots, and that they directly and indirectly regulate anti-herbivore defenses in both clades (Huffaker *et al.*, 2013): Pathogen infection induces gene expression of the precursor proteins in both Arabidopsis and maize. On the other hand, treating Arabidopsis with *AtPeps* induces expression of pathogen defense genes, such as PR-1 and PDF1.2 (Huffaker *et al.*, 2006) and

treating maize with *ZmPep1* induces PR-4 chitinase and SerPIN, which are protease inhibitors in maize (Huffaker *et al.*, 2011).

1.2.3 *AtPEPR1* and *AtPEPR2* Receptors Are Responsible for Arabidopsis Endogenous Peptide Signal (Peps) Perception and Contribute to Innate Immunity

A few months after discovering *AtPeps* family in Arabidopsis, using a photoaffinity labeling technique with synthetic homologs of *AtPep1*, a LRR-RK which is called *AtPEPR1*, identified as a receptor for *AtPeps* (Yamaguchi *et al.*, 2006). Later, *AtPEPR2* was identified and characterized as a second receptor for *AtPeps* (Krol *et al.*, 2010; Yamaguchi *et al.*, 2010). Like *AtPEPR1*, *AtPEPR2* is a plasma membrane LRR-RK; it has 76% amino acid similarity with *AtPEPR1* (Yamaguchi *et al.*, 2010). Phylogenetic studies show that *AtPEPRs* cluster in the subgroup XI of LRR-RLKs (Shiu *et al.*, 2004). This indicates a close phylogenetic similarity of *AtPEPRs* with several receptors involved in endogenous peptide signaling (Figure 1-11; Yamaguchi *et al.*, 2010).

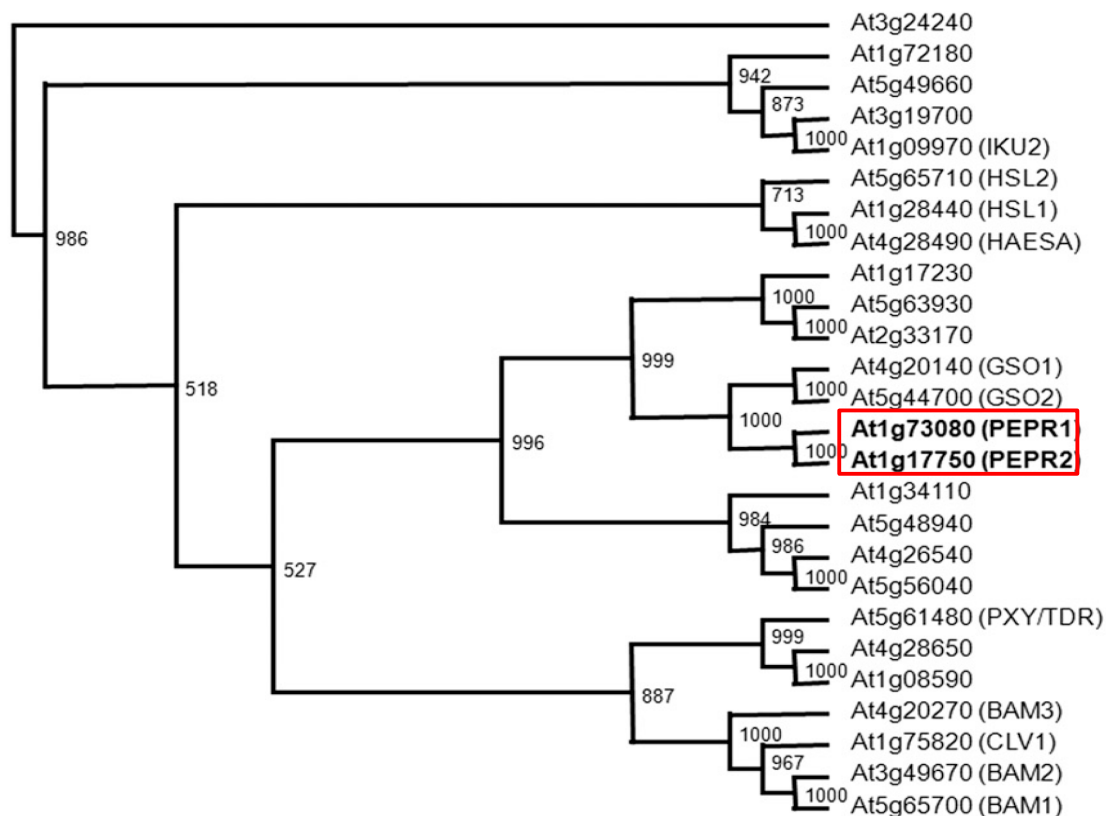


Figure 1-11. Phylogenetic analysis of the LRR XI subfamily of *Arabidopsis* LRR receptor protein kinases (Adopted from Yamaguchi *et al.*, 2010).

Both AtPEPR1 (AT1g73080) and AtPEPR2 (AT1g17750) belong to RLKs superfamily. *AtPEPR1* has 1123 amino acids while *AtPEPR2* contains 1088 amino acids. AtPEPR1 contains 28 LRR, whereas AtPEPR2 contains 26 LRR. Both have three domains including: an extracellular domain (29-769 amino acids positions for *AtPEPR1* and amino acids 27-739 for *AtPEPR2*); a helical transmembrane domain (amino acids 770-790 for *AtPEPR1* and amino acids 740-760 for *AtPEPR2*); and a cytoplasmic protein kinase domain (amino acids 791-1123 for *AtPEPR1* and amino acids 761-1088 for *AtPEPR2*).

In *AtPEPR1*, 833-841 amino acids are involved in nucleotide binding while in *AtPEPR2* the nucleotide binding site is within amino acids 800-808 and in both receptors there are two modified residues (amino acids or nucleotides that are derivatives of the standard amino acids or nucleotides are called modified residues; PDB term definition) at the cytoplasmic domain (Gou *et al.*, 2010; Pearce *et al.*, 2008; Postel *et al.*, 2010; Theologis *et al.*, 2000; Yamaguchi *et al.*, 2006). Remarkably, protein-protein interaction studies using the yeast two-hybrid assay, showed that *AtPEPR1* and *AtPEPR2*, interact with BAK1 (Postel *et al.*, 2010).

Binding assays using *AtPep* peptides and *AtPEPR1* and *AtPEPR2* indicate that *AtPEPR1* can perceive *AtPep1* to *AtPep6* while *AtPEPR2* can only perceive *AtPep1* and *AtPep2* (Yamaguchi *et al.*, 2010). Transcription of both *AtPEPR1* and *AtPEPR2* are up-regulated upon treatment with *AtPeps*, MAMP, wounding, and treatment with MeJA (Bartels *et al.*, 2013). However, it was shown experimentally that *AtPEPR1* is able to sense all eight *AtPeps*, whereas *AtPEPR2* can only recognize *AtPep1* and *AtPep2*.

These data provide evidence that *AtPEPR1* and *AtPEPR2* have differential responses to the *Pep* peptides, and therefore may have different roles in defense response signaling. However, the exact mechanisms underlying *Pep* peptides perception by *AtPEPR1* and *AtPEPR2* receptors and how they influence defense responses are largely unknown (Yamaguchi *et al.*, 2011).

Furthermore, amino acid sequence comparison between several LRR-RLKs (including AtPEPR1 and AtPEPR2) revealed a putative guanylyl cyclase (GC) catalytic domain in the AtPEPR1 and AtPEPR2 receptors; it seems that this region of the protein has GC activity, suggesting that AtPEPR1 may have a role in plants as a ligand activated GC (Figure 1-12; Ma *et al.*, 2012).

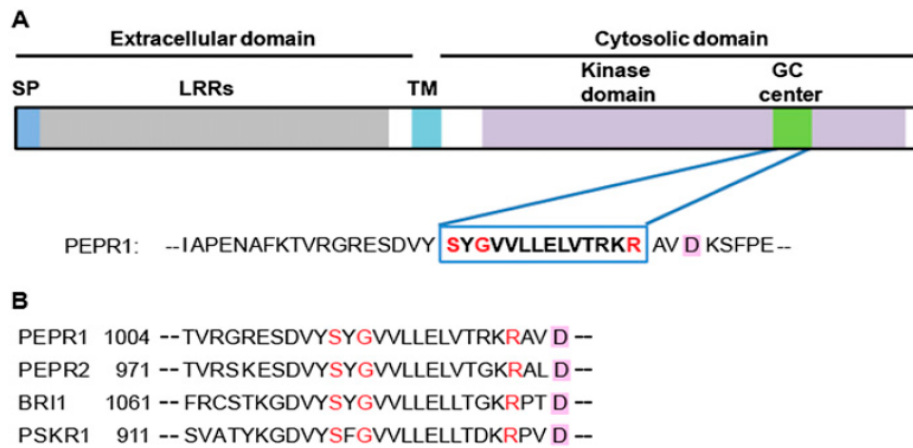


Figure 1-12. Model of the domain structure of *AtPEPR1* generalized to other LRR-RLKs. **A)** GC catalytic domain alignment; **B)** several leucine-rich repeat receptor-like kinases that contain GC domain (LRR-RLKs; Ma *et al.*, 2012).

Recently, Ma *et al.* (2014) have shown that AtPEPR2 has an important role in *AtPep1*-signaling in the roots; a transcriptional investigation has shown that the expression changes of 75% of the *AtPep1*-modulated genes in roots are dependent on the presence of the AtPEPR2 receptor.

1.2.4 BAK1 Interacts with AtPEPRs for Proper Elicitor Perception and Proper Signal Transduction

For proper perception of *AtPeps* by AtPEPR1/AtPEPR2, BAK1 (Li *et al.*, 2002; Nam and Li, 2002) is needed as a co-receptor (or, alternatively, other members of the SERKs protein family; Krol *et al.*, 2010; Gou *et al.*, 2012; Roux *et al.*, 2011; Schulze *et al.*, 2010) which subsequently activate the same downstream signaling cascade such as the MAPK cascade, oxidative burst, or induce the expression of defense-related marker genes (Huffaker *et al.*, 2006; Huffaker and Ryan, 2007; Schulze *et al.*, 2010). Based on in vitro and in vivo studies, it has also been recently reported that AtPEPR1 specifically interacts with the receptor-like cytoplasmic kinases BIK1 and PBS1-like 1 (PBL1) to trigger Pep1-induced signaling (Laluk *et al.*, 2011; Liu *et al.*, 2013; Tintor *et al.*, 2013; Zipfel, 2013).

1.2.5 Structural Basis of *AtPeps* Perception by the AtPEPR1 Receptor

Recently, the crystal structure of the ectodomain of AtPEPR1 in complex with *AtPep1* has been determined (Tang *et al.*, 2015). The crystallography results show that *AtPep1* adopts a fully extended conformation, and it binds to the inner surface of the superhelical AtPEPR1. Furthermore, biochemical assays indicate that *AtPep1* is capable of inducing AtPEPR1-BAK1 heterodimerization. It has been observed that the deletion of the last residue of *AtPep1* significantly affects *AtPep1* interaction and plays a crucial role in heterodimerization.

In that research, FLS2 (protein data bank code: 4MN8) was used as the initial search model and the electron density was used to build the model of *AtPep1* (amino acids 7-23; Figure 1-13; A). In that research, it was observed that in parallel with the central axis of the AtPEPR1 superhelix, *AtPep1* had a fully extended conformation and interacted with the inner side of the helical structure running across 15 LRR of AtPEPR1 (from LRR4 to LRR18; Figure 1-13; B).

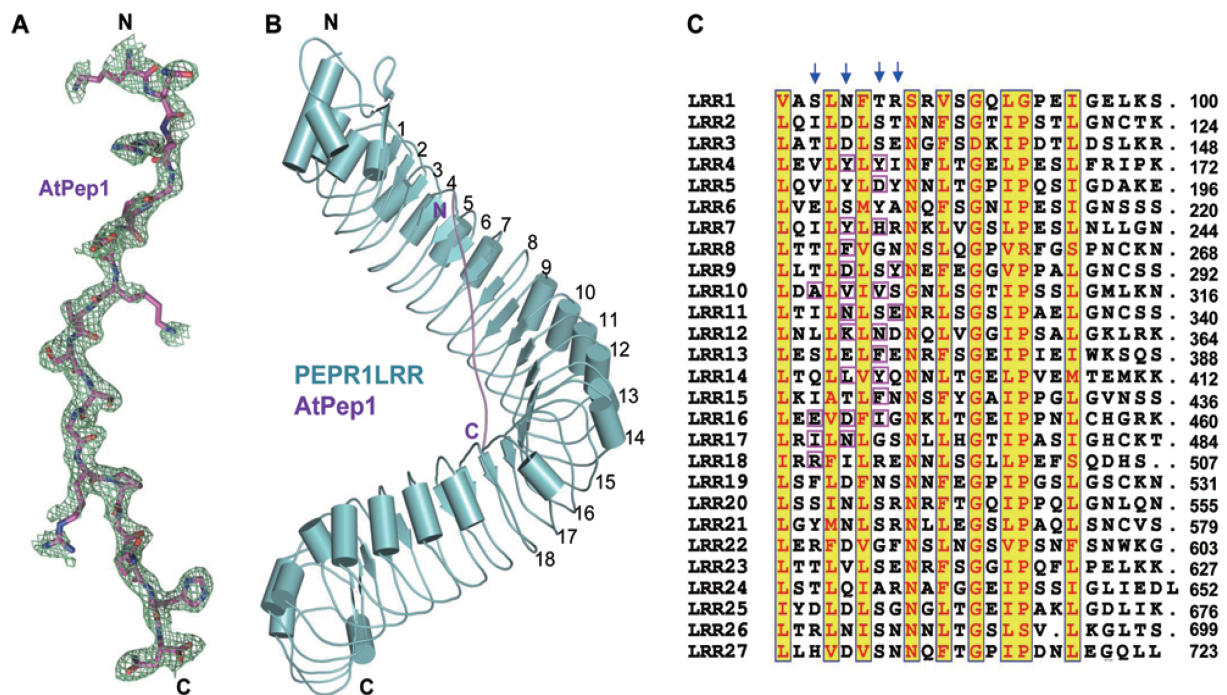


Figure 1-13. AtPep1 binds to the inner surface of PEPR1LRR superhelical structure. (A) Electron density around AtPep1 in the complex structure of the PEPR1-AtPep1 complex. (B) Structure of the PEPR1LRR-AtPep1 complex is represented in cartoon. The positions of some LRRs are indicated in number. (C) Sequence alignment of LRRs of AtPEPR1. The conserved residues are shown with yellow background. Amino acids involved in interaction with AtPep1 are highlighted. Arrows indicate the third, fifth, seventh and eighth positions of each Leucine-rich-repeat (Tang *et al.*, 2015).

As is shown in Figure 1-13; C, among the LRRs of AtPEPR1, many amino acids are highly conserved, but interestingly *AtPep1* selectively makes contacts with the variable residues on the inner surface of AtPEPR1. This indicates that these variable residues are the structural determinants for ligand specificity. It is also noteworthy that at the primary sequence level, the *AtPep1*-interacting amino acids are from the third, fifth, seventh and eighth positions of each LRR motif (Figure 1-13; C). A similar observation was also made for the binding of *flg22* to FLS2-LRR (Sun *et al.*, 2013).

In that research, it was determined that in the complex structure of the AtPEPR1-*AtPep1* interaction, the *AtPep1* closely matched the surface topology of AtPEPR1 and it bound to an elongated inner surface groove which was interspersed with cavity numbers (Figure 1-14; A). One of the most interesting findings of that study is that the ten amino acids of the C-terminal region of *AtPep1* (amino acids 14 to 23; Figure 1-14; B) form more concentrated interactions

with AtPEPR1 receptor than the seven residues at the N-terminal region (amino acids 7 to 13; Figure 1-14; C).

As it is represented in the Figure 1-14; B, amino acid 23 of *AtPep1* (Asparagine 23), which is the last amino acid of *AtPep1*, makes extensive contacts with AtPEPR1 LRR. This residue is highly conserved among *AtPeps* (Bartels *et al.*, 2013). Previously, it has been observed that deletion of this amino acid (Asn23) significantly compromised *AtPep1*-induced immune responses in *Arabidopsis* cells (Pearce *et al.*, 2008), and in the recent investigation by Tang *et al.*, (2015), it has been confirmed that deletion of Asn23 greatly affected the interaction of *AtPep1*-AtPEPR1.

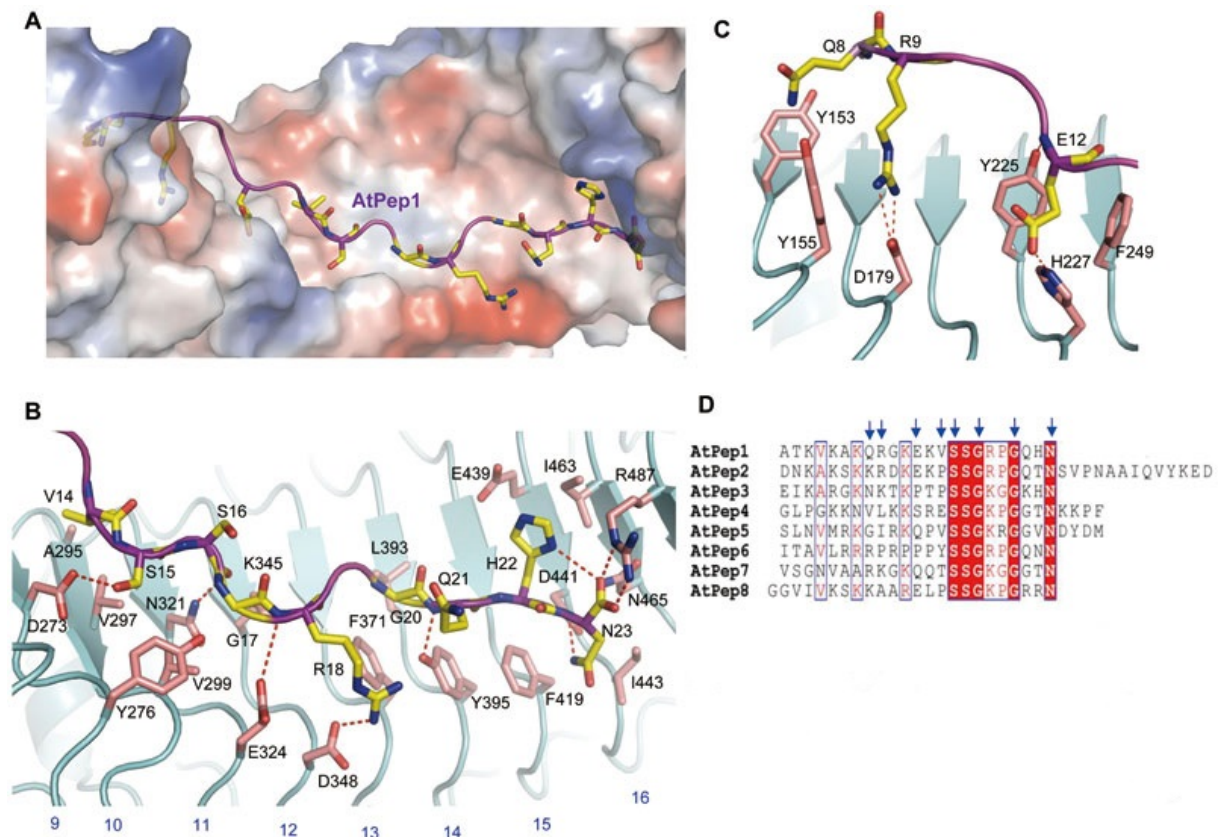


Figure 1-14. Structural basis for recognition of *AtPep1* by PEPR1LRR. **A)** *AtPep1* binds to a surface groove at the inner side of the AtPEPR1 solenoid. AtPEPR1 is represented in electrostatic surface and *AtPep1* in cartoon. White, blue and red indicate neutral, positive and negative surfaces, respectively. The side chains of some amino acids from *AtPep1* are shown (yellow and stick). **(B)** Interaction of the C-terminal part (amino acids 14-23) of *AtPep1* with AtPEPR1. The side chains of AtPEPR1 and *AtPep1* are shown in pink and yellow, orderly. Red dashed lines indicate hydrogen or salt bonds. Numbers in blue represent the positions of LRRs. **(C)** Interaction of the N-terminal portion (amino acids 7-13) of *AtPep1* with AtPEPR1 LRR. Sequence alignment of different *AtPeps* (*AtPep1*-*AtPep8*) from *Arabidopsis*. The conserved residues are represented with red background. Amino acids with arrows on the top are involved in interaction with AtPEPR1 (Adopted from Tang *et al.*, 2015).

1.2.6 Role of the Pep-PEPR System in *A. thaliana*

1.2.6.1 Downstream Events as a Consequence of Pep Perception

As the consequence of Pep perception, several events will occur. Briefly, first of all, ligand binding with AtPEPRs leads to heteromerization with their co-receptor BAK1 (Schulze *et al.*, 2010), and subsequently, downstream signaling cascades lead to the release of AtPEPR-bound BIK1 (Zhang *et al.*, 2010). Afterward, Ca^{2+} -influx is changed, and as a result, the cytosolic Ca^{2+} levels increase (Qi *et al.*, 2010). This affects the activation of the RbohD protein, which has a crucial role in the oxidative burst, i.e. the formation of ROS (Flury *et al.*, 2013; Krol *et al.*, 2010; Ranf *et al.*, 2011; Ranf *et al.*, 2014). BIK1 and also PBL1 are involved in this event (Liu *et al.*, 2013). In addition, as AtPEPRs contain a cytosolic guanylyl cyclase (GC) domain, the ligand perception may lead to production of cyclic GMP (cGMP; Kwezi *et al.*, 2007; Ma *et al.*, 2012; Qi *et al.*, 2010). In addition, the ROS that are generated may themselves have a role in different defense signaling pathways and also in membrane depolarization (Krol *et al.*, 2010; Moreau *et al.*, 2010; Baxter *et al.*, 2013). Concomitantly, phosphorylation of a MAPKs mitogen-activated protein kinases (MAPKs), specially MPK3 and MPK6 is occurring (Bartels *et al.*, 2013; Nühse *et al.*, 2000; Ranf *et al.*, 2011), which may lead to the activation of defense-related transcription factors and ultimately to the induction of many defense-related genes and an increase in the levels of the defense hormones ET, jasmonic acid and also SA (Flury *et al.*, 2013; Mishina and Zeier, 2007; Ross *et al.*, 2014). After ligand perception and signal transduction, endocytosis and degradation of the receptor may occur, in part via PUB-mediated processes (Stegmann *et al.*, 2012). In addition, in the long term, Pep perception also leads to callose deposition and seedling growth inhibition (Bartels *et al.*, 2013; Beck *et al.*, 2014) and also production of secondary metabolites (Huffaker *et al.*, 2013). Figure 1-15 provides an overview of downstream events as a consequence of Pep perception. It has been hypothesized

that Pep peptides are secreted to amplify defense responses triggered by MAMPs, based on the following observations: first, Peps and MAMPs reprogram the transcriptional level of almost the same genes (Huffaker *et al.*, 2006; Huffaker *et al.*, 2007); second, defense responses triggered by the perception of MAMPs and Peps are similar (Huffaker *et al.*, 2006; Krol *et al.*, 2010; Macho and Zipfel, 2014; Yamaguchi *et al.*, 2010; Zipfel *et al.*, 2004; Zipfel *et al.*, 2006) third, AtPEPR receptors are cell surface receptor kinases able to detect extracellular peptides (Krol *et al.*, 2010; Yamaguchi *et al.*, 2006; Yamaguchi *et al.*, 2010) and finally, overexpression of *AtPROPEPs* genes leads to constitutive defense gene expression in the absence of infection or wounding and enhances disease resistance (Huffaker *et al.*, 2006).

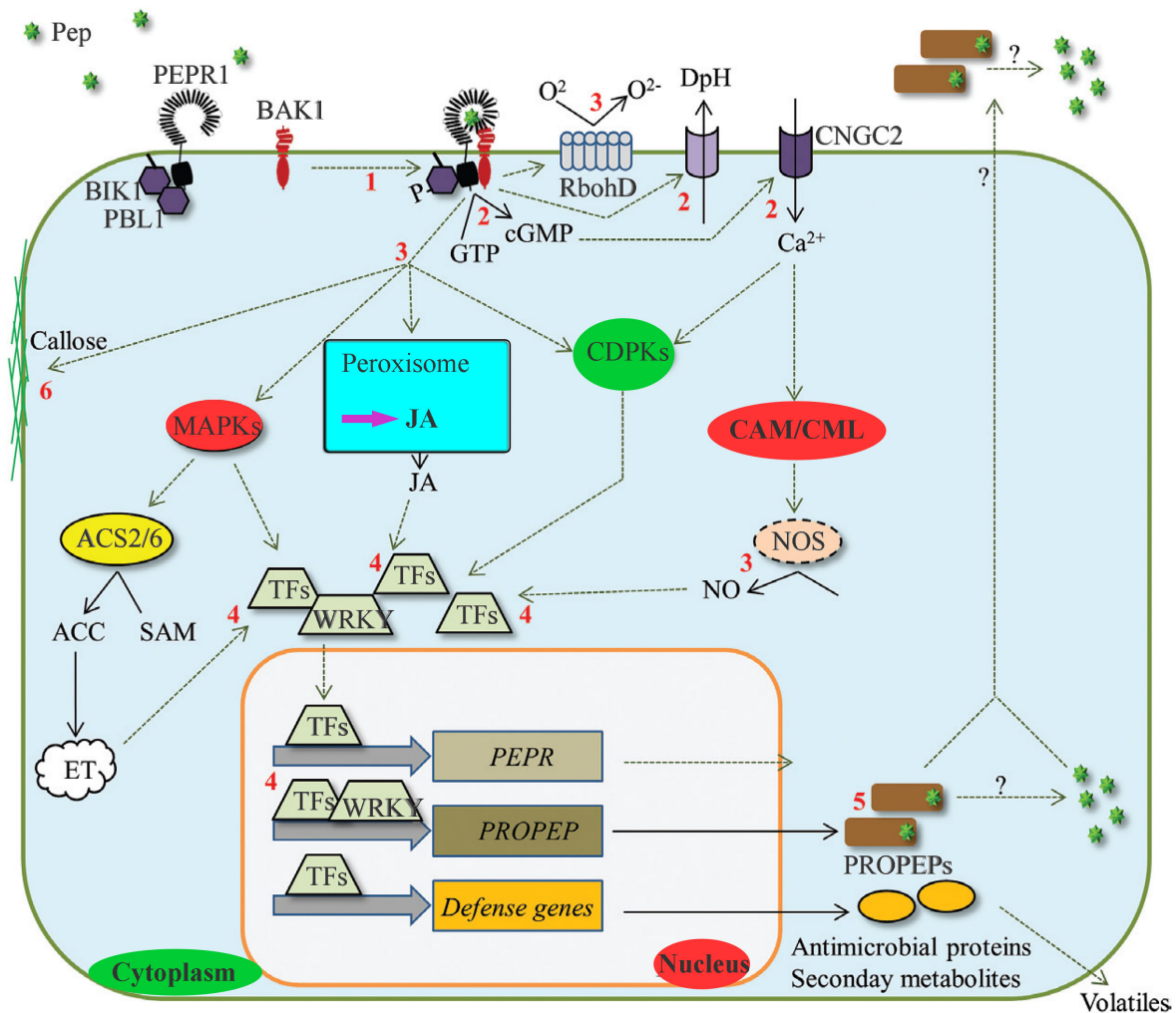


Figure 1-15. A brief overview of the downstream events as a result of Pep perception (Adopted from Bartels and Boller, 2015).

1.2.6.2 Classification of Pep Family into Two Major Groups

Recently Bartels *et al.*, (2013) showed that it is possible to subdivide the Pep family into two groups, based on their observation made using promoter-GUS reporter lines in which the promoters of the various *AtPROPEP* genes were fused with the GUS gene. The promoter activities of *AtPROPEP1*, *AtPROPEP3*, *AtPROPEP5*, and *AtPROPEP8* were partially overlapping and had a correlation with the *AtPEPR1* and *AtPEPR2*. In the other group there were *AtPROPEP4* and *AtPROPEP7* which did not show any similarities in the promoter reporter line expression with others. They also used yellow fluorescent protein (YFP) which was fused to the PROPEP proteins, to study protein localization. *AtPROPEP3* was found to be present in the cytosol, while *AtPROPEP1* and *AtPROPEP6* were observed in tonoplast. As the PROPEPs showed different expression patterns and seemed to be present and active in different regions of the cell, it can be speculated that they do have different roles and functions. As a consequence of *AtPROPEPs* processing, *AtPeps* are produced, which are about 23-aa in sequence and are present at the C-terminal part of the *AtPROPEPs* (Yamaguchi and Huffaker, 2011).

1.2.6.3 A Proposed Model for the Roles of Pep-PEPR1 and PIP-RLK7 as a Consequence of FLS2 Signal Transduction

As *AtPIP1* was recently discovered as an endogenous defense signal, Hou *et al.*, (2014) proposed a model in which FLS2 signal transduction affects both PIP1-RLK7 and also *AtPep1*-PEPR1. They proposed that both PIP1 and *AtPep1* induce their corresponding precursor and also receptor genes showing that self-amplification mechanisms act in the same signaling pathways (Figure. 1-16).

It was even observed that both induce the expression of each other's precursor and also receptor genes. Moreover, in *rlk7* mutants, the level of *AtPep1*-triggered responses was reduced (Hou

et al., 2014). These observations demonstrate that the two endogenous peptide signaling pathways are interdependent and cooperate to amplify the immune response induced by flg22 perception through FLS2.

1.2.6.4 Investigating Pep-PEPR Responses Upon Biotic Stresses

It has been shown that *AtPROPEP1* to *AtPROPEP3* are induced under biotic stresses including: microbial infections, the detection of MAMPs such as flg22 and elf18, wounding, MeJA and ET application; these observations tightly link *AtPROPEP* expression to defense responses (Huffaker *et al.*, 2006; Huffaker and Ryan 2007).

It has also been observed that *AtPep1* treatment confers resistance against *P. syringae* DC3000 (Yamaguchi *et al.*, 2010) and the overexpression of *PROPEP1* induces the resistance to the

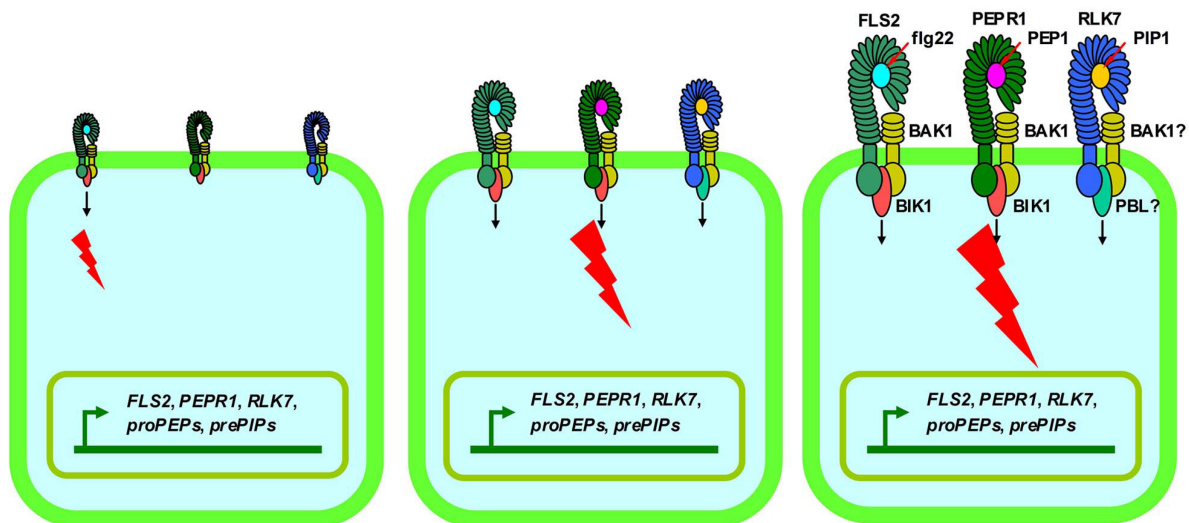


Figure 1-16. Proposed model of the roles of *AtPIP1* (perceived by RLK7) and *AtPep1* (perceived by *AtPEPR1*) in PTI signal amplification. **A)** flg22 perception by FLS2 initiates immunity and activates transcription of FLS2, *AtPEPR1*, RLK7, *AtPep1* and also prePIP1. **B)** Subsequently, *AtPep1* and *AtPIP1* peptides are generated from their precursor proteins and released into the apoplast to trigger PTI responses after recognition by the cognate receptors and act in a positive feedback loop by activating expression of gene encoding their own precursors and receptors, as well as FLS2. **C)** The level of immunity is maximized by the combined effect of FLS2, *AtPEPR1* and RLK7 (Hou *et al.*, 2014).

root pathogen *Pythium irregulare* (Yamaguchi *et al.*, 2010). As well, it has been observed that the treatment of Arabidopsis with *AtPeps* induces the accumulation of JA and its amino acid conjugate (JA-Ile; Huffaker *et al.*, 2013; Huffaker and Ryan 2007).

Logemann *et al.* (2013), identified several binding sites for WRKY TFs in the promoter regions of *AtPROPEP2* and *AtPROPEP3*, indicating a role of *AtPep*-PEPR signaling downstream of the PTI response. However, the underlying mechanisms in which *Pep* peptides and *AtPEPR1* influence defense responses are largely unknown (Yamaguchi *et al.*, 2010).

Recently, Ross *et al.* (2014) proposed a model in which *AtPeps* can become activated at the local sites upon exposure to MAMP or herbivore attack, then promote the generation and/or spread of a mobile long-distance signal(s) within the sites of pathogen attacks and also to systemic non-challenged leaves. In other words, *AtPEPRs* pathways that become activated at the sites of direct attacks, can play a role in activating systemic immunity (Figure 1-17).

Recent studies also showed that *AtPEPR1* and *AtPEPR2* and their ligands act synergistically to produce a Ca^{2+} -signal and play interdependent roles in Ca^{2+} -triggered pathogen responses against bacterial pathogens, such as basal defense, immunization, and ETI (Ma *et al.*, 2012).

It is known that wounding induces expression of *AtPROPEP1*, *AtPEPR1* and *AtPEPR2* (Yamaguchi *et al.*, 2010). Wounding activates the MAPKs, WIPK, and SIPK, likely via the perception of different DAMPs PRRs. These kinases trigger the synthesis of JA in the

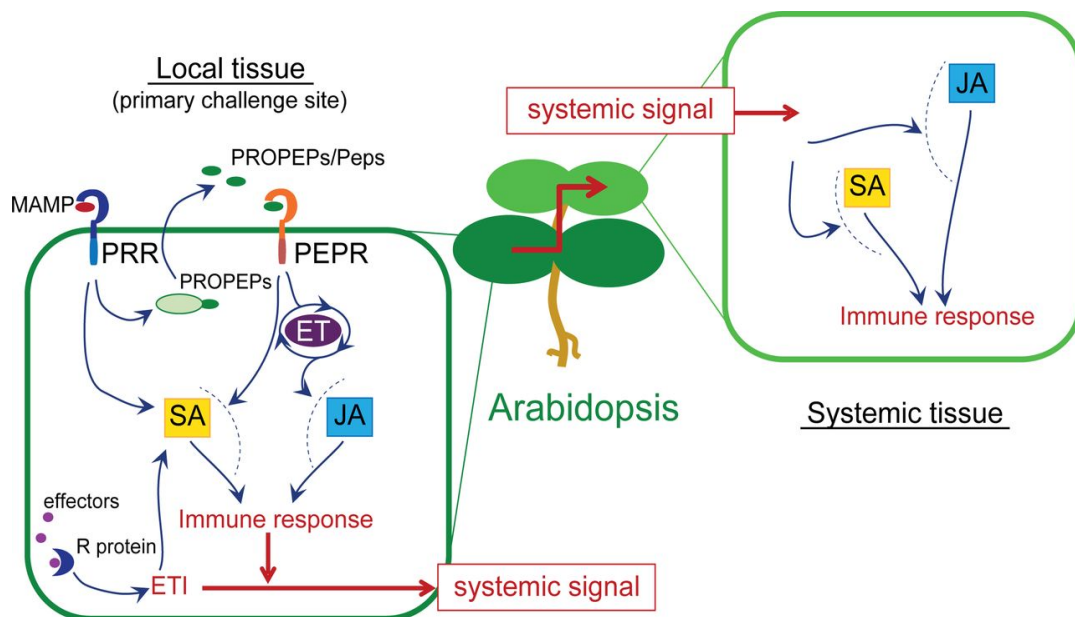


Figure 1-17. A model for the *AtPEPR* pathway in the control of local and systemic immunity. The dashed lines show molecular links between *AtPEPR*-mediated signaling and the downstream SA (salicylic acid) and JA (jasmonic acid) branches. Adopted from (Ross *et al.*, 2014).

chloroplast, which is followed by conjugation of JA to form JA-isoleucine (JA-Ile), which then interacts with its receptor.

JA-Ile specifically binds to COI1 protein and thereby promotes binding of COI1 to JASMONATE-ZIM-DOMAIN (JAZ) proteins, which represent repressors of JA-induced responses in plants. This binding event facilitates the ubiquitination of JAZs by the SCF COI1 ubiquitin ligase, which leads to the subsequent degradation of JAZs and the release of TFs, such as MYC2, and the consecutive expression of JA-responsive genes. Alternatively, Ca²⁺-influx can be triggered by the perception of extracellular ATP (eATP) by the DORN1 receptor, and this initiates the formation of ROS by NADPH oxidase, downstream MAPK signaling cascades and consecutive activation of the same genes via as-yet unknown TF (Figure 1-18; Heil and Land 2014; Savatin *et al.*, 2014; Wu and Baldwin 2010).

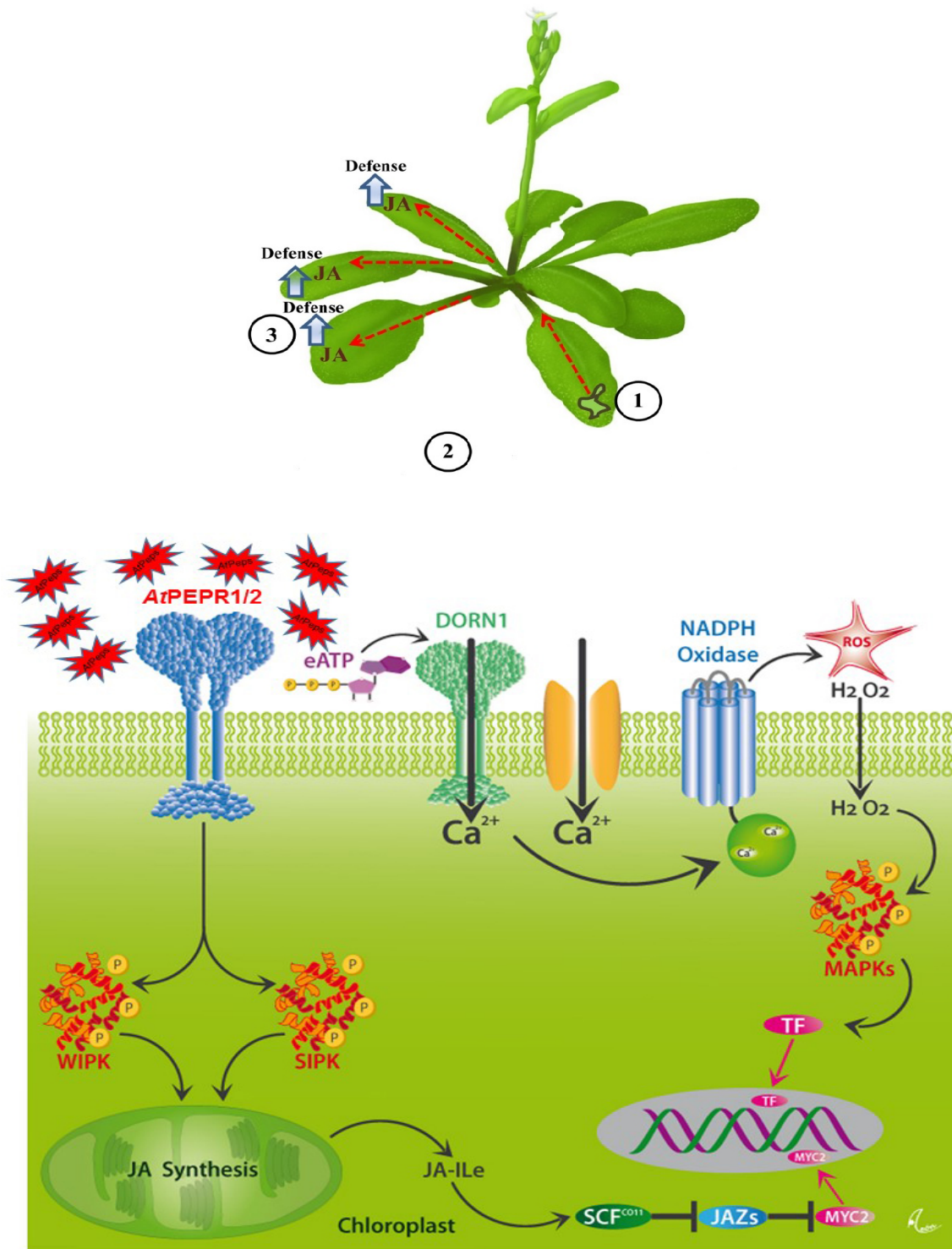


Figure 1-18. Local and systemic responses induced by wounding in *Arabidopsis*. 1) Wounding of *Arabidopsis* leaves first release AtPeps which are perceived by AtPEPR1/2 at the plasma membrane level. 2) Subsequently, elements which are involved in wound signaling include calcium channels, MAPK cascades, CDPKs, and other kinases. Cell-to-cell communication is achieved by H₂O₂ waves produced by the transmembrane NADPH oxidase RBOHD. 3) Then, alert messages are generated and systemically propagated to undamaged tissues through Jasmonic acid (JA) and WASPs. (Adopted from Heil and Land 2014; Savatin *et al.*, 2014).

1.3 The Aim of this Thesis

Despite over a decade of tremendous effort to understand the mechanisms underlying innate immunity in plants, there are still large gaps in the knowledge about the components that have functions in innate immunity. Analysis of microarray data (e.g. Zipfel *et al.*, 2004) led to the identification of several major players in flg22-triggered signaling, however, it has its limitations. Sequencing of the full transcriptome using Illumina deep sequencing technology also covers transcribed genes not present on current microarrays and thus enables the identification of hitherto unknown players. Here, I wanted to analyze transcriptome data that have been generated by treating Arabidopsis seedlings with either flg22 or AtPep1 for 30 min.

Thus the major aims of the first experimental chapter of my thesis are:

- To evaluate whether through the deep sequencing approach, is it possible to find new genes which have roles in innate immunity that are not present in a Microarray?
- To investigate whether through the reverse genetic techniques, is it possible to find novel players in the innate immune system in Arabidopsis? To answer this question, I prepared mutant lines of a selection of genes from the deep sequencing data which were highly upregulated 30 minutes after elicitor treatment.
- To investigate bacterial growth and also the early defense responses in these mutant lines and compare them to the wild type Arabidopsis to see if these mutants show enhanced susceptibility to bacterial infection compared to the wild type Arabidopsis.

In the second experimental chapter of my thesis I focused on the regulation of the Arabidopsis *AtPROPEP* gene family. It is believed that in the plant model *A. thaliana* eight small precursor proteins (*AtPROPEPs1-AtPROPEP8*) are cleaved upon perception of danger to release eight elicitor peptides known as *AtPep1 – AtPep8*, respectively. The discovery of the *AtPeps* along with their receptors, the *AtPEPRs*, has opened a new approach to understand the effect of plant endogenous peptides in plant innate immunity and with regard to their induction upon biotic

stress; it can be postulated that *At*Peps trigger immune signaling systems. However, it is still a question for researchers to evaluate whether the activation of the Pep-family is redundant or whether they have a specific function under several forms of biotic and abiotic stresses including MAMP/DAMP treatments, hormone treatments, and salt treatments. In addition, the interplay between MAMP and DAMP signaling has been a question for researchers over several years, and many theories have been proposed so far (as an example the amplifier theory, as proposed by Boller and Felix, 2009).

Although up to now, there are some fragments of information about the activation of the Pep-PEPR system, there is still a lack of information about the regulation of *At*PROPEPs and their corresponding receptors (*At*PEPR1 and *At*PEPR2) in response to biotic and abiotic stresses. Recent studies showed that *At*PROPEPs display different expression patterns and also exhibit different localizations, although all of them seem to function in similar ways by inducing defense responses (Bartels *et al.*, 2013). Although Bartels *et al.*, (2013) could classify *At*PROPEPs into different groups based on promoter reporter lines, more studies are needed to classify them and comprehensively understand the mechanisms underlying DAMP perception. Furthermore, Bartels *et al.*, (2013) reported that some of the genes encoding *At*PROPEPs are not very active upon MAMP/DAMP elicitors. Thus, whether they are redundant or have specific functions should be studied in more detail.

In the second chapter of this thesis, I particularly focused on the activation of the promoters of the *At*PROPEPs and their corresponding receptors (*At*PEPR1 and *At*PEPR2) upon biotic and abiotic stresses using promoter-GUS reporter lines.

The major aims of the second chapter of my thesis are:

- To understand and dissect the promoter activation of the *PROPEP* gene family upon biotic and abiotic stresses, for which I used the GUS promoter reporter lines including

pAtPEPR1::GUS, *pAtPEPR2::GUS*, *pAtPROPEP1::GUS*, *pAtPROPEP2::GUS*,
pAtPROPEP3::GUS, *pAtPROPEP4::GUS*, *pAtPROPEP5::GUS*,
pAtPROPEP7::GUS, and *pAtPROPEP8::GUS*;

- To characterize and classify the tissue specificity of the *AtPROPEPs* and *AtPEPR* expression regulation system in *A. thaliana* in response to biotic and also abiotic stresses.
- To find new stimuli which can activate the promoter of *AtPROPEPs* and *AtPEPR* that have not been identified yet.

My findings in this research could highlight several characteristics on the *AtPEPR* system. I could outline the new function for *AtPeps* family, their receptors and also their interaction with each other upon biotic and also abiotic stresses.

The data from *A. thaliana* as a reference plant model system, which I have presented in this dissertation work, can be extended to the endogenous peptide elicitors in other plant species.

2. Functional Genomic Studies of Previously Overlooked MAMP/DAMP-Induced Genes, Revealed by an RNA Sequencing Approach and their Possible Involvement in Innate Immunity in *A. thaliana*

2.1 Abstract

In a recent deep-sequencing expression profiling approach, a number of hitherto overlooked genes have been found to be induced in wild type *Arabidopsis* seedlings upon treatment with either flg22 (a well-known MAMP derived from bacterial flagellin), or with *AtPep1* (a peptide representing an endogenous DAMP), indicating a possible involvement in innate immunity. Here, we characterized four of them, namely AT1G56240, AT1G69900, AT1G65385, and AT2G27389, encoding proteins named PP2-B13, ACLP1, SERP1 and GRP89, respectively. PP2-B13 contains an F-Box domain and shows similarity to carbohydrate binding proteins. ACLP1 is a protein of unknown function with highest similarity to actin cross linking proteins and includes a fascin domain. SERP1 is annotated as a pseudogene but shows similarity to a serpin from citrus. Finally, GRP89 is a glycine rich protein with unknown function. Using qPCR, we verified that the genes encoding PP2-B13, ACLP1 and SERP1 were highly induced upon treatment of leaf disks with flg22. It proved to be difficult to ascertain inducibility of the *GRP89* gene by qPCR, possibly because the locus contained an additional overlapping reading frame in reverse orientation. We obtained T-DNA insertion mutants and generated homozygous mutant lines for all four of these genes. None of the mutants showed a phenotype in the absence of infection. All four mutants showed an increased susceptibility to infection by the virulent pathogen *P. syringae* pv. *tomato* DC3000 and also by the much less virulent *hrcC* mutant, as evidenced by an increased growth of the pathogen in planta. Further we present evidence that *aclp1* was deficient in ET production upon flg22 treatment, while *pp2-b13*, *serp1* and *grp89* were deficient in ROS production. In conclusion, the products of these genes may contribute to plant immunity against bacterial pathogens, although there is currently no clue for their mechanism of action.

2.2 Introduction

Plants are sessile organisms which are always under attack from different microbes (Newman *et al.*, 2013). In a co-evolutionary arms race between plants and pathogen interactions, plants initially sense the presence of microbes via perception of MAMPs by PRRs that are located on the cell surface leading to PTI (Boller and Felix, 2009). Initially, Jones and Dangle (2006) called this concept as PAMP-triggered immunity. Later on, as it was realized that this was not the most accurate term, because PAMPs would reflect that they come only from pathogens, while it seems that the elicitors which are perceived by plants come from all kinds of bacteria and fungi (e.g. flagellin from bacteria and chitin from all kinds of fungi). Therefore, MAMPs emerged as the more fitting term for the microbial molecules perceived by the plant; the designation "PTI" might fittingly be used for "pattern-triggered immunity" (Boller and Felix, 2009).

The model plant, *A. thaliana*, detects the presence of a variety of MAMPs including fungal chitin, and bacterial elicitors, like flagellin, and also EF-Tu, or their peptide surrogates flg22 and elf18, respectively (Gomez-Gomez and Boller, 2000; Zipfel *et al.*, 2006). Flagellin and EF-Tu are perceived by FLS2 and EFR receptors, respectively, and their recognition triggers several defense responses. This include the production of ROS in an oxidative burst (Chinchilla *et al.*, 2007; Torres *et al.*, 2006), the reprogramming of expression profiles at transcriptional and also post-transcriptional levels (Jones and Dangl 2006; Thilmony *et al.*, 2006; Truman *et al.*, 2006; Zipfel *et al.*, 2004), and also downstream defense responses including callose deposition (Hauck *et al.*, 2003), MAP kinase activation, synthesis of the defense hormone SA and also seedling growth inhibition (Schwessinger and Zipfel, 2008). MAMP pre-treatment resulted in enhanced resistance to adapted pathogens, and it was observed that mutants impaired in MAMP recognition displayed enhanced susceptibility, not only to adapted pathogens but also in non-adapted (Hann and Rathjen, 2007; Torres *et al.*, 2006; Zipfel *et al.*,

2004, 2006). This indicates a contribution of PTI to basal resistance and also non-host resistance, showing the importance of PTI in plant innate immunity (Gohre and Robatzek, 2008; Gimenez-Ibanez *et al.*, 2009; Shan *et al.*, 2008; Xiang *et al.*, 2008).

The proteobacterium *P. syringae* is a bacterial leaf pathogen that causes destructive chlorosis and necrotic spots in different plant species including monocots and dicots. *P. syringae* pathovars and races differ in host range among crop species and cultivars, respectively (Whalen *et al.*, 2001). Many strains of *P. syringae* are pathogenic in the model plant *A. thaliana*, and therefore *P. syringae* is widely used to investigate plant – pathogen interactions (Whalen *et al.*, 2001, Xin and He, 2013). The ability of *P. syringae* to grow in plants and to multiply endophytically is dependent on injection by the T3SS, enabling the secretion of proteins into the cytoplasm, which can suppress or, in some cases, change plant defense responses (Büttner and He, 2009). *P. syringae* encodes 57 families of different effectors injected into the plant cell by the type III secretion system (Lindeberg *et al.*, 2012). Activities of effectors inside plant cells can be recognized by R proteins which is the second level of defense known as ETI (Boller and Felix 2009; Jones and Dangl 2006).

PTI response is controlled by a complex interconnected signaling network including many TFs; interference with this network can paralyze the adequate response upon pathogen infection (Kunkel and Brooks 2002; Thatcher *et al.*, 2005; Ulker and Somssich 2005). A large percentage of genes in the plant genome respond transcriptionally to pathogen attack (Tao *et al.*, 2003; Thilmony *et al.*, 2006). In addition to reprogramming of transcription, post-transcriptional regulation also plays a role in the plant immune response (Lyons *et al.*, 2013). So far, thanks to application of new technologies, many novel players in defense signaling pathways have emerged and been identified as important components of innate immunity in *Arabidopsis*. Discovery of new proteins involved in plant immunity in *Arabidopsis* will contribute to our understanding of defense system in plant responses to pathogens.

The conserved 22-amino-acid fragment (flg22) of bacterial flagellin that is recognized by the FLS2 PRR can activate an array of immune responses in *Arabidopsis* (Felix *et al.*, 1999). In addition, it is observed that resistance to *Pst* DC3000 is induced by pre-treatment with flg22 (Gomez-Gomez and Boller 2000; Zipfel *et al.*, 2004). In previous research, it was shown that among those genes which were highly expressed upon flg22 treatment (Zipfel *et al.*, 2004), novel genes were found which have functions in the *Arabidopsis* immune pathway. Therefore, it is tempting to speculate that global transcriptome profiling of elicitor treated plants can unveil new players in the immune signaling system in *Arabidopsis*. In a study conducted by Sebastian Bartels and colleagues (personal communication), deep sequencing technology was used to monitor global transcriptome of *Arabidopsis* seedlings upon elicitor treatment. The newly observed MAMP-induced genes may encode new component proteins involved in innate immunity, and their analysis may help us to better understand cross-talk between plant immune system and pathogens.

2.3 Results

2.3.1 Global Analysis of the Gene Expression Changes Unveil Previously Overlooked MAMP/DAMP-Induced Genes in Response to Elicitor Treatment

Treatment of *Arabidopsis* seedlings with flg22 and also *AtPep1* triggers robust PTI-like responses, apparently by activating ca. 1000 genes that may have functions in PTI responses (Zipfel *et al.*, 2004). However, this list is far from being complete. Based on deep sequencing results with *Arabidopsis* seedlings, 30 minute after elicitor treatment, a major portion of genes responded to flg22 and also *AtPep1* compared to mock treated seedlings (Bartels, personal communication). A comparison of global expression profiling results generated with RNA sequencing with those based on the ATH-22k microarray uncovered many genes induced upon elicitor perception which were not present on the ATH-22k microarray (Bartels *et al.*, personal communication). Based on this information, initially, all expressed genes were selected as a

pool (Appendix 1). Later on, to investigate just those genes that had not been studied before, all genes that are present on Affymetrix ATH-22k microarray chips were discarded from this pool list. As a concurrent step, after discarding these genes, a subset of 100 "new" genes (Appendix 2) was selected based on the highest fold induction after flg22 treatment. Finally, in order to narrow down the candidate list, we decided to focus on a small set of genes that showed highest induction after flg22 treatment (Table 2-1), and we checked for the availability of T-DNA insertion mutants. Genes with available T-DNA mutants were obtained from the stock center and further analyzed.

Table 2-1. List of 20 selected candidate genes based on RNA sequence data mining (the genes of interest are highlighted in bold).

N.	Accession Number	Fold change 30 minutes after flg22 treatment	Fold change 30 minutes after AtPep1 treatment	Putative function of the gene	Consideration for subsequent study	T-DNA insertion mutant/NASC Code	Final Genotyping results confirmed by PCR
1	AT5G11140	503	141	Encodes an Arabidopsis phospholipase-like protein (PEARL1) family	---	Not available	Homozygous line
2	AT1G56240	126	120	Encodes a phloem protein 2-B13 ("PP2-B13"); function in: carbohydrate binding; F-box domain, cyclin-like, F-box domain, Skp2-like	"Consider"	detected/ SALK_144757.54.50	Homozygous line
3	AT2G32200	95	36	Encodes an unknown protein	Not considered	---	---
4	AT1G05675	72	63	Encodes an UDP-Glycosyltransferase superfamily protein	Not considered	---	---
5	AT1G65385	65	39	Encodes an pseudogene, putative serpin ("SERP1")	"Consider"	detected/ N570388, SALK_070388	Homozygous line
6	AT4G18195	60	46	Encodes the protein which is the member of a family of proteins related to PUP1, a purine transporter	Not considered	---	---
7	AT5G36925	53	55	Encodes a protein with unknown protein	Not considered	---	---
8	AT1G61470	33	21	Encodes a polynucleotidyl transferase protein which is, ribonuclease H-like superfamily protein	Not considered	---	---
9	AT4G23215	30	28	Encodes a pseudogene of cysteine-rich receptor-like protein kinase family protein pseudogene	"Consider"	detected/ N605169, SALK_105169	Homozygous line
10	AT5G09876	29	11	Encodes an unknown protein	Not considered	---	---
11	AT1G59865	28	39	Encodes an unknown protein	"Consider"	detected/ N584779, SALK _084779	Not detected
12	AT2G35658	28	22	Encodes an unknown protein	"Consider"	detected/ N825604, SAIL_600_D01	Not detected
13	AT1G24145	26	11	Encodes an unknown protein, located in: endomembrane system	"Consider"	detected/ N835081, SAIL_784_C07	Homozygous line
14	AT3G07195	24	19	Encodes a RPM1-interacting protein 4 (RIN4) family protein	Not considered	---	---
15	AT1G18300	22	9	Encodes a nudix hydrolase homolog 4 (NUDT4) protein	Not considered	---	---
16	AT2G24165	22	16	Encodes a pseudogene, similar to HerVf3 protein	Not considered	---	---
17	AT1G69900	20	10	Encodes an actin cross-linking protein; CONTAINS InterPro DOMAIN/s ("ACLP1")	"Consider"	detected/ N568692, SALK_068692 (AR)	Homozygous line
18	AT2G27389	20	13	Encodes a unknown protein ("GRP89")	"Consider"	detected/ SALK_142825.23.95	Homozygous line
19	AT4G39580	18	21	Encodes a Galactose oxidase/kelch repeat superfamily protein	Not considered	---	---
20	AT1G30755	14	13	Encodes an unknown protein	"Consider"	Not detected/ N666232, SALK_063010C	Not detected

2.3.2 Expression of the *PP2-B13*, *ACLPI*, *SERP1* and *GRP89* Genes is Induced Following flg22 Treatment

To monitor the gene expression of *PP2-B13*, *ACLPI*, *SERP1* and *GRP89* in leaves of four weeks old *Arabidopsis* plants upon elicitor perception, the quantitative polymerase chain reaction method (qRT-PCR) was used. Figure 2-1 and Supplementary Figure S1 show that expression of *PP2-B13*, *ACLPI*, and *SERP1* was strongly induced within 30 minutes after flg22 treatment. Data were normalized using the control gene ubiquitin. The copy number of *PP2-B13* mRNA increased very strongly (around 100-fold change) after 1 μ M flg22 treatment; *ACLPI* and *SERP1* expression were up-regulated almost 12-fold. This results show that these genes are strongly activated in the PTI response and also confirm the results of deep sequencing. Taken together, it seems that these genes are highly active upon flg22 treatment in seedlings (based on deep sequencing results) and also in mature leaves (qRT-PCR results). As can be seen in the Figure 2-1, the level of expression of all of these genes was strongly reduced again 2 and also 6 hours after elicitor treatment.

PP2-B13 and AT1G56242.1 (a potential natural antisense gene) have an overlap with each other (as proposed in Arabidopsis.org; Figure 2-2). Thus, to distinguish whether the qRT-PCR results were due specifically to transcription of an individual gene, or whether transcription of both genes (*PP2-B13* and AT1G56242.1) was up-regulated upon flg22 treatment, several sets of primers were designed to cover the different regions of the two genes (Table 2-2; Figure 2-2).

Table 2-2. Primer sets and *Ct* values to discriminate the transcription of *PP2-B13* and AT1G56242

Primer Name	sequence	<i>Ct</i> value of sample at Zero time point (Two individual samples)
FW-A-PP	5'-CGT GAC ACA GAC TAA ATA ATA GAT C-3'	29
RW-A-PP	5'-CCT CTG AAA TAG GGA TCA AGA TG-3'	
FW-B-PP	5'-TCA CAA TTC CAC CTT TCA GTT G-3'	27
RW-B-PP	5'-GGT GGT TGG AGA TTG AGT TAG-3'	
FW-B-PP	5'-TCA CAA TTC CAC CTT TCA GTT G-3'	27
RW-B1-PP	5'-GAT GAC TGT GGA GGC CGT AG-3'	30 \pm 1
FW-C-PP	5'-ATT GCT CCA AGA GCA GTA CG-3'	
RW-C-PP	5'-CTG ATC ACC ATC GGA TTT TTT CG-3'	

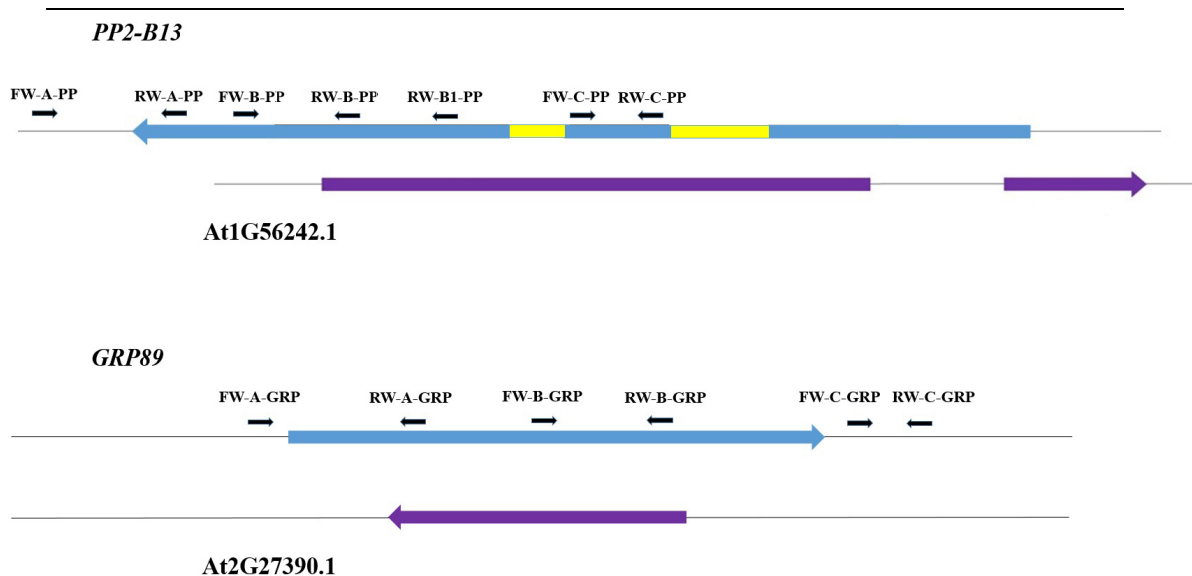


Figure 2-2. Schematic representation of *PP2-B13* which has overlap with AT1G56242.1 and also *GRP89* which has overlap with AT2G27390.1. To evaluate the transcriptome of each gene in qRT-PCR, several sets of primers were designed. Blue boxes indicate exons for *PP2-B13* and *GRP89* as presented in TAIR; violet boxes indicate the exon for AT1G56242.1 and AT2G27390.1; Yellow boxes indicate introns; Thin lines indicate untranslated regions; small arrows indicate primers; big arrows indicate the direction of the transcription of the gene. The cDNA of the leaf disc of wild type Arabidopsis without any treatment was used and the *Ct* value of each set of primers evaluated and compared.

Since the *Ct* values of the qRT-PCR using the primers FW-A-PP/RW-A-PP (*Ct* value 29), which just detects *PP2-B13* transcript, and primers FW-C-PP/RW-C-PP (*Ct* value 30), which can detect both *PP2-B13* and also AT2G56242.1 transcript, are almost same, it can be concluded that the transcription is due to the *PP2-fB13* gene (Table 2-2; Figure 2-2). Otherwise, if AT2G56242.1 was also transcribed, the *Ct* value of qRT-PCR using the primers FW-C-PP/RW-C-PP (*Ct* value 30) should be lower than for primers FW-A-PP/RW-A-PP (*Ct* value 29), which just detect *PP2-B13*.

Therefore, it seems that primers FW-C-PP/RW-C-PP could not detect expression of AT1G56242.1. On the other hand, the *Ct* value of primers FW-B-PP/RW-B-PP (*Ct* value 27) and primers FW-B-PP/RW-B1-PP (*Ct* value 27) are similar to each other. It is also possible that the intron regions of these genes that is deposited in the TAIR (www.Arabidopsis.org) are not really present. Nevertheless, from the results of *Ct* values obtained with different set of

primers, it can be concluded that the level of change of the transcriptome in this region, is specifically due to the expression of *PP2-B13*.

The *GRP89* gene, encodes a glycine-rich protein, has an overlap with the gene AT2G27390.1, which encodes a proline-rich protein. Therefore, to distinguish if the transcriptome of deep sequencing results (20 fold change upon flg22 treatment and 13 fold change upon *AtPep1* treatment) is specifically due to transcription of *GRP89* or AT2G27390, and to evaluate whether both of them are expressed upon elicitor treatment, several sets of primers were designed to cover the transcriptome of this region (Table 2-3; Figure 2-2).

Table 2-3. Primer sets and *Ct* values to discriminate the transcription of *GRP89* and AT2G27390

Primer Name	Sequence	<i>Ct</i> value of sample at Zero time point (Two individual samples)
FW-A-GRP	5'-GGC TTG ATG TTT AAG GTG TAA GG-3'	27 ±1
RW-A-GRP	5'-ACC GGC AAG CTG CCT ACG-3'	
FW-B-GRP	5'-GTG GCT CTG GAG GAA ATA AGG-3'	32
RW-B-GRP	5'-GCC TGG AAC TAC GGG AAC TTG-3'	
FW-C-GRP	5'-CCG TCT CAC ATT CTC CGT TG-3'	30
RW-C-GRP	5'-GAT AGA GGG GAC AGG AGC TG-3'	

Primers FW-A-GRP/RW-A-GRP, can detect the start region of the *GRP89* transcripts, while primers FW-B-GRP/RW-B-GRP, can detect expression of both (*GRP89* and also AT2G27390.1) and primers FW-C-GRP/RW-C-GRP can detect the untranslated region of the *GRP89* transcripts. The *Ct* value obtained using primers FW-A-GRP/RW-A-GRP, is 27 and is lower than the *Ct* value obtained using FW-B-GRP/RW-B-GRP primers (*Ct* value 32). If just the *GRP89* gene is transcribed the *Ct* value obtained using primers FW-A-GRP/RW-A-GRP should be almost similar to that obtained using primers FW-B-GRP/RW-B-GRP. Hence, as the *Ct* value obtained using the primers FW-A-GRP /RW-A-GRP is lower than that obtained using the primers FW-B-GRP/RW-B-GRP, we cannot exactly conclude that the transcription is due to only *GRP89*. Therefore, designing new sets of primers, evaluating the efficiency of these primers and also optimizing the qRT-PCR are needed to precisely determine the nature of the

upregulation observed using the in deep sequencing approach upon elicitor treatment. In addition, in order to justify this observation, subsequent studies especially using different RNA samples and also northern blot analysis for confirmation this finding are needed.

Furthermore, the temporal expression levels of *PP2-B13*, *ACLPI* and *SERP1* in three different time-points (30 minutes, at 2 hours and 6 hours after elicitor treatment) relative to the zero time-point were evaluated. While the transcription level of *PP2-B13* was set at 1 at the zero time-point, 30 minutes after flg22 treatment, *PP2-B13* transcripts increased 102 fold. 2 and 6 hours after flg22 treatment, the transcript level of elicitation, did change compared to the zero time-point.

The transcripts level of *ACLPI* gene was set at zero at the zero time-point. In the flg22 treated leaf disc *ACLPI* transcripts increased 12-fold. By 2 hours after treatment, *ACLPI* transcripts increased 3-fold. Moreover, the transcript level of *ACLPI* compared to the zero time-point, did change 6 hours after flg22 treatment.

In addition, the transcript of *SERP1* gene was set at 1 at the zero time-point. While 30 minutes after flg22 treatment it increased 10-fold. Although, 2 hours after flg22 treatment, *SERP1* transcripts increased 4-fold. At 6 hours after flg22 treatment, the transcript level of *SERP1* did not change (Figure 2-1).

To evaluate the expression profile by qRT-PCR of the *GRP89* gene upon flg22 treatment compared to the control, two sets of primers were designed (FW-A-GRP/RW-A-GRP and FW-B-GRP /RW-B-GRP, Supplementary Figure S2). Since the locus of the *GRP89* gene contained an additional overlapping reading frame in reverse orientation (AT2G27390.1), these two sets of primers, did not show the same transcriptome profile and represented unusual melting curves. Therefore, it was not easy to evaluate and monitor the expression profile of *GRP89*

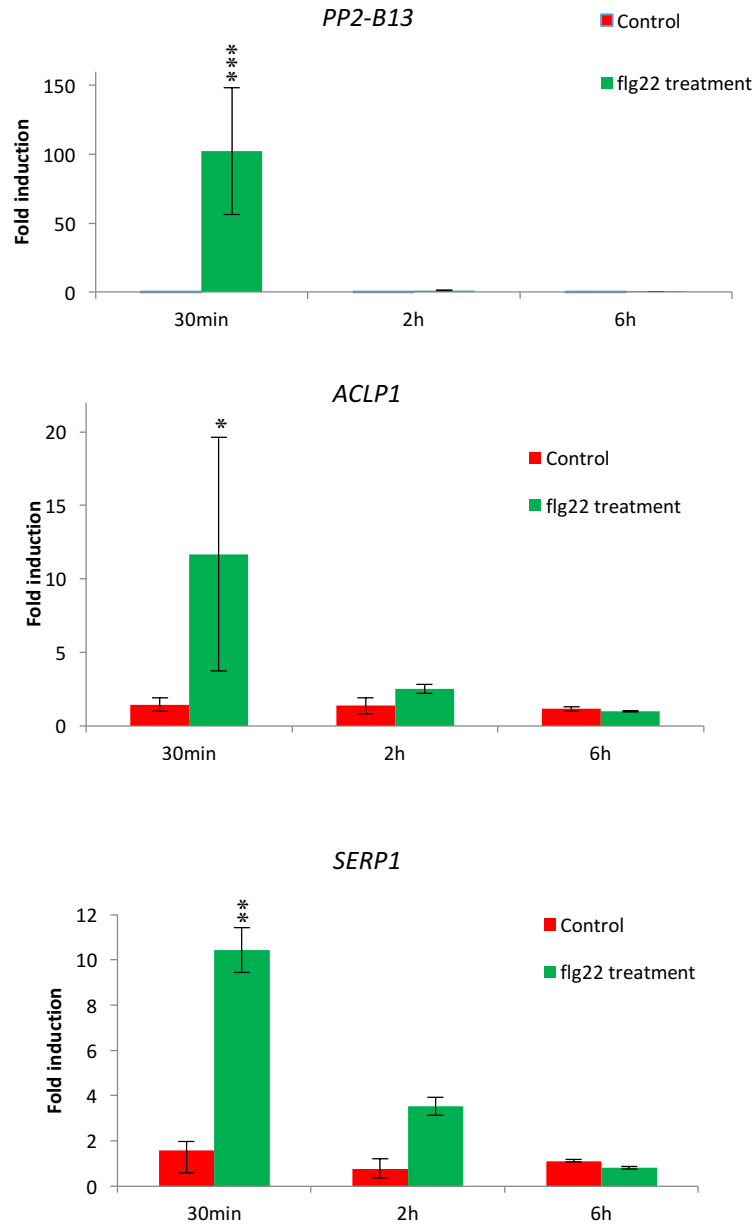


Figure 2-1. Changes in expression levels of the *PP2-B13*, *SERP1* and *ACLP1* genes after elicitor treatment. Leaf discs of five weeks old *Arabidopsis Col-0* plants were treated with 1 μ M flg22 and the expression patterns of the *PP2-B13*, *SERP1* and *ACLP1* genes were measured 30 minutes, 2 hours and 6 hours after elicitor treatment. Each bar represents the fold changes relative to mock samples. Data were obtained in two independent experiments, each with three technical replicates, and analyzed by student's *t-test*. Data were normalized using the housekeeping gene *Ubiquitin*. Error bars represent standard deviation of the mean (\pm) of 3 biological replicates with 2 technical replicates each. P values are indicated * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

gene in the graph and it was difficult to ascertain the inducibility of the *GRP89* gene by qRT-PCR. Having the correct melting curves is obligatory in qRT-PCR data analysis (Bustin and Nolan, 2004; Luo *et al.*, 2011; Mao *et al.*, 2007; Thompson *et al.*, 2014). Therefore, based on

what was observed, no conclusion can be reached at this point clearly showing whether *GRP89* is transcribed upon flg22 treatment or not. For this reason, new sets of primers need to be designed and various factors in the qRT-PCR also need to be optimized to overcome the melting curve difficulties that were observed. Nevertheless, despite all the above mentioned points which we have observed in comparisons, we have noticed that there are differences in *Ct* value compared to the untreated leaves, especially 6 hours after treatments, using primers FW-A-GRP /RW-A-GRP, which may indicate that the *GRP89* gene is induced upon elicitor treatment (Supplementary Table 2-1). Furthermore, there are many factors which significantly affect the *Ct* value such as secondary structure of the RNA, and other limiting factors in qRT-PCR experiments which should be take into account (Bustin and Nolan, 2004).

2.3.3 Identification and Characterization of T-DNA Insertion Lines in *PP2-B13*, *ACLPI*, *SERP1* and *GRP89* Genes

Among the selected candidate genes (Table 2-1) based on their availability in the Arabidopsis Stock Center (NASC), the T-DNA mutant lines AT1G56240, AT1G65385, AT4G23215, AT1G59865, AT1G24145, AT2G35658, AT1G69900, AT2G27389 and AT1G30755 were ordered. The seeds were sown in short-day under conditions (ten hours light at 21°C and 14 hours dark at 18°C, with 60% humidity) and after three weeks, the total DNA was extracted and the presence of the T-DNA insertions were checked. As the T1-populations which were ordered from the NASC were a mixture of homozygotes and heterozygotes plants, several sets of primers which can detect the T-DNA insertion line and also determine if the T-DNA insertion mutant is homozygous or heterozygous were designed to specifically detect the homozygous line (Table 2-9).

After doing these steps, toward the detection of homozygous T-DNA insertion lines, finally, we obtained T-DNA insertion mutants of six single homozygous lines bearing a disruption in the gene, including AT1G56240 (*PP2-B13*), AT1G69900 (*ACLPI*), AT1G65385 (*SERP1*),

AT2G27389 (*GRP89*), AT4G23215, and AT1G24145 (Table 2-4). The seeds of these homozygous mutant lines were sown for further study.

According to the *Arabidopsis* Information Resource (Swarbreck *et al.*, 2008), and also SIGnAL database (<http://signal.salk.edu/>), as shown in Figure 2-3, the T-DNA insertion in the mutant

Table 2-4. List of the selected homozygous mutant lines which were considered for subsequent study.

N.	Accession Number	Gene name	Fold change 30 minutes after flg22 treatment	Fold change 30 minutes after <i>AtPep1</i> treatment	Function of the gene
1	AT1G56240	<i>PP2-B13</i>	126	120	Phloem protein 2-B13 (PP2-B13); function in carbohydrate binding; F-box domain, cyclin-like, F-box domain, Skp2-like
2	AT1G65385	<i>SERP1</i>	65	39	pseudogene, putative serpin
3	AT4G23215	---	30	28	pseudogene of cysteine-rich receptor-like protein kinase family protein pseudogene
4	AT1G24145	---	26	11	Unknown protein, LOCATED IN: endomembrane system
5	AT1G69900	<i>ACLPI</i>	20	10	Actin cross-linking protein; CONTAINS InterPro DOMAIN/s
6	AT2G27389	<i>GRP89</i>	20	13	Unknown protein

line (SALK_144757.54.50) is located in the second exon of the *PP2-B13* gene, which has three exons; for the *ACLPI* gene the T-DNA insertion in the mutant line (SALK_68692.47.55) is located in the first of two exons; for the *SERP1* gene, the T-DNA insertion (070388.25.65) is located in the first part of the gene and for the *GRP89* gene, the T-DNA insertion in the mutant line (SALK_142825.23.95) is located in the middle of the exon.

The T-DNA insertion in the At4G23215 gene is in the intron region and for AT1G24145, the T-DNA insertion is located in the intron of the gene between the two exons.

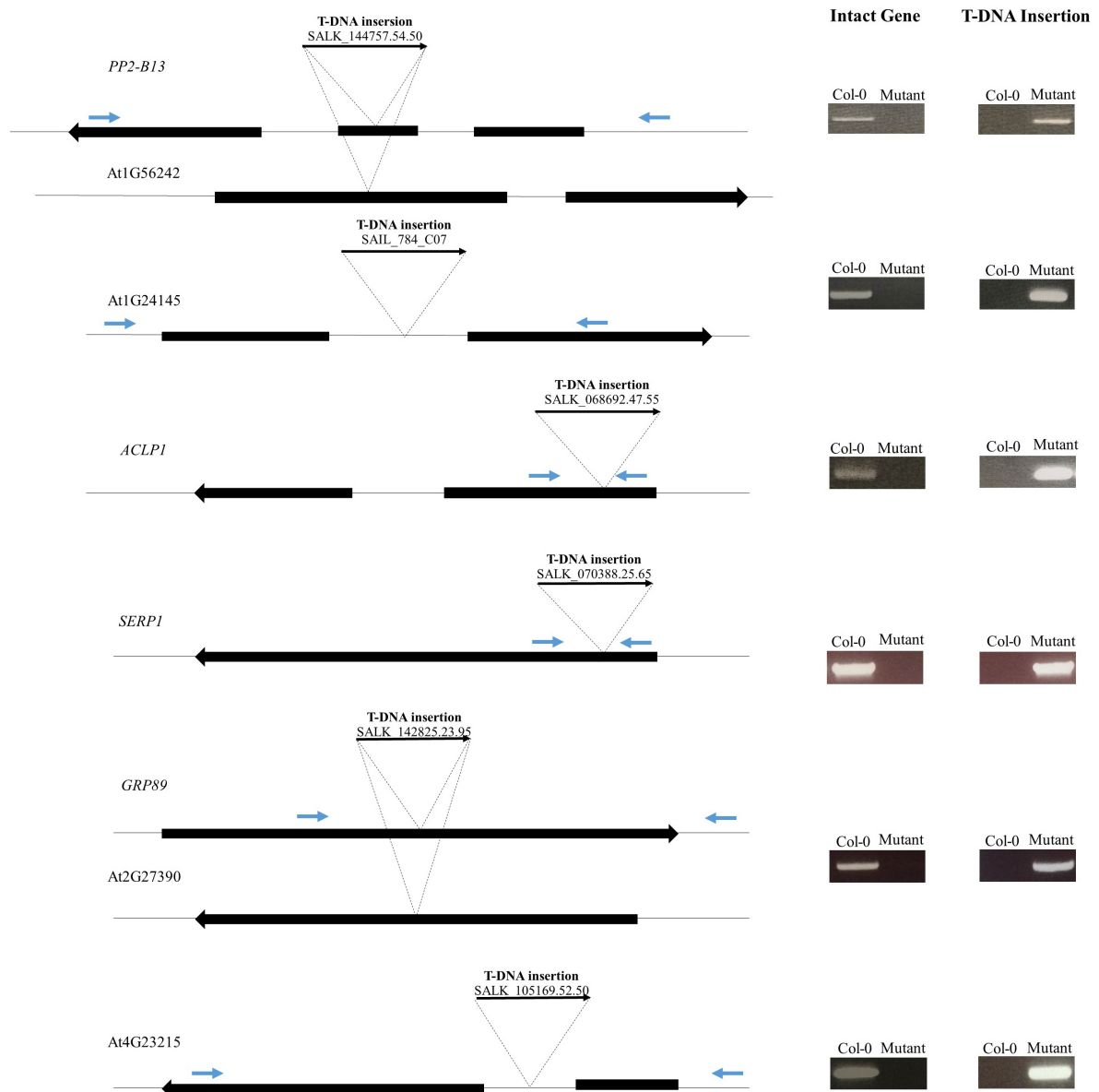


Figure 2-3. Schematic representation of homozygous T-DNA mutant lines *PP2-B13*, *ACLP1*, *SERP1* and *GRP89*. Boxes indicate exons; thin lines indicate introns; bold arrows indicate: T-DNA insertions; Arrows indicate the direction of the transcription of the gene. On the right side, the PCR results of the homozygous lines are shown, amplifying either the intact gene or the T-DNA. Small blue arrows indicate the primers position.

We compared the phenotypes of the homozygous mutant lines including AT1G69900 (*aclp1*), AT1G65385 (*serp1*), AT1G56240 (*pp2-b13*), AT1G27389 (*grp89*), AT4G23215 and AT1G24145 visually with the wild type Arabidopsis (Col0). There was no visible difference in size and shape between the mutant lines and the wild type plants at the rosette stage (Figure

2-4). The *serp1* mutant line showed some abnormality in the flowering stage compared to the wild type Arabidopsis, and the flowering stage of this mutant line was later than for wild type Arabidopsis (Supplementary Figure S3). Comparing the symptoms two days post infection with wild type Arabidopsis, these mutant lines did not show different symptoms (Supplementary Figure S4).



Figure 2-4. Phenotype of five-week old different mutant lines including AT1G69900 (*aclp1*), AT1G65385 (*serp1*), AT1G56240 (*pp2-b13*), AT1G27389 (*grp89*), AT4G23215 and AT1G24145 compared with the wild type Arabidopsis (line Col-0). Plant were grown under short-day conditions (ten hours light at 21°C and 14 hours dark at 18°C, with 60% humidity).

2.3.4 Increased Susceptibility to *P. syringae* DC3000 and also *hrcC* in *pp2-b13*, *aclp1*, *serp1* and *grp89* Mutant Lines

All of the homozygous T-DNA mutant lines were tested for bacterial growth with *P. syringae* pv. *tomato* DC3000 strains and also *P. syringae* pv. *tomato hrcC*, a strain with a defect in type T3SS. Comparing the bacterial growth titer in the mutant plants to that of wild type Arabidopsis and also the calculating the significance differences (Student's T test), only four mutant lines,

namely *pp2-b13*, *aclp1*, *serp1* and *grp89*, showed significantly more bacterial growth than Col-0 *Arabidopsis* (Figure 2-5 and Supplementary Figure S5). Thus, it seems that these genes might have a role in defense signaling.

In the following, the four gene products which might have a role in innate immunity are presented in more detail.

As can be seen in Figure 2-5 (panel A), the titer for *pp2-b13* mutant lines was 104000000 cfu/cm², which was statistically significant (P values 0.0205) compared to the counts noted in wild type plants. In addition, as can be seen in Figure 2-5 (panel C), 48 hours post inoculation of leaves with *P. syringae hrcC*, the bacterial titer for wild type *Arabidopsis*, reached 109000 cfu/cm² while for *pp2-b13* mutant lines it was 325000 cfu/cm², and compared to the wild type plants the bacterial titer for this mutant line was statistically significant (0.0261). The bacterial titer for *sid2-2* mutant plants was almost well-above 6 (804000 cfu/cm²). The protein encoded by *PP2-B13* is a phloem protein containing the F-box domain Skp2. It also has a function in carbohydrate binding, formerly was named PP2-B13 (Dinant *et al.*, 2003). This gene shows the highest similarity in sequence with AT1G56250, which formerly was reported as an F-box protein (Zaltsman *et al.*, 2010). In the region of the chromosome 1 where PP2-B13 is located, there are many genes which are activated upon biotic or abiotic stresses, such as AT1G56280. The protein product of this gene is named drought-induced protein 19 (Di19) and the expression of which increases due to progressive drought stress. This protein is a transcriptional factor (TF) which has an important role in up-regulation of pathogenesis-related *PR1*, *PR2*, and *PR5* gene expressions in response to drought stress in *Arabidopsis* (Liu *et al.*, 2013). This protein has two atypical Cys2/His2 (C2H2) zinc finger-like domains which are evolutionarily well-conserved among angiosperms indicating that it has an important function (Milla *et al.*, 2006). The protein encoded by AT1G56250 is an F-box protein that can functionally replace VirF (Zaltsman *et al.*, 2010).

The protein encoded by *ACLP1* is of unknown function with the highest similarity to actin cross-linking proteins and includes a fascin domain. As is shown in Figure 2-5 (panel A), 48 hours post inoculation of leaves with *P. syringae* DC3000, the titers of *Pst* DC3000 in wild type Arabidopsis leaves, reached 52100000 cfu/cm², while in the studied mutant lines, the bacterial counts for *aclp1* mutant plants was 104000000 cfu/cm², which is roughly double the amount of bacteria counted in Arabidopsis wild type plants and using Student's T test, was statistically significant (P values 0.0336) compared to the wild type plants. In addition, as it can be seen in Figure 2-5 (panel C), 48 hours post inoculation of leaves with *P. syringae hrcC*, the bacterial titer for wild type Arabidopsis, reached 109000 cfu/cm², while for the *aclp1* mutant line, it was 257000 cfu/cm², and compared to the wild type plants the bacterial titer for this mutant line was statistically significant (P values 0.0089).

The *SERP1* product is a pseudogene and also encodes a putative serpin located in the CLAVATA2 region of the Arabidopsis genome (Shepard and Purugganan, 2003). Since this gene showed similarity with serpins and more specifically with citrus, we named this gene *SERP1*. The role of serpins to induce resistance against blast disease (*Magnaporthe grisea*) in rice is previously confirmed in the research conducted by Chauhan *et al.*, (2002) and also Roberts and Hejgaard (2008). Furthermore, as is shown in the Figure 2-5 (panel B), 48 hours post inoculation of leaves with *P. syringae* DC3000, the bacterial titer for *serp1* mutant line was 60700000 cfu/cm²; which was also statistically significant (P values 0.0191) compared to the wild type plants which was 24500000 cfu/cm².

In addition, as can be seen in the Figure 2-5 (panel D), 48 hours post inoculation of leaves with *P. syringae hrcC*, the bacterial titer in the *serp1* mutant line, reached to well-above 63300000 cfu/cm² and was statistically significant (P value 0.0137).

The protein encoded by *GRP89* is of unknown function. This protein is glycine rich protein. Therefore we named the gene *GRP89*. As is shown in the Figure 2-5 (panel B), 48 hours post

inoculation of leaves with *P. syringae* DC3000, the bacterial titer for *grp89* mutant line was 57000000 cfu/cm²; which was also statistically significant (P 0.0456, orderly) compared to the wild type plants which was 24500000 cfu/cm². Furthermore, as can be seen in Figure 2-5 (panel D), 48 hours post inoculation of leaves with *P. syringae hrcC*, the bacterial titer in *grp89* mutant line, reached to well-above 6 which was 2270000 cfu/cm² and was statistically significant (P value 0.396).

In comparison, the T-DNA mutant lines AT1G24145 and AT4G23215 did not display any difference in susceptibility to bacterial growth assay (Table 2-5). Conclusively, these results demonstrate that PP2-B13, ACLP1, SERP1 and GRP89 have role in defense signaling and these genes are required for wild-type levels of resistance against *P. syringae* pv. *tomato* and also *hrcC*.

Table 2-5 Number of repetitions for the mutants where we have seen the response to the Bacterial infection compared to the wild type Arabidopsis.

Mutant line	Number of repetitions for the mutants where we have seen the response		Number of repetitions for the mutants where we have not seen the response	
	<i>Pst</i> DC3000	<i>Pst hrcC</i>	<i>Pst</i> DC3000	<i>Pst hrcC</i>
<i>PP2-B13</i>	2	2	1	0
<i>ACLP1</i>	2	2	1	0
<i>SERP1</i>	2	2	0	0
<i>GRP89</i>	2	2	1	0
AT4G23215	0	0	2	1
AT1G24145	0	0	2	2

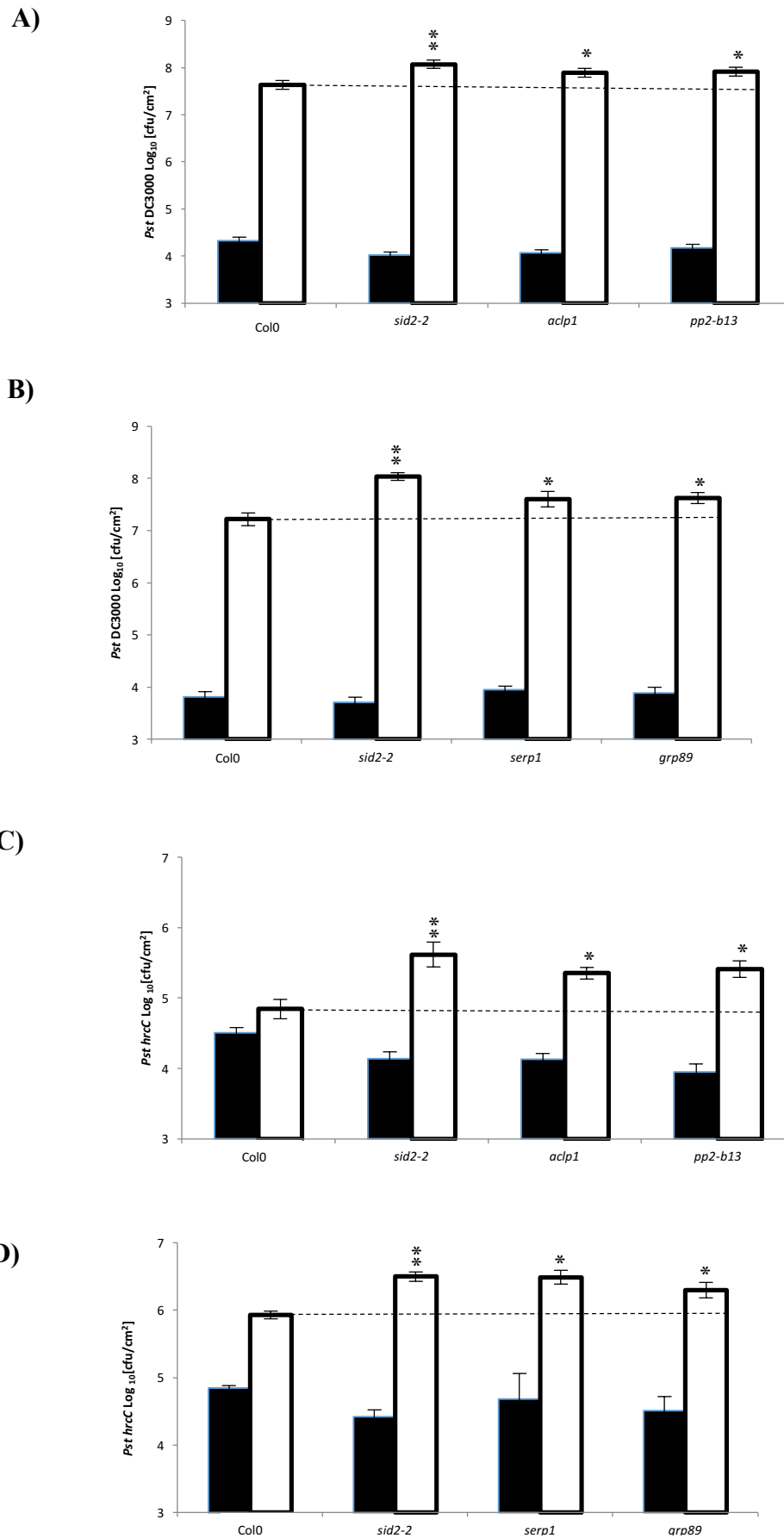


Figure 2-5. Bacterial susceptibility assay. Leaves of four-six-week-old wild type Arabidopsis (Col-0), mutant lines (including *pp2-b13*, *acp1*, *serp1* and *grp89*) and were pressure infiltrated with *Pseudomonas syringae* pv. *tomato* DC3000 and *P. syringae hrcC* (OD₆₀₀ = 0.0002, in infiltration buffer). *sid2-2* mutant plants which is deficient in salicylic acid production were used as a positive control. Open bars indicate bacterial colony from leaf discs of infected leaves just after infiltration (0 day) and filled bars represent colony-forming units (cfu/cm²) 48 hours post inoculation. Results are average ± s.e. (n=6). Similar results were observed in four independent experiments. Asterisks indicate a significant difference (*p ≤ 0.05. **p ≤ 0.01) from the wild type plants as determined by Student's *t*-test.

2.3.5 Differential ET and ROS Production in *pp2-b13*, *aclp1* *serp1* and *grp89* plants, as Compared to the Wild type Arabidopsis

To analyze the early defense responses upon elicitor treatment, we assessed ET production and also ROS in response to flg22 treatment in the mutant lines *pp2-b13*, *aclp1*, *serp1* and *grp89*. Remarkably, in comparison to wild type Arabidopsis, we observed that mutant line *aclp1* displayed a reduced ET production once these mutant lines were treated with 1 μ M flg22. We noticed that in mutant line *aclp1*, ET levels were statistically significantly lower compared with wild type Arabidopsis (P values 0.0295; Figure 2-6, Panel A and Supplementary Figure S6). It seems that ACLP1 might have a role in enhancement of ET production in response to flg22 stimulation.

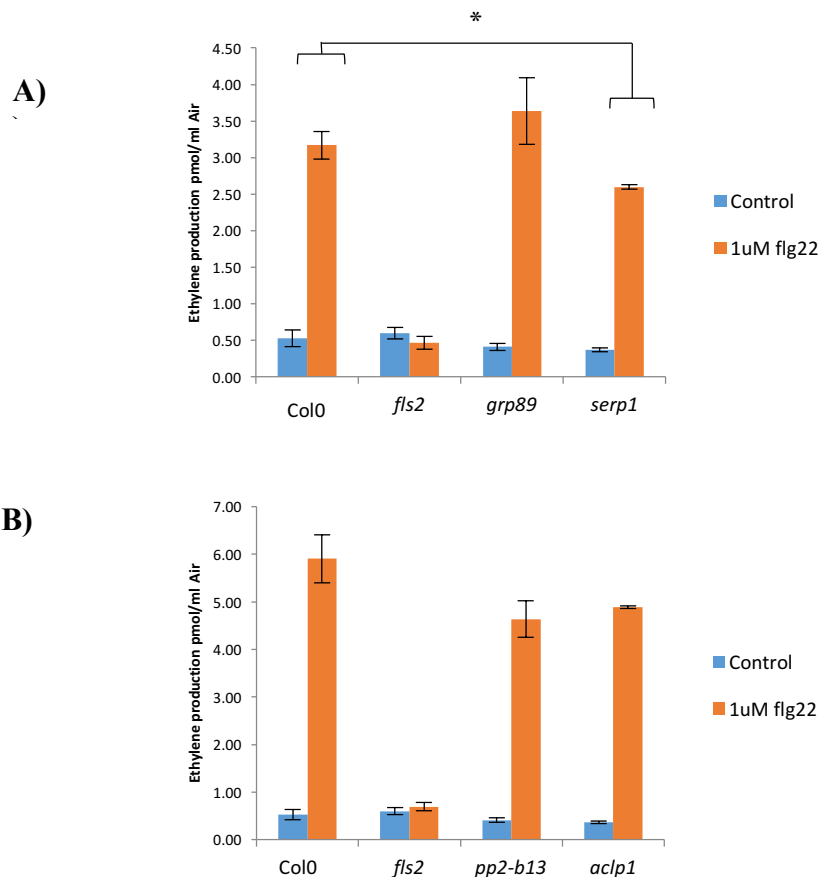


Figure 2-6. Early PTI responses upon elicitor treatment. Ethylene accumulation after elicitor treatment. Leaf discs of four to five weeks old plants of the mutant lines (*pp2-b13*, *aclp1*, *serp1* and *grp89*) and also wild type plants (Col-0) were treated with 1 μ M of the flg22 elicitor peptide or without any peptide (control). In all cases ethylene production was measured three and half hours after closing the tubes. Panel A; indicates ethylene accumulation in *pp2-b13* and *aclp1* mutant lines compared to the wild type Arabidopsis. Panel B; represents ethylene accumulation in *serp1* and *grp89* mutant lines compared to the wild type Arabidopsis. *fls2* mutant line was used as a negative control. Columns represent averages of detected ethylene values of five biological replicates. Error bars indicate standard deviation with n=6. Similar results were obtained in at least six independent biological replicates. *T-test* was performed comparing the responses of the control treatment to the elicitor treatments; P values are indicated * $p \leq 0.05$.

One of the early responses triggered by MAMPs and DAMPs is the production of apoplastic ROS by the Arabidopsis NADPH-oxidases RbohD and RbohF protein (Torres *et al.*, 2006). Surprisingly, in the treated leaf discs upon flg22 perception, *pp2-b13* displayed differently in ROS production and it produced lower than wild type Arabidopsis (Figure 2-7, Panel A and Supplementary Figure S7-A).

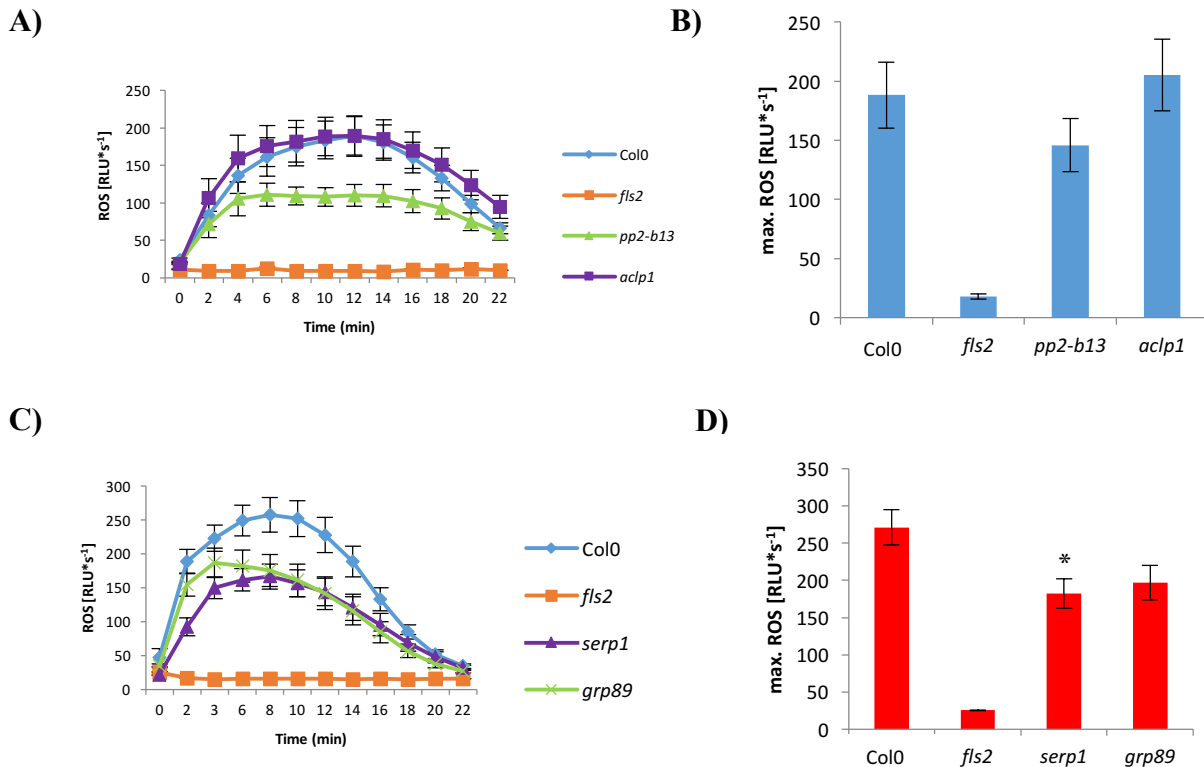


Figure 2-7. ROS production after treatment with flg22. Leaf discs were treated with indicated 1 μ M flg22 or without any peptide (control). Panel A; indicates ROS production in *pp2-b13* and *aclp1* mutant lines compared to the wild type Arabidopsis. Panel B; represents maximum ROS production in *pp2-b13* and *aclp1* mutant lines compared to the wild type Arabidopsis. Panel C; shows ROS production in *serp1* and *grp89* mutant lines compared to the wild type Arabidopsis. Panel D; represents maximum ROS production in *serp1* and *grp89* mutant lines compared to the wild type Arabidopsis. *fls2* mutant line was used as a negative control. Graphs display average of 12 replicates. Error bars indicate standard error (SE) of the mean. The experiment was repeated four times with similar results. RLU= relative light units. *T-test* was performed comparing the responses of the control treatment to the elicitor treatments; P values are indicated * $p \leq 0.05$.

Thus, PP2-B13, may have role in early PTI to enhance the oxidative burst in response to the flg22 treatments. On the other hand, although *aclp1* exhibited deficiency in ET production

upon flg22 perception, this mutant line exhibited robust enhancement of ROS production, to the same levels for wild type Arabidopsis. Intriguingly, the mutant lines *serp1* and *grp89*, responded more weakly than wild type Arabidopsis (Figure 2-7, Panel C and Supplementary Figure S7-B). Since RbohD and RbohF are the main producers of apoplastic ROS in response to elicitor treatment and also the presence of pathogens (Torres *et al.*, 2006), it can be concluded that as *pp2-b13*, *serp1* and *grp89* displayed altered response to ROS burst compared to the same treatment of wild type Arabidopsis. It seems that the proteins encoded by *PP2-B13*, *SERP1* and *GRP89* might have a function in ROS production. It might be possible that *PP2-B13*, *SERP1* and *GRP89* protein products have interact with RbohD and RbohF or other components which have role in flg22-triggered ROS burst, such as peroxidases and polyamine oxidases. In addition, as can be seen in the Figure 2-6, the negative control treatment using flg22 on *fls2* mutant plants did not induce ROS production.

Furthermore, we also have compared maximum ROS production of all mutant lines and compared them with wild type Arabidopsis. Overall, while we have seen differences in ROS generation between various mutant lines and wild type Arabidopsis, except for the *serp1* mutant line (Figure 2-7, Panel D), the maximum ROS generation for other mutant lines including *pp2-b13*, *serp1* and *grp89* in response to flg22 treatment compared to the wild type Arabidopsis, were not statistically significant. Even for the *serp1* mutant line, despite the difference in ROS production, in other repeat experiments, the maximum ROS generation compared to the wild type Arabidopsis was not statistically significantly different in several repeats (Figure S7). Therefore, it can be concluded that these proteins do not have important roles in ROS production.

2.3.6 FLS2 Receptor Abundance in *pp2-b13* and *ac1p1* Mutants were Similar to the Wild Type Arabidopsis

The *PP2-B13* and *ACLPI* genes were strongly induced upon elicitor treatment as seen in deep sequencing and qPCR studies. Additionally, both mutant lines were deficient in early PTI responses (ET and ROS measurement). Hence it might be conceivable that the products of the *PP2-B13* and *ACLPI* genes affect the abundance of FLS2 receptor. However, Figure 2-7 shows that both mutant lines exhibit a similar pattern of FLS2 accumulation compared to the wild type Arabidopsis. Thus it appears that these genes do not have a role in regulating the abundance of the FLS2 receptor.

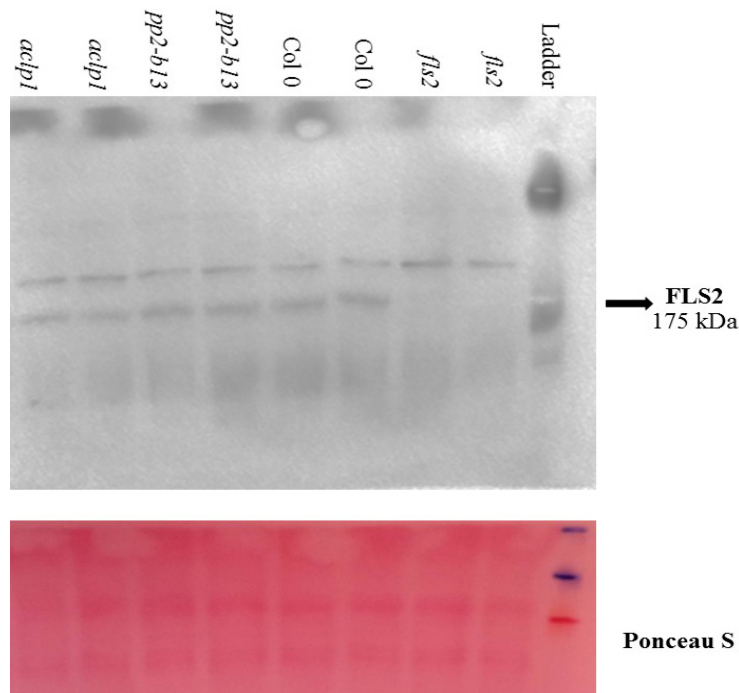


Figure 2-7. FLS2 protein levels. FLS2 protein levels of the mutant lines *pp2-b13* and *ac1p1* as detected by western blot analysis using a FLS2-specific antibody. *fls2* mutant plant, is used as negative control. Ponceau S staining was used as loading control.

2.4 Discussion

Plants are constantly under exposure of microbial signals from potential pathogens, potential commensals and also mutualists but the plant cell immune sensors are able sense these signals and expand the defense against pathogens (Nishimura and Dangl, 2014). Host-pathogen interactions encompass a complex set of events which are dependent on the nature of the interacting partners, developmental stage, and also environmental conditions (Cecchini *et al.*, 2015; Katagiri *et al.*, 2002). They are regulated through diverse signaling pathways that induce or repress gene expression (Huang *et al.*, 2010; Zipfel *et al.*, 2004). Global gene expression profiling of wild type Arabidopsis seedlings resulted in the identification of a large number of genes induced by flg22 and AtPep that had not been detected by the ATH-22 array technology (Bartels, personal communication). Among them, we focused on four, namely *PP2-B13*, *ACLPI*, *SERP1* and *GRP89*. Upon flg22 treatment, expression of these genes is rapidly upregulated in wild type Arabidopsis (Figure 2-1) and reverse-genetic studies of *PP2-B13*, *ACLPI*, *SERP1* and *GRP89* genes showed that these genes are required for wild-type levels of resistance to infection by the bacterial pathogens *P. syringae* pv. *tomato* DC3000 and also *Pst hrcC* (Figure 2-5). Our results demonstrate that the deep sequencing approach performed by Bartels *et al.* (personal communication) can help us to find new players in early defense responses in innate immunity in Arabidopsis after treatment of the Arabidopsis plants with elicitors. It might also be possible to find more new players in innate immunity by extending the time points of the elicitor treatment.

2.4.1 PP2-B13

PP2-B13 is an F-Box protein with homology to PP2-B14, which was formerly reported as an F-Box protein (Zaltsman *et al.*, 2010). The F-Box domain of PP2-B13 is close to the N-terminus of the protein. Zhang *et al.*, (2011) showed that proteins PP2-B13 and also PP2-B14 were highly abundant in phloem upon aphid infection, and as these genes are present in a cluster

of defense-related genes, it seems that these proteins play a role in the defense signaling network.

To obtain protein structural information on PP2-B13, its sequence was sent to the Raptor X server (Källberg *et al.*, 2012). Based on the output of the server, two domains for PP2-B13 were predicted (Supplementary Figure S8). The N-terminal region of the PP2-B13 protein (from residue 4-46) is an F-box domain, while the PP2 domain is at the C-terminal region (residue 93-280). PP2-domain proteins are one of the most abundant and enigmatic proteins in the phloem sap of higher plants (Dinant *et al.*, 2003). Recently it was reported that lectin domain proteins are important in plant defense responses, and so far 10 membrane-bound lectin type PRRs, which are involved in plant defense signaling and symbiosis, have been identified (Lannoo and Damme, 2014). Lectins are proteins containing at least one non-catalytic domain which enables them to selectively recognize and bind to specific glycans that are either present in a free form or are part of glycoproteins and glycolipids and may help the plants to sense the presence of pathogens; as a defense response they use a broad variety of lectin domains to interact with pathogens (Van Damme *et al.*, 2011). To determine if the *PP2-B13* gene is conserved among plant species, we have done a phylogenetic analysis. Interestingly, we have noticed that this gene is conserved among different plant species. Therefore, we propose that this gene may have the same function in other plant species as was observed in *Arabidopsis* (Supplementary Figure S9).

2.4.2 ACLP1

ACLP1 is an actin cross-linking protein with 397-aa. In order to obtain protein structural information on ACLP1, its amino acid sequence was sent to the Raptor X server (Källberg *et al.*, 2012). The output of the server, predicted two domains for ACLP1 (Supplementary Figure S10). A Fascin motif is predicted to be present in both N-terminal and C-terminal domains of this protein (from amino acid 18-70 and 229-318, respectively). The fascins are a structurally

unique and evolutionarily conserved group of actin cross-linking proteins. Fascins function in the organization of two major forms of actin-based structures: dynamic, cortical cell protrusions and cytoplasmic microfilament bundles (Habazettl *et al.*, 1992; Jayo and Parsons, 2010; Opassiri *et al.*, 2007).

The cytoskeleton is dynamic and plays very important roles in functions basic to all cell types. In mammalian cells, it defines the shape and size of cells through its highly organized structure. The cytoskeleton of mammalian cells includes three filament types: F-actin microfilaments, intermediate filaments (like vimentin, keratin and desmin), and polymeric alpha- and beta-tubulin composing the microtubules.

As dynamic structural proteins with multiple functions, actins are the most abundant structural protein in eukaryotes which play diverse roles in cell functions (Sol *et al.*, 2014). They have roles in cytoskeleton formation, cell division, motility, adhesion, and also signaling, and exist in the cells as monomers (globular, G-actin) or as polymers, which are called actin filaments or F-actin (Rottner and Stradal, 2011). These actin species are transformed into one another by different factors. G-actin is polymerized into F-actin by an increase in monovalent or divalent cation concentration and by specific positively charged proteins and peptides.

2.4.3 SERP1

We have found that *SERP1* is highly induced upon flg22 treatment. Deep sequencing data also showed that *SERP1* is highly induced after flg22 treatment and also *AtPep1* treatment. Furthermore, we have observed that the *serp1* mutant line showed increased susceptibility to bacterial infection both with *Pst* DC3000 and *Pst hrcC*. We also provide evidence that the *serp1* mutant line is deficient in ROS production compared to wild type Arabidopsis. In addition to the role of SERP1 in defense regulation, as was shown in the Supplementary Figure S3, we also observed that SERP1 affected the flowering stage. Thus, SERP1 may have a role

in the flowering stage besides its role in innate immunity. An interaction between flowering and defense also has been observed in several independent studies such as LEAFY (Winter *et al.*, 2011), SIZ1 (Jin *et al.*, 2008) and ENHANCED DOWNY MILDEW2 (Tsuchiya *et al.*, 2010), which are mediators of both flowering and bacterial defense. It also was reported that PLANT U BOX PROTEIN (Li *et al.*, 2012) and HOPW1-1-INTERACTING (Wang *et al.*, 2011) have roles in the different developmental stages beside their role in innate immunity. Therefore, there may be a link between immunity and the flowering stage function of SERP1 at the molecular level, and it might be interesting to evaluate the interconnection and coordination between innate immunity and development in plants.

2.4.4 GRP89

In the current research, we have observed that the *grp89* mutant line showed increased susceptibility to bacterial infection both with *Pst* DC3000 and *Pst hrcC*. In addition, we also provide evidence that *grp89* mutant line is deficient in ROS production compared to wild type *Arabidopsis*. Beside these, deep sequencing data also showed that *GRP89* is highly induced after flg22 treatment, which was increased 20-fold compared to the untreated seedling and also after *AtPep1* treatment, a 13-fold increase was observed compared to the untreated seedling (Bartels, personal communication). Due to the overlap of *GRP89* with AT2G27389, we could not ascertain the inducibility of *GRP89* upon elicitor treatment and their overlap made their expression study more complex. Therefore, new sets of primers should be designated to evaluate the efficiency of these primers and optimize qRT-PCR, and other experiments such as northern blotting should be done to exactly determine the upregulation that we have observed in the deep sequencing approach upon elicitor treatment.

In plants, Mangeon *et al.*, (2010) have reported that GRPs have a role in plant defense as several GRPs are reported so far that have functions in inducing immunity against microbial infection. Our observation for GRP89, is in the line with the previous studies. This protein has also 51%

similarity to a Glycine-rich cell wall structural protein (GRP1) which has a role in the plasticity of the cell wall (Keller *et al.*, 1988).

GRP89 protein shows 60.2% similarity with the keratin type I cytoskeletal 9 of humans (*Homo sapiens*) and also 56.1% with the keratin type II cytoskeletal 2 epidermal of mouse (*Mus musculus*). These proteins have important roles in epidermal diseases (Hennies *et al.*, 1994; Reis *et al.*, 1994) and recently the interaction of keratin with *Staphylococcus aureus* was studied (An *et al.*, 2015).

2.4.5 Conclusions and Outlook

A very rapid, but transient induction of *PP2-B13*, *ACLPI*, *SERP1* and *GRP89* was observed within 30 minutes of elicitor treatment (Figure 2-1). Most interestingly, *PP2-B13* was induced >100-fold 30 min after treatment of wild-type plants with flg22. Based on the susceptibility to *P. syringae* pv. *tomato* DC3000 observed in the current research, subsequent studies are needed to determine whether the protein products of these genes are targets of pathogen effectors or whether they interact with other defense components in innate immunity.

Using a mutant approach, we provide evidence that defect in these genes can affect the early PTI responses including ET and ROS measurements (Figure 2-6 and Figure 2-7). We could show a defect in activation of ET production for *aclp1* plants and also attenuated ROS generation in *pp2-b13*, *serp1* and *grp89* plants in response to flg22 treatment. ROS accumulation is regarded as an early PTI event occurring a few minutes after *Pst* inoculation (Boller and Felix 2009). These findings suggest that these genes might have a function through interaction with PTI signaling pathways during bacterial infection. However, we cannot yet determine at what point of the MAMP signaling cascade the products of these genes function. Therefore, subsequent studies are needed to determine the relationship of these genes in MAMP recognition and other signaling cascades in innate immunity.

In conclusion, based on what we have observed in different experiments, it can be concluded that *PP2-B13*, *ACLPI*, *SERP1* and *GRP89* genes have a role in innate immunity. It is likely that the protein products of these genes can have multiple functions in innate immunity in Arabidopsis, as has been reported for BAK1 protein which has multiple function in different pathways in Arabidopsis (Jaouannet *et al.*, 2014; Pedro *et al.*, 2015; Wang *et al.*, 2008; Zhou *et al.*, 2015).

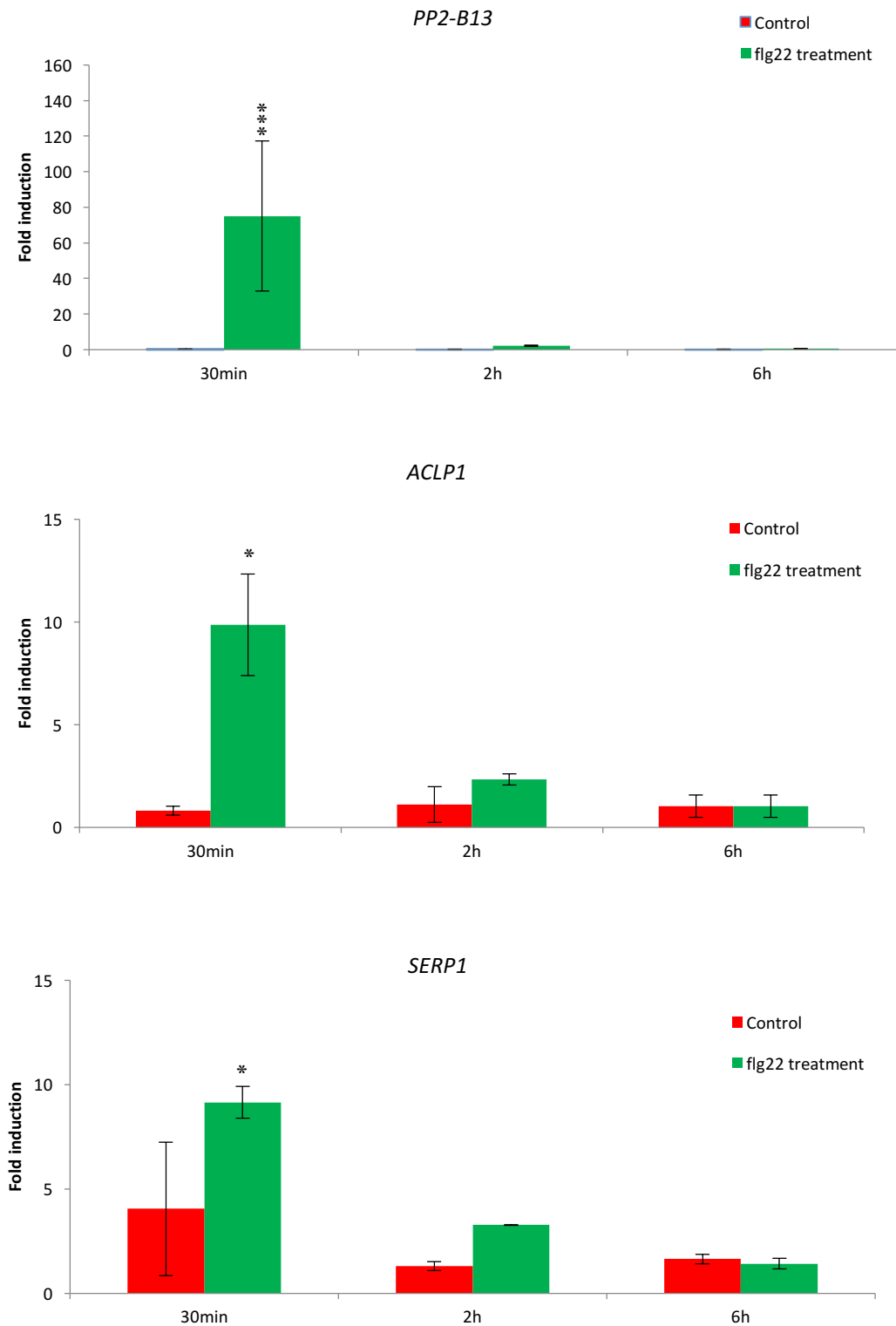
The MAPK cascades have diverse roles in innate immunity (Asai *et al.*, 2002; Taj *et al.*, 2010). Therefore, as the expression of many defense related genes is affected by MAPK3 and also MAPK6, it would be interesting to examine the expression of *PP2-B13*, *ACLPI*, *SERP1* and *GRP89* genes in *mpk3* and *mpk6* mutant plants. It is also possible that the proteins encoded by the four genes have functions in different levels of innate immunity and also in regulating other proteins involved in this scenario, either at elevating their mRNA or at the protein level, in response to flg22 treatment, pathogen attack, and also even in abiotic stresses.

It has been previously reported that the genes which have a function in innate immunity in Arabidopsis can also have role in resistance against abiotic stress (Rejeb *et al.*, 2014). Therefore, it is interesting to see the response of these mutant plants upon abiotic stresses (such as salt stress, cold stress, drought stress) and also monitor transcriptional changes and signal transduction of these genes upon abiotic stresses in wild type Arabidopsis.

Having identified new players in innate immunity in Arabidopsis on chromosome 1 and 2, the next step is to clone these genes to determine their function in MAMP recognition. As yet, we have not produced transgenic plants expressing *PP2-B13*, *ACLPI*, *SERP1* and *GRP89* under the control of the 35S promoter to determine the phenotype of over expression of the genes and also to monitor their response to MAMP perception and also bacterial infection.

2.5 Supplementary Figures

Functional Genomic Studies of Previously Overlooked MAMP/DAMP-Induced Genes, Revealed by an RNA Sequencing Approach and their Possible Involvement in Innate Immunity in *A. thaliana*



Supplementary Figure S1. Changes in expression levels of the *PP2-B13*, *SERP1* and *ACLP1* genes after elicitor treatment.

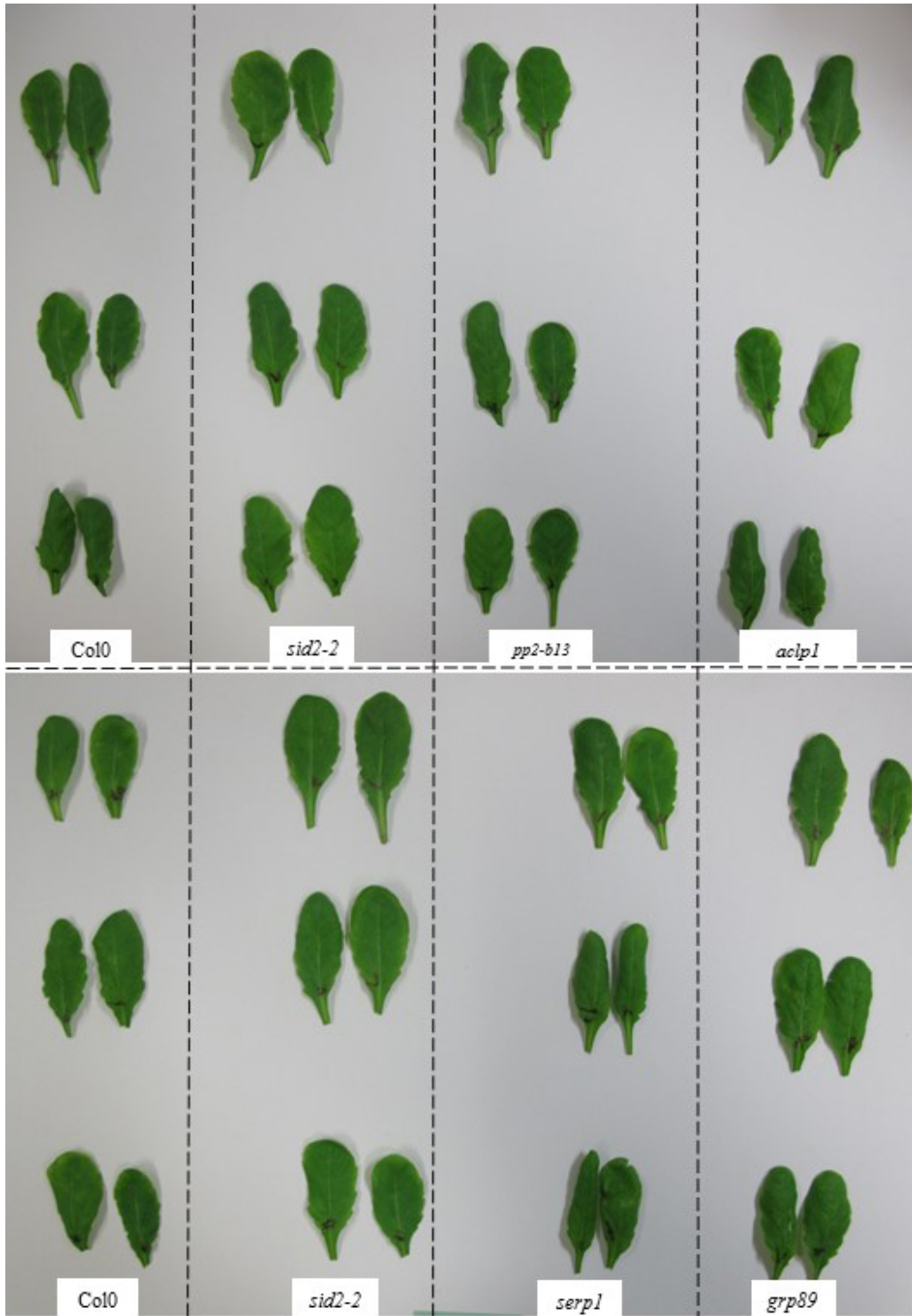
caatcaaagcaaggaagcttcagcctttgaaagtcgcattagaaatattcaacgcaagagtaaagttataaaatgatttgattaatcacacgcggttact
 tagaatcgtttaataaagtgtggttgatggttaagggttaagggttttgatatatacaaaATCGAAATCCAAATGCGTAGCATAATGTTTCATCTATTT
 CTATCCCTTCTGATTACACGCAATCCAAGCCGTTGGTTCACCTTGATCAAGCTTCTGTGCATCTATTGATGTAAAACGTCAATCTTTAACGGTAGAAA
 CGATGCCGTTTTTCGGGTGATTTTGTGCGTAGGCAGCTTGCCGGTGGAGGTGGTTCCTCAGGTGGAGGAGGGGGAGGCGGAGGCTCTCGAGGTGGTCTTC
 AGGTGGAGGAAGCAGTGGTGGAGGAAGTCGTGGAAGTGGAGGCGGTGAAAAAGTGGAGGAGGAAATCATAAACCGTGGTGGCAAGCGGTGGCTCTGGAGGA
 AATAAGGCTGAAAAGGTGGTGGCAGTCGTGGTGGCGATGATGGTGACGGTGGTGGAGATGGTGGTGGTGGATAGCGGAAGTAGTGAAAACACTAGAGGTG
 GAGGACAACAAGTTCCGTAGTTCAGGCCGCGAGTTTCCAAGCGATGGTGTCCGAATTGAATATTCTTTAGTGTGTTTCATCTTCATGACATGTTTAGT
 TATGGGATCATGTTTTCTTAGactccaaattttaatttgtaacctatctctctctctctctcttttagtttgtaattatcttgaataacaatta

FW-A-GRP →
 ← RW-A-GRP
 → FW-B-GRP
 ← RW-B-GRP

Supplementary Figure S2. Primers designed for evaluation the transcript of *GRP89* in qRT-PCR experiment. Arrows means the designated primers; Blue boxes means translational start/stop codon; Orange color in big letters indicate Exon, small letters mean Intron; Red and black colors mean untranslated regions.

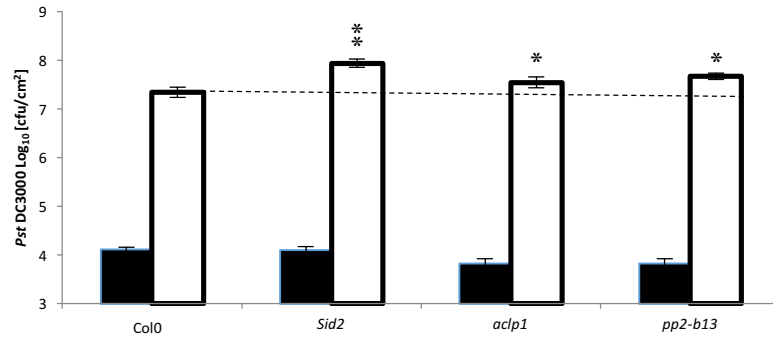


Supplementary Figure S3. *serp1* mutant line showed abnormality at the flowering stage compared with wild type Arabidopsis.

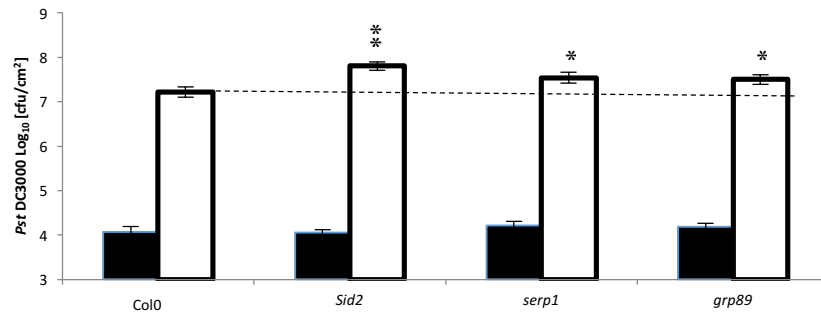


Supplementary Figure S4. Symptoms of leaves infiltrated with *P. syringae hrcC* two days post infiltration, comparing mutant lines *sid2-2*, *pp2-b13*, *aclp1*, *serp1* and *grp89* with wild type Arabidopsis.

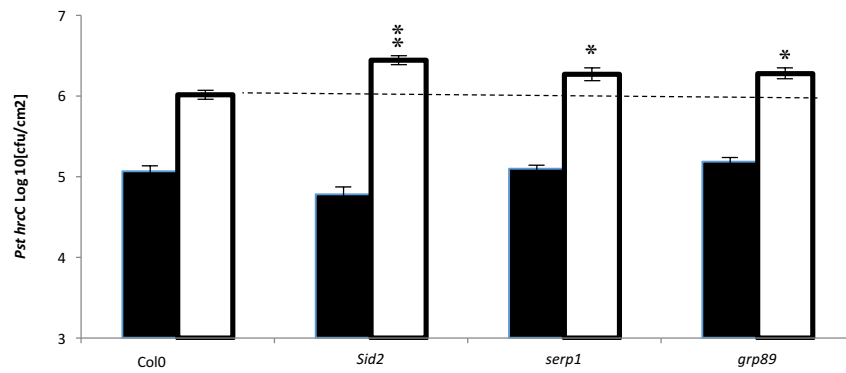
A)



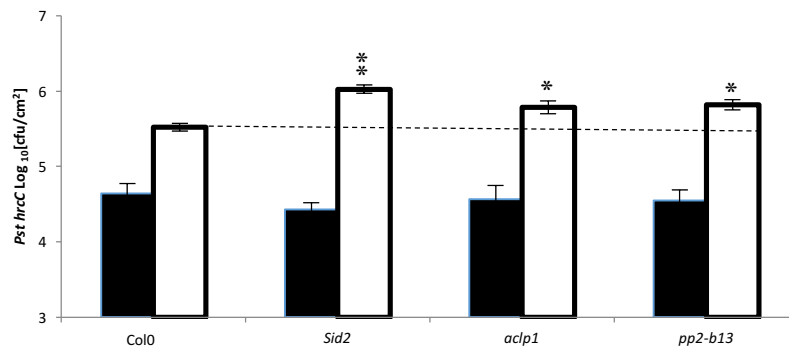
B)



C)



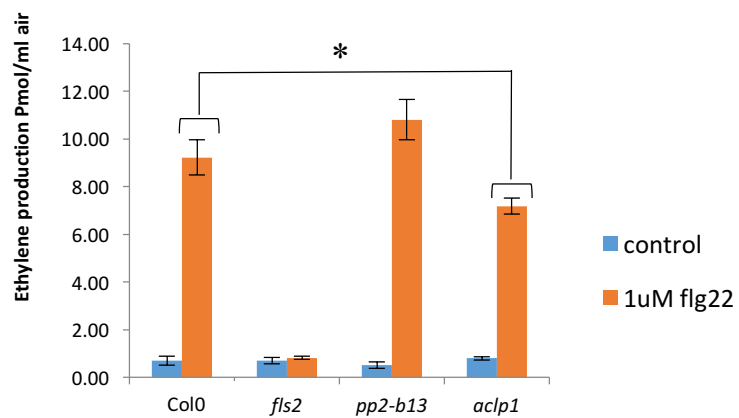
D)



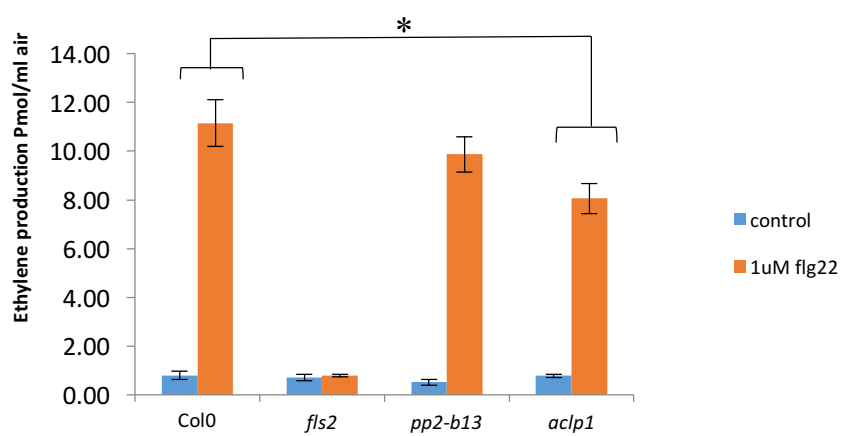
Supplementary Figure S5. Bacterial susceptibility assay. Leaves of four to six-week-old wild type (Col-0), mutant lines and also *sid2-2* mutant plants were pressure infiltrated with *Pseudomonas syringae* pv. *tomato* DC3000 and *P. syringae hrcC* ($OD_{600} = 0.0002$, in infiltration buffer). Open bars indicate bacterial colony from leaf discs of infected

leaves just after infiltration (0 day) and filled bars represent colony-forming units (cfu/cm²) 48 hours post inoculation. Results are average \pm s.e. (n=6). Similar results were observed in four independent experiments. Asterisks indicate a significant difference (* $p \leq 0.05$, ** $p \leq 0.01$ from the wild type plants as determined by Student's *t-test*).

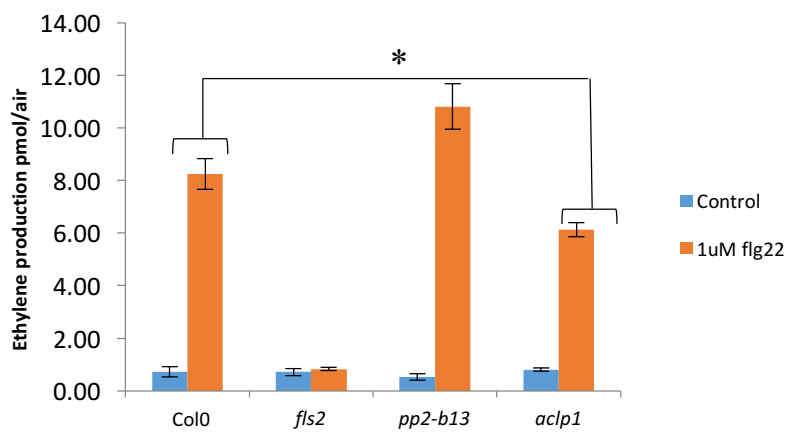
A)



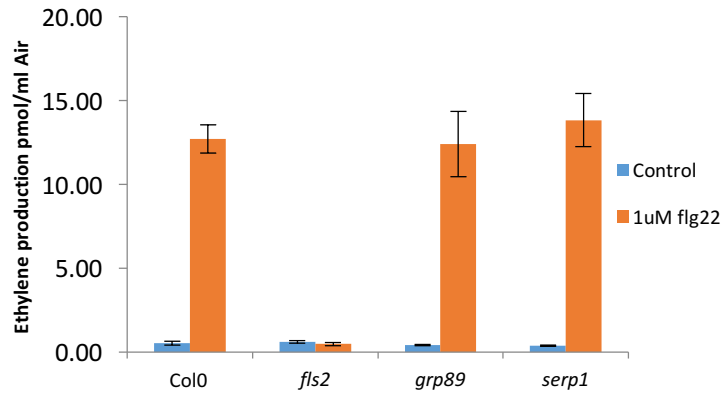
B)



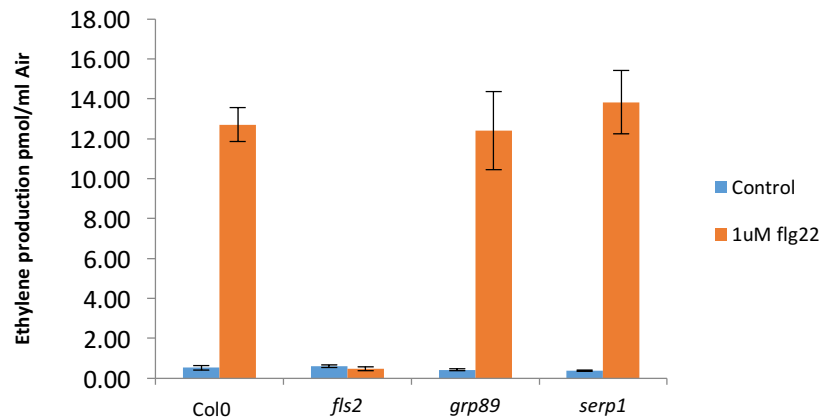
C)



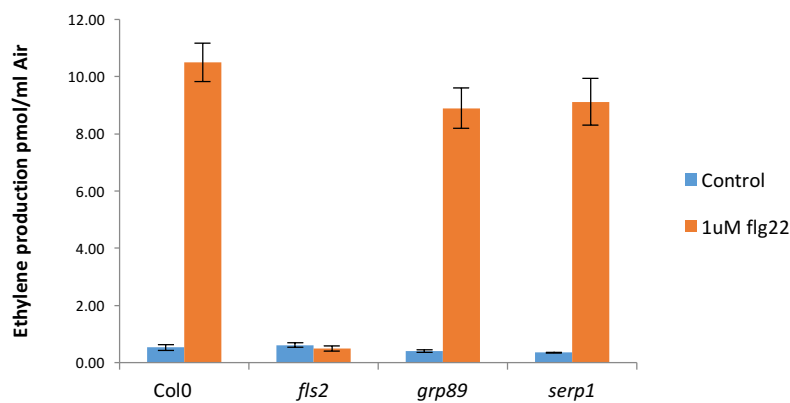
D)



E)

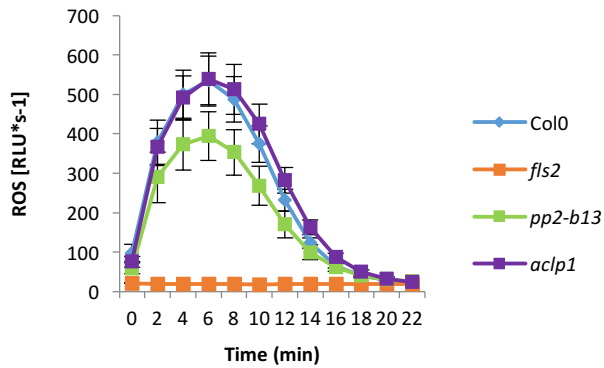


F)

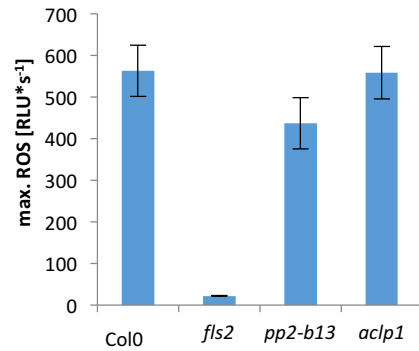


Supplementary Figure S6. Ethylene accumulation after elicitor treatment. Leaf discs of four to five weeks old of the mutant lines (*pp2-b13*, *aclp1*) and also wild type plants were treated with 1 μ M of the flg22 elicitor peptide or without any peptide (control). *fls2* mutant line were used as a negative control. In all cases ethylene production was measured three and half hours after closing tubes. Panel A; B and C; indicate ethylene accumulation in *pp2-b13* and *aclp1* mutant lines compared to the wild type Arabidopsis in three independent biological repeats. Panel D; E and F represent ethylene accumulation in *serp1* and *grp89* mutant lines compared to the wild type Arabidopsis in three independent biological repeats. *fls2* mutant line was used as a negative control. Columns represent averages of detected ethylene values of five biological replicates. Columns represent averages of detected ethylene values of five biological replicates. Error bars indicate standard deviation with n=6. Similar results were obtained in at least six independent biological replicates. T-test was performed comparing the responses of the control treatment to the elicitor treatments; P values are indicated * $p \leq 0.05$.

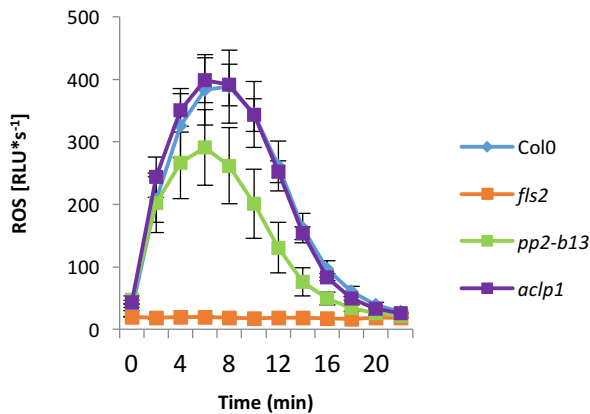
A)



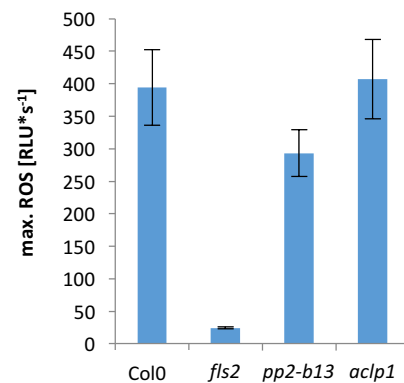
B)



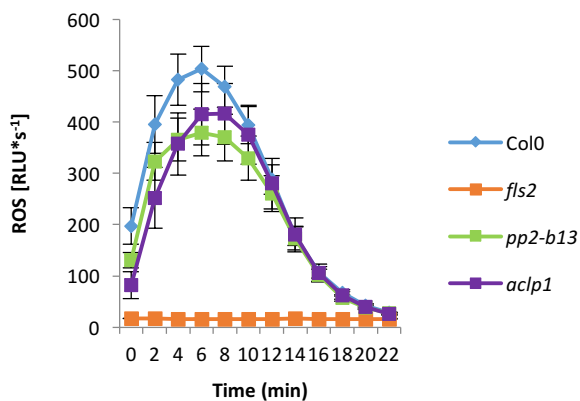
C)



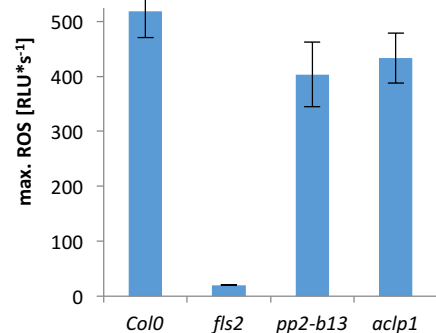
D)



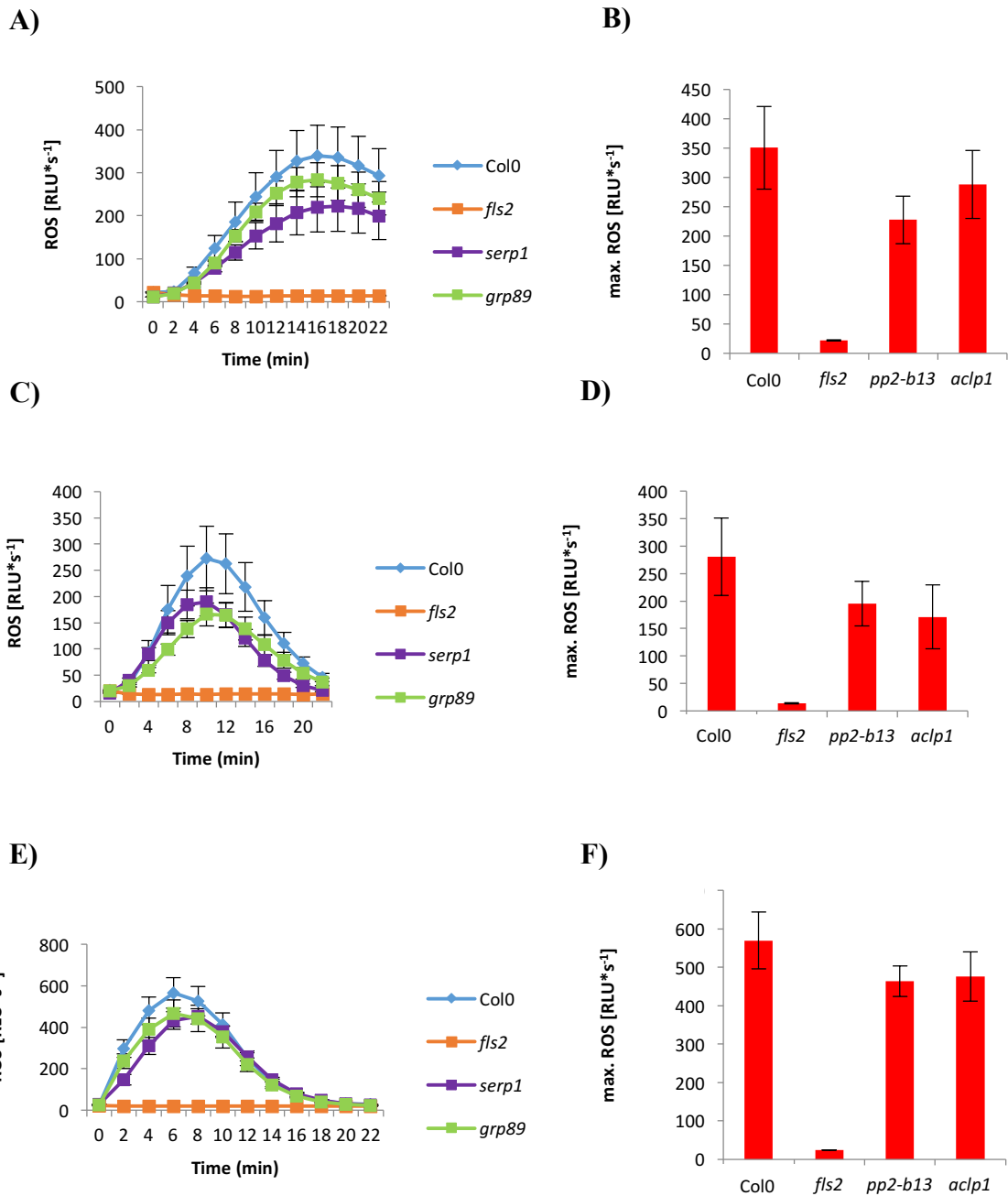
E)



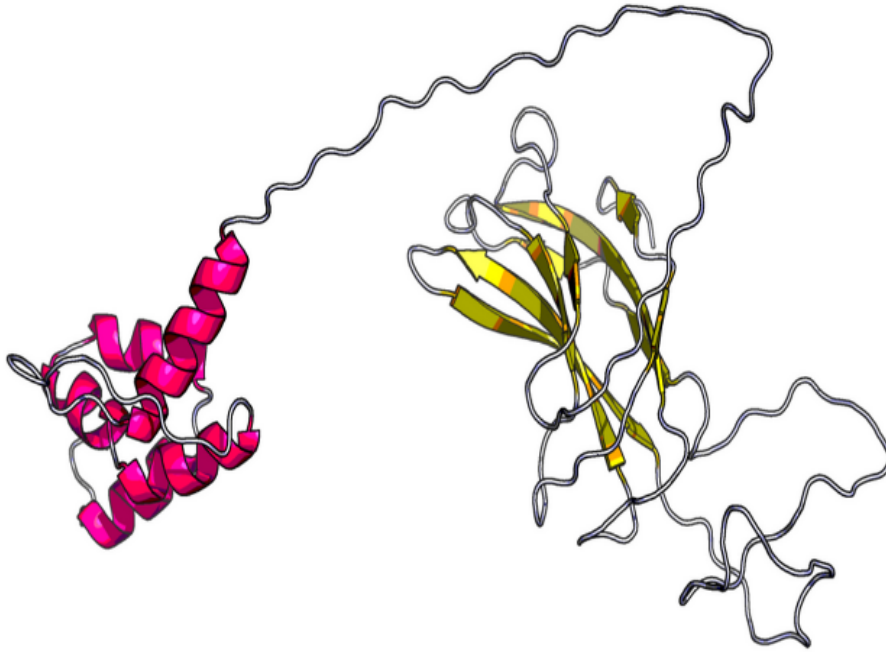
F)



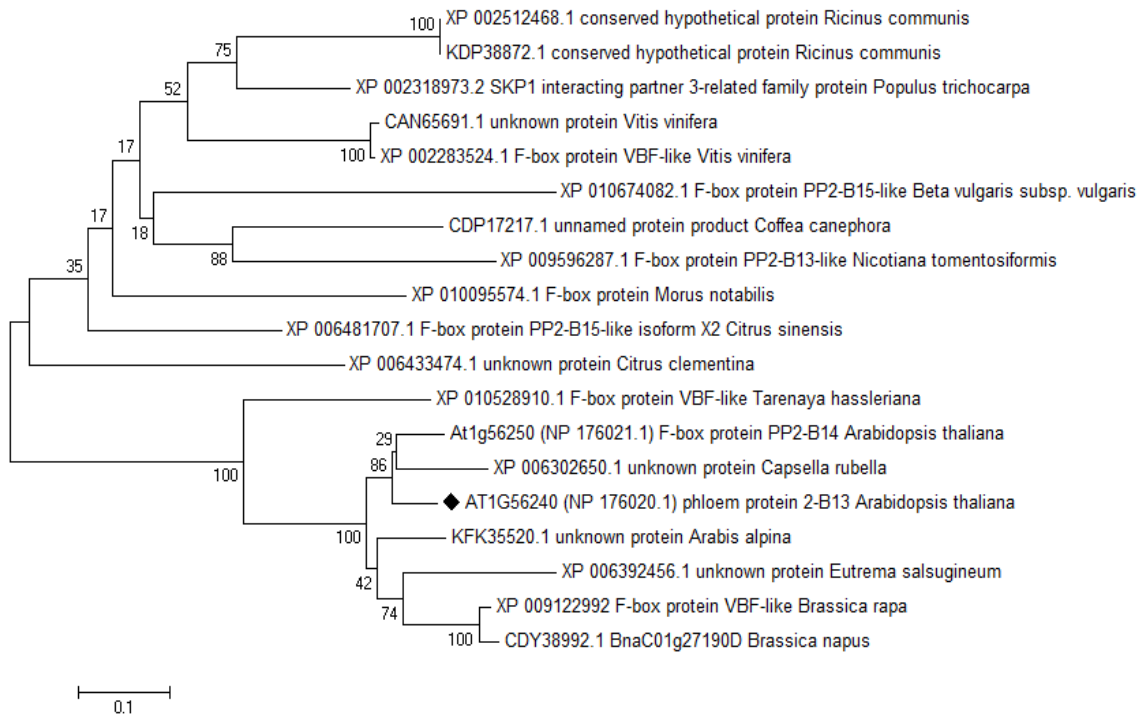
Supplementary Figure S7-A. ROS production after treatment with flg22. Leaf discs were treated with indicated 1 μ M flg22 or without any peptide (control). Panel A; C and E; indicate ROS production in *pp2-b13* and *aclp1* mutant lines compared to the wild type Arabidopsis in three independent biological replicates in three independent biological replicates. Panel B; D and F; represent maximum ROS production in *pp2-b13*, *aclp1* mutant lines compared to the wild type Arabidopsis in three independent biological replicates. *fls2* mutant line was used as a negative control. Graphs display average of 12 replicates. Error bars indicate standard error (SE) of the mean. The experiment was repeated four times with similar results. RLU= relative light units. *T-test* was performed comparing the responses of the control treatment to the elicitor treatments; P values are indicated * $p \leq 0.05$.



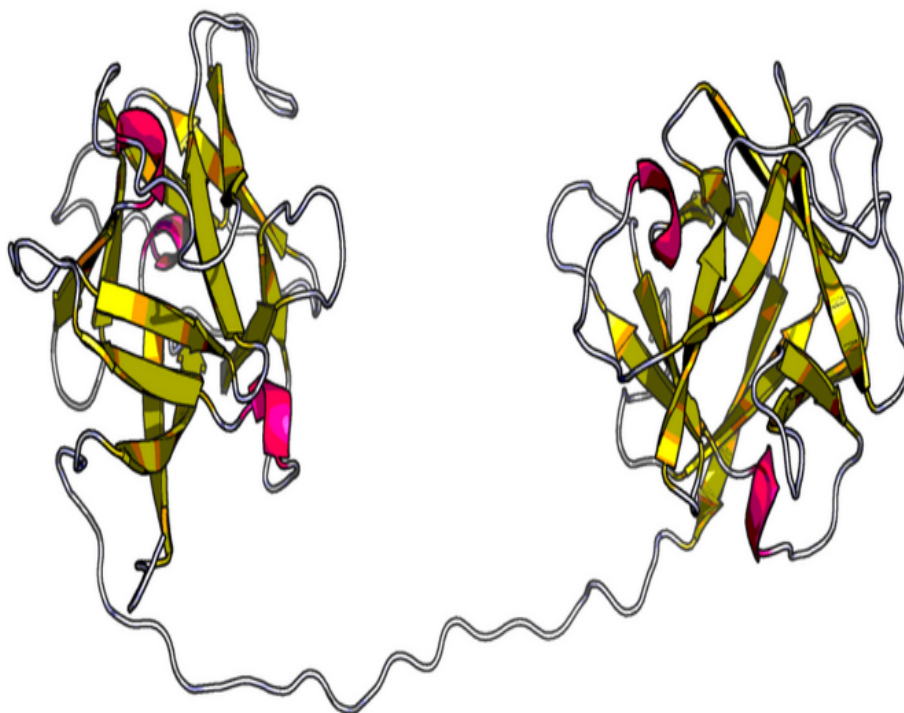
Supplementary Figure S7-B. ROS production after treatment with flg22. Leaf discs were treated with indicated 1 μ M flg22 or without any peptide (control). Panel A; C and E; indicate ROS production in *serp1* and *grp89* mutant lines compared to the wild type Arabidopsis in three independent biological replicates in three independent biological replicates. Panel B; D and F; represent maximum ROS production in *serp1* and *grp89* mutant lines compared to the wild type Arabidopsis in three independent biological replicates. *fls2* mutant line was used as a negative control. Graphs display average of 12 replicates. Error bars indicate standard error (SE) of the mean. The experiment was repeated four times with similar results. RLU= relative light units. *T-test* was performed comparing the responses of the control treatment to the elicitor treatments; P values are indicated * $p \leq 0.05$.



Supplementary Figure S8. Structure of PP2-B13 protein determined by Raptor X (Källberg *et al.*, 2012).



Supplementary Figure S9. Phylogenetic analysis from protein sequences of PP2-B13 proteins in *Arabidopsis* and other plant species. Sequences for comparisons were obtained from GenBank and the affiliation of each isolates is also indicated.



Supplementary Figure S10. Structure of ACLP1 protein determined by Raptor X (Källberg *et al.*, 2012).

Supplementary table 2-1. *Ct* value obtained for two sets of primers which were designed to evaluate the expression of *GRP89* upon flg22 treatment in different time points (0 hour, 30 minutes, 2 hours, 6 hours). For each time-point two different samples with three technical replicates were used (for Zero time point, three samples were used).

Primer name	FW-A-GRP	RW-A-GRP	FW-B-GRP	RW-B-GRP	
Position in the Plate	Treatment repeat-timepoint	<i>Ct</i> value	Position in the Plate	Treatment-timepoint	<i>Ct</i> value
A1	Zero 1	29	E1	Zero 1	20
A2	Zero 1	29	E2	Zero 1	21
A3	Zero 1	27	E3	Zero 1	20
A4	Zero 2	29	E4	Zero 2	20
A5	Zero 2	30	E5	Zero 2	20
A6	Zero 2	29	E6	Zero 2	20
A7	Zero 3	30	E7	Zero 3	20
A8	Zero 3	29	E8	Zero 3	20
A9	Zero 3	28	E9	Zero 3	20
A10	-flg22-1-30min	30	E10	-flg22-1-30min	20
A11	-flg22-1-30min	29	E11	-flg22-1-30min	20
A12	-flg22-1-30min	29	E12	-flg22-1-30min	20
B1	-flg22-2-30min	29	F1	-flg22-2-30min	19
B2	-flg22-2-30min	27	F2	-flg22-2-30min	19
B3	-flg22-2-30min	28	F3	-flg22-2-30min	18
B4	+flg22-1-30min	29	F4	+flg22-1-30min	19
B5	+flg22-1-30min	27	F5	+flg22-1-30min	19
B6	+flg22-1-30min	29	F6	+flg22-1-30min	18
B7	+flg22-2-30min	27	F7	+flg22-2-30min	19
B8	+flg22-2-30min	27	F8	+flg22-2-30min	19
B9	+flg22-2-30min	27	F9	+flg22-2-30min	19
B10	-flg22-1-2h	29	F10	-flg22-1-2h	20
B11	-flg22-1-2h	31	F11	-flg22-1-2h	20
B12	-flg22-1-2h	27	F12	-flg22-1-2h	21
C1	-flg22-2-2h	29	G1	-flg22-2-2h	20
C2	-flg22-2-2h	30	G2	-flg22-2-2h	20
C3	-flg22-2-2h	28	G3	-flg22-2-2h	20
C4	+flg22-1-2h	28	G4	+flg22-1-2h	19

Continued

Primer name	FW-A-GRP	RW-A-GRP	FW-B-GRP	RW-B-GRP	
Position in the Plate	Treatment repeat-timepoint	Ct value	Position in the Plate	Treatment-timepoint	Ct value
C5	+flg22-1-2h	28	G5	+flg22-1-2h	19
C6	+flg22-1-2h	27	G6	+flg22-1-2h	18
C7	+flg22-2-2h	28	G7	+flg22-2-2h	19
C8	+flg22-2-2h	29	G8	+flg22-2-2h	19
C9	+flg22-2-2h	29	G9	+flg22-2-2h	19
C10	-flg22-1-6h	30	G10	-flg22-1-6h	21
C11	-flg22-1-6h	28	G11	-flg22-1-6h	21
C12	-flg22-1-6h	28	G12	-flg22-1-6h	20
D1	-flg22-2-6h	30	H1	-flg22-2-6h	20
D2	-flg22-2-6h	30	H2	-flg22-2-6h	20
D3	-flg22-2-6h	29	H3	-flg22-2-6h	20
D4	+flg22-1-6h	27	H4	+flg22-1-6h	19
D5	+flg22-1-6h	26	H5	+flg22-1-6h	19
D6	+flg22-1-6h	27	H6	+flg22-1-6h	19
D7	+flg22-2-6h	29	H7	+flg22-2-6h	19
D8	+flg22-2-6h	28	H8	+flg22-2-6h	19
D9	+flg22-2-6h	28	H9	+flg22-2-6h	19

2.6 Materials and Methods

2.6.1 Plant Material and Growth Conditions

2.6.1.1 *In vitro* Conditions for *A. thaliana*

All genotypes Arabidopsis accessions were derived from the wild- type accession Columbia-0 (Col-0). Arabidopsis seeds first was washed with 99% ethanol supplemented with 0.5% Triton for one minute; and washed again with 50% ethanol supplemented with 0.5% Triton for one minute; and finally washed with 100% ethanol for two minutes. Then, the seeds were sown on Murashige and Skoog salt solid medium containing 1% sucrose and 0.8% agar at pH 5.7. Then in order to vernalize the seeds, the plates were kept for two days at 4°C in the dark before transferring them to continuous light at 20°C for germination.

2.6.1.2 *A. thaliana* “short-day” Conditions

Seeds were sown in soil and then vernalized for two days at 4°C in the dark. Then the pots were placed under the short-day conditions (ten hours light at 21°C and 14 hours dark at 18°C with 60% humidity). One week after germination, plants were grown as one plant per pot. Plants were grown in these conditions for four weeks.

2.6.1.3 “Long day” Conditions for *A. thaliana*

After sowing the seeds, the pots were kept for two days in the dark at 4°C for vernalization then transferred in 16 hours light at 21°C and 8 hours dark at 18°C with 55% humidity. Plants were grown as one plant per pot.

2.6.2. Genotyping T-DNA Mutants

2.6.2.1 Data analysis

From deep sequencing data, genes which were up-regulated (gene expression profile after 30 minutes of treatment of Arabidopsis seedling with 1 μ M flg22 and also 1 μ M *AtPep1*) initially were selected as a pool (Appendix 1). Subsequently, based on the Microarray data (Zipfel *et al.*, 2004) those genes which previously were reported and also those genes which previously were studied were discarded from the list. At the next step a subset of 100 genes (Appendix 2) were chosen and first classified based on the following criteria: 1) high induction of transcription after flg22 treatment, 2) not present on Affymetrix ATH-22k microarray chips, 3) no published function or at least not connected to defense, and 4) not a member of a large gene family (in order to avoid potential redundancy, so that in the absence of the gene it is compensated by other gene family members). Finally 20 of them are selected as the candidate genes (Table 2-1) and T-DNA insertion lines available at the NASC (<http://signal.salk.edu/cgi-bin/tdnaexpress>) were ordered.

2.6.2.2 Analysis of T-DNA Insertion Mutants

Based on the genomic sequence surrounding the T-DNA insertion, primers LP and RP were designed. The LBE primer is based on the left border of the T-DNA insertion site. Alleles with T-DNA insertion site were amplified using the BP and LB primers, whereas alleles without any insertion in the gene were amplified just using the LB and RB primers. The plant were regarded as homozygous mutants, if there was a PCR product with BP/LB primers indicating the presence of the T-DNA insertion; but not any product with the LB/RB primers (Figure 2-7).

After grinding leaf material in liquid nitrogen, total DNA was extracted using EDM-Buffer

(200 mM Tris pH7.5; 250 mM NaCl, 25 mM EDTA; 0.5% SDS), and using a PCR assay (2-6), all of the T-DNA insertion mutants were genotyped (Table 2-1). Nine of them were found to have insertions and homozygous lines were obtained.

2.6.2.3 Primers Designing

In order to design the specific primers and also discard any primer dimer, Oligo Calc primer designer (<http://www.basic.northwestern.edu/biotools/oligocalc.html>) was used with target $T_m = 60^\circ\text{C} \pm 1^\circ\text{C}$; primer length range from 18 to 23 base pairs. Primers were ordered from Microsynth AG (Balgach, Switzerland) without any modifications at the sequence and diluted in ddH₂O to reach 100 μM . The sequence of all primers are listed in Table 2-9.

2.6.2.4 Analysis of Nucleic Acid

A 1% agarose gel containing 0.1 $\mu\text{g/ml}$ Ethidium bromide (EtBr) was prepared and nucleic acids were loaded on to the gel. After electrophoresis in TAE buffer (Tris-HCl 50 mM, pH 8.0, acetic acid 20 mM, EDTA 0.5 mM) depending on the size, amplified DNA fragments were

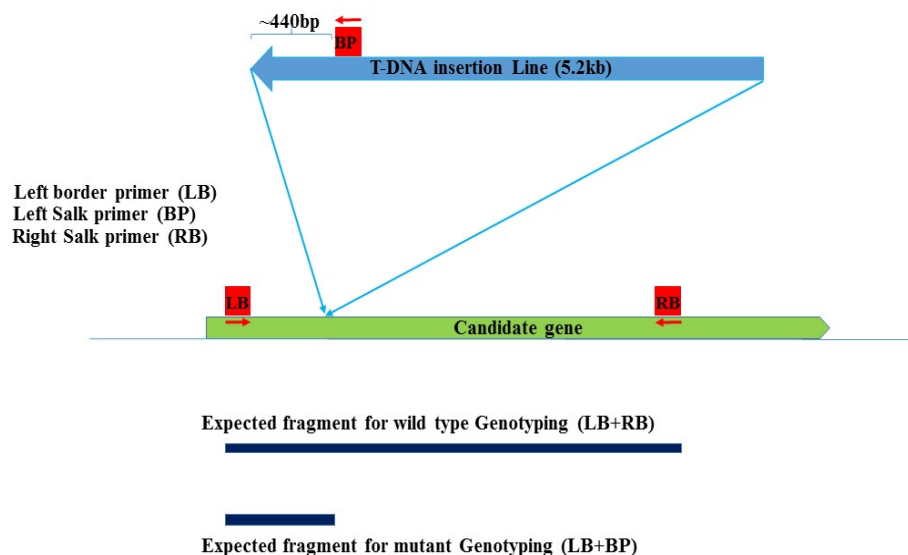


Figure 2.7- Schematic representation to identify homozygous T-DNA insertion mutants

detected under ultraviolet light and sizes were determined by comparison with a commercial DNA gene ruler (GeneRuler™ 1kb DNA Ladder, Fermentas).

2.6.2.5 Quantification of Nucleic Acid

NanoDrop 2000 (Thermo-Scientific) was used for quantification and verification of purity of DNA or RNA, according to the manufacturer's instructions.

Table 2-6. Reagents and PCR program for Mutagenesis study

PCR reagents		PCR program			
DNA	5 µl	steps	Temp (°C)	Time	Cycle
Forward primer (5 µM)	0.5 µl	--	95	∞	--
Reverse primer (5 µM)	0.5 µl	--	95	3'	--
buffer FirePol	1 µl	--	95	30'	--
dNTP (10 µM)	0.5 µl	denaturing	95	30''	10
Taq DNA polymerase	0.25 µl	hybridization	62	30''	10
ddH ₂ O	16.25 µl	elongation	72	1':30''	10
		denaturing	95	30''	28
		hybridization	55	30''	28
		elongation	72	1':30''	28
		Final elongation	72	5'	--
		--	8	∞	--

2.6.3 Quantitative RT-PCR

2.6.3.1 Treatment of Arabidopsis Leaf Disk

Discs of leaves of four weeks old Arabidopsis plants were cut out using a sterile cork borer (d=7mm) and placed overnight in 5 cm Petri dish. In the morning, 1 µM flg22 was added to each Petri dish in a time course, including: 30 min, 2 hours and 6 hours. BSA solution (1 mg/mL bovine serum albumin and 0.1 M NaCl) was used as a control.

2.6.3.2 Quantitative RT-PCR

Total RNA from leaves of four weeks *Arabidopsis* plants was extracted using the NucleoSpin RNA plant extraction kit (Macherey-Nagel) and treated with rDNase according to the manufacturer's extraction protocol. After RNA quality of all samples was assessed using NanoDrop and in each PCR reaction, 10 ng of RNA was used to synthesize the cDNA with oligo (dT) primers using AMV reverse transcriptase according to the manufacturer's instructions (Promega). Using a GeneAmp 7500 Sequence Detection System (Applied Biosystems), quantitative RT-PCR was performed in a 96-well format. Expression of *UBQ10* (AT4G05320) which is validated for gene expression profiling upon flg22 treatment (Flury *et al.*, 2013; Wyrsh *et al.*, 2015) was used as the reference gene and based on the obtained C_T values and also normalized expression to the *UBQ10* (AT4G05320), the expression profile for each candidate gene was calculated using the qGene protocol (Muller *et al.*, 2002).

2.6.4 Bacterial Growth Assay

Pseudomonas syringae pathovar *tomato* DC3000 and also *hrcC*- were grown in 20 ml liquid YEB medium supplemented with 50 µg/ml Rifampicin on a shaker at 28°C overnight. Then the bacterial suspension was centrifuged at 20 °C for 10 min at 4000 rpm in a Megafuge 1.0R (Heraeus). Afterwards, the pellet was resuspended in infiltration buffer (10 mM MgCl₂, 10 mM MES, pH 5.6). In order to reach the optical density equivalent to OD₆₀₀ = 0.0002, using a Biophotometer (Eppendorf), the OD was measured between 0.1 and 1 and then the suspension was diluted. Leaves of 4-5 weeks old *Arabidopsis* plants were used for the infiltration assay. One hour before infiltration, plants were watered and leaves were sprayed with sterile water. Using a syringe without a needle, the plants were pressure-infiltrated with a bacterial suspension. The *sid2-2* mutant plants (do not accumulate salicylic acid) were used as a positive control (Wildermuth *et al.*, 2001). After infiltration, plants were again watered. Mock-infected

plants were similarly treated with infiltration buffer. One hour after infiltration, plants were directly transferred to growth chambers (Sanyo, Japan) and were maintained at 23°C with 12h/12h light/dark cycle. In order to determine the bacterial titers at day 0, as soon as leaves were dry (about one to one and half hours post-infiltration) using a sterile hole puncher (d = 7mm), two leaf disks from two different leaves were harvested. Parts of the main vein were discarded. This first harvest was regarded as day 0. In order to determine the bacterial titers, the leaf disks were transferred to a 1.5 ml Eppendorf tube containing 200 µl infiltration buffer. Then the leaves were ground for 10 second (s), vortexed vigorously and ground again for 10 s. After centrifugation using a 96 well plate, dilutions were performed by adding 10 µl of the supernatant to 90 µl buffer in the well and then making serial dilutions. For this, bacterial suspensions were vortexed and 20 µl of the sample was added to 80 µl of 10 mM MgCl₂. Each time the suspension was mixed by pipetting up and down and the pipette tips were changed between each dilution step.

From each individual dilution, 20 µl of each sample was plated on solid YEB containing 50 µg/ml Rifampicin and 50 µg/ml of the fungicide Nystatin. To allow distinction of the different samples, tissue culture dishes with a 20 mm grid were used, and one sample was plated per square. Plates were left open under sterile conditions until they were dry. Afterward, the plates were incubated for 36 hours (h) at 28 °C and colonies were counted using a Binocular (Olympus SZX12). The method for harvest and dilution procedure was the same for the harvests on day 0, day 2 and day 4.

2.6.5 Peptides

Peptides were used as elicitors, including Flg22 (QRLSTGSRINSAKDDAAGLQIA), and *AtPep1* (ATKVKAKQRGKEKVSSGRPGQHN), which were obtained from EZBiolabs (<http://www.ezbiolab.com/>), and were dissolved in a solution containing 1 mg/mL bovine serum albumin (BSA) and 0.1 M NaCl, and were kept in -20°C.

2.6.6 Measurement of ET Production

Leaf material of Arabidopsis plants was cut into discs of 10 mm² using a sterile cork borer, in the evening. After mixing leaf strips from several plants, six leaf strips were placed together in a 6 ml glass vial containing 0.5 ml of ddH₂O. Vials with leaf strips were incubated overnight in the dark in a short-day room. A day later (approximately after 16 hours), elicitor peptide (1 μM final concentration) was added to the desired final concentration and vials were closed with air-tight rubber septa and put in the short-day room. ET accumulating in the free air space was measured by gas chromatography (GC-14A Shimadzu) after 4 hours of incubation with elicitor or without elicitor.

2.6.7 Measurement of ROS Generation

Using a sterile cork borer, leaf discs of approximately 5 mm² were cut from several plants. One leaf disc per well was floated overnight in darkness in 96-well plates (LIA White, Greiner Bio-One) on 100 ul ddH₂O at 18°C. ROS was released by adding horseradish peroxidase (1 μg final concentration), luminol (100 μM final concentration) and also the elicitor peptide (1 μM final concentration). Using a plate reader (MicroLumat LB96P, Berthold Technologies) directly after addition of the elicitor peptides, quantification of light emission of oxidized luminol in the presence of peroxidase was determined over 30 minutes (min).

2.6.8 SDS-PAGE and Western Blotting

Proteins were extracted from plant material by the following method: 150 mg of leaf material was directly shock frozen and ground in liquid nitrogen. To this ground material 200 μl Lämmli buffer containing 50 mM β-mercaptoethanol was added and the ground homogenate was further mixed with the tissue powder by vortexing.

Proteins were denatured by boiling for 10 min at 95 °C. Debris was pelleted by centrifugation for 5 min at 13,000 rpm. A mini format SDS-PAGE -polyacrylamide gel (7 %; Table 2-7) was

Table 2-7. Polyacrylamid gel compositions.

	Stacking Gel	Running Gel
Acrylamide mix	1%	7%
SDS	0.0104 %	0.1 %
Tris pH 8.8	130mM	375 mM
TEMED	0.08 %	0.08 %
10 % APS	0.1 %	0.1 %

prepared and the electrophoresis cell was filled with SDS running buffer (Table 2-8). Then, 50 μ l of supernatant was mixed with loading buffer and loaded into the wells. A commercial molecular weight protein standard was used as a ladder. The electrophoresis was run for 2.5 hours at 110V.

2.6.9 Transferring the Protein to the Membrane

First, the gel was transferred to the 1x transfer buffer (Table 2-8) and then the cassette was assembled, as the gel was in between the membrane, two sheets of Whatman paper and two sponges with the membrane facing towards the positive charge of the tank. Then the cassette was transferred to the tank containing an ice block and was electrophoresed for 2 hours in 110V.

Table 2-8. Reagents were used for western blotting.

Buffer	Reagents	Solution	pH
	Tris-HCl	25 mM	
Running buffer: Tris/Glycine/SDS	Glycine	0.2 M	8.3
	SDS	0.1 %	
	SDS	2 %	
	0.125 M Tris-HCl	50 mM	
Loading Buffer: 2x Laemmli buffer	Bromophenol blue	50 mM	6.8
	Glycerol	10 %	
	2-Mercaptoethanol ¹	5 %	
	Ponceau S	1 % (w/v)	
Ponceau S staining Solution	Glacial acetic acid	5 %	
	NaCl	140 mM	
PBS Phosphate Buffer	Na ₂ HPO ₄	10 mM	pH 7.3
	KCl	2.7 mM	
	KH ₂ PO ₄	1.8 mM	
	Methanol	40 %	
Coomassie blueStaining solution	Coomassie Brilliant Blue	0.25 %	
	Glacial acetic acid	10 %	
Blocking buffer	Bovine serum albumin (BSA) in PBS buffer	3%	---
Transfer buffer	Tris-HCl	25mM	
	Methanol	20%	8.3
	Glycine	190mM	
1) 2-Mercaptoethanol should be add to the buffer directly before use			

2.6.10 Antibody Incubation

The cassette was opened, and to evaluate the transfer quality, the membrane was stained in Ponceau solution (2-8) and then rinsed off again with three wash-steps with buffer. The membrane was blocked with 2% BSA in PBS buffer for one hour at room temperature. The blocking buffer was removed and then the primary antibody, which was diluted in the blocking buffer (1:10000), was added and the membrane plus antibody probing solution were incubated overnight at 4°C. In the morning the membrane was washed 3-4 times with 1xPBS buffer for 5 minutes each time. Then secondary antibody was diluted in blocking buffer (1:20000) and was incubated with the membrane in a shaker at room temperature for one hour. Then, the membrane was washed five times for 5 minutes each times in PBS buffer and again one time with AP-Buffer for 5 minutes. Finally, AP-Buffer containing 6 µl CDP (Chemiluminescent Substrate; CDP-Star®) was added to each membrane, after which the membrane was photographed using Azure biosystems c300.

Table 2-9. List of the oligonucleotides which were used.

Purpose	Forward Primer Sequence (5' – 3')	Reverse Primer Sequence (5' – 3')
AT1G69900 (qRT-PCR, gene expression)	GGA ATA TTT CCA TCG CCG ATA C	GAT CCT GGG TCA CTT GTA TCA G
AT1G65385 (qRT-PCR, gene expression)	CCC ATA TCA AGA GCA CAA CCG	CGT TTT GAA TCA AGT GGG AGT AC
AT1G56240 (qRT-PCR, gene expression)	TCC TTT CAC CTC CGT AAG ACT C	GGG TGG TTG GAG ATT GAG TTA G
AT1G56240 (qRT-PCR, copy number determination)	CTA CGG CCT CCA CAG TCA TC	GCG GAG ACA TCG GTT AAA TC
AT1G56240 (qRT-PCR, copy number determination)	CGT GAC ACA GAC TAA ATA ATA GAT C	CCT CTG AAA TAG GGA TCA AGA TG
AT1G56240 (qRT-PCR, copy number determination)	TCA CAA TTC CAC CTT TCA GTT G	GGT GGT TGG AGA TTG AGT TAG
AT4G23215 Mutagenesis	TCCAGCTATCATACGACTTGTC	CATCTCTGATAGCGTTGCTCTG
AT1G56240 Mutagenesis	TCAATCTCCAACCACCCGTC	GGT CAG CAG AAA TAT GCC AAT GAT CAC T
AT1G69900 Mutagenesis	GAGACCGACGAGTTAAAAGTAC	TAAACCAAAATTCATACGTCCTCAAG
AT1G65385 Mutagenesis	TC TTGATGAGATTATTGGTGTGATC	CAAAACGACGTCGCGATGATC
AT1G24145 Mutagenesis	GACCGAGTAAACGCTAAGATCCAC	GAG GAG GTT GTG GAA GTG AGG ATG
AT2G27389 Mutagenesis	CTCAAACCGTGGTGGCAGC	GAGCTGCGTTTAGGCACTAC
SALK_LB1	TGGTTCACGTAGTGGGCC	---
A-PP (evaluation the expression of <i>PP2-B13</i> and AT1G56242)	5'-CGT GAC ACA GAC TAA ATA ATA GAT C-3'	5'-CCT CTG AAA TAG GGA TCA AGA TG-3'
B-PP (evaluation the expression of <i>PP2-B13</i> and AT1G56242)	5'-TCA CAA TTC CAC CTT TCA GTT G-3'	5'-GGT GGT TGG AGA TTG AGT TAG-3'
B1-PP (evaluation the expression of <i>PP2-B13</i> and AT1G56242)	5'-TCA CAA TTC CAC CTT TCA GTT G-3'	5'-GAT GAC TGT GGA GGC CGT AG-3'
C-PP (evaluation the expression of <i>PP2-B13</i> and AT1G56242)	5'-ATT GCT CCA AGA GCA GTA CG-3'	5'-CTG ATC ACC ATC GGA TTT TTT CG-3'
A-GRP (evaluation the expression of <i>GRP89-B13</i> and AT2G27390)	5'-GGC TTG ATG TTT AAG GTG TAA GG-3'	5'-ACC GGC AAG CTG CCT ACG-3'
B-GRP (evaluation the expression of <i>GRP89-B13</i> and AT2G27390)	5'-GTG GCT CTG GAG GAA ATA AGG-3'	5'-GCC TGG AAC TAC GGG AAC TTG-3'
C-GRP (evaluation the expression of <i>GRP89-B13</i> and AT2G27390)	5'-CCG TCT CAC ATT CTC CGT TG-3'	5'-GAT AGA GGG GAC AGG AGC TG-3'
SALK_RB	TCATGCGAAACGATCCAG	---
SALK_LB2	GCGTGGACCGCTTGCTGCAACT	---
Pro35S	CGCACAATCCCACTATCCTTCGC	---
SAIL-Pdap101_LB1	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC	---
<i>UBQ10</i> (AT4G05320) (qRT-PCR, copy number determination)	GGCCTTGATAATCCCTGATGAATAAG	AAAGAGATAACAGGAACGGAACATAG

3. Promoter Activation of the *AtPROPEP* and *AtPEPR* Genes and their Regulation under Biotic and Abiotic Stress, Studied with Promoter-GUS-reporter Constructs in *A. thaliana*

3.1 Abstract

In *A. thaliana* *AtPEPR1* and *AtPEPR2* act as the receptors for the endogenous *AtPROPEP*-derived Pep peptides and subsequently initiate defense-signaling cascades. In previous work, the expression pattern of the genes encoding the PEPR receptors and the PROPEP peptide precursor proteins was studied using promoter-GUS reporter constructs (Bartels *et al.*, 2013). Here, using the same constructs to study their expression pattern under biotic and abiotic stress, including *AtPep1*, flg22, MeJA, and NaCl treatments, we observed that in response to *AtPep1* and flg22, the activation of *AtPEPR1* promoter was different from *AtPEPR2*. We also observed that these promoters were differentially activated in response to NaCl. Remarkably, we observed that it is possible to classify the genes of the *AtPROPEP* family, based on the response of their promoters to the various stimuli employed: thus, we classify *AtPROPEP1* and in one group; *AtPROPEP2* and *AtPROPEP3* in a second group; *AtPROPEP4* and *AtPROPEP7* and *AtPROPEP8* in a third group and *AtPROPEP5* in a fourth group.

3.2 Introduction

In plants, PRRs recognize microbe-derived signature components known as MAMPs and also DAMPs which are host-derived danger signals that subsequently trigger plant immunity (Boller and Felix, 2009; Macho and Zipfel, 2015). Some of the well-studied PRRs are FLS2, EFR and CERK1 from *A. thaliana*, perceive the conserved N-terminal portion of flg22, EF-Tu, and fungal cell wall component chitin, respectively (Gomez-Gomez and Boller, 2000; Miya *et al.*, 2007; Zipfel *et al.*, 2006).

The family of *AtPeps*, peptides which were initially identified as endogenous peptide elicitors in *A. thaliana* (Huffaker *et al.*, 2006), are considered to be DAMPs because they may play a critical role in defense signaling, but they may also be involved in development (Bartels and Boller, 2015). *AtPep1* and its homologs (*AtPep2-8*) originate from the conserved C-terminal portion of their respective precursors *AtPROPEP1-8*, respectively (Bartels *et al.*, 2013; Huffaker *et al.*, 2006). They are perceived through two plant cell surface PRRs, namely *AtPEPR1* and *AtPEPR2* (Newman *et al.*, 2013; Yamaguchi *et al.*, 2010; Yamaguchi and Huffaker, 2011).

Perception of Peps by the PEPRs, like perception of MAMPs by the corresponding PRRs, leads to the activation of downstream defense cascades including ion fluxes across the plasma membrane (e.g. increase in Ca^{2+} influx) within 30 seconds to 2 minutes (Qi *et al.*, 2010). The Ca^{2+} elevation leads to the activation of the RbohD protein, which has crucial role in the production of ROS which occurs in 2 to 3 minutes (Baxter *et al.*, 2013; Gomez-Gomez and Boller, 2000). Concomitantly, phosphorylation of MAPKs (particularly of MPK3, MPK4 and MPK6) via MAP kinase cascades which occurs a few minutes after elicitor perception (Asai *et al.*, 2002; Felix *et al.*, 1999; Ranf *et al.*, 2011). In addition, stomatal closure is accrued after a few minutes upon elicitor perception (Mott *et al.*, 2014). As well, ET biosynthesis is enhanced through activation of ACC synthase within 30 minutes (ACS; Liu and Zhang, 2004).

Furthermore, jasmonic acid responsive genes involved in plant defense get activated in a few hours (Huffaker and Ryan 2007; Ross *et al.*, 2014). Also, the accumulation of the plant defense hormone SA, which occurs in a few hours, is a subsequent event after elicitor perception (Huffaker and Ryan 2007; Huffaker *et al.*, 2011; Ross *et al.*, 2014).

DAMP-triggered signaling cascades also leads to massive transcriptional reprogramming of the host cell, which may subsequently fine-tune effective and adequate defense responses (Ross *et al.*, 2014).

It has been observed that treatments of *Arabidopsis* plants with *AtPeps* induce the transcription of their own precursor genes and also other defense genes (Bartels and Boller 2015; Ross *et al.*, 2014). Studies showed that the expression of the *AtPROPEP1* gene is induced upon flg22 treatment, *AtPep1* itself, wounding, ET, and also MeJA (Huffaker *et al.*, 2006). It was also observed that WRKY TFs, which are the major regulators of MAMP-induced genes, have a role in expression of *AtPROPEP2* and *AtPROPEP3* expression (Logemann *et al.*, 2013). It seems that there is a positive feedback between Peps perception and activation of the immune system (Ryan *et al.*, 2007). It has also been observed that perception of Peps can increase resistance against bacterial or fungal infections in *Arabidopsis* and also in maize (Huffaker *et al.*, 2011, 2013; Tintor *et al.*, 2013) and it has recently been shown that *AtPEPR1* and *AtPEPR2* may play a crucial role in inducing local and systemic immunity (Ross *et al.*, 2014).

The PRRs *AtPEPR1* and *AtPEPR2* have been identified as the receptors for *AtPeps*. Both belong to the RLKs with extracellular LRR motifs (Yamaguchi *et al.*, 2010; Yamaguchi and Huffaker, 2011). *AtPEPR1* and *AtPEPR2* have different preferences for *AtPeps* perception: *AtPep1* to *AtPep8* are perceived by *AtPEPR1*, and they have similar activity in inducing *AtPEPR1*-mediated plant immune responses, while *AtPEPR2* is only responsive to *AtPep1* and *AtPep2* (Bartels *et al.* 2013; Yamaguchi *et al.*, 2010).

Despite numerous attempts to determine the role of each *AtPROPEP* and also *AtPEPR1* and *AtPEPR2*, the exact role of these elicitor and of the corresponding receptors is still unknown (Bartels and Boller, 2015). Bartels *et al.* (2013) used promoter-GUS fusion lines to study the expression pattern of the *AtPROPEP* genes. They observed that different *AtPROPEPs* promoters represented diverse expression patterns and found that although *AtPeps* are redundant, the activation pattern of *AtPROPEPs* are different. They proposed that *AtPROPEPs* can be classified into two groups, based on the expression patterns of GUS in the various promoter-GUS fusion lines. In that study, they classified *AtPROPEP1*, *AtPROPEP3*, *AtPROPEP5*, and *AtPROPEP8* in one group and *AtPROPEP4* and *AtPROPEP7* in the other group. Furthermore, in that study, they showed that expression of the *AtPROPEP1*, *AtPROPEP2*, and *AtPROPEP3* genes was related to plant defense, while *AtPROPEP5* expression was related to plant reproduction. The classification that they proposed in that study, was essentially based on the effects of wounding and plant development on *AtPROPEPs* promoter expression patterns. Using promoter- β -glucuronidase fusion constructs including: *pAtPEPR1::GUS*, *pAtPEPR2::GUS*, *pAtPROPEP1::GUS*, *pAtPROPEP2::GUS*, *pAtPROPEP3::GUS*, *pAtPROPEP4::GUS*, *pAtPROPEP5::GUS*, *pAtPROPEP7::GUS*, and *pAtPROPEP8::GUS*, we attempted to further evaluate and dissect the promoter activities of the genes encoding these endogenous peptide defense signals and also their corresponding receptors under different biotic and abiotic stresses and subsequently, to characterize and classify them into different groups.

3.3 Results

3.3.1 Differential, and Tissue Specific Activation of the Promoters *pAtPEPR1* and *pAtPEPR2* Studied in promoter-GUS Reporter Lines in Seedlings of *A. thaliana* in Response to *AtPep1* and *flg22* Treatment

We first studied the activities of the *pAtPEPR1* and *pAtPEPR2* promoters in seedlings harboring promoter-GUS fusions after elicitor treatment and compared them with mock-treated controls. We fixed the seedlings after 0, 1, 6, and 24 h and then performed a GUS staining.

We noticed that *pAtPEPR1* and *pAtPEPR2* promoters exhibited different expression patterns in response to these elicitor treatments. The *pAtPEPR1* promoter was activated already one hour after the *AtPep1* treatment in cotyledons, in shoots and in roots, but neither in the hypocotyls nor in the root tips. Compared to the mock-treated control seedlings, 6 hours after the *AtPep1* treatment, the *pAtPEPR1* promoter became activated even more higher level in cotyledon leaves and root tip. We did not observe the activation of *pAtPEPR1* promoter in hypocotyls. Within 24 hours post treatment, compared to the mock-treated control seedlings, we noticed that the *pAtPEPR1* promoter was activated in the in shoot, but its activation was weaker than 6 hours post treatment (Figure 3-1; Table 3-1).

On the other hand, one hour post treatment with *flg22*, the *pAtPEPR1* promoter became activated in cotyledons but its activation remained more and less the same after 6 and 24 hours post treatment.

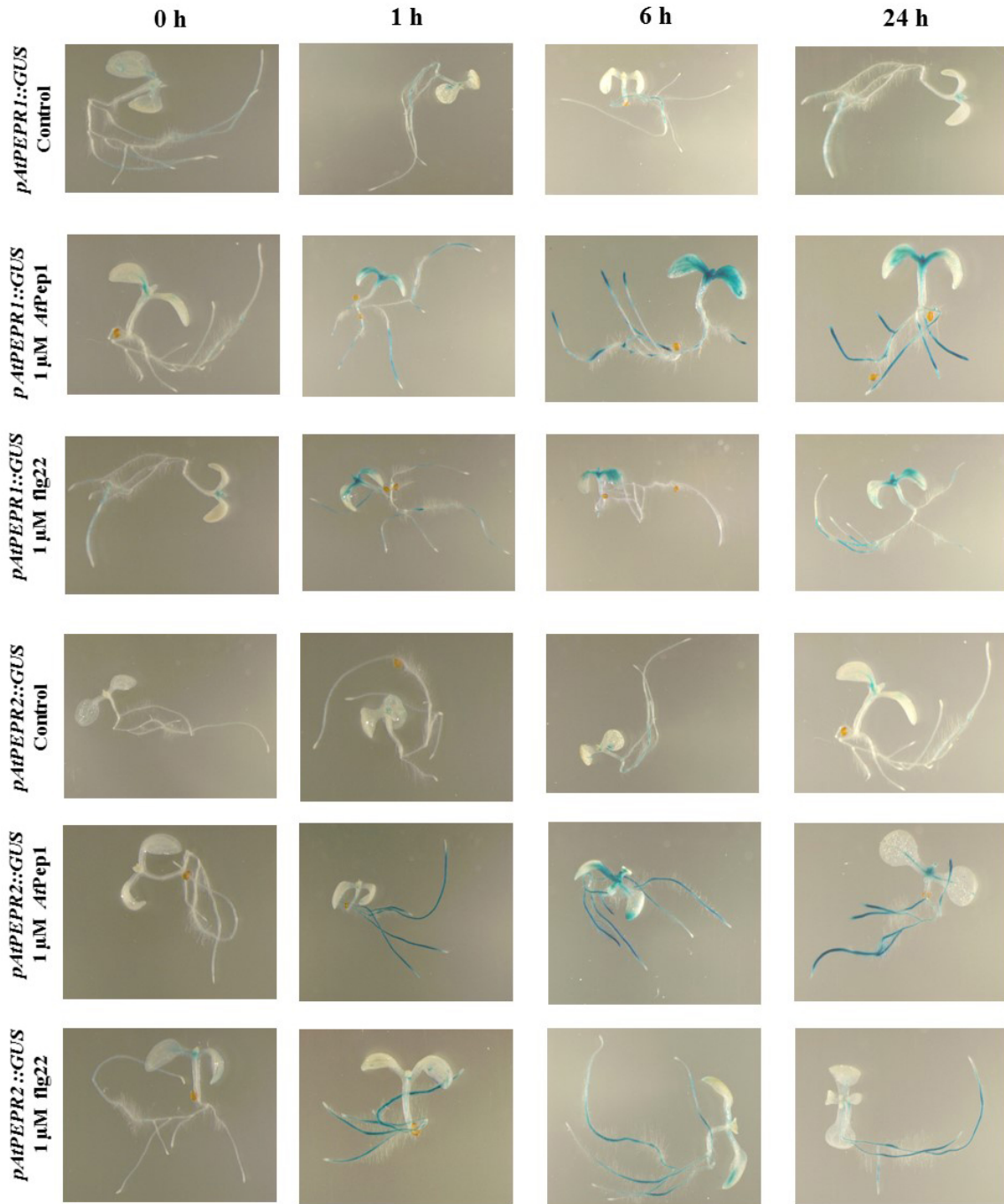


Figure 3-1. Patterns of GUS staining in seedlings of Arabidopsis carrying *pAtPEPR1::GUS* and *pAtPEPR2::GUS* reporter constructs, treated with 1 μ M flg22 and 1 μ M AtPep1. 0 h: 0 time point; 1 h: one hour after treatment; 6 h: six hours after treatment; 24 h: twenty-four hours after treatment.

Table 3-1. *pAtPEPR1::GUS* and *pAtPEPR2::GUS* activity in response to *AtPep1* and *flg22* treatments.^a

<i>AtPEPRs</i>	Treatment	Time point	Shoot			Root		
			Shoot meristem	Hypocotyl	Cotyledon	Tip	Lateral root	Ground Tissues
<i>pAtPEPR1</i>	<i>AtPep1</i>	1 h	+	-	+	-	-	+
		6 h	+	-	+	+	-	+
		24 h	+	-	+	+	-	+
	<i>flg22</i>	1 h	+	-	+	-	-	-
		6 h	+	-	+	-	-	-
		24 h	+	-	+	-	-	+
<i>pAtPEPR2</i>	<i>AtPep1</i>	1 h	-	-	-	-	-	+
		6 h	+	-	-	-	-	+
		24 h	+	-	-	-	-	+
	<i>flg22</i>	1 h	-	-	-	-	-	+
		6 h	-	-	-	-	-	+
		24 h	-	-	-	-	-	+

^aThe signs in the table signify the following:

+: promoter is activated.

-: promoter is not activated.

For the *pAtPEPR2* promoter, one-hour post treatment with *AtPep1*, compared to the mock-treated control seedlings, we noticed that the promoter became activated in roots one hour after treatment. The response at 6 and 24 hours was the same in roots but the promoter also became activated in the shoot meristem in response to *AtPep1*. We observed that 6 hours post treatment

with *AtPep1*, the promoter activation of *pAtPEPR2* was more robust than 24 hours post treatment. Compared to the mock-treated control seedlings in response to flg22, the promoter *pAtPEPR2* became activated in the root one hour post treatment.

We observed the same response at 6 and 24 hours post treatment but the response was a bit weaker compared to the one hour treatment. We also did not observe any activation of the promoter for *pAtPEPR2* in shoots in response to flg22 and they were similar to mock-treated control seedlings. In a nutshell, it seems that the *pAtPEPR1* promoter can be activated in different parts of the plant while *pAtPEPR2* promoter expression is more restricted to the root.

3.3.2 Activation of the Promoters of the *AtPROPEPs* and *AtPEPR* Genes in Response to MeJA in Leaves

We used the transgenic Arabidopsis lines described earlier, carrying promoter-GUS-reporter constructs for each of the *AtPROPEPs* and *AtPEPR* genes available (Bartels *et al.*, 2013). All promoters were activated by a treatment with the phytohormone MeJA, as visualized by an increase of GUS expression, compared to a mock-treated control leaves. Interestingly, we noticed that all promoter reporter lines which we used in this study, became activated in response to MeJA treatment but overall, upon MeJA treatment, the levels of their activation were different from each other; therefore, it was also possible to differentiate the Pep family according to the response of their promoters upon MeJA treatment.

As it can be seen in the Figure 3-2, in response to MeJA compared to the mock-treated control leaves, *pAtPEPR1* promoter was strongly get activated and the level of activation was almost the same at different concentrations (1 μ M, 10 μ M, 100 μ M; Figure 3-2, Table 3-2). This indicates that even a low concentration of MeJA (1 μ M) is adequate to activate the *pAtPEPR1* promoter. This promoter was also slightly activated in mock-treated control leaves, especially 24 hours post treatment. On the other hand, as can be seen in the Figure 3-2, the promoter for the *pAtPEPR2* gene is just activated in veins and vascular tissues, and the level of the

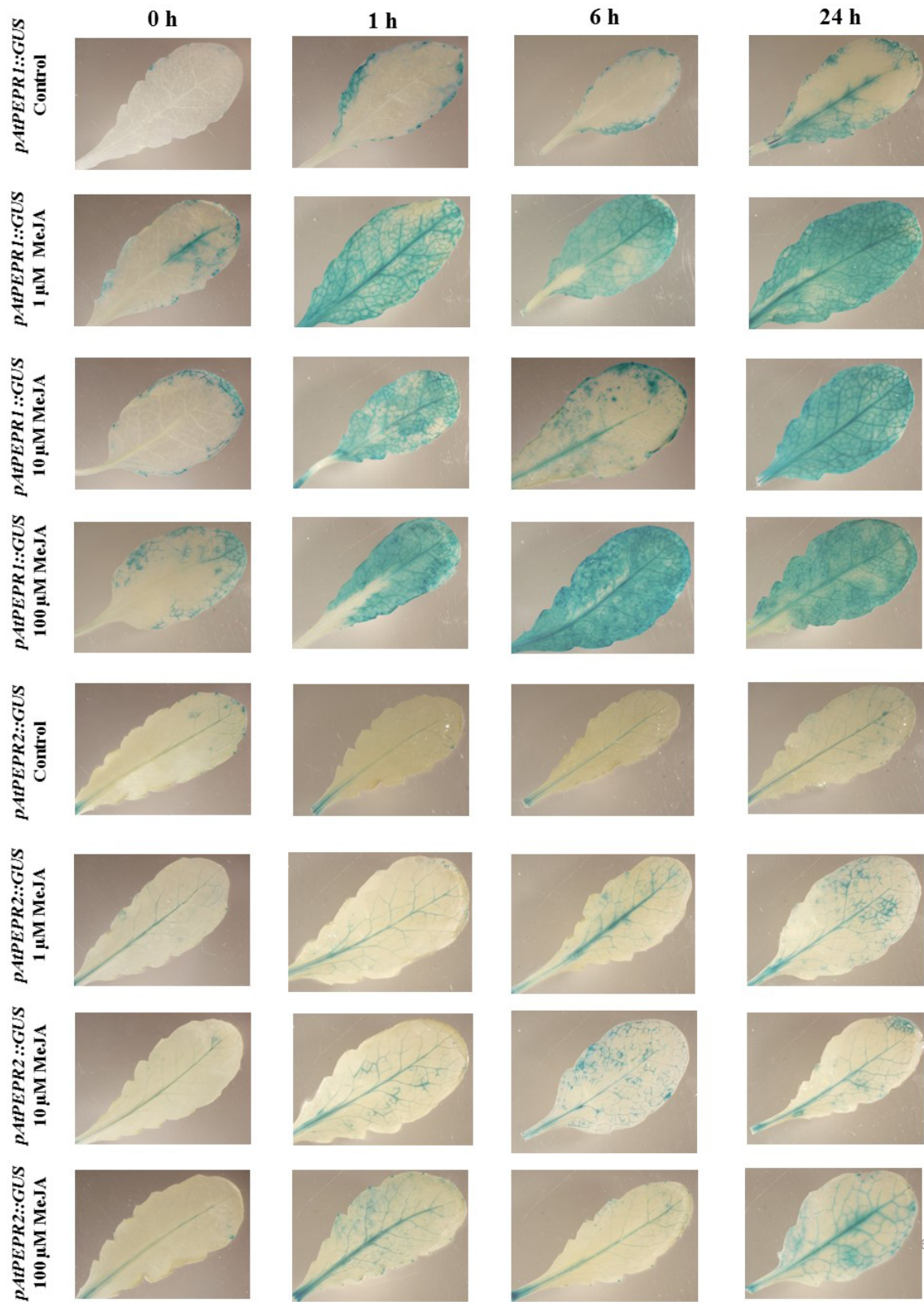


Figure 3-2. Patterns of GUS staining in seedlings of *Arabidopsis* carrying *pAtPEPR1::GUS* and *pAtPEPR2::GUS* reporter constructs, treated with Methyl Jasmonate (MeJA). 0 h: 0 time point; 1 h: one hour after treatment; 6 h: six hours after treatment; 24 h: twenty-four hours after treatment.

expression of these gene is similar at different time points (1 hour, 6 hours and 24 hours), and also at different concentrations of MeJA (1 μ M, 10 μ M, 100 μ M). Remarkably, we observed that just 1 μ M of MeJA activated the *pAtPEPR1* promoter 1 hour post treatment and the level of activation of this promoter increased over time as 24 hours post treatment with 1 μ M of MeJA the stained leaves were almost completely blue (Figure 3-2). Treatment of the *pAtPEPR1* promoter reporter line with 10 μ M MeJA also led to the robust activation of this promoter; the level of activation of this promoter, comparing the 1 μ M and 10 μ M of MeJA treatments, was almost the same. We also observed that 100 μ M of MeJA induced a very strong activation of this promoter, as the leaves were almost blue, especially 6 hours after treatment. On the other hand, we observed that the *pAtPEPR2* promoter was not so active compared to the *pAtPEPR1* promoter. In other words, as can be seen in Figure 3-2, the level of activation of these two promoters (*pAtPEPR1* and *pAtPEPR2*) was completely different from each other. Intriguingly, the *pAtPEPR2* promoter, in response to MeJA, was only activated in the main vascular tissue. As shown in Figure 3-2, the *pAtPEPR2* promoter was also a little active without any stimuli, but MeJA get activate this promoter slightly more than the control treatment. As also shown in Figure 3-2, 100 μ M of MeJA treatment activated this promoter in the main vascular tissue clearly more than 1 μ M and 10 μ M of MeJA treatment. Cumulatively, these results indicate that there is a specific and also differential activation pattern between *pAtPEPR1* and *pAtPEPR2* promoters in response to MeJA compared to the control treatment.

Concerning the promoters of the *AtPROPEP* genes, we observed that the *pAtPROPEP1* to *pAtPROPEP8* promoter activities were different from each other, in response to MeJA and also compared to the control. Table 3-2 schematically represents the activation of these promoters in response to the MeJA. Specifically, as can be seen in Figure 3-3, the *pAtPROPEP1* promoter was activated by MeJA mainly in the main vascular tissue of the leaf. This hormone also activateed the promoter in additional parts of the leaves beside the vascular tissue, leading to

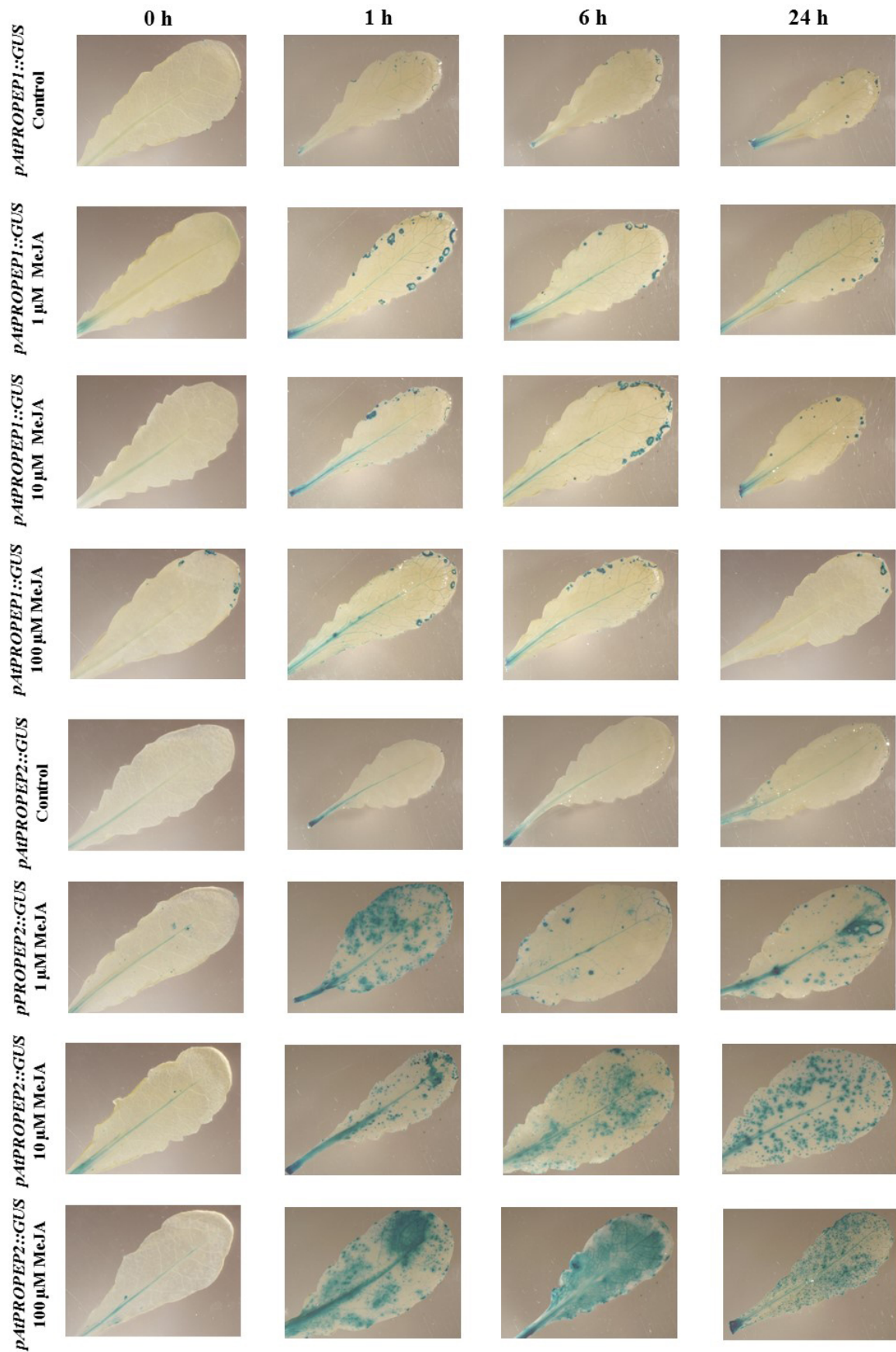


Figure 3-3. Patterns of GUS staining in leaves of *Arabidopsis* carrying *pAtPROPEP1::GUS* and *pAtPROPEP2::GUS* reporter constructs, treated with Methyl Jasmonate (MeJA). 0 h: 0 time point; 1 h: one hour after treatment; 6 h: six hours after treatment; 24 h: twenty-four hours after treatment.

“blue patch” GUS staining. On the other hand, MeJA treatment activated the *pAtPROPEP2* promoter in different parts of the leaves and also in the vascular tissue.

Remarkably, the pattern of the *pAtPROPEP2* promoter activation was exacerbated at higher concentrations of MeJA (Figure 3-3). Interestingly, this promoter was almost more active after 1 hour after treatment than after 24 hours after treatment. One of the most interesting findings in this study is the high activation of *pAtPROPEP3* in response to MeJA treatment. As can be seen in the Figure 3-4 and also Table 3-2, the *pAtPROPEP3* promoter is activated in the vascular tissues and also additional parts of the leaves. We have observed that the *pAtPROPEP3* promoter is highly active 1 hours after treatment in response to 10 μ M and also 100 μ M MeJA treatment but we have not seen such a strong activation in treatment with 1 μ M MeJA treatment. It seems that the concentration of MeJA is important for the activation of the *pAtPROPEP3* promoter. Furthermore, as it can be seen in the figure 3-4, the *pAtPROPEP3* promoter is also slightly active without any treatments and its activation is increased over the time of treatment. It seems that *pAtPROPEP3* promoter activity is highly dynamic upon MeJA treatment. This observation is in line with the former investigations indicating that the *AtPROPEP3* promoter region is highly active and dynamic (Logemann *et al.*, 2013). Note that we saw a slight activation of *pAtPROPEP3* in some of the mock-treated control leaves.

On the other hand, as is represented in the Figure 3-4, compared to the control leaves the *pAtPROPEP4* promoter was just activated in the main vascular tissue, and the pattern of the activation of this promoter line was very different compared to *pAtPROPEP3* (Figure 3-4; Table 3-2).

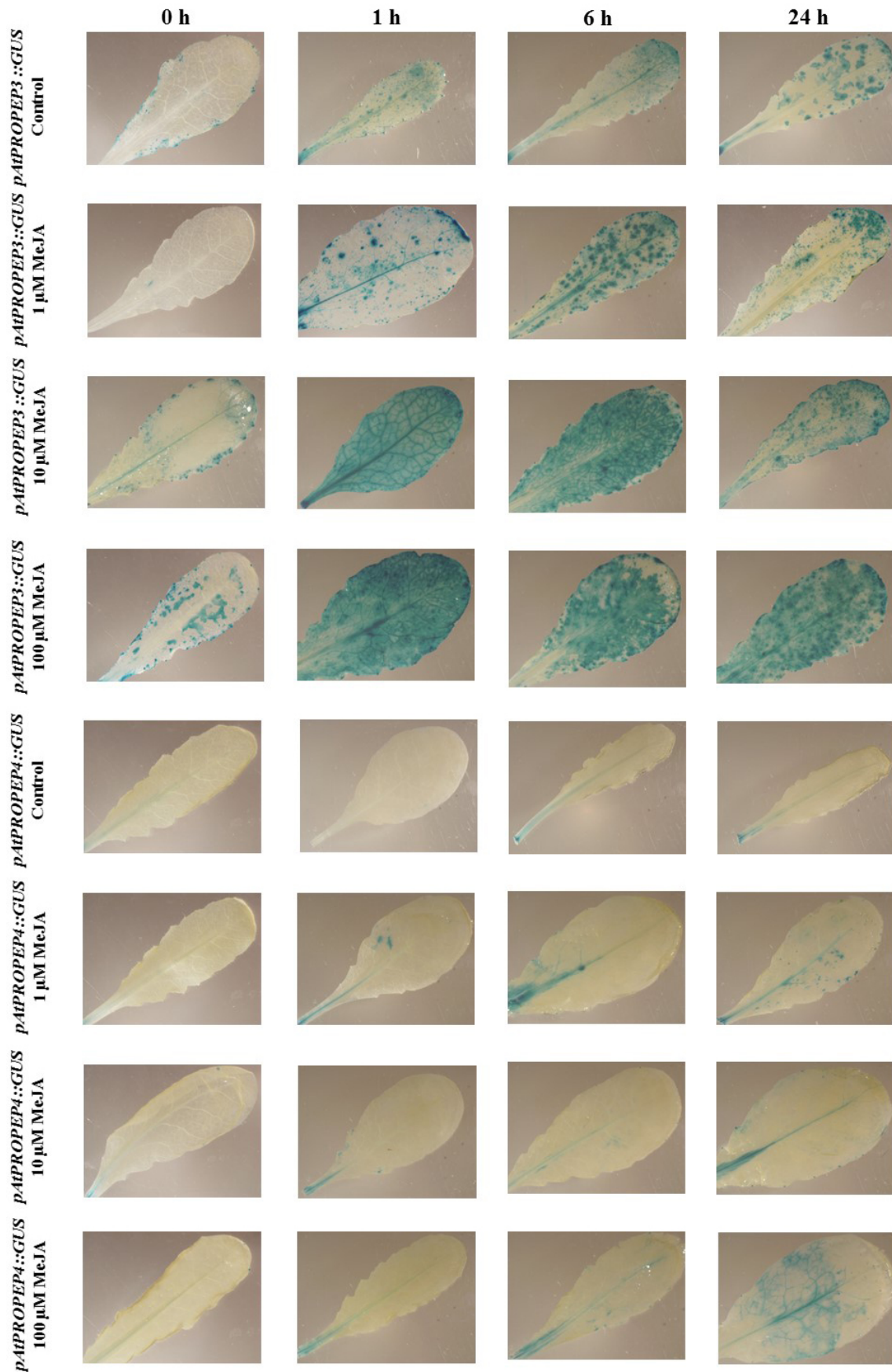


Figure 3-4. Patterns of GUS staining in leaves of Arabidopsis carrying *pAtPROPEP3::GUS* and *pAtPROPEP4::GUS* reporter constructs, treated with Methyl Jasmonate (MeJA). 0 h: 0 time point; 1 h: one hour after treatment; 6 h: six hours after treatment; 24 h: twenty-four hours after treatment.

As illustrated in the Figure 3-5, the *pAtPROPEP5* promoter is highly inducible in all vascular tissues of the leaves compared to the mock-treated control leaves, and activation of this promoter increased at higher concentrations of MeJA. But as can be seen in the Figure 3-5, the pattern of induction of the *pAtPROPEP5* promoter is different compared with other promoters; it becomes activated in all vascular tissues in leaves but not in additional parts of the leaves. In addition, comparing the different concentrations, we observed that the higher the concentration of MeJA, the more this promoter was activated.

We observed that in response to MeJA and compared to the mock-treated control leaves, the *pAtPROPEP7* promoter was activated only in the main vascular tissue. We noticed that the *pAtPROPEP7* promoter was more responsive to 10 μ M MeJA. As it can be seen in Figure 3-5 and in Table 3-2, compared to the mock-treated control leaves, the *pAtPROPEP7* promoter responded almost in the same way as *pAtPROPEP4*. Concerning the *pAtPROPEP8* promoter, we did not observe high activity of this promoter in response to MeJA treatment when compared to the mock-treated control leaves; this promoter was just active in the main vascular tissues (Figure 3-6; Table 3-2) and responded almost the same way to MeJA as the *pAtPROPEP4* and *pAtPROPEP7* promoters.

In conclusion, in response to MeJA and compared to the mock-treated control leaves, we have noticed that it is possible to classify the Pep family promoter activation in response to MeJA (Figure 3-2 to 3-6; Table 3-2). We can classify *pAtPROPEP1* in one group as it expressed almost uniformly; *pAtPROPEP2* and *pAtPROPEP3* in the second group as they are highly activated in response to MeJA treatment; *pAtPROPEP4*, *pAtPROPEP7* and *pAtPROPEP8* in the third group; and as *pAtPROPEP5* promoter did respond differentially comparing with others, we grouped it in the fourth group.

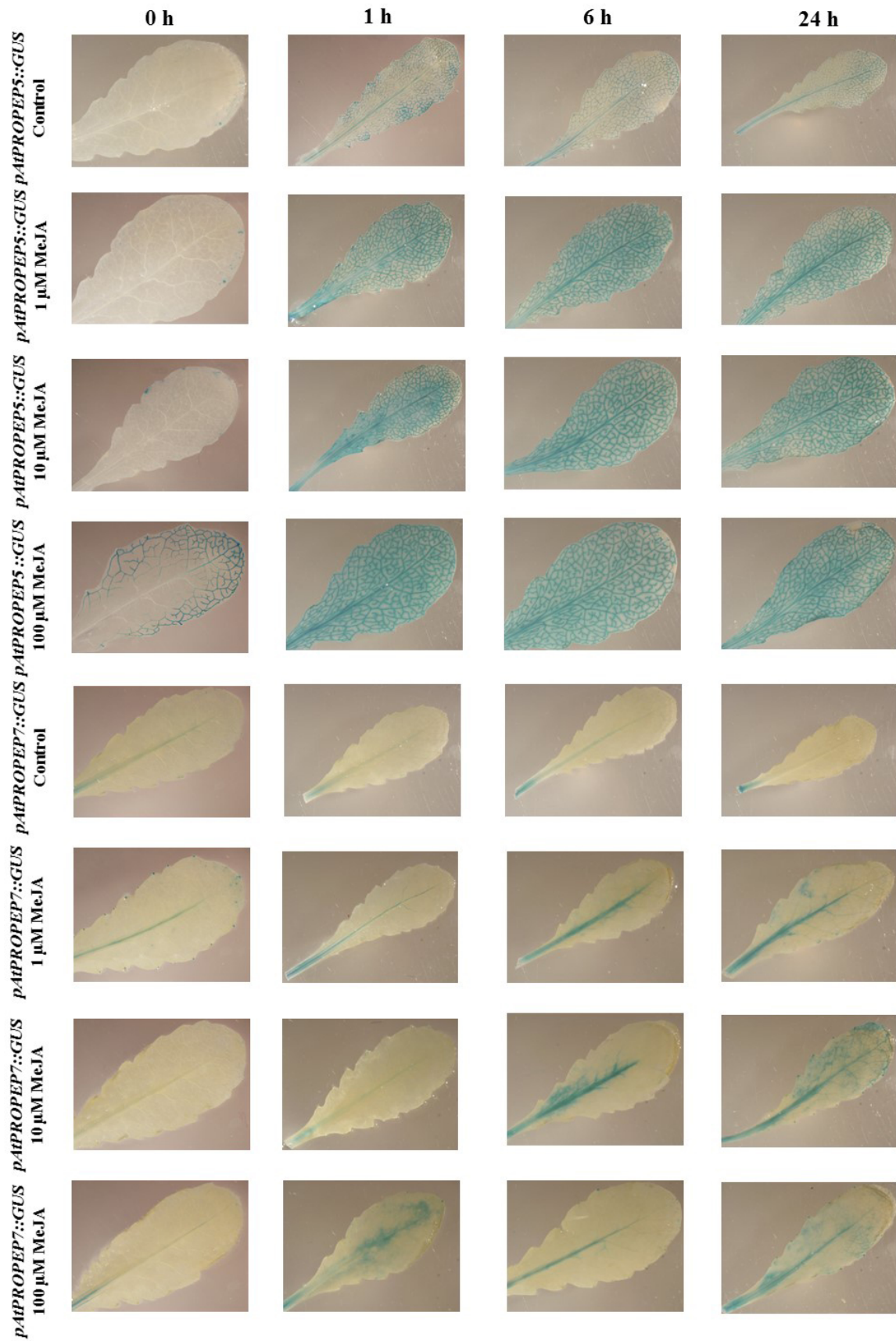


Figure 3-5. Patterns of GUS staining in leaves of Arabidopsis carrying *pAtPROPEP5::GUS* and *pAtPROPEP7::GUS* reporter constructs, treated with Methyl Jasmonate (MeJA). 0 h: 0 time point; 1 h: one hour after treatment; 6 h: six hours after treatment; 24 h: twenty-four hours after treatment.

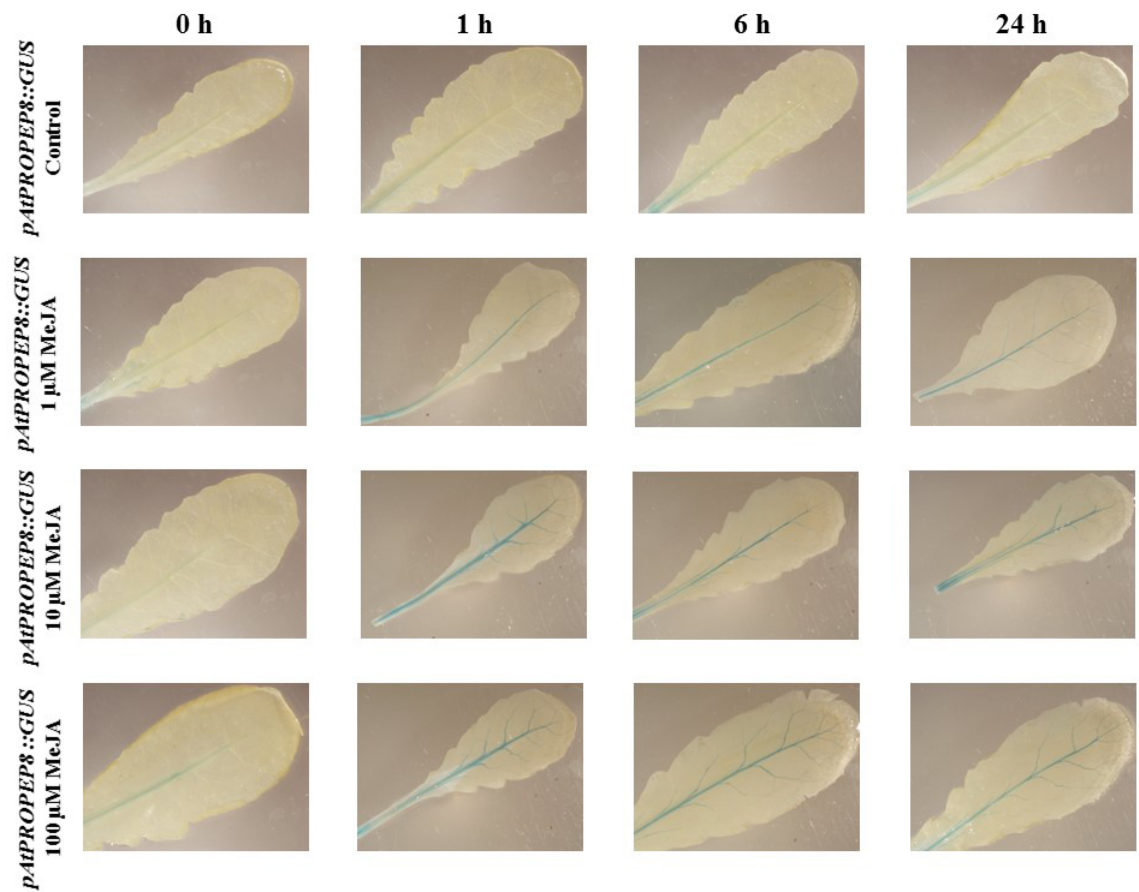


Figure 3-6. Patterns of GUS staining in leaves of Arabidopsis carrying *pAtPROPEP8::GUS* reporter construct, treated with Methyl Jasmonate (MeJA). 0 h: 0 time point; 1 h: one hour after treatment; 6 h: six hours after treatment; 24 h: twenty-four hours after treatment.

3.3.3 The *AtPROPEPs* and *AtPEPR* Promoters are induced in Response to *AtPep1* and *flg22*

We observed that the promoters of *AtPROPEP* 1-8 and also of the genes encoding the corresponding receptors (*AtPEPR1* and *AtPEPR2*) responded differently in treatment with *AtPep1* and also *flg22* compared to the mock treatments. Briefly, we observed that in response to *AtPep1*, the *pAtPEPR1* promoter, is highly activated already after 1 hour of treatment, while we did not observe a robust activation of the *pAtPEPR2* promoter in response to *AtPep1* treatment and this promoter was most active in the main vascular tissue and the highest activation of this promoter was 24 hours post treatment (Figure 3-7; Table 3-2). We also observed that the *pAtPEPR2* promoter was almost inactive in control untreated leaves.

One of the interesting findings of this research was that *flg22* differentially activated the *pAtPEPR1* promoter and to a lower extent the *pAtPEPR2* promoter. We observed that the pattern of activation of these two promoters was different. More specifically, the *pAtPEPR1* promoter was active in both the vascular tissues and also additional parts of the leaves but the *pAtPEPR2* promoter was only active in the main vascular tissues. We also observed that after 24 hours of treatment the highest activation of both promoters occurred, compared to the mock-treated control leaves (Figure 3-7). This observation is in line with former studies indicating that *flg22* and Pep system work as a positive loop.

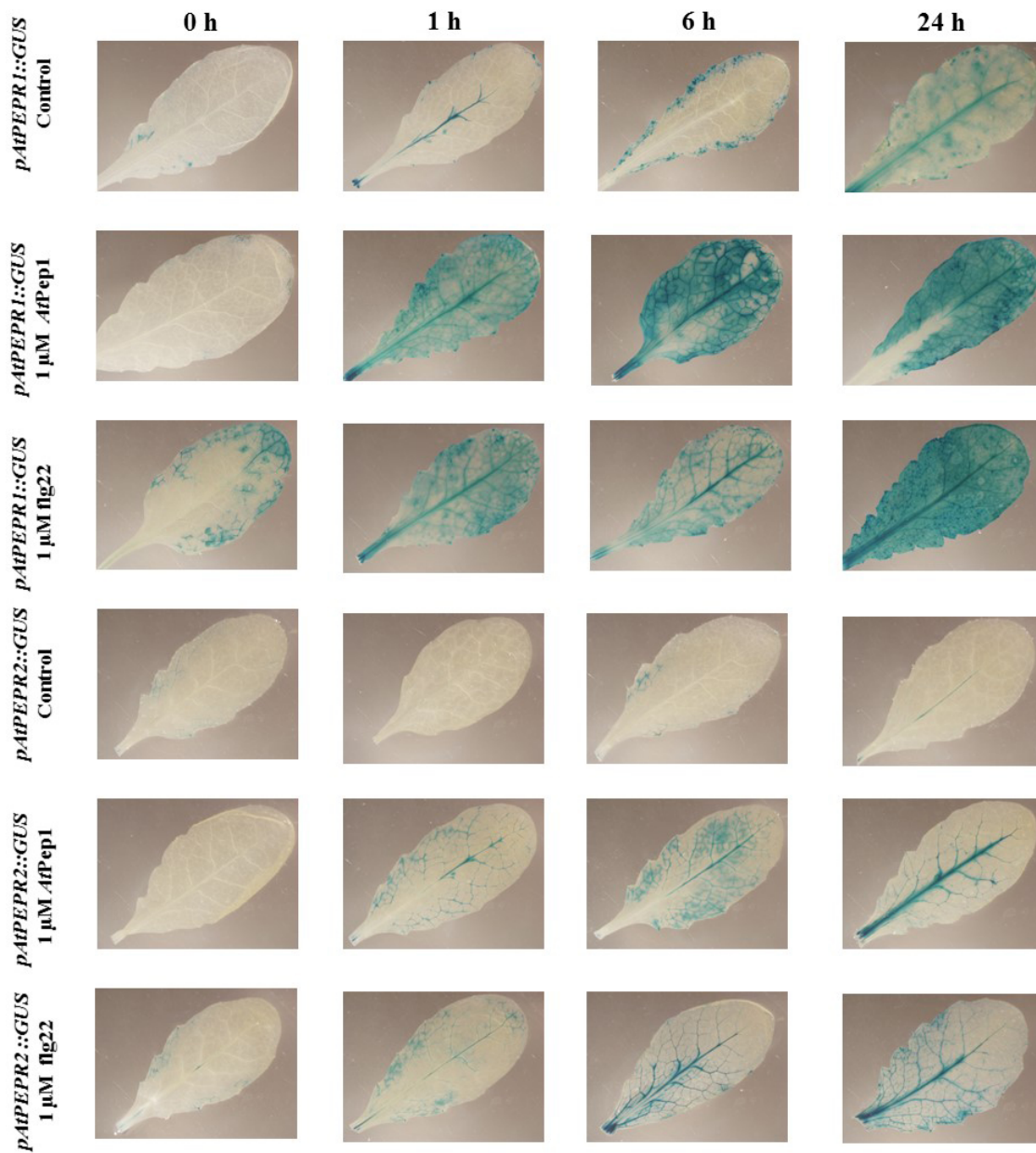


Figure 3-7. Patterns of GUS staining in leaves of *Arabidopsis* carrying *pAtPEPR1::GUS* and *pAtPEPR2::GUS* reporter constructs, treated with 1 μ M *AtPep1* and 1 μ M flg22. 0 h: 0 time point; 1 h: one hour after treatment; 6 h: six hours after treatment; 24 h: twenty-four hours after treatment.

On the other hand, contrary to expectations, *AtPep1* did not highly activate its own promoter (Figure 3-8; Table 3-2). However, *AtPep1* activated the *pAtPROPEP3* promoter and to a lower extent the *AtPROPEP2* promoter.

Interestingly, we observed that in response to *flg22* and also *AtPep1*, the *pAtPROPEP2* promoter was not activated in the main vascular tissue but it was activated in additional parts of the leaves. A similar observation was made for the *pAtPROPEP3* promoter, with the exception that 24 hours post treatment with *AtPep1*, beside additional parts of the leaves, we observed the activation of this promoter in the main vascular tissue too (Figure 3-9).

The *pAtPROPEP4* promoter activated at a very low level in response to *AtPep1* and also *flg22* (Figure 3-9). Concerning the *pAtPROPEP7* promoter, we did not observe any activation of this promoter in response to *AtPep1* and *flg22*.

Regarding the *pAtPROPEP5* promoter, we observed a very low activation of this promoter in response to *AtPep1* and *flg22*. There were differential responses to *AtPep1*. As can be seen in Figure 3-10, the *pAtPROPEP5* promoter was activated to similar degree at 1, 6 and 24 hours post treatment with *AtPep1*, while the activation of this promoter by *flg22* showed a different time course; *flg22* activated this promoter mainly 6 hours after treatment. It seems that in response to *AtPep1*, the activation of the *pAtPROPEP5* promoter is higher than in response to *flg22*. For the *pAtPROPEP8* promoter, as shown in Figure 3-10, we did not observe an activation of this promoter in response to *flg22* and *AtPep1*, except for the main vein. Briefly, *AtPep1* activated this promoter in the main vascular tissue, and the highest activation of this promoter was 24 hours post treatment. *Flg22* similarly activated this promoter in the main vascular tissue 6 and 24 hours post treatment.

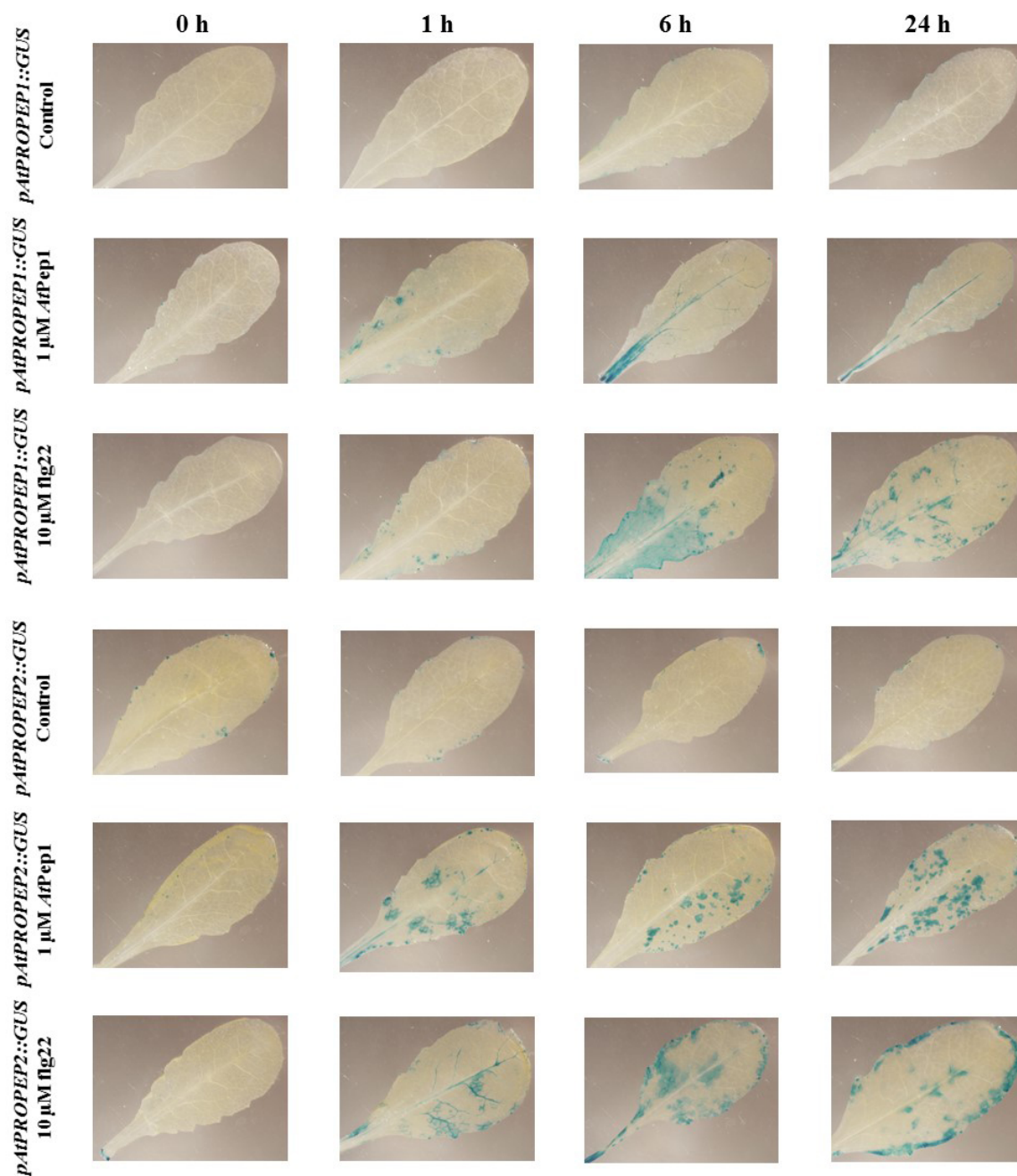


Figure 3-8. Patterns of GUS staining in leaves of Arabidopsis carrying *pAtPROPEP1::GUS* and *pAtPROPEP2::GUS* reporter constructs, treated with 1 μ M *AtPep1* and 1 μ M flg22. 0 h: 0 time point; 1 h: one hour after treatment; 6 h: six hours after treatment; 24 h: twenty-four hours after treatment.

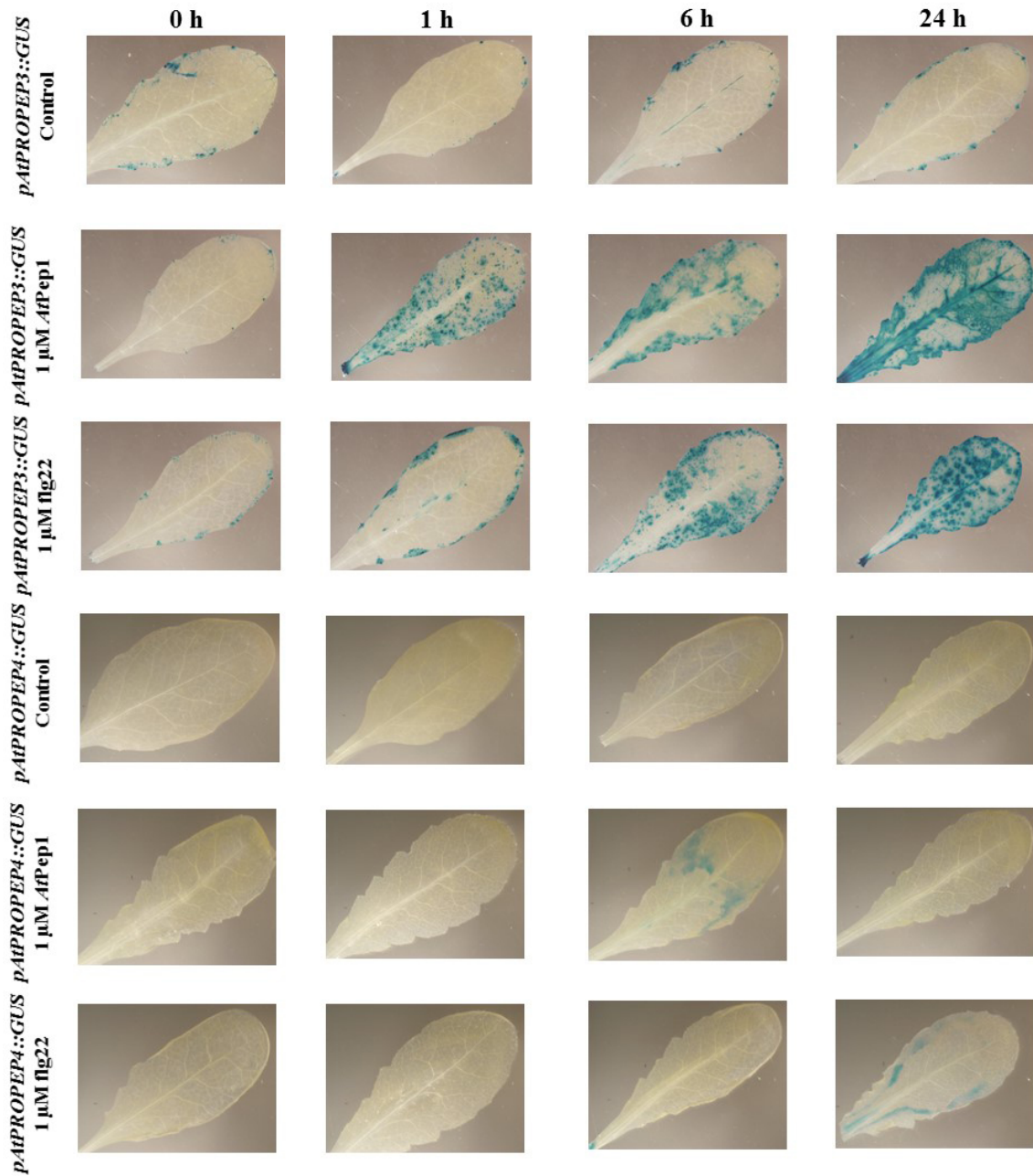


Figure 3-9. Patterns of GUS staining in leaves of *Arabidopsis* carrying *pAtPROPEP3::GUS* and *pAtPROPEP4::GUS* reporter constructs, treated with 1 μ M *AtPep1* and 1 μ M flg22. 0 h: 0 time point; 1 h: one hour after treatment; 6 h: six hours after treatment; 24 h: twenty-four hours after treatment.

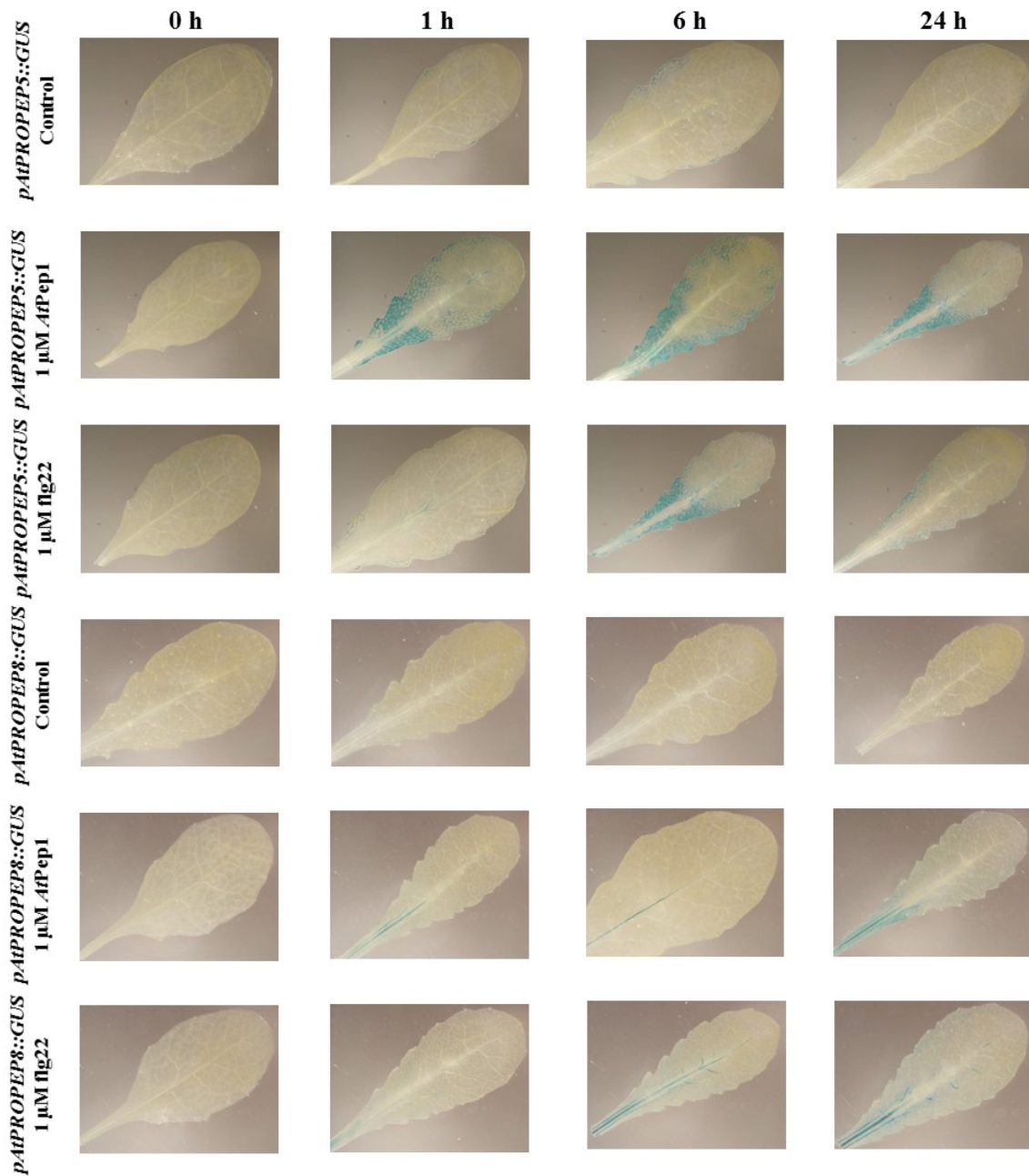


Figure 3-10. Patterns of GUS staining in leaves of Arabidopsis carrying *pAtPROPEP5::GUS* and *pAtPROPEP8::GUS* reporter constructs, treated with 1 μ M *AtPep1* and 1 μ M flg22. 0 h: 0 time point; 1 h: one hour after treatment; 6 h: six hours after treatment; 24 h: twenty-four hours after treatment.

3.3.4 *pAtPROPEP1*, *pAtPROPEP3* and *pAtPEPR1* Promoters are Induced in Response to NaCl Treatment

We observed that compared to the mock-treated control leaves, treatment with NaCl (100 mM and 250 mM concentration) strongly activated the *pAtPROPEP1*, *pAtPROPEP3* and also *pAtPEPR1* promoters in leaves (Figure 3-11 and 3-12). Briefly, as it can be seen in the Figure 3-11, 24 hours post treatment with NaCl, the *pAtPROPEP1* promoter was activated in response to 100 mM NaCl. As is illustrated in the Figure 3-11, the level of activation of this promoter increased once the leaves were treated with 250 mM NaCl. In addition, as can be seen in the Figure 3-11, the level of the *pAtPROPEP1* promoter activation in response to 250 mM NaCl treatment was increased over different time points.

Remarkably, we noticed that the *pAtPROPEP3* promoter produced a stronger GUS staining compared to the *pAtPROPEP1* promoter. As presented in Figure 3-11, compared to the mock-treated control leaves, 1 hour after treatment with 100 mM NaCl, the *pAtPROPEP3* promoter was activated, and activation of this promoter increased with time. Furthermore, treatment with 250 mM NaCl led to stronger activation of *pAtPROPEP3* promoter. At 24 hours post treatment with 250 mM NaCl, the *pAtPROPEP3* promoter was activated in the main vascular tissue and also in additional parts of the leaf.

Cumulatively, as can be seen in Figure 3-11 and also in Figure 3-12, the activation of these promoters (*pAtPROPEP1*, *pAtPROPEP3* and *pAtPEPR1*) was increased 24 hours after treatment. We observed an increased activity of the *pAtPROPEP3* promoter in all parts of the leaves but for the *pAtPROPEP1* promoter it was activated only in some parts of the leaves.

Interestingly, the *AtPEPR1* promoter was also activated in response to NaCl treatment. As can be seen in Figure 3-12, treatment with 100 mM NaCl led to the activation of the *AtPEPR1* promoter one hour after treatment and the activation of this promoter increased over time. Furthermore, 250 mM NaCl treatment strongly activated the *AtPEPR1* promoter, especially 24

hours post treatment. In summary, treatment with NaCl activated the *pAtPROPEP1*, *pAtPROPEP3* and *pAtPEPR1* promoters in a time- and concentration-dependent manner. The promoters of the other *AtPROPEP* genes were not activated in response to NaCl treatment.

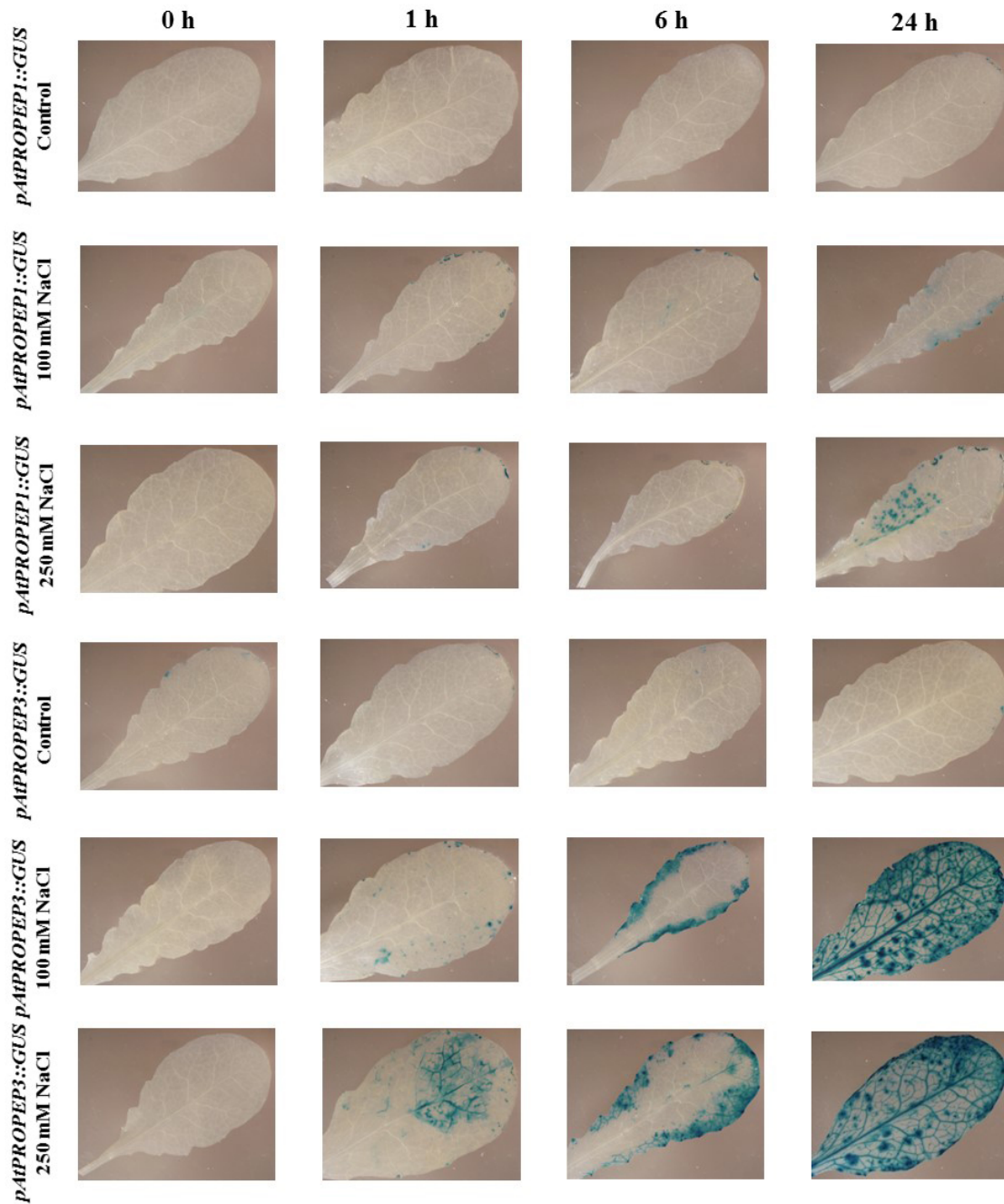


Figure 3-11. Patterns of GUS staining in leaves of Arabidopsis carrying *pAtPROPEP1::GUS* and *pAtPROPEP3::GUS* reporter constructs, treated with 100 mM and 250 mM NaCl. 0 h: 0 time point; 1 h: one hour after treatment; 6 h: six hours after treatment; 24 h: twenty-four hours after treatment.

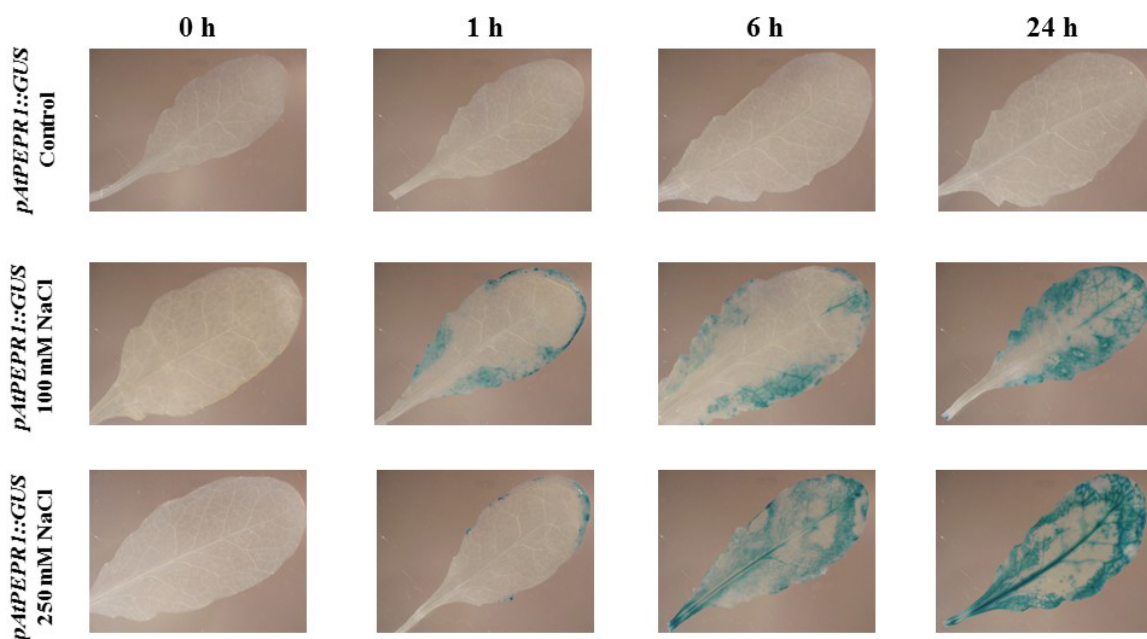


Figure 3-12. Patterns of GUS staining in leaves of Arabidopsis carrying *pAtPEPR1::GUS* reporter construct, treated with 100 mM and 250 mM NaCl. 0 h: 0 time point; 1 h: one hour after treatment; 6 h: six hours after treatment; 24 h: twenty-four hours after treatment.

3.3.5 Activation of the Promoters *pAtPEPR1* and *pAtPEPR2* by NaCl Treatment in Seedlings

We also studied promoter activities of our transgenic plants in seedlings treated with NaCl at 100 mM and 250 mM. We observed that in seedlings, the *pAtPEPR1* and *pAtPEPR2* promoters were activated in response to NaCl. We fixed the seedlings after 0, 1, 6 and 24 hours treatment with NaCl and observed that the *pAtPEPR1* and *pAtPEPR2* promoters became activated in response to this stimulus. However, these two promoters exhibited different expression patterns in response to NaCl treatment. As is illustrated in Figure 3-13, compared to the mock-treated control seedlings, in response to the NaCl treatment (100 mM and 250 mM concentration), 1 hour after treatment, the *pAtPEPR1* promoter was activated in leaves and also in roots. In addition, 6 hours after treatment, the *pAtPEPR1* promoter became strongly activated in shoots

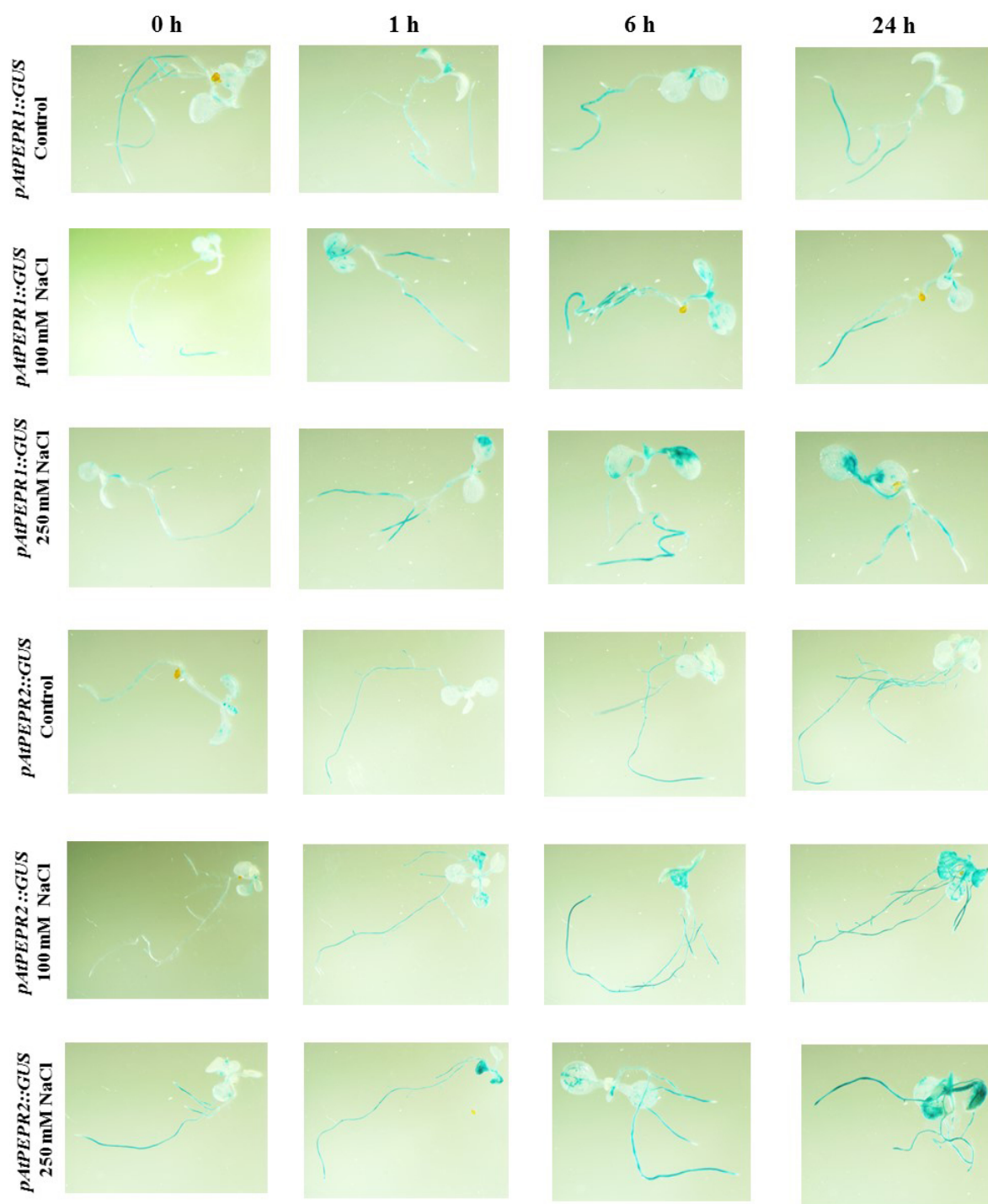


Figure 3-13. Patterns of GUS staining in leaves of *Arabidopsis* carrying *pAtPEPR1::GUS* and *pAtPEPR2::GUS* reporter construct, treated with 100 mM and 250 mM NaCl. 0 h: 0 time point; 1 h: one hour after treatment; 6 h: six hours after treatment; 24 h: twenty-four hours after treatment.

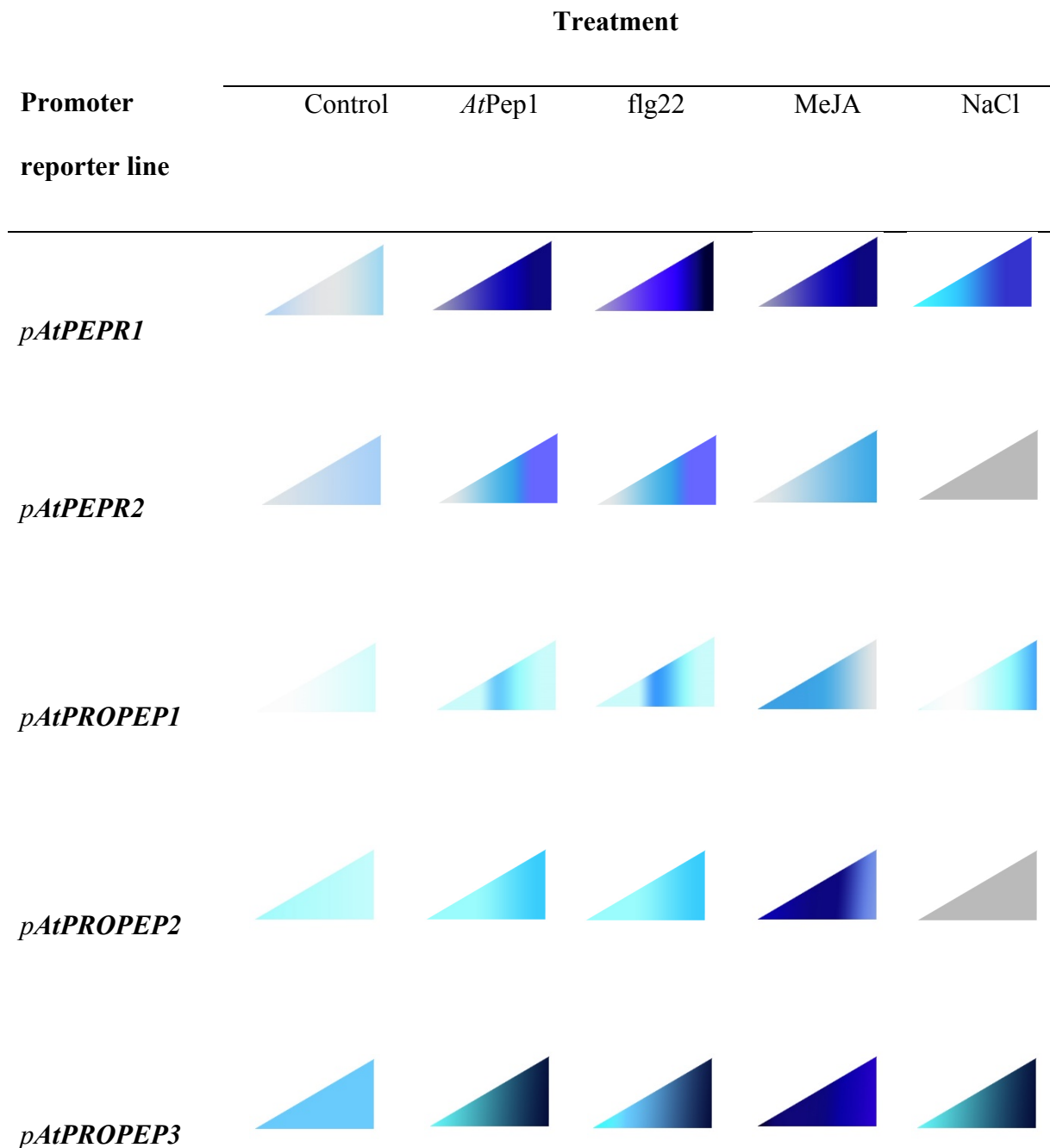
and roots tissue, and this activation was higher in the treatment with the higher NaCl concentration. Importantly, treatment with NaCl led to the activation of *pAtPEPR1* promoter in the hypocotyls within 24 hours of treatment. However, we did not observe an activation of the *pAtPEPR1* promoter in the root tips. NaCl treatment also induced the activity of the *pAtPEPR2* promoter in different parts of the seedling (Figure 3-13). Briefly, compared to the mock-treated control seedlings, activation of the *pAtPEPR2* promoter was observed in the NaCl-treated seedlings, and this activation increased over time. In addition, 24 hours after treatment with 100 mM NaCl resulted in a strong activation of the *pAtPEPR2* promoter in different parts of the seedling. In addition, we observed that the *pAtPEPR2* promoter was strongly activated in roots compared to the mock-treated control seedling. Interestingly, in response to NaCl treatment, the *pAtPEPR2* promoter also was activated in the root tips while we did not observe such an activation of *pAtPEPR1* promoter in the root tips.

3.3.6 Comparative Analysis of Pep-family Promoters in Response to Different Treatments

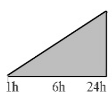
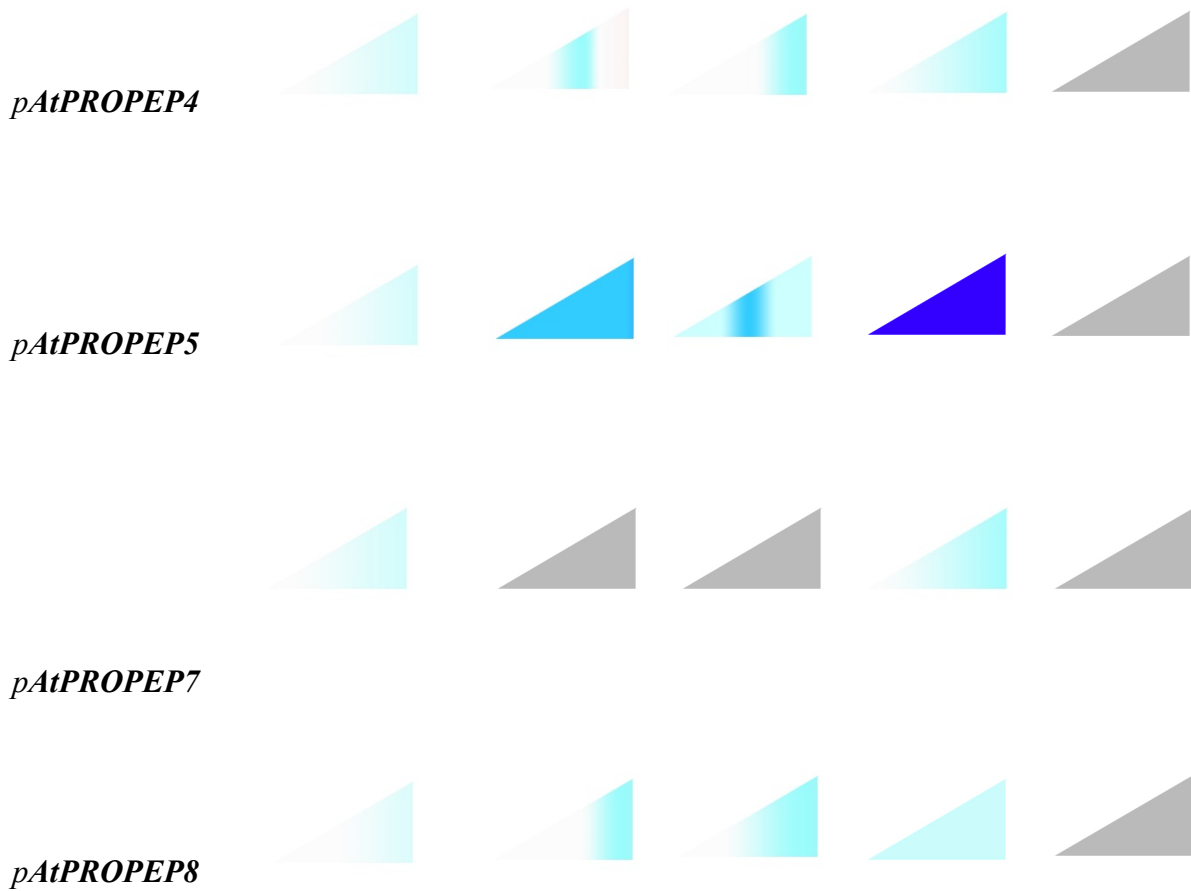
In Table 3-2, we compared the activation of the *pAtPEPR1*, *pAtPEPR2*, *pAtPROPEP1*, *pAtPROPEP2*, *pAtPROPEP3*, *pAtPROPEP4*, *pAtPROPEP5*, *pAtPROPEP7* and *pAtPROPEP8* promoters in leaves upon different treatments including *AtPep1*, flg22, MeJA, and NaCl. This table highlights several features of Pep-family promoter activation. As can be seen in this Table, the *pAtPEPR1* and *pAtPROPEP3* promoters were highly active upon *AtPep1*, flg22, MeJA, and NaCl treatments. Interestingly, in response to different stimuli, the activation of these two promoters increased over time. The *pAtPROPEP2* promoter also followed the same pattern of activation; the difference is that the response of this promoter was weaker compared to the *pAtPROPEP3* promoter. On the other hand, *pAtPEPR2* is not highly active compared to *pAtPEPR1*. It was almost only active in main vascular tissue on one hand and on the other hand, the activation of this promoter did not increase over time.

As shown in Table 3-2, *pAtPROPEP1* was activated only slightly in response to *AtPep1* and also flg22, and it is worth mentioning that the activation of this promoter upon different stimuli did not follow the same pattern as it was highly active 6 hours after flg22 treatment but for NaCl treatment it was more active 24 hours post treatment. As illustrated in Table 3-2, *pAtPROPEP1* responded differentially upon different treatments, and it was highly active in response to MeJA but differentially respond to *AtPep1* and also flg22 treatment. It is worth mentioning that the *pAtPROPEP4*, *pAtPROPEP7* and *pAtPROPEP8* promoters responded very weakly and similarly to these stimuli. Nevertheless, their response over time after treatment was somehow different. The highest activation of the *pAtPROPEP4* promoter in response to *AtPep1* occurred 6 hours after treatment while for the *pAtPROPEP8* promoter, it was 24 hours after treatment.

Table 3-2. Schematic representation of the promoter activation of *pAtPEPR1*, *pAtPEPR2*, *pAtPROPEP1*, *pAtPROPEP2*, *pAtPROPEP3*, *pAtPROPEP4*, *pAtPROPEP5*, *pAtPROPEP7* and *pAtPROPEP8*: GUS in leaves of *Arabidopsis thaliana* in response to elicitor treatments¹.



Continued



1: First angle of the triangle represents 1 hour after treatment, middle part of the triangle shows 6 hours post treatments and the right angle of the triangle indicates 24 hours after elicitor treatment. Dark blue color indicates the robust promoter activation; Light blue color represent slight promoter activation; Grey triangle means that the activation of the promoter is not observed.

It is interesting to note that, the *pAtPROPEP7* promoter showed the weakest response to different stimuli compared to the other promoters studied here. In addition, as it is represented in Table 3-2, almost all promoters had some activity without any stimuli but the level of their activity were very low.

3.4 Discussion

Endogenous host molecules, termed as DAMPs, trigger a plant defense immune system through PRRs, which are released as a result of tissue damage (Boller & Felix, 2009). DAMP signaling cascades intensify or prolong the stereotypical defense responses that are also triggered by MAMPs (Yamaguchi and Huffaker, 2011).

Using promoter reporter lines, we were able to confirm a strong induction of the *pAtPROPEP* promoters upon biotic and abiotic stresses. We observed a great similarity in the activation of *pAtPROPEP1* and *pAtPROPEP3* in response to these stimuli (*AtPep1*, flg22, MeJA, and NaCl). In other words, whatever stimuli can activate the *pAtPROPEP1* promoter, can also activate the *pAtPROPEP3* promoter. It seems that there is a positive feedback in the activation of these two promoters. Interestingly, in all treatments, the *pAtPROPEP3* promoter activation was much more robust, and the pattern of induction was also different.

Furthermore, we have not observed a robust induction of *pAtPROPEP4*, *pAtPROPEP7* and also *pAtPROPEP8*. This observation is in line with the finding of Bartels *et al.* (2013). They also did not observe an induction of the *pAtPROPEP4* and *pAtPROPEP7* promoters in response to any induction or wounding.

Although studies on Pep research focused on their role in the plant immune system (Albert, 2013; Ferrari *et al.*, 2013; Yamaguchi and Huffaker, 2011), there is emerging evidence indicating that the Pep family also has a role in the development of the plant (Gully *et al.*, 2015; Ma *et al.*, 2014). Ma *et al.* (2014) have reported that the *pAtPEPR2* promoter has strong activity in the vascular tissues of the roots. In transcriptional profiling analysis, they have shown that expression of 75% of *AtPep1*-modulated genes in roots is fully dependent on *pAtPEPR2*. They have shown that Pep affects the regulation of *GLUTAMINE DUMPER* (*GDU*) genes. Remarkably, they also have shown that *atpepr2* mutants exhibit shorter root

phenotype. Our observation is in line with their findings, as we also observed that *AtPEPR2* was highly active in roots. Remarkably, we have noticed that *pAtPEPR2* promoter is strongly activated in response to *AtPep1* perception. Ma *et al.*, (2014), have also observed that *AtPEPR2* mediates most of the *AtPep1*-induced transcriptional responses in the roots. Based on what we have found in this research, coupled with Ma *et al.*, (2014), it seems that the *pAtPEPR2* has an important role in the *AtPep1*-mediated signaling in the roots and taken together, we postulate that *pAtPEPR2* may have a role in root development of Arabidopsis.

Gully *et al.* (2015) also have found a new possible function of Peps apart from their presumed major role in the plant immune system. They showed that dark-induced leaf senescence was affected by the presence of Peps. They also observed that this response was dependent on ET signaling and inhibited by the addition of growth hormone cytokinins. Interestingly, flg22 or elf18, both of which are potent inducers of PTI, did not induce an early start of leaf senescence. Based on their finding, it seems that Pep-perception affects and accelerates dark/starvation-induced senescence via an early induction of chlorophyll degradation and autophagy which is unrelated to PTI.

Concerning the activity of these promoters without any stimuli, Bartels *et al.* (2013) also showed that these promoters were not active even after 24 hours of staining and they barely observed a visible blue precipitate in the leaves. Klauser *et al.* (2015) also showed that promoters of the *AtPROPEP* family were inactive in the absence of biotic and abiotic stresses. It seems that without any stress or stimuli, the activity of these genes are very low. This observation is in line with our finding indicating that the promoters of the *AtPROPEP* and *AtPEPR* gene families (*pAtPEPR1*, *pAtPEPR2*, *pAtPROPEP1*, *pAtPROPEP2*, *pAtPROPEP3*, *pAtPROPEP4*, *pAtPROPEP5*, *pAtPROPEP7* and *pAtPROPEP8*) are almost inactive in the absence of any treatments. Furthermore, they also observed that MeJA has positive effect on the activation of the Pep family. In addition, it was shown previously that the Pep family is a

positively affected with the phytohormone jasmonic acid (Ross *et al.*, 2014). We have also observed MeJA has positive affect in the activation Pep family promoters.

Host transcriptional responses are considered as a vital and crucial component of the plant defense system, and so far, several TFs have been identified as important regulators of plant immunity (reviewed by Eulgem, 2005; Moore *et al.*, 2011). Recently, Logemann *et al.* (2013) described cis-regulatory modules (CRMs) of the *AtPROPEP2* and *AtPROPEP3* promoters, using *Petroselinum crispum* protoplasts and transgenic *A. thaliana* plants harboring promoter-reporter constructs that report MAMP responsiveness. They also detected a specific TF inducing transcription of the *AtPROPEP2* and also *AtPROPEP3* by chromatin immunoprecipitation and also DNA interaction studies. They identified conserved regions of the *AtPROPEP3* locus in different Brassicaceae species. They confirmed that WRKY-type TFs played the most important role in regulating transcriptional outputs of the *AtPROPEP2* and *AtPROPEP3*. They observed that in the *AtPROPEP3* promoter, the CRM contained six W boxes. This observation may explain why the *pAtPROPEP3* promoter is so active upon biotic and also abiotic stresses. However, although Logemann *et al.* (2013) have observed the same regulatory element for *AtPROPEP2* and *AtPROPEP3* promoter, we have not observed a similarly strong activation for the *AtPROPEP2* promoter: Activation of the *AtPROPEP2* promoter was much weaker than that of the *AtPROPEP3* promoter. It seems that there are other factors affecting the activation of these promoters that must be studied in more detail.

As it is now known that Peps are not limited to the model plant *A. thaliana* (Huffaker *et al.*, 2011, 2013), and that they are present and also active in other angiosperms, we can extend our observations to other plant species.

Although the members of the *AtPep* family show similarity in amino acid sequence (Figure 3-14), the pattern of expression of their precursors is quite different. As an example, it has been



Figure 3-14: Comparison of the consensus of highly conserved Pep family. Twenty-four amino acid sequences from C-terminal region of AtPROPEPs (AtPROPEP1-AtPROPEP8) were used for comparison. The letters mean amino acid sequence. The size of the letters indicates more sequence conservation at that position.

observed that the promoter of *AtPROPEP1* responds to infection with pathogens while the promoter of *AtPROPEP3* is activated upon herbivory attack (Huffaker *et al.*, 2011, 2013; Klauser *et al.*, 2015; Liu *et al.*, 2013). Therefore, as *AtPROPEPs*, *AtPEPR1* and *AtPEPR2* promoter activation is very different, it seems that the *AtPROPEPs*, and also *AtPEPR1* and *AtPEPR2* have different roles. For instance, we have observed that the *AtPROPEP3* promoter is strongly activated by various stress treatments, while we have not seen such an activation for others and especially the promoters of *AtPROPEP4* and *AtPROPEP7*, which were almost inactive. It should be noted, however, that former studies have shown that almost all *AtPROPEPs* are activated by wounding and MeJA (Bartels *et al.*, 2013; Huffaker and Ryan, 2007; Huffaker *et al.*, 2011, 2013; Ross *et al.*, 2014).

Recently, Bartels and Boller (2015) summarized the transcriptional landscape of the Arabidopsis *AtPEPRs* and *AtPROPEPs* genes in a review based on previous publications. They compared the activation of the various *AtPROPEP* promoters in different tissues of *A. thaliana*

in response to various treatment including MAMPs, Peps, hormones, oral secretion of herbivores, pathogens, wounding and also darkness. Our findings have some differences and similarities with their report. Briefly, they summarized that, based on former publications, the *AtPEPR1* and *AtPEPR2* promoters were activated in response to flg22, *AtPeps* (*AtPep1* to 6 for *AtPEPR1* and *AtPep1*, 2 and 4 for *AtPEPR2*), elf18, MeJA, ET, and wounding. Our observation is in line with their review for the *AtPEPR* receptors with respect to the statement that the *AtPEPR1* promoter is more active than the *AtPEPR2* promoter. In their review they also state that the promoters of *AtPROPEP1*, *AtPROPEP2* and *AtPROPEP3* are activated by flg22, *AtPeps*, elf18, MeJA, ET, and wounding. We also have confirmed that these three promoters are indeed strongly activated by the treatments mentioned. However, it is worth mentioning that there are some differences in the robustness of activation of the *AtPROPEP1*, *AtPROPEP2* and *AtPROPEP3* promoters. In comparison between the *AtPROPEP1*, *AtPROPEP2* and *AtPROPEP3* in response to MeJA, *AtPep1*, flg22 and NaCl, we found that the *AtPROPEP1* promoter was little active in response to MeJA, while *AtPROPEP2* promoter responded strongly to MeJA stimulus but the activity of *AtPROPEP2* promoter was less than the *AtPROPEP3* promoter in response to MeJA. In response to the *AtPep1* treatment, the *AtPROPEP1* promoter was more restricted to the vascular tissue while we observed that in response to *AtPep1* treatment, the *AtPROPEP2* promoter was activated in additional parts of the leaves but not in main vascular tissue. On the other hand, in response to *AtPep1* treatment, the *AtPROPEP3* promoter was strongly activated in the main vascular tissues and also additional parts of the leaves (especially 24 hours post treatment). Furthermore, NaCl treatment activated the promoters of the *AtPROPEP1* and also *AtPROPEP3*, but we did not see an activation of the *AtPROPEP2* promoter in response to NaCl treatment. In conclusion, it seems that there is the specific activation of these promoters (*AtPROPEP1*, *AtPROPEP2* and *AtPROPEP3*) in response to different stimuli.

Bartels and Boller (2015) have summarized that the *AtPROPEP4*, *AtPROPEP5* promoters are not activated in response to flg22, *AtPeps* and MeJA while we have shown that they respond to these stimuli differentially. In addition, they have not shown any data about the *AtPROPEP7* and *AtPROPEP8* promoters, while we present evidence that both the *AtPROPEP7* and *AtPROPEP8* promoters are active upon MeJA treatment but their activation is not strong and they were mainly active in the main vascular tissues. In comparison, in response to flg22 treatment and also *AtPep1* treatment the *AtPROPEP8* promoter respond slightly while *AtPROPEP7* promoter did not respond to these stimuli (flg22 and also *AtPep1* treatment). In addition, none of them responded to NaCl treatment. However, we have not done any evaluation of these promoters in response to wounding.

The observations confirm the former findings about the activation of the *AtPEPR1* and *AtPEPR2* genes. However, there are some differences among the robustness of their activation, as the *AtPEPR1* promoter has proven to be highly active in response to different stimuli (Table 3-2), while the activation of the *AtPEPR2* promoter in response to these stimuli was less robust, and restricted essentially to the main vascular tissue.

In addition, comparing the FLS2 receptor and *AtPEPRs*, FLS2 receptor can only perceive flg22 and closely related peptides (Boller and Felix, 2009), while *AtPEPR1* and *AtPEPR2* can perceive elicitors with more divergent amino acid sequences (*AtPEPR1* can perceive *AtPep1* to *AtPep8*; *AtPEPR2* can perceive *AtPep1* and *AtPep2*). It seems that *AtPEPR1* and *AtPEPR2* can perceive the broad spectrum of ligand in amino acid sequence in contrast with the FLS2 receptor. Therefore, it may be speculated that *AtPEPR1* and *AtPEPR2* might have more functions in addition to their role in immunity, as recently suggested by Bartels and Boller (2015).

From the discovery of the Pep family in Arabidopsis in 2006, and up to now that these

endogenous peptide elicitors have been discovered in other plant species, little is known about the specific activation and function of Pep family. This study was an initial attempt to determine the expression of Pep family in response to biotic and abiotic stresses in *A. thaliana*. Based on what we have found, and comparing our results with former investigations, we can say that different AtPEPRs and AtPROPEPs might have different roles and functions in addition to their role in immunity, in development (Ma *et al.*, 2014) and also other biological process from germination to flowering and seed production. As an internal alarm, they might control the activation or repression of other genes. Cumulatively, they also might have specific functions such as a role in defense and also in development. In addition, it seems that their activity is distinct from each other in different tissues. As an example, *AtPEPR2* promoter is highly active in the root even in the root tips but *AtPEPR1* is more active in the leaves but not in the root tips. Therefore, it seems that the activation of the Pep family genes is regulated under special physiological and environmental circumstances. Hence, subsequent studies are needed to determine their multiple functions.

3.6. Material and Methods

3.6.1 Plant Material and Growth Conditions

3.6.1.1 In vitro Conditions for *A. thaliana*

All *Arabidopsis* accessions were derived from the wild-type accession Columbia-0 (Col-0). *Arabidopsis* seeds were first washed with 99% ethanol supplemented with 0.5% Triton for one minute; then washed with 50% ethanol supplemented with 0.5% Triton for one minute, and finally with 100% ethanol for two minutes. Then seeds were sown on Murashige and Skoog salt solid medium containing 1% sucrose and 0.8% agar at pH 5.7. In order to carry out vernalization, the plates were kept two days at 4°C in the dark. Then, they were transferred to continuous light at 20°C for germination. Since the *GUS* reporter transgenic lines were harbored resistance to BASTA and the seeds were heterozygous, the Phosphinothricin which is the basic compound for BASTA was added to the medium to keep only seedlings which harboring the promoter-*GUS* reporter. For *GUS* staining, a 1/1000 solution of phosphinothricin (Duchefa Biochemie) was added to the solid medium.

3.6.1.2 *A. thaliana* “short-day” Conditions

Seeds were sown in soil and then vernalized for two days at 4°C in the dark. Then pots were placed in short-day conditions (ten hours light at 21°C and 14 hours dark at 18°C with 60% humidity). One week after germination, plants were grown as one plant per pot. Plants were grown under these conditions for four weeks. As the *GUS* reporter transgenic lines were BASTA resistance and the seeds were heterozygous, the plants at the seedling stage were sprayed with 0.1% BASTA to keep only the promoter-*GUS* reporter lines.

3.6.1.3 “Long day” Conditions for *A. thaliana*

After sowing seeds, pots were kept two days in the dark at 4°C for vernalization, then transferred to 16 hours light at 21°C and 8 hours dark at 18°C with 55% humidity. Plants were grown as one plant per pot.

3.6.2 Peptides

Peptides used as elicitors were flg22 (QRLSTGSRINSAKDDAAGLQIA), and *AtPep1* (ATKVKAKQRGKEKVSSGRPGQHN), which were obtained from EZBiolabs (<http://www.ezbiolab.com/>) and were dissolved in a BSA solution containing 1 mg/mL bovine serum albumin and 0.1 M NaCl and were kept in -20°C. The BSA treatment is used as the control and regarded as the mock-treated control.

3.6.3 Elicitor Treatment and GUS Staining

Nine transgenic lines containing different promoter-GUS-reporter constructs and a resistance gene against BASTA were obtained from S. Bartels, namely: *pAtPEPR1::GUS*, *pAtPEPR2::GUS*, *pAtPROPEP1::GUS*, *pAtPROPEP2::GUS*, *pAtPROPEP3::GUS*, *pAtPROPEP4::GUS*, *pAtPROPEP5::GUS*, *pAtPROPEP7::GUS*, *pAtPROPEP8::GUS*. Seedlings and mature leaves of *Arabidopsis* were used for GUS staining. For seedlings, one week old *Arabidopsis* plantlets were used. Two seedlings were placed in each well of a 24-well plate containing 200µl ddH₂O. For leaf staining, in the evening, leaves of four-five weeks old *Arabidopsis* plants (two leaves per plant), which had been kept under short-day conditions, were harvested. All leaves were pooled to get a random mixture, and then two of these leaves per assay were transferred into a 20ml Eppendorf tube containing 7ml ddH₂O and placed overnight in short-day condition. In the morning the leaves were treated with elicitors *AtPep1* and flg22 (1 µM); MeJA (1 µM, 10 µM, 100 µM), NaCl (100 mM NaCl and 250 mM) for different times in order to produce a time course: Zero, 1 hour after treatment, 6 hours after treatment, and 24 hours after treatment. The experiment has been done two times with four replicates for each time point. As a control for *AtPep1* and flg22 treatment, the solvent for the peptides was used, i.e. a BSA solution and regarded as the mock-treated control (1 mg/mL bovine serum albumin and 0.1 M NaCl). As a control for the MeJA treatment, a solvent control

consisting of DMSO was used, and as a control for the NaCl treatment, the same amount of ddH₂O was added. Then the water containing elicitors was discarded and the tissue was fixed using ice-cold 90 % acetone for 20 minutes. Then GUS staining buffer (1 mM 5-bromo-4-chloro-3-indolyl β -d-glucuronide cyclohexyl ammonium salt monohydrate (Biosynth AG, Switzerland), 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) was added to each tube and placed at 37 °C for 24 hours. Plant tissue was washed and cleared with 75% (v/v) ethanol for two times then photographed with Olympus SZX12 binocular in attached with an Olympus DP72 camera and the CellSens imaging software (Olympus America, Pennsylvania, USA).

3.6.4 Comparison Consensus of the AtPROPEPs

In order to generate sequence logos of AtPROPEPs, twenty-four amino acid sequences from C-terminal region of AtPROPEPs (AtPROPEP1-AtPROPEP8) were sent to the WeLogo (<http://www.weblgo.berkeley.edu/>) and graphical representations of consensus sequences were visualized.

4. General Discussion

Plants have a highly sophisticated immune system for which a dysfunction of one gene can result in inadequate defense responses. Recent research on plant immunity has been focused strongly on the interaction between *Pst* DC3000 and the model plant *A. thaliana*, which can be investigated from early PTI responses to complete infections. In this sophisticated interaction, many proteins from both sides play different roles and interact with each other. Some proteins play crucial roles, while others just have minimal roles. So far, the functions of many of the major players in this complex interaction have been identified as described in different reviews (Bigeard *et al.*, 2015; Boller and Felix, 2009; Macho and Zipfel, 2014; Newman *et al.*, 2013). Nevertheless, there are probably still many unknown actors in this scenario and I want to go after these unknown actors.

The current research investigated several new players in innate immunity in *A. thaliana*, a model plant system, the data from which can be extended to other plant species. Although the absence of *PP2-B13*, *ACLPI*, *SERP1* and *GRP89* genes all resulted in enhanced susceptibility to bacterial infection, the functions of *PP2-B13*, *ACLPI*, *SERP1* and *GRP89* proteins might be completely different from each other. We could just observe that *PP2-B13*, *ACLPI*, *SERP1* and *GRP89* genes have a role in innate immunity, but determining the function(s) of their protein products requires further intensive studies and many experiments are needed to determine not only their function in innate immunity, but also other possible functions that they might have during the Arabidopsis life cycle. The question is then what is the function of *PP2-B13*, *ACLPI*, *SERP1* and *GRP89* proteins? In which pathway/pathways do they have a role? Do they have synergistic effects with each other or do they work independently?

4.1 Possible Functions which can be Suggested for PP2-B13, ACLP1, SERP1 and GRP89 Proteins in Innate Immunity

We showed that PP2-B13, ACLP1, SERP1 and GRP89 all have a role in innate immunity. Briefly, what we have observed is that *pp2-b13*, *aclp1*, *serp1* and *grp89* showed an increased susceptibility to infection by *P. syringae* pv. *tomato* DC3000 and also by the *hrcC* mutant. In addition, we also have observed that *aclp1* was deficient in ET production upon flg22 treatment and *pp2-b13*, *serp1* and *grp89* mutant lines were deficient in ROS production.

As we still do not know in which step in innate immunity the protein products of *PP2-B13*, *ACLP1*, *SERP1* and *GRP89* genes have roles, in order to hypothesize the functional partnerships between PP2-B13 at the core of a complex in cellular interaction, the amino acid sequence of this protein was sent to the STRING database, which hypothetically determines protein–protein interactions based on computational prediction methods. Interestingly, STRING predicted that PP2-B13 has direct and indirect interactions with several major players in innate immunity, specifically with WRKY33 protein and also TDR1, which is the transcriptional regulator of defense response 1 (Figure 4-1; Çevik *et al.*, 2012; Denoux *et al.*, 2008). Remarkably, STRING predicted that PP2-B13 interacts with SZF1, which is a salt-inducible, zinc-finger protein and has a role in salt stress responses (LeBlanc *et al.*, 2013). STRING predicted both WRKY11 as a functional partner for ACLP1, which has an important role in early PTI responses and defense (Larroque *et al.*, 2013), and also DRB2, which has an important role in microRNA biogenesis related to different defense responses (Clavel *et al.*, 2015; Eamens *et al.*, 2012).

STRING also predicted metacaspase 9 (MC9) and RD21 as functional interaction partners for the SERP1. MC9 has role in pathogen-induced cell death in plants (Kim *et al.*, 2013), while RD21 has role in stress responses and defense (Boex-Fontvieille *et al.*, 2015; Figure 4-2). In

any event, all of above-mentioned observations are just predictions and confirming them needs many subsequent studies.

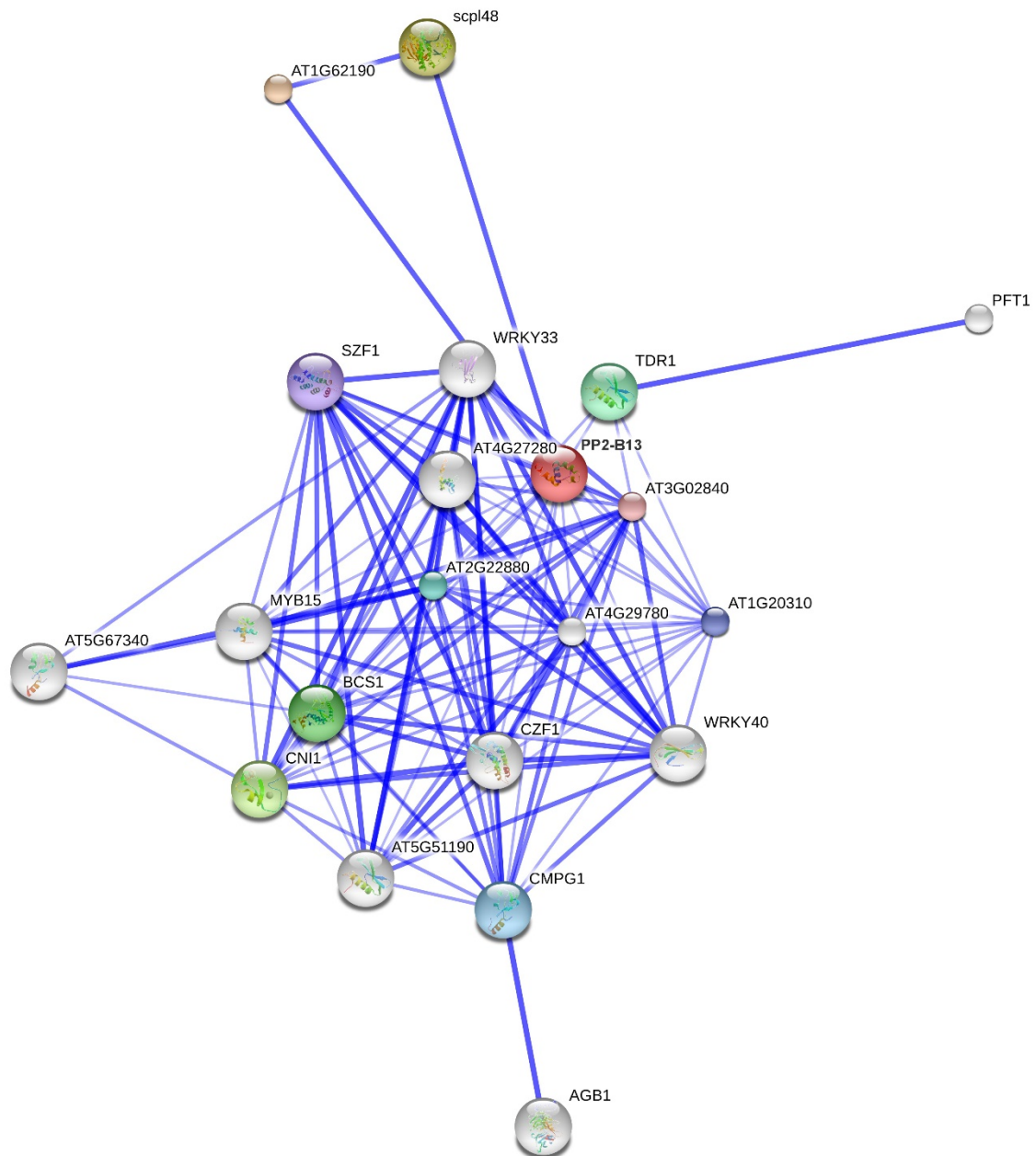


Figure 4-1. Predicted functional partners for PP2-B13 protein.

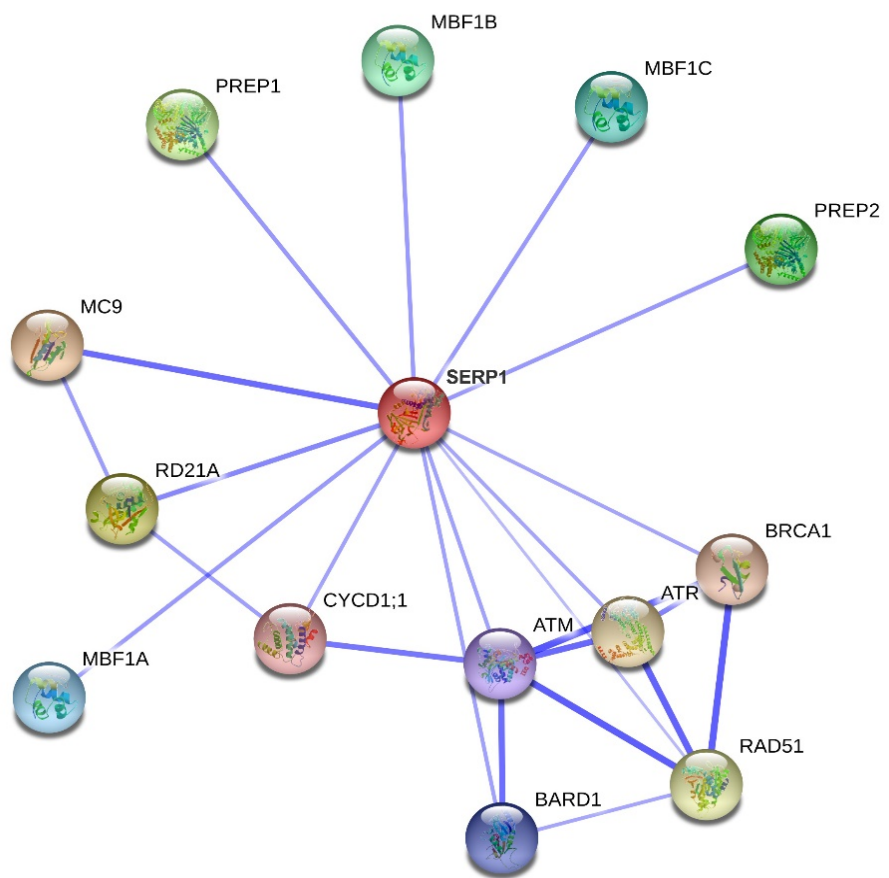
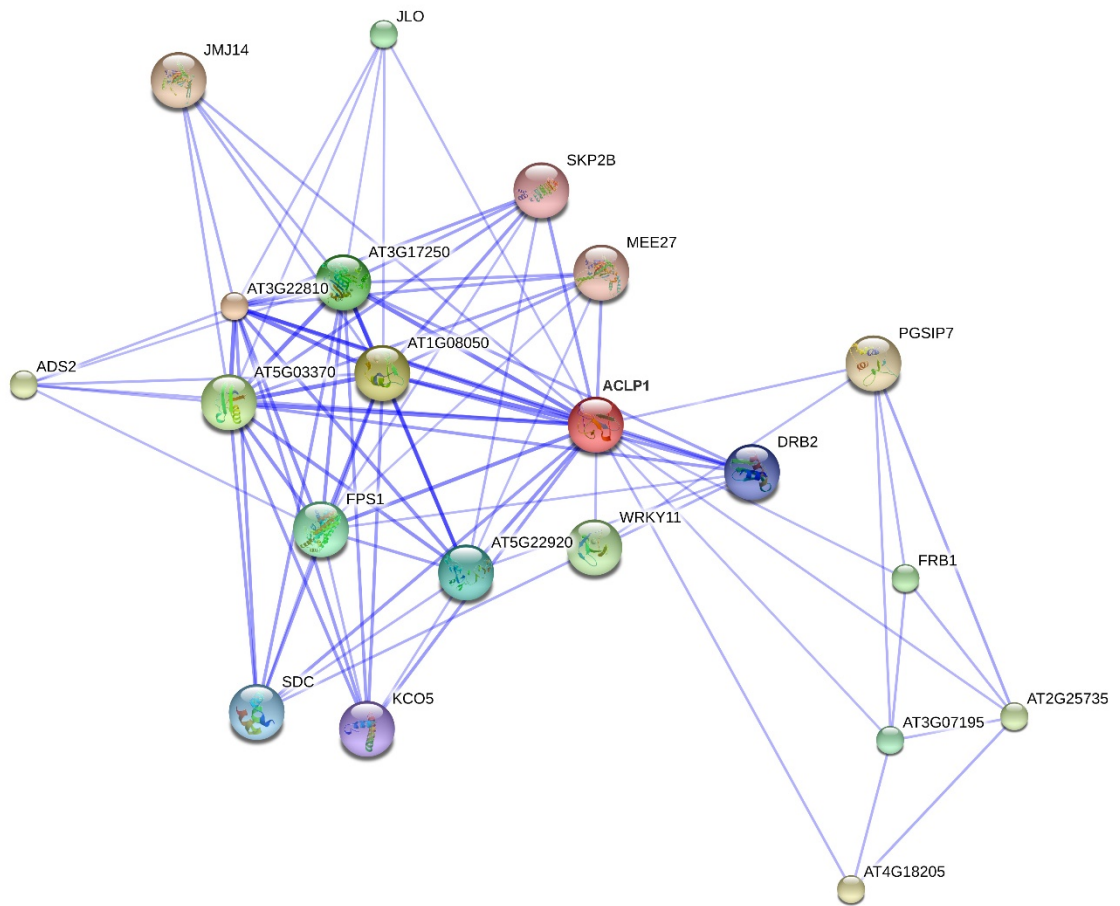


Figure 4-2. Predicted functional partners for ACLP1 and SERP1 proteins.

Recently, Bigeard *et al.* (2015) proposed a model in which they illustrate signaling mechanism upon flg22 perception that leads to PTI responses. In this model, they highlighted 21 different steps which finally lead to adequate defense responses (Figure 4-3). Based on what we found and the hypothetical protein interaction predictions, we decided to hypothesize in which steps proposed by Bigeard *et al.* (2015) that PP2-B13, ACLP1, SERP1 and GRP89 can have a role. Briefly, flg22 perception by FLS2 receptor induces rapid immune receptor complex formation at the plasma membrane that subsequently resulted in different auto and transphosphorylations of different proteins (step 1). Subsequently, BIK1 protein quickly becomes phosphorylated and released from the PRR complex (step 2). As there have been many different studies in these two steps toward a better understanding of flg22/FLS2 perception, and many participants in these two steps have been determined so far (Boller and Felix, 2009; Macho and Zipfel, 2014), therefore, it is less likely that PP2-B13, ACLP1, SERP1 and GRP89 proteins have a role in step 1 and step 2. According to Bigeard *et al.* (2015), at the concurrent step, as a consequence of BIK1 phosphorylation, Ca²⁺ burst would accrue in 30 seconds to 2 minutes after flg22 perception and reach a peak at around 4 to 6 minutes (step 3). Ca²⁺ influx leads to the opening of other membrane channels including influx of H⁺, and efflux of K⁺, Cl⁻ and nitrate. This event leads to an extracellular alkalization after 1 minute and a depolarization of the plasma membrane in 1 to 3 minutes (step 4).

In parallel, the ROS burst rapidly occurs in 2 to 3 minutes after flg22 perception through RBOHD protein, which reaches a peak at around 10 to 14 minutes. It is worth noting that full activation of RBOHD protein requires phosphorylation by BIK1 and Ca²⁺ induced CDPKs (calcium-dependent protein kinase; step 5).

As a result of this process, RBOHD produces O₂⁻ in the apoplast, which is converted into hydrogen peroxide (H₂O₂) by superoxide dismutases (SOD; step 5 and step 6). It is also worth

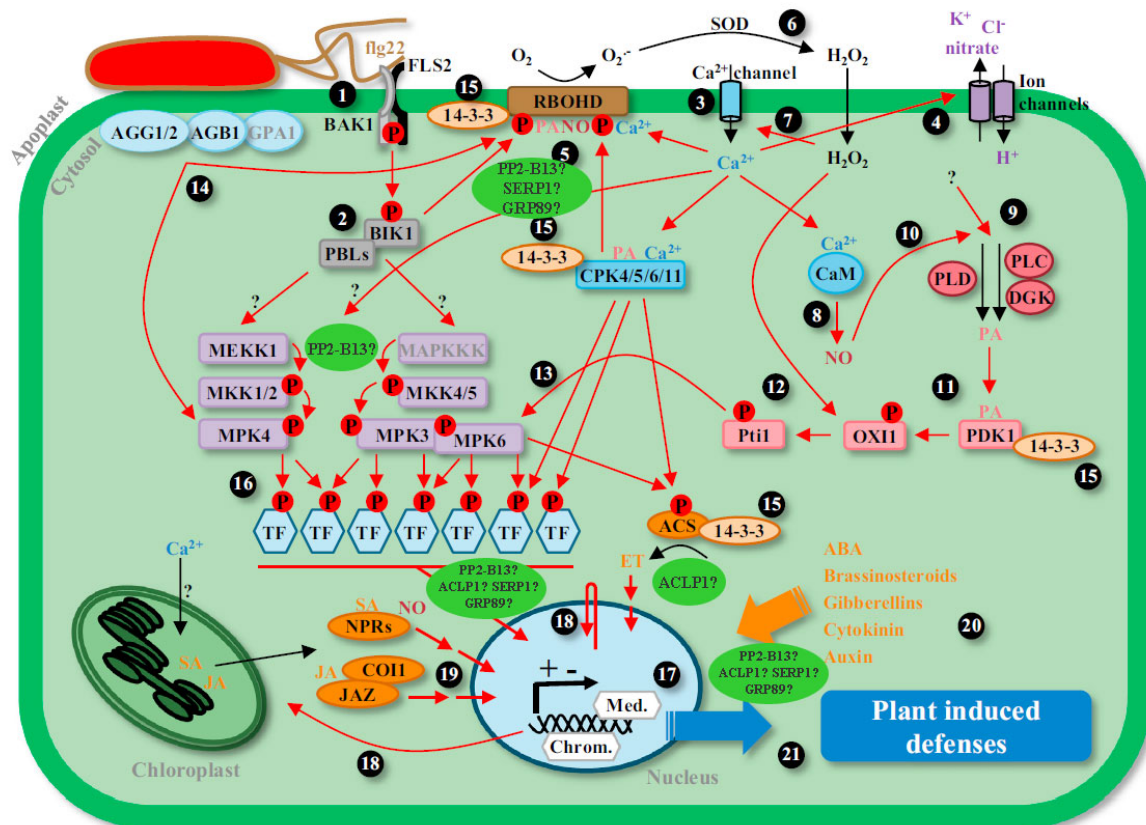


Figure 4-3. Signaling Mechanisms in PTI leads to defense responses. Possible function/functions for PP2-B13, ACLP1, SERP1 and GRP89 proteins is represented. Black arrows indicate enzymatic pathways or transport, and red arrows represent regulation (direct or indirect activation/inhibition). Question marks indicate unidentified or unclear events. (Adopted from Bigeard *et al.*, 2015).

noting that as *pp2-b13*, *serp1* and *grp89* mutant lines did show deficiency in ROS production in response to flg22 treatment (Figure 2-6 and Supplementary Figure S7-B), it might be possible that the protein products of the *PP2-B13*, *SERP1* and *GRP89* genes have roles in this step in interaction with other factors in ROS production. Hydrogen peroxide production in the cytosol is capable of inducing cytosolic Ca^{2+} elevation (step 7). Ca^{2+} elevation also induces CaM that leads to production of NO, which regulates NPR1 and RBOHD through cysteine S-nitrosylation (step 8). PA production by PLD protein and also PLC/DGK is dramatically elevated in 2 minutes to 8 minutes (step 9). Remarkably, NO production is partly required for PA generation (phosphatidic acid; step 10). PA can also affect the activity of CDPKs, PDK1, and RBOHD proteins. PA activates OXI1 in a PDK1-dependent manner (step 11). In parallel,

hydrogen peroxide can also activate Pti1 kinases in an OXI1-dependent pathway (step 12). Pti1 kinases regulate MAPKs activation (step 13). Since STRING predicted that PP2-B13 interacts with WRKY33, which is one of the major pathogen-inducible TFs (Mao *et al.*, 2011), it might be possible that the PP2-B13 protein has a role in this step. It was also reported previously that WRKY33 is a key transcriptional regulator against *Botrytis cinerea* infection (Birkenbihl *et al.*, 2012). In addition, in this model that Bigeard *et al.* (2015) proposed, there are several steps which are highlighted with question marks, especially in the interaction of PBLs protein with MPKs proteins. It might be possible that PP2-B13 has a role in this step. Bigeard *et al.* (2015) indicated that AGB1, AGG1, and AGG2 G proteins are also partly needed for ROS burst and MPK4 activation (step 14), but it is less likely that PP2-B13, ACLP1, SERP1 and GRP89 proteins have a role in this step. 14-3-3 proteins also have a role in the activity of RBOHD, CDPKs, PDK1, and several isoforms of ACS (step 15). MAPK activation happens in a few minutes, probably as part of the Ca²⁺ burst and also in a BIK1/PBLs dependent manner. Ca²⁺ induces CDPKs, which phosphorylate a large number of substrates such as ACS isoforms, which as a result leads to ET synthesis, and also activation of TFs (step 16). As has been seen, the *aclp1* mutant line responded differentially in ET production (Figure 2-6 and Supplementary Figure S6). Thus, the protein product of the *ACLP1* gene might have a role in this step in interaction with other players in ET production. TFs activation regulate several thousand genes (step 17). The most important ones are those genes for which their protein products have role in SA, JA, and ET signaling, synthesis of antimicrobial compounds and also other transcription regulatory factors (step 18). Then SA, JA, and ET signaling cascade have roles in regulation of different gene products (step 19). As SA, JA, and ET phytohormones are playing crucial roles in defense and their mode of activation is very diverse, it might be possible that PP2-B13, ACLP1, SERP1 and GRP89 proteins might have a role in phytohormone production. It is also conceivable that PP2-B13, ACLP1, SERP1 and GRP89 either have roles in SA, JA, and ET

production, as well as in signaling pathways of these phytohormones, or contribute to regulation of those genes which have roles in phytohormone production. As Bigeard *et al.* (2015) proposed, crosstalk with other phytohormones would be the next step (step 20).

Finally, this sophisticated signaling cascades leads to the accomplishment of plant-induced defenses, such as the production and also secretion of antimicrobial compounds and the production of toxic ROS (step 21). Therefore, it also might be conceivable that PP2-B13, ACLP1, SERP1 and GRP89 proteins have roles in the production of antimicrobial compounds that are normally produced against microbial infections.

Moreover, since we have observed that the mutant line of *PP2-B13*, *ACLP1*, *SERP1* and *GRP89* showed susceptibility to *Pst* DC3000 infection, it is conceivable that PP2-B13, ACLP1, SERP1 and GRP89 have interactions with effectors that are produced upon infection by *Pst* DC3000. It is also conceivable that PP2-B13, ACLP1, SERP1 and GRP89 have roles in the biogenesis of microRNAs that have roles in defense responses. In any event, at present many mechanisms and pathways in defense responses are still a mystery and many players in this scenario are still unknown (Bigeard *et al.*, 2015). Therefore, it is possible that PP2-B13, ACLP1, SERP1 and GRP89 proteins have roles in pathways which are not yet characterized. Hence, many experiments and evaluations are needed to determine the functions of PP2-B13, ACLP1, SERP1 and GRP89 proteins in PTI responses.

4.2 Have PP2-B13, ACLP1, SERP1 and GRP89 Proteins other Functions Besides in Innate Immunity?

Despite our observations and the hypothetical protein partners which are predicted for PP2-B13, ACLP1, and SERP1, we still do not know in which pathway these genes products can have roles and functions. Figure 4-4, briefly represents the possible functions of the *PP2-B13*, *ACLP1*, *SERP1* and *GRP89* genes products that can be postulated among 22 major roles for

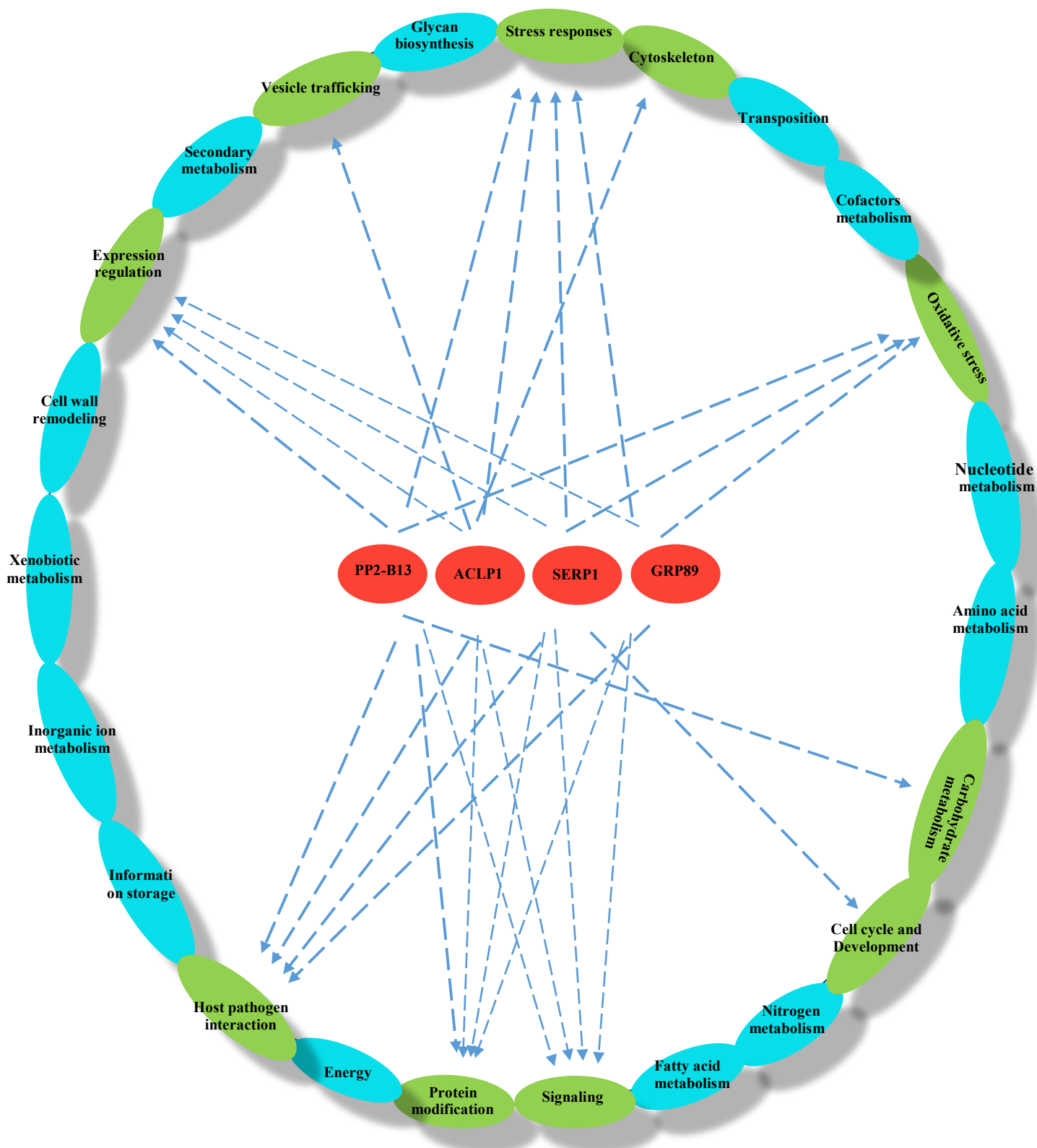


Figure 4-4. Possible function which can be speculated for PP2-B13, ACLP1, SERP1 and GRP89 proteins. The arrows indicate the possible function(s) for each individual protein based on what we have found, hypothetical protein interaction and predicted protein domain. Weight of the arrow shows that there is more possibility for each individual interaction. Green oval, indicates that the possible function(s) which can be proposed. Blue oval represents that there is less possibility to propose these functions for PP2-B13, ACLP1, SERP1 and GRP89 proteins.

any particular gene product in *A. thaliana* including: stress response, secondary metabolism, oxidative stress, cytoskeleton, transposition, glycan biosynthesis, vesicle trafficking, carbohydrate metabolism, amino acid metabolism, fatty acid metabolism, signal transduction, energy, protein modification, expression regulation, cell wall remodeling, host pathogen interaction, inorganic ion metabolism, information storage and processing, xenobiotic metabolism, cell cycle, development and morphogenesis, nucleotide metabolism, nitrogen metabolism and cofactors metabolism (www.Arabidopsis.org). From what we have found in this research, from prediction of protein domain and what can be hypothesized for potential partners of *PP2-B13*, *ACLPI* and *SERP1* proteins (Figure 4-1 and 4-2), we can hypothesize that these gene products might have multiple roles beside their roles in innate immunity. Therefore, hypothetical functions of *PP2-B13*, *ACLPI*, *SERP1* and *GRP89* gene products are illustrated in Figure 4-4. Briefly, based on what we have found in the current research (susceptibility to bacterial infection and deficiency in ROS production), *PP2-B13* could have roles in defense responses and host pathogen interactions. In addition, as *PP2-B13* has an F-box conserved domain, it also might have role in protein modification. In addition, as Raptor X also determined the carbohydrate binding domain for *PP2-B13* (Supplementary Figure S8), it might be possible that *PP2-B13* has also role in carbohydrate metabolism. It is also conceivable that *PP2-B13* might have roles in oxidative stresses as it showed deficiency in ROS production. Furthermore, protein partner prediction indicated that *PP2-B13* interacts with WRKY33 which is one of the most important TFs. Therefore, *PP2-B13* might have a role in expression regulation. For *ACLPI*, several functions can be suggested. Based on what we found in this research, *ACLPI* might have a role in defense responses and also in host-pathogen interactions. Since *ACLPI* is an actin cross-linking protein, it might also have roles in vesicle trafficking, vesicle formation and also cytoskeleton formation. Since we observed that the *aclp1* mutant line was deficient in ET production, it might be possible that the protein product

of this gene has a role in ET production. *SEPR1* is a pseudogene and also a putative serpin (NCBI determine protein for *SEPR1*; Acc. No AAO43395.1), which might have roles in defense responses. Beside these, since we observed a deficiency in the flowering stage in the *serp1* mutant line, it might also have roles in cell cycle and development (Supplementary Figure S3). It is also conceivable that SERP1 has a role in oxidative stress, as we saw differential ROS production in the *serp1* mutant line. In addition, as SEPR1 is a putative serpin, it might have a role in protein modification. Based on what we found in the current research, GRP89 protein can have functions in stress responses and host pathogen interactions. In addition, as we saw differential ROS production in the *grp89* mutant line, it is also conceivable that GRP89 has role in oxidative stress (Figure 4-4). As many proteins with different domains have roles in expression regulation, signaling and protein modifications, it might be possible that PP2-B13, ACLP1, SERP1 and GRP89 might have roles in expression regulation, signaling and protein modifications. In any event, PP2-B13, ACLP1, SERP1 and GRP89 proteins might have roles in other pathways. Anyway, all of the mentioned speculated functions for PP2-B13, ACLP1, SERP1 and GRP89 proteins are just predictions and to confirm them, many different experiments are needed to determine the function(s) of these proteins.

4.3 *AtPROPEPs* and *AtPEPR1/2* Promoter Reporter lines Differentially Respond to Biotic and Abiotic Stimuli

As the first Arabidopsis endogenous peptide signal which was investigated to regulate plant anti-microbial defenses, the *AtPeps* family is considered as the best model to study endogenous peptide elicitors, despite the discovery of systemin long time ago (Huffaker *et al.*, 2006). In addition, the discovery of their corresponding receptors AtPEPR1 and AtPEPR2 has opened a unique approach to study Pep perception system in more detail such as the effect of each individual receptor in this scenario and also their interaction with other receptors and co-

receptors. Beside these, the possibility to do mutagenesis study of each individual member of the Pep family, makes it possible for researchers to study each individual member of the Pep family in more detail. Furthermore, the possibility to artificially produce the C-terminal Pep-like peptides which responds similar to protein extracted from the leaf tissue would allow a comprehensive study in the Pep-PEPR system. Beside these, as a recent finding showed that AtPEPR2 may have a role in development (Ma *et al.*, 2012), it might be possible that AtPEPR1 and also AtPROPEPs also have specific role in Arabidopsis development. Therefore, at the moment DAMPs perception in plants is a new topic in Arabidopsis research that is still at its beginning. On the other hand, since that AtPROPEPs homologue proteins are present within all angiosperms (Huffaker *et al.*, 2013; Lori *et al.*, 2015), the more they are identified in other plant species, the greater is the need to study them in more detail. Altogether, these observations make the Pep-PEPR system in *A. thaliana* an exciting field.

With regard to all above mentioned points, there are still many questions in the Pep-PEPR system in Arabidopsis which are not answered yet. One question for all researchers in this field is whether AtPROPEPs and their corresponding receptors are redundant or each has specific functions? And also whether gene activation of the Pep family is tissue specific? In addition, it is still unclear if AtPROPEPs and their corresponding receptors play different roles in Arabidopsis development?

Therefore, the first step to answer these questions is to classify the activation pattern of *AtPROPEPs* and also *AtPEPRs* based on their promoter activation. In this thesis, using promoter reporter constructs of *AtPROPEPs* genes and also their corresponding receptors fused to the *GUS* gene we could characterize and classify activation patterns of the Pep-family. We have observed that despite the similarity in the AtPROPEPs family especially in their C-terminal region, the activation patterns of their promoter are totally different in response to the biotic and abiotic stresses due to the difference in their promoter sequence region. We have

observed that stresses including *AtPep1*, *flg22*, hormone treatment MeJA, and NaCl treatment, can differentially activate promoters of *AtPROPEP* genes and also *AtPEPR1* and *AtPEPR2*. We have shown that although *AtPEPR1* and *AtPEPR2* are similar in sequence, their responses upon different biotic and abiotic stresses were totally different. We also have shown that it is possible to classify the promoters of *AtPROPEPs* and their correspondence receptors into different group based on their response upon MeJA treatment.

Previously Huffaker *et al.* (2006) have speculated that *AtPeps* peptides do their function during wounding and they are released once the plant cell is damaged. In other words, they hypothesized that the cell should be damaged and these elicitors should be released to perceive by their receptors. But are they released indeed outside of the cell to be perceived by their corresponding receptors? In other words, it is still unclear how are they released into the apoplast be perceived by their correspondence receptors. Answering to this question would be interesting, as hormones and other treatment activate Pep-family without damaging the cell. In the current research, we just treated the plant materials with different stimuli without damaging the leaf surface or seedlings but we have seen the activation of the promoter reporter lines of these endogenous peptide elicitors and also their corresponding receptors.

Unfortunately, we do not have GUS promoter reporter constructs for *AtPROPEP6* to evaluate its response to the stimuli which we have done on other *AtPROPEPs*. The result of the *AtPROPEP6* promoter reporter line would be interesting as it is located in another chromosome. Therefore, it is suggested to make a GUS reporter construct of *AtPROPEP6* promoter reporter line and compare the results with others.

4.4 A Proposed Model for Pep-PEPR Activation in Response to Different Stimuli

Different studies showed that *AtPROPEPs* and their correspondence receptors are differentially induced upon different stimuli such as *flg22* and *elf18* treatment (Yamaguchi *et al.*, 2010), oral

secretions of herbivores (Klauser *et al.*, 2015), viral infections (Kørner *et al.*, 2013), SA (Huffaker *et al.*, 2007), wounding (Bartels *et al.*, 2013; Yamaguchi *et al.*, 2010), pathogens (Huffaker *et al.*, 2007; Logemann *et al.*, 2013), darkness (Gully *et al.*, 2015), NaCl (chapter three of this Thesis), and MAMPs (Bartels *et al.*, 2013; Yamaguchi *et al.*, 2010).

Therefore, it seems that different stimuli directly or indirectly can activate the Pep-PEPR system through the activation of their corresponding genes without releasing Pep outside the cell. It seems that the Pep family are fine-tuning the interactive roles in response to many diverse stimuli over the different stages of development in Arabidopsis. It might be possible that the Pep family responds to many other stimuli which are not identified so far. Therefore, we propose a model in which different stimuli, directly and indirectly affect the activation of the Pep family which subsequently leads to differential activation of *AtPROPEP* genes and also *AtPEPRI/2* genes that resulted in differential reprogramming of the transcriptome (Figure 4-5).

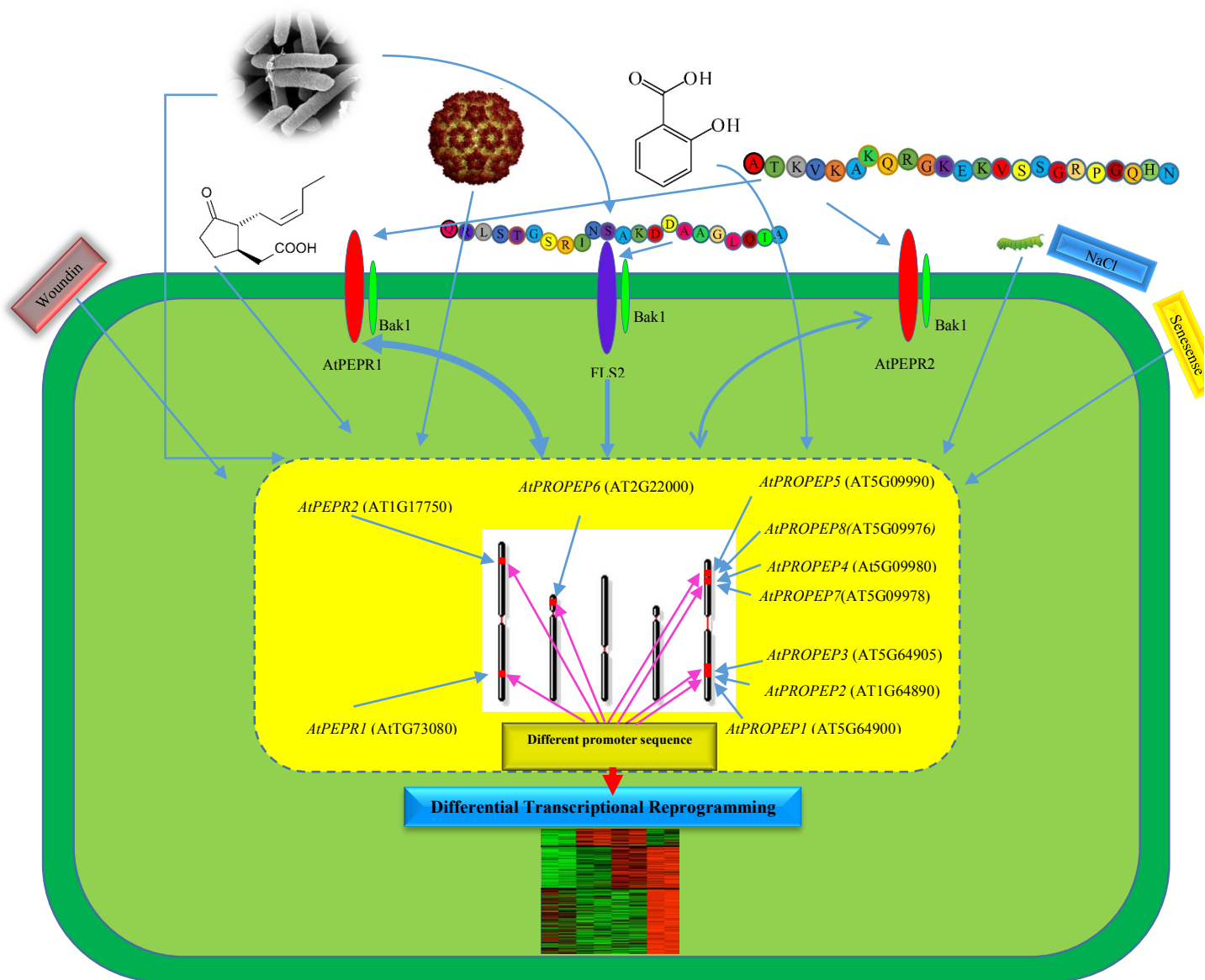


Figure 4-5. A proposed model in which Pep-PEPR system can get activated in response to different stimuli and subsequently reprogram the transcriptome of the genes.

4.6 Outlook

As the recognition of MAMPs provides the basis for pathogen resistance, any genes which play a role in this story should be identified. The characterization of *PP2-B13*, *ACLPI*, *SERP1* and *GRP89* genes, can help us not only to better understand plant-microbe interactions but also formulate resistance strategy. Our study which is based on a deep sequencing approach, provides a new way for other researchers to find other novel components in innate immunity in Arabidopsis. In addition, the large quantity of deep sequencing data can be used as a reference for establishment of the innate immune system network in Arabidopsis.

4.6.1 Possible Approach to Determine the Function of *PP2-B13*, *ACLPI*, *SERP1* and *GRP89* Genes Product

Despite what we have found and predicted potential partner of *PP2-B13*, *ACLPI*, *SERP1* and *GRP89*, our knowledge toward better understanding the role of these genes product in innate immunity, is very low. Therefore, many different experiments will need to be done.

In order to determine whether over-expression of *PP2-B13*, *ACLPI*, *SERP1* and *GRP89* genes can exacerbate their response upon infection and also monitor the differential innate immunity response in overexpressed transgenic plants compared to the wild type *Arabidopsis*, we suggest overexpressing these genes (*PP2-B13*, *ACLPI*, *SERP1* and *GRP89*) under a 35S promoter in *A. thaliana* plants and evaluating these transgenic plants in innate immunity responses using the bacterial infiltration assay, ET measurement and ROS production. In addition, by overexpressing *PP2-B13*, *ACLPI*, *SERP1* and *GRP89* genes, the morphology can be compared to wild type *Arabidopsis* to determine if overexpression of *PP2-B13*, *ACLPI*, *SERP1* and *GRP89* leads to abnormalities in the transgenic plants as it does for some genes under the control of the 35S promoter. For example, overexpression of *BAKI* leads to production of abnormal plants (Marta Kiss-Papp, 2014). Therefore, we also suggested expressing *PP2-B13*, *ACLPI*, *SERP1* and *GRP89* genes under the control of the XVE system and subsequently evaluating these transgenic plants for responses to biotic stresses. Further studies of *pp2-b13*, *aclp1*, *serp1* and *grp89* mutant lines including complementation assays and transcript analysis should be used to analyze the functions of their gene products in more detail.

In order to determine if *PP2-B13*, *ACLPI*, *SERP1* and *GRP89* proteins have roles in proper perception of other elicitors such as *AtPeps* and *elf18* proper defense responses, we recommend investigating *pp2-b13*, *aclp1*, *serp1* and *grp89* mutant plants for their changes in ET and ROS levels in response to *AtPeps* and also *elf18* perception to monitor if they have similar responses compared to wild type *Arabidopsis*.

In order to assess the subcellular localization of PP2-B13, ACLP1, SERP1 and GRP89 proteins within the cell, it is suggested to fuse these proteins with a GFP-based reporter protein to determine which parts of the cell they are localized in and monitor the trafficking of PP2-B13, ACLP1, SERP1 and GRP89 GFP-fusion within the cell before and after flg22 treatment.

In addition, via a GFP-based reporter protein approach, it is also possible to determine whether PP2-B13, ACLP1, SERP1 and GRP89 proteins are activated in specialized cells upon elicitor treatment or bacterial infection. In addition, we can evaluate their involvement in cell-cell communication especially for PP2-B13 protein; since Phloem protein 2 (PP2) are previously reported as one of the most abundant and enigmatic proteins in the phloem sap (Dinant *et al.*, 2003).

Previously, it was reported that a GFP-tag can indirectly determine the function of the protein (Albert *et al.*, 2007). As an example, those proteins which travel from the cytoplasm to the nucleus once the cell is exposed to growth factor, indicate that these proteins have roles in gene expression regulation (Albert *et al.*, 2007). Therefore, we suggest using an expression vector harboring *PP2-B13*, *ACLP1*, *SERP1* and *GRP89* fused to GFP or m-cherry, to make transgenic plants and subsequently treat these transgenic plants with either flg22 or infect them with *Pst* DC3000 and then determine in which part of the cell these proteins are localized. In this regard, we can expand our understanding by treatment of these transgenic plants with other elicitors such as *AtPeps* and *elf18*.

In order to determine the tissue specificity activation of *PP2-B13*, *ACLP1*, *SERP1* and *GRP89* genes and expand our knowledge toward a better understanding of the tissue-specific activation of *PP2-B13*, *ACLP1*, *SERP1* and *GRP89*, we suggest producing native promoter reporter constructs of *PP2-B13*, *ACLP1*, *SERP1* and *GRP89* genes fused with *GUS* gene and subsequently characterize their promoter activation patterns in response to different stimuli to

determine with or without any stimuli in which tissues *PP2-B13*, *ACL1*, *SERP1* and *GRP89* genes are active.

We still do not have either any information about the phenotypes of *pp2-b13*, *aclp1*, *serp1* and *grp89* mutant lines in the roots, or any data about the activation patterns of *PP2-B13*, *ACL1*, *SERP1* and *GRP89* genes in the roots. Therefore, in order to analyze the involvement of *PP2-B13*, *ACL1*, *SERP1* and *GRP89* genes in roots, a comprehensive study of these genes in the roots such as growth of roots upon flg22 treatment in *pp2-b13*, *aclp1*, *serp1* and *grp89* mutant lines and analysis of GUS promoter reporter construct fused with *PP2-B13*, *ACL1*, *SERP1* and *GRP89* genes should be made to understand the roles of these genes in the root tissues.

In order to determine whether PP2-B13, ACL1, SERP1 and GRP89 proteins can affect to the function of other major players in innate immunity, it would be interesting to prepare and study double mutants by crossing our mutant lines (*pp2-b13*, *aclp1*, *serp1* and *grp89*) with mutants of major players in innate immunity in Arabidopsis such as FLS2, SID2-2, and PAD4, to determine and evaluate their synergistic effect on PTI responses. We also recommend making double mutants between the mutant lines of *pp2-b13*, *aclp1*, *serp1* and *grp89* genes comparing the PTI responses with single mutants, to evaluate whether PP2-B13, ACL1, SERP1 and GRP89 proteins have synergistic effect with each other.

In order to monitor response of *pp2-b13*, *aclp1*, *serp1* and *grp89* mutant lines to other stimuli including abiotic stresses (such as salt stress, osmotic stress, etc.), and other biotic stresses such as viral infection, fungal infection and also herbivores attack, evaluation and treatments of *pp2-b13*, *aclp1*, *serp1* and *grp89* mutant lines with biotic and abiotic stresses can help us to determine if PP2-B13, ACL1, SERP1 and GRP89 proteins have roles in responding to other stimuli or if they just have roles in bacterial infection.

Since BAK1 contributes in defense against many responses, it would be interesting to make double mutants of *pp2-b13*, *aclp1*, *serp1* and *grp89* mutant lines with a *bak1* mutant line to determine if they have synergistic effect in response to bacterial infection and early PTI responses.

qRT-PCR can be used to evaluate the quantity of the major players during innate immunity in *pp2-b13*, *aclp1*, *serp1* and *grp89* mutant lines compared to the wild type Arabidopsis, in response to elicitor treatment, to determine if they response differentially or similarly.

In order to determine with which proteins PP2-B13, ACLP1, SERP1 and GRP89 have interactions, at the first step it is suggested using FLAG fused to *PP2-B13*, *ACLP1*, *SERP1* and *GRP89* genes and subsequently using antibodies raised against to FLAG precipitate PP2-B13, ACLP1, SERP1 and GRP89 proteins and sequence them to determine the functional partner of these protein. If this approach does not work, we strongly recommend producing antibodies against PP2-B13, ACLP1, SERP1 and GRP89 proteins and also determine the abundance of PP2-B13, ACLP1, SERP1 and GRP89 proteins under different stress conditions in wild type Arabidopsis. In addition, using this approach beside the yeast two hybrid assay, can help us to determine the interaction partners of PP2-B13, ACLP1, SERP1 and GRP89.

4.6.2 Pep Family Promoter Activation Pattern Prospect View

Based on former studies and what we have found, it is hypothesized that *AtPep-AtPEPR* are universal element which integrate in diverse defense responses against biotic and abiotic stresses at different stages of development in Arabidopsis. Besides having a function in fine-tuning role in defense, they might have other functions in a specific developmental stage as was observed for *AtPEPR2* (Ma *et al.*, 2013). Although there are some information about the Pep family in fertilization (Bartels *et al.*, 2013), we still indeed do not know if *AtPROPEPs* or *AtAtPEPR1* have a role in other biological process at different stages of development in Arabidopsis. Therefore, many studies to answer these questions is needed.

In addition, as we have seen the robust activation of the promoter line of *AtPROPEPs* and their corresponding receptors in response to MeJA, it might be possible that it has an important role in biogenesis of these proteins and activation of *AtPROPEP* genes and also *AtPEPR1/2* genes. Therefore, any connection between MeJA biogenesis and the Pep family should be investigated using mutant lines on both sides.

In addition, it would be very interesting to evaluate the interaction of *AtPROPEPs* and *AtPEPR1/2* genes at the global transcriptome level.

The result that we have presented regarding the GUS activity of these promoter reporter line was based on what we have observed under magnification range of binocular. It might be possible that the promoter of *AtPROPEPs* and *AtPEPR1/2* genes get activated in response to these stimuli in a very low level that we cannot see under this magnification range. Therefore, it is conceivable to measure quantitatively the transcriptome of *AtPROPEPs* and *AtPEPR1/2* genes with qRT-PCR and compare the results with GUS activity that we have found. It is also suggested to quantitatively measure the GUS protein activity in response to these stimuli that we have tested to see if there is a correlation with what we have found.

4.7 Final Conclusion

As we have seen there is a role for PP2-B13, ACLP1, SERP1 and GRP89 proteins in innate immunity in Arabidopsis, it might be possible that there is a functional link between PP2-B13, ACLP1, SERP1 and GRP89 proteins and the Pep family. Therefore, further research is needed to determine the link between them. Making double mutant lines between *pp2-b13*, *aclp1*, *serp1*, and *grp89* with *AtPROPEPs* and *AtPEPR1/2* and subsequently evaluating their response in innate immunity compared to single mutant would be interesting to study.

In addition, although we have found that flg22 can activate promoter reporter line of the Pep family, the exact relationship and also the interaction between MAMP and DAMP signaling should be studied at more detail.

Altogether, the results which are presented here, can open a new way for better understanding innate immune system in Arabidopsis and expand our knowledge to formulate a new innovative approach to find novel players in the innate immunity in the model plant *A. thaliana*. In addition, we can monitor how these endogenous peptide signals can differentially become activated to overcome diverse challenges during the different stages in *A. thaliana*.

5. List of Abbreviations

aa: Amino Acid

At/A.t.: *Arabidopsis thaliana*

avr: avirulence gene or protein

BAK1: BRASSINOSTEROIDE INSENSITIVE 1-ASSOCIATED KINASE 1

BIK1: BOTRYTIS-INDUCED KINASE 1

BR: brassinosteroid

BRI1: BRASSINOSTEROID INSENSITIVE 1

BSA: Bovine Serum Albumin

CaMV: Cauliflower mosaic virus

cDNA: complementary DNA

CDPKs: Calcium-Dependent Protein Kinases

cfu: colony forming unit

CLV2: CLAVATA 2

CERK1: CHITIN ELICITOR RECEPTOR KINASE 1

Col-0: Arabidopsis ecotype Columbia; it is also referred as wild type

DAMP: Damage-Associated Molecular Patterns

DMSO: Dimethyl Sulfoxide

ddH₂O: double-distilled water

EDTA: Ethylenediaminetetraacetic acid

EDS: Enhanced Disease Susceptibility

ET: Ethylene

DNA: Deoxyribonucleic acid

dpi: day(s) post-inoculation

EFR: ELONGATION FACTOR TU RECEPTOR

EF-Tu: elongation factor Tu

elf: EF-Tu peptide

EMS: ethyl methanesulphonate

ER: endoplasmic reticulum

ETI: effector-triggered immunity

EtOH: ethanol

E3: ubiquitin ligase

flg22: flagellin 22

FLS2: flagellin sensing 2

GC: Gas chromatography

GUS: β -Glucuronidase

HR: hypersensitive response

IP: immunoprecipitation

kDa: kilodalton

LRR: leucine-rich repeat

LRR-RKs: Leucine-rich repeat receptor kinases

LRR-RLKs: Leucine-rich repeat receptor like kinases

MAMP: microbe-associated molecular pattern

MAP: Mitogen-activated protein

MAPK: mitogen-activated protein kinase

MeJA: Methyl jasmonate

PA: phosphatidic acid

PAMP: pathogen-associated molecular pattern

Pep: Danger peptide

PEPR: Pep RECEPTOR

PP2: Phloem protein 2

PR: Pathogenesis-related

AtPROPEP: Precursor of *Arabidopsis thaliana* AtPep

PRR: pattern recognition receptor

Pst DC3000: *Pseudomonas syringae* pv. *tomato* strain DC3000

PTI: pattern-triggered immunity

PUB: PLANT U-BOX

pv: Pathovar

qRT-PCR: Quantitative Real Time PCR

R gene/protein: resistance gene/protein

RbohD: Respiratory burst oxidase homologue D

RbohH: Respiratory-burst oxidase homologue

RLK: receptor-like kinase

RLP: receptor-like protein

ROS: reactive oxygen species

RPS5: RESISTANT TO *P. SYRINGAE* 5

SA: salicylic acid

SAR: Systemic acquired resistance

SCF: Skp1-Cullin-F-box

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SERK: SOMATIC EMBRYOGENESIS RECEPTOR KINASE

T-DNA: transfer-DNA

TF: transcription factor

T3SS: Type Three Secretion System

UBQ: Ubiquitin

WRKY: a 60-amino acid-protein domain which is defined by the conserved sequence WRKYGQK together with a zinc-finger-like motif

WT: wild type

Xa21: A rice resistance locus which confers disease resistance to *Xanthomonas oryzae* pv. *Oryzae*

Zm: *Zea mays*

6. Literature Cited:

- Albert, M. (2013). Peptides as triggers of plant defence. *Journal of Experimental Botany* **64**: 5269-5279.
- Alfano, J.R., and Collmer, A. (1997). The type III (Hrp) secretion pathway of plant pathogenic bacteria: trafficking harpins, Avr proteins, and death. *Journal of Bacteriology* **179**: 5655-62.
- Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2007). *Molecular Biology of the Cell*, 4th edition. New York; Garland Science; 1392 pp.
- An, J., Li, Z., Dong, Y., Wu, J., and Ren, J. (2015). Complement activation contributes to the anti-methicillin-resistant *Staphylococcus aureus* effect of natural anti-keratin antibody. *Biochemical and Biophysical Research Communications* **461**:142-7.
- Asai, T., Tena, G., Plotnikova, J., Willmann, M.R., Chiu, W.L., Gomez-Gomez, L., Boller, T., Ausubel, F.M., and Sheen, J. (2002). MAP kinase signalling cascade in Arabidopsis innate immunity. *Nature* **415**: 977-983.
- Ascencio-Ibanez, J.T., Sozzani, R., Lee, T.J., Chu, T.M., Wolfinger, R.D., Cella, R., and Hanley-Bowdoin, L. (2008). Global analysis of arabidopsis gene expression uncovers a complex array of changes impacting pathogen response and cell cycle during geminivirus infection. *Plant Physiology* **148**: 436-454.
- Autelitano, D.J., Rajic, A., Smith, A.I., Berndt, M.C., Ilag, L.L., and Vadas, M. (2006). The cryptome: A subset of the proteome, comprising cryptic peptides with distinct bioactivities. *Drug Discovery Today* **11**: 306-314.
- Bartels, S., and Boller, T. (2015). Quo vadis, Pep? Plant elicitor peptides at the crossroads of immunity, stress and development. *Journal of Experimental Botany* [Epub ahead of print].

- Bartels, S., Lori, M., Mbengue, M., van Verk, M., Klauser, D., Hander, T., Rainer, B., 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* **64**: 5309-5321.
- Baxter, A., Mittler, R., and Suzuki, N. (2013). ROS as key players in plant stress signalling. *Journal of Experimental Botany* **65**: 1229-40.
- Beck, M., Wyrsh, I., Strutt, J., Wimalasekera, R., Webb, A., Boller, T., and Robatzek, S. (2014). Expression patterns of flagellin sensing 2 map to bacterial entry sites in plant shoots and roots. *Journal of Experimental Botany* **65**: 6487-6498.
- Bellia, F., Pietropaolo, A., and Grasso, G. (2013). Formation of insulin fragments by insulin-degrading enzyme: The role of zinc (II) and cystine bridges. *Journal of Mass Spectrometry* **48**: 135-140.
- Bigeard, J., Colcombet, J., and Hirt, H. (2015). Signaling mechanisms in pattern-triggered immunity (PTI). *Molecular Plant* **8**: 521-39.
- Birkenbihl, R.P., Diezel, C., and Somssich, I.E. (2012). Arabidopsis WRKY33 is a key transcriptional regulator of hormonal and metabolic responses toward Botrytis cinerea infection. *Plant Physiology* **159**: 266-85.
- Block, A., and Alfano, J.R. (2011). Plant targets for Pseudomonas syringae type III effectors: virulence targets or guarded decoys? *Current Opinion in Microbiology* **14(1)**: 39-46.
- Boex-Fontvieille, E., Rustgi, S., von Wettstein, D., Reinbothe, S., and Reinbothe, C. (2015). Water-soluble chlorophyll protein is involved in herbivore resistance activation during greening of Arabidopsis thaliana. *Proceedings of the National Academy of Sciences* **112**: 7303-8.

- Boller, T., and Felix, G. (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 He, S.Y. (2009). Innate immunity in plants: an arms race between pattern recognition receptors in plants and effectors in microbial pathogens. *Science* **324**: 742-4.
- Bustin, S.A. and Nolan, T. (2004). Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. *Journal of Biomolecular Technology* **15**: 155-66.
- Büttner, D., and He, S.Y. (2009). Type III protein secretion in plant pathogenic bacteria. *Plant Physiology* **4**: 1656-64.
- Çevik, V., Kidd, B.N., Zhang, P., Hill, C., Kiddle, S., Denby, K.J., Holub, E.B., Cahill, D.M., Manners, J.M., Schenk, P.M., Beynon, J., and Kazan, K. (2012). MEDIATOR25 acts as an integrative hub for the regulation of jasmonate-responsive gene expression in Arabidopsis. *Plant Physiology* **160**:541-55.
- Cecchini, N.M., Jung, H.W., Engle, N.L., Tschaplinski, T.J., and Greenberg, J.T. (2015). ALD1 Regulates Basal Immune Components and Early Inducible Defense Responses in Arabidopsis. *Molecular Plant Microbe Interaction* **28**: 455-66.
- Chauhan, R.S., Farman, M.L., Zhang, H.B., and Leong, S.A. (2002). Genetic and physical mapping of a rice blast resistance locus, Pi-CO39(t), that corresponds to the avirulence gene AVR1-CO39 of *Magnaporthe grisea*. *Molecular Genetics and Genomics* **267**: 603-12.
- Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nuernberger, T., Jones, J.D., Felix, G., and Boller, T. (2007). A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature* **448**: 497-500.

- Chisholm, S.T., Coaker, G., Day, B., and Staskawicz, B.J. (2006). Host-microbe interactions: shaping the evolution of the plant immune response. *Cell* **124**: 803-14.
- Chuang, T.H., and Ulevitch, R.J. (2004). Triad3A, an E3 ubiquitin-protein ligase regulating Toll-like receptors. *Nature immunology* **5**: 495-502.
- Clark, S.E., Running, M.P., and Meyerowitz, E.M. (1993). CLAVATA1, a regulator of meristem and flower development in Arabidopsis. *Development* **119**: 397-418.
- Clavel, M., Péliissier, T., Descombin, J., Jean, V., Picart, C., Charbonel, C., Saez-Vásquez, J., Bousquet-Antonelli, C., and Deragon, J.M. (2015). Parallel action of AtDRB2 and RdDM in the control of transposable element expression. *BMC Plant Biology* **15**: 70.
- Colcombet, J., and Hirt, H. (2008). Arabidopsis MAPKs: a complex signalling network involved in multiple biological processes. *Biochemistry Journal* **413**: 217-26.
- Constabel, C.P., Yip, L., and Ryan, C.A. (1998). Prosystemin from potato, black nightshade, and bell pepper: primary structure and biological activity of predicted systemin polypeptides. *Plant Molecular Biology* **36**: 55-62.
- Cornelis, G.R., and Van Gijsegem, F. (2000). Assembly and function of type III secretory systems. *Annual Review of Microbiology* **54**: 735-74.
- Crooks, G.E., Hon, G., Chandonia, J.M., and Brenner, S.E. (2004). WebLogo: a sequence logo generator. *Genome Research* **14**: 1188–1190.
- Cui, H., Tsuda, K., and Parker, J.E. (2015). Effector-Triggered Immunity: from pathogen perception to robust defense. *Annual Review of Plant Biology* **66**: 487-511.
- Dangl, J.L., Horvath, D.M., and Staskawicz, B.J. (2013). Pivoting the plant immune system from dissection to deployment. *Science* **16**: 746-51.

- Dardick, C., and Ronald, P. (2006). Plant and animal pathogen recognition receptors signal through non-RD kinases. *PLoS Pathogen* **2**: e2.
- Denoux, C., Galletti, R., Mammarella, N., Gopalan, S., Werck, D., De Lorenzo, G., Ferrari, S., Ausubel, F.M., and Dewdney, J. (2008). Activation of defense response pathways by OGs and Flg22 elicitors in *Arabidopsis* seedlings. *Molecular Plant* **1**: 423-45.
- Devine, D.A., and Hancock, R.E. (2002). Cationic peptides: distribution and mechanisms of resistance. *Current Pharmaceutical design* **8**: 703-714.
- Dinant, S., Clark, A.M., Zhu, Y., Vilaine, F., Palauqui, J.C., Kusiak, C., and Thompson, G.A. (2003). Diversity of the superfamily of phloem lectins (phloem protein 2) in angiosperms. *Plant Physiology* **13**: 114-28.
- Dodds, P.N., and Rathjen, J.P. (2010). Plant immunity: towards an integrated view of plant–pathogen interactions. *Nature Reviews Genetics* **11**: 539-548.
- Duckworth, W.C., Fawcett, J., Tsui, B.T., Bennett, R.G., and Hamel, F.G. (2004). *Biochemical and Biophysical Research Communications* **318**: 1019-1024.
- Dunning, F.M., Sun, W., Jansen, K.L., Helft, L., and Bent, A.F. (2007). Identification and mutational analysis of *Arabidopsis* FLS2 leucine-rich repeat domain residues that contribute to flagellin perception. *Plant Cell* **19**: 3297-3313.
- Duplan, V., and Rivas, S. (2014). E3 ubiquitin-ligases and their target proteins during the regulation of plant innate immunity. *Frontiers in plant science* **13**: 42.
- Eamens, A.L., Kim, K.W., Curtin, S.J., and Waterhouse, P.M. (2012). DRB2 is required for microRNA biogenesis in *Arabidopsis thaliana*. *PLoS One* **7**: e35933.

- Eulgem, T., 2005. Regulation of the Arabidopsis defense transcriptome. *Trends in Plant Science* **10**: 71-78.
- Felix, G., Duran, J.D., Volko, S., and Boller, T. (1999). Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant Journal* **18**: 265-276.
- Ferrari, S., Savatin, D.V., Sicilia, F., Gramegna, G., Cervone, F., and Lorenzo, G.D. (2013). Oligogalacturonides: plant damage-associated molecular patterns and regulators of growth and development. *Frontiers in Plant Science* **4**: 1-9.
- Flury, P., Klauser, D., Schulze, B., Boller, T., and Bartels, S. (2013). The anticipation of danger: microbe-associated molecular pattern perception enhances AtPep-triggered oxidative burst. *Plant Physiology* **161**: 2023-35.
- Gassmann, W., and Bhattacharjee, S. (2012). Effector-triggered immunity signaling: from gene-for-gene pathways to protein-protein interaction networks. *Molecular Plant Microbe Interaction* **25**: 862-8.
- Gimenez-Ibanez, S., D.R., Hann, V., Ntoukakis, E., Peptutchnig, V., Lipka, and Rathjen, J. (2009). AvrPtoB Targets the LysM Receptor Kinase CERK1 to Promote Bacterial Virulence on Plants. *Current Biology* **19**: 423-429.
- Goldberg, M.B. (2001). Actin-based motility of intracellular microbial pathogens. *Microbiology and Molecular Biology Review* **65**: 595-626.
- Gomez-Gomez, L., Bauer, Z., and Boller, T. (2001). Both the extracellular leucine-rich repeat domain and the kinase activity of FLS2 are required for flagellin binding and signaling in Arabidopsis. *Plant Cell* **13**: 1155-1163.

- Gomez- Gómez, L., and Boller, T. (2000). FLS2: An LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. *Molecular Cell* **5**: 1003-11.
- Gou, X., He, K., Yang, H., Yuan, T., Lin, H., Clouse, S.D., and Li, J. (2010). Genome-wide cloning and sequence analysis of leucine-rich repeat receptor-like protein kinase genes in *Arabidopsis thaliana*. *BMC Genomics* **11**: 11-19.
- Gou, X., Yin, H., He, K., Du, J., Yi, J., Xu, S., Lin, H., Clouse, S.D., and Li, J. (2012). Genetic evidence for an indispensable role of somatic embryogenesis receptor kinases in brassinosteroid signaling. *PLoS Genetic* **8**: e1002452.
- Göhre, V., and Robatzek, S. (2008). Breaking the barriers: microbial effector molecules subvert plant immunity. *Annual Review of Phytopathology* **46**: 189-215.
- Göhre, V., Spallek, T., Häweker, H., Mersmann, S., Mentzel, T., Boller, T., de Torres, M., Mansfield, J.W., and Robatzek, S. (2008). Plant pattern-recognition receptor FLS2 is directed for degradation by the bacterial ubiquitin ligase AvrPtoB. *Current Biology* **9**: 1824-32.
- Greeff, C., Roux, M., Mundy, J., and Petersen, M. (2012). Receptor-like kinase complexes in plant innate immunity. *Frontiers in plant science* **3**: 209-2012.
- Grennan, A.K. (2006). Plant Response to Bacterial Pathogens. Overlap between Innate and Gene-for-Gene Defense Response. *Plant Physiology* **142**: 809-811.
- Guiney, D.G., and Lesnick, M. (2005). Targeting of the actin cytoskeleton during infection by *Salmonella* strains. *Clinical Immunology* **114**: 248-55.

- Gully, K., Hander, T., Boller, T., and Bartels, S. (2015). Perception of Arabidopsis AtPep peptides, but not bacterial elicitors, accelerates starvation-induced senescence. *Frontiers in Plant Science* **6**: 1-10.
- Habazettl, J., Schleicher, M., Otlewski, J., and Holak, T.A. (1992). Homonuclear three-dimensional NOE-NOE nuclear magnetic resonance/spectra for structure determination of proteins in solution. *Journal of Molecular Biology* **228**: 156-69.
- Hann, D.R., and Rathjen, J.P. (2007). Early events in the pathogenicity of *Pseudomonas syringae* on *Nicotiana benthamiana*. *Plant Journal* **49**: 607-18.
- Hauck, P., Thilmony, R., and He, S.Y. (2003). A *Pseudomonas syringae* type III effector suppresses cell wall-based extracellular defense in susceptible Arabidopsis plants. *Proceedings of the National Academy of Sciences* **100**: 8577-82.
- He, P., Shan, L., Lin, N.C., Martin, G.B., Kemmerling, B., Nurnberger, T., and Sheen, J. (2006). Specific bacterial suppressors of MAMP signaling upstream of MAPKKK in Arabidopsis innate immunity. *Cell* **125**: 563-575.
- Heese, A., Hann, D.R., Gimenez-Ibanez, S., Jones, A.M., He, K., Li, J., Schroeder, J.I., Peck, S.C., and Rathjen, J.P. (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.
- Heil, M. (2014). Herbivore-induced plant volatiles: targets, perception and unanswered questions. *New Phytologist* **204**: 297-306.
- Heil, M., and Land, W.G. (2014). Danger signals-damaged-self recognition across the tree of life. *Frontiers in plant science* **578**: 1-16.

- Hennies, H.C., Zehender, D., Kunze, J., Kuster, W., and Reis, A. (1994). Keratin 9 gene mutational heterogeneity in patients with epidermolytic palmoplantar keratoderma. *Human Genetics* **93**: 649-654.
- Hou, S., Wang, X., Chen, D., Yang, X., Wang, M., Turra, D., Pietro, A.D., and Zhang, W. (2014). The Secreted Peptide PIP1 Amplifies Immunity through Receptor-Like Kinase 7. *PLoS Pathogens* **10**: 1-15.
- Huang, T., Liu, L., Qian, Z., Tu, K., Li, Y., and Xie, L. (2010). Technical Note Using GeneReg to construct time delay gene regulatory networks. *BMC Research Notes* **3**:142.
- Hueck, C.J., (1998). Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiology and Molecular Biology Reviews*. **62**: 379-433.
- Huffaker, A., Dafoe, N.J., and Schmelz, E. (2011). ZmPep1, an Ortholog of Arabidopsis Elicitor Peptide 1, Regulates Maize Innate Immunity and Enhances Disease Resistance. *Plant Physiology* **155**: 1325-1338.
- Huffaker, A., Pearce, G., and Ryan, C.A. (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., Pearce, G., Veyrat, N., Erb, M., Turlings, T., Sartor, R., Shen, Z., Briggs, S., Vaughan, M., Alborn, H., Teal, P., and Schmelz, E. (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 Ryan, C.A. (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.

- Jaouannet, M., Rodriguez, P.A., Thorpe, P., Lenoir, C.A., Macleod, R., Escudero-Martinez, C., and Bos, J.I. (2014). Plant immunity in plant-aphid interactions. *Frontiers in Plant Science* **5**: 663.
- Jayo, A., and Parsons, M. (2010). Fascin: a key regulator of cytoskeletal dynamics. *The International Journal of Biochemistry and Cell Biology* **42**: 1614-7.
- Jin, J.B., Jin, Y.H., Lee, J., Miura, K., Yoo, C.Y., Kim, W.Y., Van Oosten, M., Hyun, Y., Somers, D.E., Lee, I., Yun, D. J., Bressan, R.A., and Hasegawa, P.M. (2008). The SUMO E3 ligase, AtSIZ1, regulates flowering by controlling a salicylic acid-mediated floral promotion pathway and through affects on FLC chromatin structure. *Plant Journal* **53**: 530-40.
- Jones, J.D.G., and Dangl, J.L. (2006). The plant immune system. *Nature* **444**: 323-329.
- Katagiri, F., Thilmony, R., and He, S.Y. (2002). The *Arabidopsis thaliana*-*Pseudomonas syringae* interaction. *Arabidopsis Book*. **1**: e0039.
- Kayes, J.M., and Clark, S.E. (1998). CLAVATA2, a regulator of meristem and organ development in *Arabidopsis*. *Development* **125**: 3843-3851.
- Källberg, M., Wang, H., Wang, S., Peng, J., Wang, Z., Lu, H., and Xu, J. (2012). Template-based protein Structure modeling using the RaptorX web server. *Proceedings of the National Academy of Sciences* **7**: 1511-22.
- Keller, B., Sauer, N., and Lamb, C.J. (1988). Glycine-rich cell wall proteins in bean: gene structure and association of the protein with the vascular system. *EMBO Journal* **7**: 3625-33.
- Kemmerling, B., Schwedt, A., Rodriguez, P., Mazzotta, S., Frank, M., Qamar, S.A., Mengiste, T., Betsuyaku, S., Parker, J.E., Mussig, C., Thomma, B.P., Albrecht, C., de Vries, S.C.,

- Hirt, H., and Nurnberger, T. (2007). The BRI1-associated kinase 1, BAK1, has a brassinolide-independent role in plant cell-death control. *Current Biology* **17**: 1116-1122.
- Kim, S.M., Bae, C., Oh, S.K., and Choi, D. (2013). A pepper (*Capsicum annuum* L.) metacaspase 9 (Camc9) plays a role in pathogen-induced cell death in plants. *Molecular Plant Pathology* **14**: 557-66.
- Kiss-Papp, M. (2014). The role of BRI1-associated kinase 1 (BAK1) in the regulation of plant innate immunity: functional and genetic characterization of BAK1 overexpression in *Arabidopsis thaliana*. PhD Thesis. University of Basel pp: **114**.
- Klauser, D., Desurmont, G.A., Glauser, G., Vallat, A., Flury, P., Boller, T., Turlings, T.C.J., and Bartels, S. (2015). The *Arabidopsis* Pep-PEPR system is induced by herbivore feeding and contributes to JA-mediated plant defence against herbivory. *Journal of Experimental Botany* [Epub ahead of print].
- Kobe, B., and Deisenhofer, J. (1995). A structural basis of the interactions between leucine-rich repeats and protein ligands. *Nature* **374**: 183-6.
- Kørner, C., Klauser, D., Niehl, A., Dominguez-Ferreras, A., Chinchilla, D., Boller, T., Heinlein, M., and Hann, D.R. (2013). The immunity regulator BAK1 contributes to resistance against diverse RNA viruses. *Molecular Plant-Microbe Interactions* **26**: 1271-1280.
- Krol, E., Mentzel, T., Chinchilla, D., Boller, T., Felix, G., Kemmerling, B., Postel, S., Arents, M., Jeworutzki, E., Al-Rasheid, K., Becker, D., and Hedrich, R. (2010). Perception of the *Arabidopsis* danger signal peptide 1 involves the pattern recognition receptor *AtPEPR1* and its close homologue *AtPEPR2*. *Journal of Biological Chemistry* **285**: 13471-13479.
- Kunkel, B.N., and Brooks, D.M. (2002). Cross talk between signaling pathways in pathogen defense. *Current Opinion in Plant Biology* **5**: 325-31.

- Kwezi, L., Meier, S., Mungur, L., Ruzvidzo, O., Irving, H., and Gehring, C. (2007). The *Arabidopsis thaliana* brassinosteroid receptor (*AtBRI1*) contains a domain that functions as a guanylyl cyclase *in vitro*. *PLoS One* **2**: e449.
- Laluk, K., Luo, H., Chai, M., Dhawan, R., Lai, Z., and Mengiste, T. (2011). Biochemical and genetic requirements for function of the immune response regulator BOTRYTIS-INDUCED KINASE1 in plant growth, ethylene signaling, and PAMP-triggered immunity in *Arabidopsis*. *Plant Cell* **23**: 2831-49.
- Lannoo, N., and Van Damme, E.J. (2014). Lectin domains at the frontiers of plant defense. *Frontiers in Plant Science* **13**: 397.
- Larroque, M., Belmas, E., Martinez, T., Vergnes, S., Ladouce, N., Lafitte, C., Gaulin, E., and Dumas, B. (2013). Pathogen-associated molecular pattern-triggered immunity and resistance to the root pathogen *Phytophthora parasitica* in *Arabidopsis*. *Journal Experimental Botany* **64**: 3615-25.
- Lee, S.W., Han, S.W., Sririyanyum, M., Park, C.J., Seo, Y.S., and Ronald, P.C. (2009). A type I-secreted, sulfated peptide triggers XA21-mediated innate immunity. *Science* **326**: 850-853.
- Li, W., Ahn, I.P., Ning, Y., Park, C.H., Zeng, L., Whitehill, J.G., Lu, H., Zhao, Q., Ding, B., Xie, Q., Zhou, J.M., Dai, L., and Wang, G.L. (2012). The U-Box/ARM E3 Ligase PUB13 Regulates Cell Death, Defense, and Flowering Time in *Arabidopsis*. *Plant Physiology* **159**: 239-250.
- Li, J., Wen, J., Lease, K.A., Doke, J.T., Tax, F.E., and Walker, J.C. (2002). BAK1, an *Arabidopsis* LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling. *Cell* **110**: 213-222.

- LeBlanc, M., Kim, G., Patel, B., Stromberg, V., and Westwood, J. (2013). Quantification of tomato and Arabidopsis mobile RNAs trafficking into the parasitic plant *Cuscuta entagona*. *New Phytologist* **200**: 1225-33.
- Lindeberg, M., Cunnac, S., and Collmer, A. (2012). *Pseudomonas syringae* type III effector repertoires: last words in endless arguments. *Cell* **20**: 199-208.
- Liu, Y., Burch-Smith, T., Schiff, M., Feng, S., and Dinesh-Kumar, S.P. (2004). Molecular Chaperone Hsp90 Associates with Resistance Protein N and Its Signaling Proteins SGT1 and Rar1 to Modulate an Innate Immune Response in Plants. *Journal of Biological Chemistry* **16**: 2101-2108.
- Liu, Z., Wu, Y., Yang, F., Zhang, Y., Chen, S., Xie, O., Tian, X., and Zhou, J.M. (2013). BIK1 interacts with PEPRs to mediate ethylene-induced immunity. *Proceedings of the National Academy of Sciences* **110**: 6205-10.
- Liu, W.X., Zhang, F.C., Zhang, W.Z., Song, L.F., Wu, W.H., and Chen, Y.F. (2013). Arabidopsis Di19 functions as a transcription factor and modulates PR1, PR2, and PR5 expression in response to drought stress. *Molecular Plant* **6**: 1487-502.
- Liu, Y., and Zhang, S. (2004). Phosphorylation of 1-aminocyclopropane-1-carboxylic acid synthase by MPK6, a stress-responsive mitogen-activated protein kinase, induces ethylene biosynthesis in Arabidopsis. *Plant Cell* **16**: 3386-99.
- Logemann, E., Birkenbihl, R.P., Rawat, V., Schneeberger, K., Schmelzer, E., and Somssich, I.E. (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* **198**: 1165-1177.

- Lori, M., Verk, M.C., Hander, T., Schatowitz, H., Klauser, D., Flury, P., Gehring, C.A., Boller, T., and Bartels, S. (2015). Evolutionary divergence of the plant elicitor peptides (Peps) and their receptors: interfamily incompatibility of perception but compatibility of downstream signalling. *Journal of Experimental Botany* [Epub ahead of print].
- Lu, D., Lin, W., Gao, X., Wu, S., Cheng, C., Avila, J., Heese, A., Devarenne, T.P., He, P., and Shan, L. (2011). Direct ubiquitination of pattern recognition receptor FLS2 attenuates plant innate immunity. *Science* **332**: 1439-1442.
- Lu, D., Wu, S., He, P., and Shan, L. (2010). Phosphorylation of receptor-like cytoplasmic kinases by bacterial flagellin. *Plant Signal Behavior* **5**: 598-600.
- Luo, T., Jiang, L., Sun, W., Fu, G., Mei, J., and Gao, Q. (2011). Multiplex real-time PCR melting curve assay to detect drug-resistant mutations of *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology* **49**: 3132-8.
- Lyons, R., Iwase, A., Gansewig, T., Sherstnev, A., Duc, C., Barton, G.J., Hanada, K., Higuchi-Takeuchi, M., Matsui, M., Keiko Sugimoto, Kazan, K., Simpson, G.G., and Shirasu, K. (2013). The RNA-binding protein FPA regulates flg22-triggered defense responses and transcription factor activity by alternative polyadenylation. *Scientific reports* **9**: 2866.
- Ma, C., Guo, J., Kang, Y., Doman, K., Bryan, A.C., Tax, F.E., Yamaguchi, Y., and Zhi, Q. (2014). AtPEPTIDE RECEPTOR2 mediates the AtPEPTIDE1 - induced cytosolic Ca₂p rise, which is required for the suppression of Glutamine Dumper gene expression in *Arabidopsis* roots. *Journal of Integrative Plant Biology* **56**: 684-694.
- Ma, Y., Walker, R.K., Zhao, Y., and Berkowitz, G. (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.

- Macho, A.P., and Zipfel, C. (2014). Plant PRRs and the Activation of Innate Immune Signaling. *Molecular Cell* **54**: 263-272.
- Macho, A.P., and Zipfel, C. (2015). Targeting of plant pattern recognition receptor-triggered immunity by bacterial type-III secretion system effectors. *Current Opinion in Microbiology* **23**: 14-22.
- Mangeon, A., Junqueira, R. M., and Sachetto-Martins, G. (2010). Functional diversity of the plant glycine-rich proteins superfamily. *Plant Signal. Behaviour* **5**: 99-104.
- Mao, F., Leung, W.Y., and Xin, X. (2007). Characterization of EvaGreen and the implication of its physicochemical properties for qPCR applications. *BMC Biotechnology* **9**: 76.
- Mao, G., Meng, X., Liu, Y., Zheng, Z., Chen, Z., and Zhang, S. (2011). Phosphorylation of a WRKY transcription factor by two pathogen-responsive MAPKs drives phytoalexin biosynthesis in Arabidopsis. *Plant Cell* **23**: 1639-53.
- McGurl, B., Pearce, G., Orozco-Cardenas, M., and Ryan, C.A. (1992). Structure, expression, and antisense inhibition of the systemin precursor gene. *Science* **255**: 1570-1573.
- Melotto, M., Underwood, W., Koczan, J., Nomura, K., and He, S.Y. (2006). Plant stomata function in innate immunity against bacterial invasion. *Cell* **126**: 969-980.
- Milla, M.A., Townsend, J., Chang, I.F., and Cushman, J.C. (2006). The Arabidopsis AtDi19 gene family encodes a novel type of Cys2/His2 zinc-finger protein implicated in ABA-independent dehydration, high-salinity stress and light signaling pathways. *Plant Molecular Biology* **61**: 13-30.
- Mishina, T.E., and Zeier, J. (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.

- Mishina, T.E., and Zeier, J. (2007). Bacterial non-host resistance: interactions of Arabidopsis with non-adapted *Pseudomonas syringae* strains. *Physiologia Plantarum* **131**: 448-61.
- Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., Narusaka, Y., Kawakami, N., Kaku, H., and Shibuya, N. (2007). CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis. *Proceedings of the National Academy of Sciences* **104**: 19613-18.
- Monaghan, J., and Zipfel, C. (2012). Plant pattern recognition receptor complexes at the plasma membrane. *Current Opinion in Plant Biology* **15**: 349-357.
- Moore, J., W.G.J., Loake, and Spoel, S.H. (2011). Transcription Dynamics in Plant Immunity. *Plant Cell* **23**: 2809-2820.
- Moreau, M., Lindermayr, C., Durner, J., and Klessig, D.F. (2010). NO synthesis and signaling in plants--where do we stand? *Physiologia Plantarum* **138**: 372-383.
- Mostowy, S. (2014). Multiple roles of the cytoskeleton in bacterial autophagy. *PLoS Pathogen* **10**: e1004409.
- Mott, G.A., Middleton, M.A., Desveaux, D., and Guttman, D.S. (2014). Peptides and small molecules of the plant-pathogen apoplastic arena. *Frontiers in Plant Science* **28**: 677.
- Muller, P. Y., Janovjak, H., Miserez, A. R., and Dobbie, Z. (2002). Processing of gene expression data generated by quantitative real-time RT-PCR. *BioTechniques* **32**: 1372-1374.
- Nam, K.H., and Li, J. (2002). BRI1/BAK1, a receptor kinase pair mediating brassinosteroid signaling. *Cell* **110**: 203-12.

- Navarro, L., Zipfel, C., Rowland, O., Keller, I., Robatzek, S., Boller, T., and Jones, J.D. (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.
- Newman, M.A., Sundelin, T., Nielsen, J.T., Erbs, G. (2013). MAMP (microbe-associated molecular pattern) triggered immunity in plants. *Frontiers in Plant Science* **16**: 139.
- Nicaise, V., Roux, M., and Zipfel, C. (2009). Recent Advances in PAMP-Triggered Immunity against Bacteria: Pattern Recognition Receptors Watch over and Raise the Alarm. *Plant Physiology* **150**: 1638-1647.
- Nishimura, M.T., and Dangl, J.L. (2014). Plant science. Paired plant immune receptors. *Science* **18**: 267-8.
- Nurnberger, T., Brunner, F., Kemmerling, B., and Piater, L. (2004) Innate immunity in plants and animals: striking similarities and obvious differences. *Immunology Review* **198**: 249-266.
- Nurnberger, T., and Lipka, V. (2005). Non-host resistance in plants: new insights into an old phenomenon. *Molecular Plant Pathology* **6**: 335-345.
- Nühse, T.S., Peck, S.C., Hirt, H., and Boller, T. (2000). Microbial elicitors induce activation and dual phosphorylation of the *Arabidopsis thaliana* MAPK 6. *Journal of Biologicstegal Chemistry* **275**: 7521-7526.
- Opassiri, R., Pomthong, B., Akiyama, T., Nakphaichit, M., Onkoksoong, T., Ketudat Cairns, M., Ketudat, and Cairns, J.R. (2007). A stress-induced rice (*Oryza sativa* L.) beta-glucosidase represents a new subfamily of glycosyl hydrolase family 5 containing a fascin-like domain. *Biochemistry Journal* **408**: 241-9.

- Ogawa, M., Shinohara, H., Sakagami, Y., and Matsubayashi, Y. (2008). Arabidopsis CLV3 peptide directly binds CLV1 ectodomain. *Science* **18**: 294.
- Pearce, G., Bhattacharya, R., Chen, Y.C., Barona, G., Yamaguchi, Y., and Ryan, C.A. (2009). Isolation and characterization of hydroxyproline-rich glycopeptides signals in black nightshade leaves. *Plant Physiology* **150**: 1422-1433.
- Pearce, G., Moura, D.S., Stratmann, J., and Ryan, C.A. (2001). Production of multiple plant hormones from a single polyprotein precursor. *Nature* **411**: 817-820.
- Pearce, G., and Ryan, C.A. (2003). Systemic signaling in tomato plants for defense against herbivores: isolation and characterization of three novel defense-signaling glycopeptide hormones coded in a single precursor gene. *Journal of Biological Chemistry* **278**: 30044-30050.
- Pearce, G., Siems, W.F., Bhattacharya, R., Chen, Y.C., and Ryan, C.A. (2007). Three hydroxyproline-rich glycopeptides derived from a single petunia polyprotein precursor activate defensin I, a pathogen defense response gene. *Journal of Biological Chemistry* **282**: 17777-84.
- Pearce, G., Strydom, D., Johnson, S., and Ryan, C.A. (1991). A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. *Science* **253**: 895-897.
- Pearce, G., Yamaguchi, Y., Barona, G., and Ryan, C.A. (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.
- Pearce, G., Yamaguchi, Y., Munske, G., and Ryan, C.A. (2008). Structure-activity studies of AtPep1, a plant peptide signal involved in the innate immune response. *Peptides* **29**: 2083-9.

- Pedro, J.M., Wei, Y., Sica, V., Maiuri, M.C., Zou, Z., Kroemer, G., and Levine, B. (2015). BAX and BAK1 are dispensable for ABT-737-induced dissociation of the BCL2-BECN1 complex and autophagy. *Autophagy* **11**: 452-9.
- Postel, S., Küfner, I., Beuter, C., Mazzotta, S., Schwedt, A., Borlotti, A., Halter, T., Kemmerling, B., and Nurnberger, T. (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., Verma, R., Gehring, C., Yamaguchi, Y., Zhao, Y., Ryan, C.A., and Berkowitz, G. (2010). Ca²⁺ signaling by plant *Arabidopsis thaliana* Pep peptides depends on AtPepR1, a receptor with guanylyl cyclase activity, and cGMP-activated Ca²⁺ channels. *Proceedings of the National Academy of Sciences* **107**: 21193-21198.
- Ramos, H.C., Rumbo, M., and Sirard, J.C. (2004). Bacterial flagellins: mediators of pathogenicity and host immune responses in mucosa. **12**: 509-517.
- Ranf, S., Eschen-Lippold, L., Frohlich, K., Westphal, L., Scheel, D., and Lee, J. (2014). Microbe-associated molecular pattern-induced calcium signaling requires the receptor-like cytoplasmic kinases, PBL1 and BIK1. *BMC Plant Biology* **14**: 374.
- Ranf, S., Eschen-Lippold, L., Pecher, P., Lee, J., and Scheel, D. (2011). Interplay between calcium signalling and early signalling elements during defence responses to microbe- or damage-associated molecular patterns. *The Plant Journal* **68**: 100-113.
- Reis, A., Hennies, H.C., Langbein, L., Digweed, M., Mischke, D., Drechsler, M., Schröck, E., Royer-Pokora, B., Franke, W.W., Sperling, K., and Küster, W. (1994). Keratin 9 gene mutations in epidermolytic palmoplantar keratoderma (EPPK). *Nature Genetics* **6**: 174-9.
- Rejeb, I.B., Pastor, V., and Mauch-Mani, B. (2014). Plant Responses to Simultaneous Biotic and Abiotic Stress: Molecular Mechanisms. *Plants* **3**: 458-475.

- Roberts, T.H., Hejgaard, J., (2008). Serpins in plants and green algae. *Functional Integrative Genomics* **8**: 1-27.
- Ross, A., Yamada, K., Hiruma, K., Yamashita-Yamada, M., Lu, X., Takano, Y., Tsuda, K., and Saijo, Y. (2014). The Arabidopsis PEPR pathway couples local and systemic plant immunity. *The EMBO Journal* **33**: 62-75.
- Rottner, K., and Stradal, T.E. (2011). Actin dynamics and turnover in cell motility. *Current Opinion in Cell Biology* **23**: 569-578.
- Roux, M., Schwessinger, B., Albrecht, C., Chinchilla, D., Jones, A., Holton, N., Malovsky, F., Tor, M. De Vries, S., and Zipfel, C. (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.
- Ryan, C.A., Huffaker, A., and Yamaguchi, Y. (2007). New insights into innate immunity in Arabidopsis. *Cellular Microbiology* **9**: 1902-1908.
- Ryan, C.A., and Pearce, G. (1998). Systemin: A polypeptide signal for plant defensive genes. *Annual Review of Cell and Developmental Biology* **14**: 1-17.
- Samir, P., and Link, A.J. (2011). Analyzing the cryptome: Uncovering secret sequences. *AAPS Journal* **13**: 152-158.
- Savatin D.V., Gramegna, G., Modesti, V., and Cervone, F. (2014). Wounding in the plant tissue: the defense of a dangerous passage. *Frontiers in Plant Science* **16**: 470.
- Schmelz, E.A., Carroll, M.J., LeClere, S., Phipps, S.M., Meredith, J., Chourey, P.S., Alborn, H.T., and Teal, PE. (2006). Fragments of ATP synthase mediate plant perception of insect attack. *Proceedings of the National Academy of Sciences* **103**: 8894-8899.

- Schmelz, E.A., LeClere, S., Carroll, M.J., Alborn, H.T., and Teal, P.E. (2007). Cowpea chloroplastic ATP synthase is the source of multiple plant defense elicitors during insect herbivory *Plant Physiology* **144**: 793-805.
- Schulze, B., Mentzel, T., Jehle, A.K., Mueller, K., Beeler, S., Boller, T., Felix, G., and Chinchilla, D. (2010). Rapid heteromerization and phosphorylation of ligand-activated plant transmembrane receptors and their associated kinase BAK1. *Journal of Biological Chemistry* **285**: 9444-51.
- Schwessinger, B., and Zipfel, C. (2008). News from the frontline: recent insights into PAMP-triggered immunity in plants. *Current Opinion in Plant Biology* **11**: 389-395.
- Segonzac, C., and Zipfel, C. (2011). Activation of plant pattern-recognition receptors by bacteria. *Current Opinion in Microbiology* **14**: 54-61.
- Serrano, M., Coluccia, F., Torres, M., L'Haridon, F., and Metraux, J.P. (2014). The cuticle and plant defense to pathogens. *Frontiers in Plant Science* **5**: 274.
- Shan, L., He, P., Li, J., Heese, A., Peck, S., Nuernberger, T., Martin, G., and Sheen, J. (2008). Bacterial effectors target the common signaling partner BAK1 to disrupt multiple MAMP receptor-signaling complexes and impede plant immunity. *Cell Host and Microbe* **4**: 17-27.
- Shepard, K.A., and Purugganan, M.D. (2003). Molecular population genetics of the *Arabidopsis* CLAVATA2 region. The genomic scale of variation and selection in a selfing species. *Genetics* **163**: 1083-95.
- Shiu, S.H., and Bleecker, A.B. (2003). Expansion of the receptor-like kinase/Pelle gene family and receptor-like proteins in *Arabidopsis*. *Plant Physiology* **132**: 530-543.

- Shiu, S.H., Karlowski, W.M., Pan, R., Tzeng, Y.H., Mayer, K.F., and Li, W.H. (2004). Comparative analysis of the receptor-like kinase family in Arabidopsis and rice. *Plant Cell* **16**: 1220-34.
- Sol, A., Skvirsky, Y., Nashef, R., Zelentsova, K., Burstyn-Cohen, T., Blotnick, E., Muhrad, A., and Bachrach, G. (2014). Actin enables the antimicrobial action of LL-37 peptide in the presence of microbial proteases. *The Journal of Biological Chemistry* **289**: 22926-41.
- Spoel, S.H., and Dong, X. (2012). How do plants achieve immunity? Defence without specialized immune cells. *Nature Review Immunology* **12**: 89-100.
- Stahl, E.A., Dwyer, G., Mauricio, R., Kreitman, M., and Bergel, J. (1999). Dynamics of disease resistance polymorphism at the *Rpm1* locus of Arabidopsis. *Nature* **400**: 667-671.
- Stegmann, M., Anderson, R.G., Ichimura, K., Pecenkova, T., Reuter, P., Zarsky, V., McDowell, J.M., Shirasu, K., and Trujillo, M. (2012). The ubiquitin ligase PUB22 targets a subunit of the exocyst complex required for PAMP-triggered responses in Arabidopsis. *The Plant Cell* **24**: 4703-4716.
- Sun, Y., Li, L., Macho, A.P., Han, Z., Hu, Z., Zipfel, C., Zhou, J., and Chai, J. (2013). Structural Basis for flg22-Induced Activation of the *Arabidopsis* FLS2-BAK1 Immune Complex. *Science* **6158**: 624-628.
- Swarbreck, D., Wilks, C., Lamesch, P., Berardini, T.Z., Garcia-Hernandez, M., Foerster, H., Li, D., Meyer, T., Muller, R., Ploetz, L., Radenbaugh, A., Singh, S., Swing, V., Tissier, C., Zhang, P., and Huala, E. (2008). The Arabidopsis Information Resource (TAIR): gene structure and function annotation. *Nucleic Acids Research* **36**: D1009-14.

- Taj, G., Agarwal, P., Grant, M., and Kumar, A. (2010) MAPK machinery in plants: recognition and response to different stresses through multiple signal transduction pathways. *Plant Signal Behavior* **5**: 1370-8.
- Tang, J., Han, Z., Sun, Y., Zhang, H., Gong, X., and Chai, J. (2015). Structural basis for recognition of an endogenous peptide by the plant receptor kinase PEPR1. *Cell Research* **25**: 110-120.
- Tao, Y., Xie, Z.Y., Chen, W.Q., Glazebrook, J., Chang, H.S., Han, B., Zhu, T., Zou, G.Z., and Katagiri, F. (2003). Quantitative nature of Arabidopsis responses during compatible and incompatible interactions with the bacterial pathogen *Pseudomonas syringae*. *The Plant Cell* **15**: 317-330.
- Thatcher, L., Anderson, J., and Singh, K. (2005). Plant defence responses: what have we learnt from arabidopsis. *Functional plant biology* **32**: 1-19.
- Theologis, A., Ecker, J.R., Palm, C.J., Federspiel, N.A., Kaul, S., White, O., Alonso, J., Altafi, H., Araujo, R., Bowman, C.L., Brooks, S.Y., Buehler, E., Chan, A., Chao, Q., Chen, H., Cheuk, R.F., Chin, C.W., Chung, M.K., Conn, L., Conway, A.B., Conway, A.R., Creasy, T.H., Dewar, K., Dunn, P., Etgu, P., Feldblyum, T.V., Feng, J., Fong, B., Fujii, C.Y., Gill JE, Goldsmith, A.D., Haas, B., Hansen, N.F., Hughes, B., Huizar, L., Hunter, J.L., Jenkins, J., Johnson-Hopson, C., Khan, S., Khaykin, E., Kim, C.J., Koo, H.L., Kremenetskaia, I., Kurtz, D.B., Kwan, A., Lam, B., Langin-Hooper, S., Lee, A., Lee, J.M., Lenz, C.A., Li, J.H., Li, Y., Lin, X., Liu, S.X., Liu, Z.A., Luros, J.S., Maiti, R., Marziali, A., Militscher, J., Miranda, M., Nguyen, M., Nierman, W.C., Osborne, B.I., Pai, G., Peterson, J., Pham, P.K., Rizzo, M., Rooney, T., Rowley, D., Sakano, H., Salzberg, S.L., Schwartz, J.R., Shinn, P., Southwick, A.M., Sun, H., Tallon, L.J., Tambunga, G., Toriumi, M.J., Town, C.D., Utterback, T., Van Aken, S., Vaysberg, M., Vysotskaia, V.S., Walker, M., Wu, D., Yu, G.,

- Fraser, C.M., Venter, J.C., and Davis, R.W. (2000). Sequence and analysis of chromosome 1 of the plant *Arabidopsis thaliana*. *Nature* **408**: 816-20.
- Thilmony, R., Underwood, W., and He, S.Y. (2006). Genome-wide transcriptional analysis of the *Arabidopsis thaliana* interaction with the plant pathogen *Pseudomonas syringae* pv. tomato DC3000 and the human pathogen *Escherichia coli* O157:H7. *Plant Journal* **46**: 34-53.
- Thompson, R.E., Duncan, G., and McCord, B.R. (2014). An investigation of PCR inhibition using Plexor(®) -based quantitative PCR and short tandem repeat amplification. *Journal of Forensic Sciences* **59**: 1517-29.
- Tintor, N., Ross, A., Kanehara, K., Yamada, K., Fan, L., Kemmerling, B., Nuernberger, T., Tsuda, K., and Saijo, Y. (2013). Layered pattern receptor signaling via ethylene and endogenous elicitor peptides during *Arabidopsis* immunity to bacterial infection. *Proceedings of the National Academy of Sciences* **110**: 6211-6216.
- Torii, K.U. (2004). Leucine-rich repeat receptor kinases in plants: structure, function, and signal transduction pathways. *International Review of Cytology* **234**: 1-46.
- Torii, K.U. (2012). Mix-and-match: ligand-receptor pairs in stomatal development and beyond. *Trends in Plant Sciences* **17**: 711-9.
- Torres, M.A., Jones, J.D., and Dangl, J. (2006). Reactive oxygen species signaling in response to pathogens. *Plant Physiology* **141**: 373-378.
- Truman, W., Bennett, M.H., Kubigsteltig, I., Turnbull, C., and Grant, M. (2006). *Arabidopsis* systemic immunity uses conserved defense signaling pathways and is mediated by jasmonates **104**: 1075-1080.

- Tsuchiya, T., and Eulgem, T. (2010). The Arabidopsis defense component EDM2 affects the floral transition in an FLC-dependent manner. *Plant Journal* **62**: 518-528.
- Tsuda, H., Han, S.M., Yang, Y., Tong, C., Lin, Y.Q., Mohan, K., Haueter, C., Zoghbi, A., Harati, Y., Kwan, J., Miller, M.A., and Bellen, H.J. (2008). The Amyotrophic Lateral Sclerosis 8 Protein VAPB Is Cleaved, Secreted, and Acts as a Ligand for Eph Receptors. *Cell* **133**: 963-977.
- Ueki, N., Someya, K., Matsuo, Y., Wakamatsu, K., and Mukai, H. (2007). Cryptides: functional cryptic peptides hidden in protein structures. *Peptide Science* **88**: 190-198.
- Ulker, B., and Somssich, I.E. (2005). WRKY transcription factors: from DNA binding towards biological function. *Current Opinion in Plant Biology* **7**: 491-8.
- Van Damme, E.J.M., Fouquaert, E., Lannoo, N., Vandenborre, G., Schoupe, D., and Peumans, W.J. (2011). Novel concepts about the role of lectins in the plant cell, in *The Molecular Immunology of Complex Carbohydrates-3*, Vol. 705 ed. A. M. Wu (New York: Springer), 295-324.
- Van der Biezen, E.A., and Jones, J.D. (1998). Plant disease-resistance proteins and the gene-for-gene concept. *Trends in Biochemical sciences* **23**: 454-6.
- Wang, X., Kota, U., He, K., Blackburn, K., Li, J., Goshe, M.B., Huber, S.C., and Clouse, S.D. (2008). Sequential transphosphorylation of the BRI1/BAK1 receptor kinase complex impacts early events in brassinosteroid signaling. *Developmental Cell* **15**: 220-35.
- Wang, G.F., Seabolt, S., Hamdoun, S., Ng, G., Park, J., and Lu, H. (2011). Multiple roles of WIN3 in regulating disease resistance, cell death, and flowering time in Arabidopsis. *Plant Physiology* **156**: 1508-19.

- Whalen, C., Innes, R.W., Bent, A.F., and Staskawicz, B.J. (2001). Identification of *Pseudomonas syringae* Pathogens of Arabidopsis and a Bacterial Locus Determining Avirulence on Both Arabidopsis and Soybean Maureen. *Plant Cell* **3**: 49-59.
- Wildermuth, M.C., Dewdney, J., Wu, G., and Ausubel, F.M. (2001). Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* **29**: 562-5.
- Winter, C.M., Austin, R.S., Blanvillain-Baufumé, S., Reback, M.A., Monniaux, M., Wu, M.F., Sang, Y., Yamaguchi, A., Yamaguchi, N., Parker, J.E., Parcy, F., Jensen, S.T., Li, H., and Wagner, D. (2011). LEAFY target genes reveal floral regulatory logic, cis motifs, and a link to biotic stimulus response. *Developmental Cell* **20**: 430-43.
- Wu, J., and Baldwin, I.T. (2010). New insights into plant responses to the attack from insect herbivores. *Annual Review of Genetics* **44**: 1-24.
- Wyrsh, I., Domínguez-Ferreras, A., Geldner, N., and Boller, T. (2015). Tissue-specific FLAGELLIN-SENSING 2 (FLS2) expression in roots restores immune responses in Arabidopsis fls2 mutants. *New Phytologist* **206**:774-84.
- Xiang, Y., Tang, N., Du, H., Ye, H., and Xiong, L. (2008). Characterization of OsbZIP23 as a key player of the basic leucine zipper transcription factor family for conferring abscisic acid sensitivity and salinity and drought tolerance in rice. *Plant Physiology* **148**: 1938-1952.
- Xin, X.F., and He, S.Y. (2013). *Pseudomonas syringae* pv. tomato DC3000: a model pathogen for probing disease susceptibility and hormone signaling in plants. *Annual Review of Phytopathology* **51**: 473-98.
- Yamaguchi, Y., and Huffaker, A. (2011). Endogenous peptide elicitors in higher plants. *Current Opinion in Plant Biology* **14**: 351-357.

- Yamaguchi, Y., Huffaker, A., Bryan, A., Tax, F., and Ryan C.A. (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., Pearce, G., and Ryan C.A. (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.
- Yonekura, K., Maki-Yonekura, S., and Namba, K. (2003). Complete atomic model of the bacterial flagellar filament by electron cryomicroscopy. *Nature* **424**: 643-650.
- Zaltsman, A., Krichevsky, A., Loyter, A., and Citovsky, V. (2010). Agrobacterium Induces Expression of a Host F-Box Protein Required for Tumorigenicity. *Cell Host Microbe* **7**: 197-209.
- Zhang, J., Li, W., Xiang, T., Liu, Z., Laluk, K., Ding, X., Zou, Y., Gao, M., Zhang, X., Chen, S., Mengiste, T., Zhang, Y., and Zhou, J.M. (2010). Receptor-like cytoplasmic kinases integrate signaling from multiple plant immune receptors and are targeted by a *Pseudomonas syringae* effector. *Cell Host Microbe* **22**: 290-301.
- Zhang, J., Shao, F., Li, Y., Cui, H., Chen, L., Li, H., Zou, Y., Long, C., Lan, L., Chai, J., Chen, S., Tang, X., and Zhou, J.M. (2007). A *Pseudomonas syringae* effector inactivates MAPKs to suppress PAMP-induced immunity in plants. *Cell Host Microbe* **1**: 175-185.
- Zhang, C., Shi, H., Chen, L., Wang, X., Lü, B., Zhang, S., Liang, Y., Liu, R., Qian, J., Sun, W., You, Z., and Dong, H. (2011). Harpin-induced expression and transgenic overexpression of the phloem protein gene AtPP2-A1 in Arabidopsis repress phloem feeding of the green peach aphid *Myzus persicae*. *BMC Plant Biology* **11**: 11.

- Zhou, J., Lu, D., Xu, G., Finlayson, S.A., He, P., and Shan, L. (2015). The dominant negative ARM domain uncovers multiple functions of PUB13 in Arabidopsis immunity, flowering, and senescence. *Journal of Experimental Botany* **66**: 3353-66.
- Zhou, N., Tootle, T.L., Tsui, F., Klessig, D.F., and Glazebrook, J. (1998). PAD4 functions upstream from salicylic acid to control defense responses in *Arabidopsis*. *Plant Cell* **10**: 1021-1030.
- 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. (2014). Plant pattern-recognition receptors. *Trends in Immunology* **35**: 345-51.
- Zipfel, C., and Felix, G. (2005). Plants and animals: a different taste for microbes? *Current Opinion in Plant Biology* **8**: 353-360.
- Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J.D., Boller, T., and Felix, G. (2006). Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. *Cell* **125**: 749-760.
- Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E., Jones, J.D., Felix, G., and Boller, T. (2004). Bacterial disease resistance in Arabidopsis through flagellin perception. *Nature* **428**: 764-767.

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