*At*Peps as danger signals in Arabidopsis - their release from PROPEP proteins by highly specific Metacaspases

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ABBREVIATIONS

aa	Amino Acid
ACC	1-Aminocyclopropane-1-carboxyclic acid
A.th.	*Arabidopsis thaliana*
ACC	1-Aminocyclopropane-1-carboxylic acid
ACCS	ACC-Synthase
ALE	ABNORMAL LEAF SHAPE
*At*Pep	Arabidopsis thaliana Pep, C-terminal part of PROPEP
BAK1	BRI1 associatedKinase 1
BKK1	BAK1-LIKE 1
BRI1	Brassinosteroid Insensitive 1
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium
CaMV35S	Cauliflower Mosaic Virus 35S
cDNA	complementary DNA
CDPK	Calcium-Dependent Protein Kinase
CEP1	C-terminally encoded peptide 1
CERK1	Chitin Elicitor Receptor Kinase 1
CLE	Clavata/Endosperm Surrounding Region
CLV1	CLAVATA1
CLV3	CLAVATA3
Col-0	*Columbio-0* Ecotype of *A.th.*
DAMP	Damage-Associated Molecular Pattern
DORN1	Does not Respond to Nucleotides 1
Ds	Double stranded
eATP	Extracellular Adenosine triphosphate
EDTA	Ethylendiamintetraacetatic acid
EGF	Epidermal Growth Factor
EFR	Elongation Factor TU Receptor
EPIP	Extended PIP
ER	EndoplasmicReticulum
ETI	Effector-Triggered Immunity
ETS	Effector-triggered Susceptibility
flg22	conserved N-terminal region of flagellin
FLS2	Flagellin Sensing 2
GFP	Green Fluorescent Protein
GmSubPep	*Glycine max* subtilase peptide
GSO	GASSHO
GUS	ß-Glucoronidase
HAMP	Herbivore-Associated Molecular Pattern

HR	Hypersensitive Response
HypSys	Hydroxyproline-richsystemins
IL (R/F)	Interleukin (receptor/family)
JA	Jasmonicacid
kDa	kiloDalton
LRR	Leucine Rich Repeat
LysM	Lysine Motif
MAMP	Microbe-Associated Molecular Pattern
MAPK	Mitogen-Activated Protein Kinase
MC	Metacaspase
mRNA	messengerRNA
mtDNA	mitochondrial DNA
MS	Murashige & Skoog Medium
N.b.	*Nicotiana benthamiana*
NLR/NB-LRR	Nucleotide Binding LRR
OGAs	Oligogalacturonides
PAMP	Pathogen-Associated Molecular Pattern
PCR	Polymerase Chain Reaction
PEP	Lant Elicitor Peptide
PEPR	*At*PepReceptor
PGN	Peptidoglycan
PP	PROPEP
PROPEP	Precursorof*At*Pep
PRR	Pattern Recognition Receptor
PSK	Phytosulfokine
Pst	*Pseudomonas syringae* pathovar *tomato* DC3000
PSY1	Plant peptide containing sulfate tyrosine 1
PTI	PAMP/Pattern-Triggered Immunity
RALF	Rapid alklinizationFactor
RLK	Receptor Like Kinase
RLP	Receptor Like Protein
ROS	Reactive Oxygen Species
SA	SalicylicAcid
SPE	Soätzle Processing Enzyme
ss	Single stranded
T-DNA	Transfer DNA
TLR	Toll Like Receptor
OS	Oral Secretions
WAK1	Wall Associated Kinase 1
WT	Wild Type
YFP	Yellow Fluorescent Protein
*Zm*PEP	*Zea mays* PEP

SUMMARY

Microbial pathogens and herbivores are some of the key drivers of evolutionary adaptations by plants. As sessile organisms plants have to react quickly and strongly with defense responses to repel any invading organism. Besides preformed structures like thick cell walls and long thorns plants can activate innate immune responses that in a complex way lead to the activation of very efficient countermeasures. These include measurable changes on the plants hormone and gene expression levels but also plenty of secondary metabolites can be produced that directly have antimicrobial or herbivore repellent activity. Key to the timely initiation of defense responses is the perception of the invader and its detrimental activity. Plants carry highly specific pattern recognition receptors (PRR) to detect microbial or herbivore specific molecular signatures, so called microbe- or herbivore-associated molecular patterns (MAMP/HAMP). Less specific but equally efficient plant defenses can also be activated by the perception of self-molecules that behave differently once cell damage occurs. So called damage-associated molecular patterns (DAMP) are released passively or actively from damaged cells and serve as strong indicators of an infection or the presence of an herbivore.

In this work the mechanisms around expression, activation and activity of recently described DAMPs, the family of plant elicitor peptides (PEPs), were investigated in more detail. PEPs are perceived by the plant they are released from via specific PEP receptors (PEPRs) and thereby trigger defense responses. PEPs are expressed as larger PROPEPs, and we first investigated the expression of seven formerly known and a newly identified eighth *PROPEP* and that of the two *PEPR*s in Arabidopsis tissues using the promoter-GUS fusion technique. We were able to show that expression of *PROPEP*s 1-3, 5 and 8 mostly overlapped and correlated with the expression of both *PEPR*s, whilst *PROPEP4* and *7* were only weakly expressed in small areas of the roots. *In silico* analysis unveiled the influences of biotic stresses on the *PROPEP* expression patterns and showed that PROPEP 1-3 are most strongly regulated by defense-associated mechanisms. To determine the subcellular localization of a selection of PROPEPs we observed PROPEP 1, 3 and 6 fused to Yellow Fluorescent Protein (YFP) within the cells and found PROPEP1 and 6 to be localized to the tonoplast membrane,

whilst PROPEP3 showed a cytoplasmic localization. Despite the apparent different expression and localization patterns of PROPEPs, the elicitation activity of the mature PEPs was very similar, even though all eight *At*PEPs were perceived by *At*PEPR1 while *At*PEPR2 was activated exclusively by *At*PEP1 and 2.

Even though a lot of research has been already done on the responses induced after PEP elicitation the circumstances and the mechanism leading to PEP genesis from the PROPEP precursor has not been uncovered so far. Here, we observed the rapid formation of Arabidopsis PEP1 from PROPEP1 upon cell damage. Cleavage of PROPEP1 depended on the presence of the conserved arginine 69 and was impaired by chelating Ca^{2+} ions or addition of a metacaspase-specific inhibitor. This led to the identification of the arginine-specific cysteine protease *At*Metacaspase 4 (MC4). MC4 activation correlated with PEP1 formation, MC4 was able to cleave PROPEP1 *in vitro,* and lack of MC4 impaired PROPEP1 cleavage *in vivo*. Furthermore, laser ablation experiments revealed damage-induced relocalization of PROPEP1 that was dependent on MC4 activity. Notably, PEPR1 internalization in cells adjacent to the site of laser ablation indicated PEP1 release. Thus MC4 is the bona fide protease for PROPEP1 processing and thereby enables PEP1 relocalization to first the cytosol and, depending on the cellular integrity, the extracellular space.

In a third project we gained knowledge about the conservation of the PROPEP-Pep-PEPR system across the plant kingdom. We identified new PEPs in Brassicaceae, Solanaceae and Poaceae species with elicitor activity being limited to the plant family of their origin. We deduced Brassicaceae, Solanaceae and Poaceae specific amino acid motifs within the respective PEP families that are required for intra-family elicitor activity and seem to explain the interfamily incompatibility. In addition we identified a large number of PEPRs outside Arabidopsis and cloned the coding sequences of *Zea mays* PEPR and *Solanum Lycopersicum* PEPR for further characterization. Expression of these newly identified receptors in *Nicotiana benthamiana* demonstrated their functionality upon perception of the corresponding PEPs. Thus, contrary to PROPEPs, the PEPRs are interspecies compatible.

In summary with this study valuable new data on the characteristics and ubiquity of the PROPEP-PEP-PEPR system in general and the PROPEPs in particular were generated. Importantly, light was shed on the hitherto unknown processing of PROPEPs that not only significantly advanced PEP research but also the work on plant proteases which is struggling to identify *in vivo* substrates. Finally, this work might soon be recognized as the foundation to define the first plant cytokines.

1. INTRODUCTION

1.1 GENERAL INTRODUCTION

As the primary producers of organic energy, the photosynthetically active plants are an obligate food source for a huge variety of organisms. From the smallest microbe to the largest elephant plants serve as a main source of resources and energy needed for survival. Via their roots plants obtain nutrients and water from the soil needed to run photosynthesis in the green aboveground parts, rendering them anchored to the ground at a given place and taking away the escape options from uncomfortable conditions like appearing herbivores. These biotic as well as abiotic stresses put plants under evolutionary pressure and drive the development of diverse adaptations including phenotypic adaptations like a waxy cuticula or thorns as well as molecular adaptations like the storage of secondary metabolites (Malinovsky et al., 2014). Some adaptations serve as physical barriers against biotic invaders, making the plant indigestible to some herbivores or less vulnerable and therefore reduce the likelihood of pathogen entry into the plant body at the site of wounding. A second and more specific line of defense especially against pathogens is formed by the plants` inducible innate immune system (Muthamilarasan and Prasad, 2013). It relies on a specific recognition machinery that allows the perception of whole classes of biotic stressors via their molecular fingerprints (Zipfel, 2014). For example the molecular fingerprints of microbes are based on greatly conserved molecules, so called microbe-associated molecular patterns (MAMPs), which are required by these microbes for their lifestyle and hence their survival (Mackey and McFall, 2006; Boller and Felix, 2009). These molecules allow a specific discrimination between self (plant-born molecules) and non-self (microbe-born molecules, MAMPs), a prerequisite for a specific response. Notably, plants are able to perceive their own molecules as well, for example in cases of danger or damage situations. So called damage-associated molecular patterns (DAMPs) are released by dying or wounded cells and are perceived by neighboring cells as danger signals (Heil et al., 2012; Heil and Land, 2014).

MAMPs and DAMPs are perceived by specific plasma membrane bound pattern recognition receptors (PRRs) and thereby trigger signal transduction pathways for the

activation of defense reactions known as pattern-triggered immunity (PTI) (Chinchilla et al., 2006; Zipfel et al., 2006; Krol et al., 2010; Macho and Zipfel, 2014). These include rapidly induced direct defense responses like the production of anti-microbial compounds, repellent molecules, and volatile organic compounds (VOC) but also long-term adaptations like altered gene expression patterns, strengthening of the cell wall, and shifted growth patterns (Boller and Felix, 2009). The perception of MAMPs signals the presence of potentially pathogenic microbes whereas the perception of DAMPs follows either mechanical damage or established infections which are accompanied by frequent cell death or damage to the host (Heil et al., 2012).

Most DAMP and MAMP PRRs interact with a specific co-receptor upon binding of a specific ligand and thereby initiate a multitude of downstream responses including the induction of basal defense responses that subsequently lead to non-host resistance against an invading pathogen or the induction of wound healing (Heil et al., 2012; Zipfel, 2014). Involved components of PTI signaling are shown in figure 1.1 and will be described in more detail in the individual chapters.

PTI represents the first layer of plant innate immunity. Nevertheless pathogens evolved specific strategies to overcome this first line of defense of their host. Pathogenic effectors, also called virulence factors, are able to inhibit crucial steps in PTI signaling (Deslandes and Rivas, 2012; Maffei et al., 2012). Effectors can either be injected into the host cells or be secreted into the extracellular space where they lead to blockage of crucial PTI signaling events. During *Pseudomonas syringae* pv. DC3000 infections on Arabidopsis, the pathogen injects the effectors AvrPto and AvrPtoB via a type III secretion system into the host cell. The two effectors directly interfere with receptor kinases that are crucial for PTI signaling (Xing et al., 2007; Gimenez-Ibanez et al., 2009). Effectors can also act indirectly on the induction of PTI. The effector AprA of *P. syringae* is able to degrade monomers of the bacterial protein flagellin, a very strong elicitor of PTI, and thereby makes the bacterium “invisible” to the very first detection by the PRR FLAGELLIN SENSING 2 (FLS2) (Bardoel et al., 2011). Ultimately effectors serve the

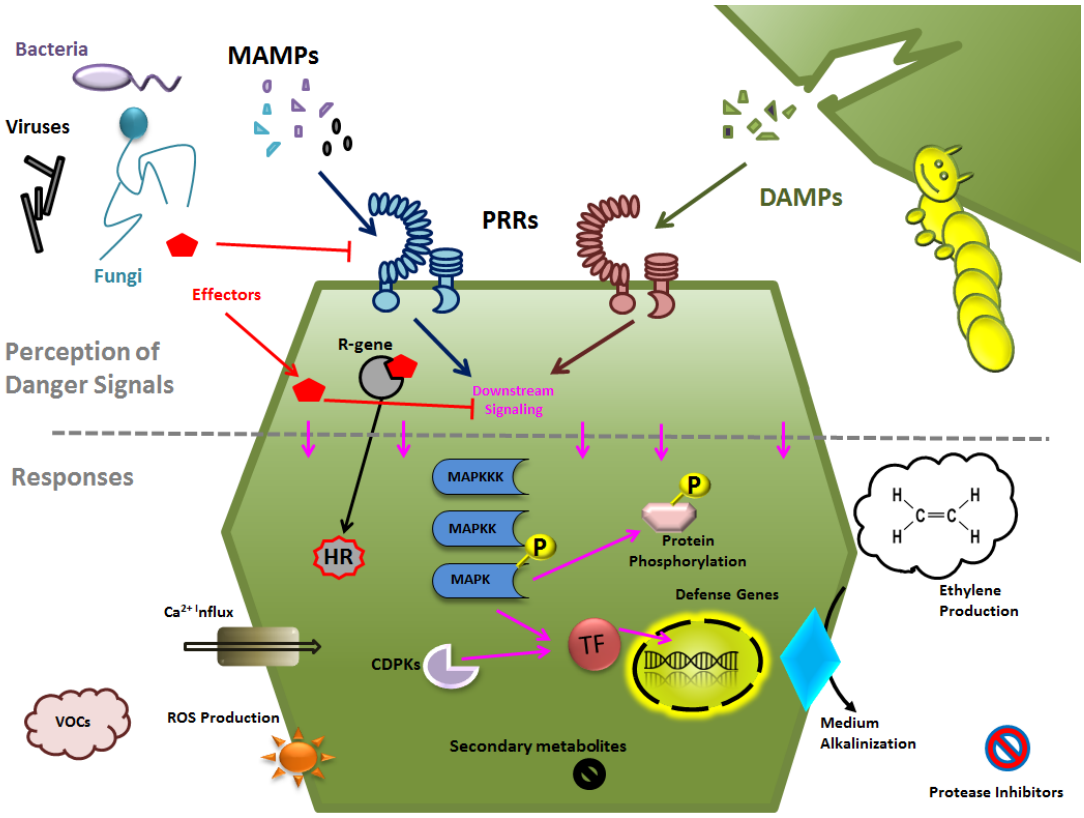

Figure 1.1. Perception of molecular danger signals in plants and the induced downstream responses. MICROBE OR DAMAGE-ASSOCIATED MOLECULAR PATTERNs (MAMPs/ DAMPs) can be sensed by each individual plant cell via highly specific PATTERN-RECOGNITION RECEPTORs (PRRs) at the cell surface. Activation of the respective PRRs by ligand binding induces various downstream responses of which some are specifically induced upon only MAMP or DAMP perception, whereas the majority of responses are induced upon elicitor perception in general. Induction of defense mechanisms by PRRs finally leads to increased resistance against pathogens and is called pattern-triggered immunity (PTI). Volatile organic compounds (VOCs), protease inhibitors (PI) and further secondary metabolites are especially important as defense components that directly act against an invader but can also serve as signaling molecules. Microbes as well as herbivores developed effectors to inhibit recognition or defense mechanism by the plant. Effectors in turn can be recognized by specific resistance proteins (R proteins), that induce PTI responses on a much stronger level and in most cases lead to a hypersensitive reaction (HR) causing cell death and blocking of pathogen spreading. Defense mediated by microbial effectors is called effector-triggered immunity (ETI)

purpose to suppress the host immune responses to ensure successful infection thereby leading to so called effector triggered susceptibility (ETS) of the host (Deslandes and Rivas, 2012; Zhou et al., 2014). Hence, in addition to the basal immune system of plants including PTI, plants in turn evolved strategies to counteract ETS directly. This adaptation is referred to as effector-triggered immunity (ETI) and acts as a second layer of defense against pathogen infections (Jones and Dangl, 2006). ETI relies on specific immune receptors that sense effectors either directly or indirectly detect effector activity. Recognition is executed by resistance (R) proteins, intracellular nucleotide-binding leucine-rich repeat (NB-LRR) proteins that initiate a set of defense responses, which partially differ from the one induced by PTI. Defense responses initiated by the

activation of R proteins are mostly rapidly induced on a generally high level and often lead to a so called hypersensitive response (HR) (Mur et al., 2008). HR is a specific type of programmed cell death that is induced in pathogen infected tissue upon ETI and also in the surrounding areas, leading to necrosis in this area. HR is a characteristic mechanism in ETI and effectively blocks the spread of biotrophic pathogens from the infection side (Mengiste, 2012).

PTI is a rather static building block of the innate immune system. It relies on the perception of highly conserved microbial structures, which cannot be easily altered by the microbe without effecting its survival or virulence. Effectors are in contrast to most MAMPs not essential for microbial survival and may therefore be altered by the pathogen to evade from ETI, or to more efficiently suppress PTI (Jones and Dangl, 2006; Hein et al., 2009). In the same way R proteins require adaptation by the plant to keep up ETI. Since effector and R protein are both encoded in the respective genome it has been found that a given cultivar of a plant shows resistance to some strains of a given pathogen but not to other strains and vice versa some strains are able to infect some cultivars but not others (Boyd et al., 2013). When observed as a single situational snapshot, one effector interacts with one R protein and therefore this process has initially been described as “gene for gene” interaction. Due to co-evolution of host and pathogen this mechanism by which ETS and ETI interact is rather flexible and driven by the selective pressure ETS and ETI force to the respective suffering organism. Thus this dynamic interplay of PTI, ETS and ETI has been described in the "zigzag model" (figure 1.2) as a sort of arms race of plants and pathogens (Jones and Dangl, 2006). Seen in an evolutionary context, pathogens get selected against the perception by the plant’s immune system and vice versa the plant’s immune system gets selected for the perception of invading pathogens.

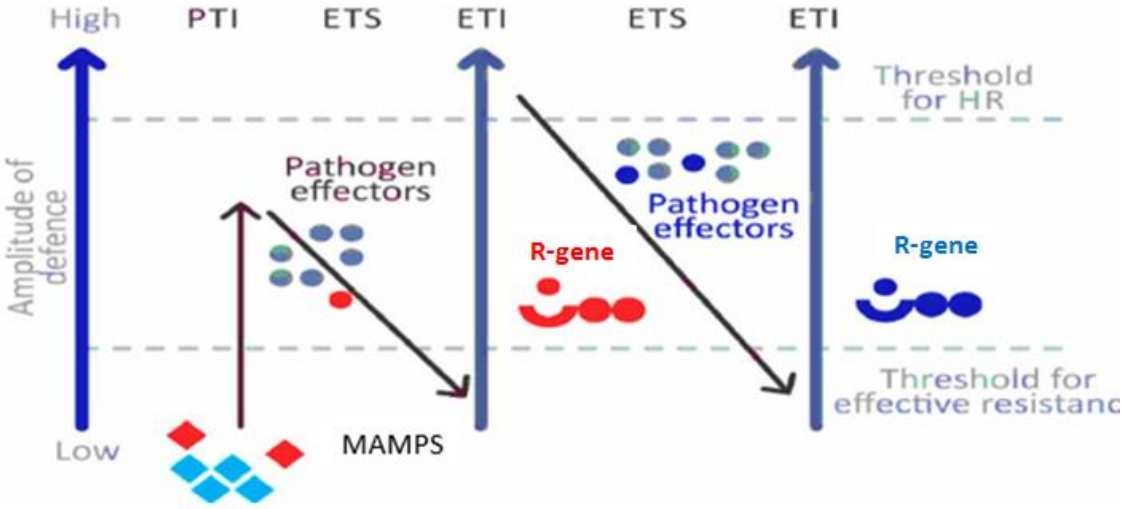

Figure 1.2. The "Zigzag" model to co-evolution of plants defense mechanisms and pathogen countermeasures. Detection of MAMPs induces basal defense responses in plants that lead to PTI. Pathogens can evade from PTI or block its activation by carrying specific effectors that attenuate defense responses and render the plant susceptible to the pathogen (ETS). Recognition of effectors by plant *R*-genes induces enhanced defense responses stronger than basal PTI responses, thereby leading to the cell death-like HR and finally to ETI. Alterations of effectors as well as *R*-genes are seen as a sort of arms-race between pathogens and the host for ETS and ETI (adapted from (Jones and Dangl, 2006)).

1.2 PLANT DEFENSE AND PERCEPTION OF DANGER SIGNALS

"It is easier to resist at the beginning than at the end."

— Leonardo da Vinci

Since plants lack an adaptive immune system, eradication of a manifested infection is rather difficult and therefore the above quote gives a fitting description of the mode of operation of the plants` defense machinery and emphasizes the need for a rapid detection of all kinds of pathogens. Thus the perception of molecular signatures like MAMPs and DAMPs is integral for the timely initiation of defense responses. In the following chapter these exogenous as well as endogenous elicitors will be described in more detail.

1.2.1 Microbe-associated Molecular Patterns (MAMPs)

Formerly described as pathogen-associated molecular patterns (PAMPs), these molecules have the ability to elicit defense responses upon their perception by other species. Since they are not exclusively restricted to be carried by pathogens the term Microbe-associated Molecular Patterns turned out to be more appropriate (Boller and Felix, 2009). As mentioned before MAMPs are highly conserved and crucial structures often found in a whole clade of microbes. Examples are peptidoglycans, the building blocks of the bacterial cell wall, Elongation factors that are essential for pathogen survival, or the flagellin monomer, which is required for movement by motile bacteria (Newman et al., 2013). The necrosis and ethylene-inducing peptide 1-like proteins (Nep1/NLP) are proteins secreted not only by bacteria but by many plant pathogens, ranging from fungi and oomycetes to bacteria and trigger defenses responses in Arabidopsis (Oome et al., 2014).

Bacterial MAMPs

Bacterial flagellin is the paradigm of a peptide MAMP (Felix et al., 1999). Flagellin is an essential protein to motile bacteria since it builds up the bacterial flagellum (Taguchi

et al., 2008). Some parts of the protein like the N- and C- termini are highly conserved whereas the middle part, which is exposed to the outside of the flagellum, is highly diverse in its amino acid (aa) sequence. Nanomolar concentrations of a conserved part of 22 aa from the N-terminus of *Pseudomonas syringae pv. tomato* (*Pst* DC3000) flagellin have been found to be sufficient to elicit PTI in plants (Felix et al., 1999; Smith et al., 2003). This so called flg22 epitope is perceived by most plant species through the leucine-rich repeat receptor like kinase (LRR-RLK) FLAGELLIN SENSING 2 (FLS2) (Gomez-Gomez et al., 1999; Bauer et al., 2001; Chinchilla et al., 2006). In *Arabidopsis thaliana* pretreatment with exogenously applied flg22 had a vaccination like effect to the treated plants and rendered them more resistant to subsequent infections with *Pst* DC3000, whilst the *fls2* mutant, which is "blind" to flagellin, was found to be more susceptible than wild type plants (Zipfel et al., 2004). Sensing of the flagellin protein is also present in mammals by the cell surface receptor Toll-like receptor 5 (TLR5) that, in contrast to FLS2, perceives an epitope at the C-terminal end of flagellin (Hayashi et al., 2001; Smith et al., 2003). There are also other epitopes of flagellin which are not as universal as the flg22 epitope like flgII-28, which is only perceived by solanaceous species and CD2-1, a C-terminal epitope perceived by rice (Cai et al., 2011; Veluchamy et al., 2014; Katsuragi et al., 2015).

As described by the "Zigzag"- model it would be beneficial for bacteria to evade from this perception for example by alteration of the amino acid sequence of the flagellin protein. Indeed the *Agrobacterium tumefaciens* flagellin sequence is altered in such a way that it is not perceived by Arabidopsis FLS2 and therefore does not induce defense reactions (Zipfel et al., 2004). Symbiotic bacteria like *Rhizobium meliloti* have also been found to evade defense responses by alteration of the flagellin protein (Felix et al., 1999). Another strategy to overcome PRR perception of flagellin was developed by P. syringae that expresses and releases the alkaline protease AprA, which specifically degrades flagellin and thus prevents detection by FLS2 (Bardoel et al., 2011).

PRR mediated perception of bacterial microbes is furthermore not restricted to flagellin. In the group of protein MAMPs there are some more prominent examples. ELONGATION FACTOR THERMO UNSTABLE (EF-Tu) is a very abundant protein in bacteria and it is essential for elongation during protein synthesis (Jeppesen et al., 2005). EF-Tu shows 90% sequence homology between hundreds of bacterial species,

and a highly conserved 18 or 26 aa (elf18/26) epitope of the N-terminus can be detected by Brassicaceae via the PRR EF-Tu RECEPTOR (EFR) (Kunze et al., 2004; Zipfel et al., 2006). Surprisingly, when transformed with the EFR coding sequence, other species like *Nicotiana benthamiana*, naturally insensitive to EF-Tu, become responsive to elf18 treatment (Lacombe et al., 2010). This highlights the apparent conservation of the PRR-operated downstream signaling pathways involved in PTI. Other proteinaceous bacterial MAMPs are the enigmatic MAMP of *Xanthomonas* (eMax) and cold-shock proteins (Felix and Boller, 2003; Jehle et al., 2013). But also non-proteinaceous MAMPs like peptidoglycan (PGN), β-glucans, and lipopolysaccharides (LPS) have been identified as elicitors of PTI in various studies (Erbs et al., 2010; Proietti et al., 2014; Zipfel, 2014; Gust, 2015).

Fungal MAMPs

Besides bacterial pathogens plants also need to be properly protected against fungal pathogens. As for bacterial MAMPs, plants carry PRRs that sense specific conserved fungal structures. Potent elicitors are the cell wall and membrane components chitin and ergosterol, which are again important building blocks of the fungus itself (Granado et al., 1995; Ferreira et al., 2007; Klemptner et al., 2014). But also factors that are essential for fungal virulence like the toxin cerato-platanin of *Botrytis cinerea*, or endo-polygalacturonases and pectinases, which are secreted by many fungi to destroy the plant`s cell wall, are perceived as MAMPs by tobacco and Arabidopsis, respectively (Klarzynski et al., 2000; Frias et al., 2011; Zhang et al., 2014b).

Viral MAMPs

Viruses are obligate parasites and hijack the host translation machinery to reproduce themselves (Nelson and Citovsky, 2005). Viruses are often transmitted through vector organism like aphids or nematodes, which wound the plant by sucking or feeding on it and thereby transmit the virus into wounded plant cells (Bragard et al., 2013). Viral infections play important roles in the context of yield loss in agriculture and are therefore intensively studied. The potential to recognize virus-specific molecular patterns is rather restricted due to the very few building blocks a virus is composed of.

In several studies nuclear binding (NB)- domain leucine-rich repeat (LRR) receptors (NLR) have been identified that sense virus-specific coat proteins, movement proteins or replicases (Padmanabhan and Dinesh-Kumar, 2014). Furthermore, also nucleic acid combinations that are unusual to the ones present within healthy plant cells are perceived. These are for example virus specific double stranded (ds) RNA, single stranded (ss) RNA or free ds DNA that are sensed by different classes of receptors (Peisley and Hur, 2012; Berke et al., 2013). Again these elicitors are essential, virus specific components. But in contrast to other MAMPs, viral elicitors induce, if at all, not only PTI but especially virus-specific defense responses like activation of the RNA silencing machinery, and more often a HR is induced in comparison to PTI responses to restrict viral spread (Padmanabhan and Dinesh-Kumar, 2014). Perception of viruses as well as that of damage signals can induce another important mechanism, the so called systemic acquired resistance (SAR) and the slightly different induced systemic resistance (ISR), which makes tissues distant from the perception (infection) site more resistant to following viral infections (Choudhary et al., 2007; Yi et al., 2013). ISR and SAR effectively inhibit the infection with and the spread of viruses within the plant.

1.2.2 Herbivore-associated Molecular Patterns (HAMPs)

Herbivores are not classified as microbes and thus their molecular patterns are referred to as herbivore-associated molecular patterns (HAMP). As mentioned before, herbivores can directly serve as transporters for the spread of viruses between plants in addition to the damage caused by feeding on a plant (Bragard et al., 2013). Protection against herbivore feeding or sucking is thus very important to plants. The perception of herbivores can occur on two levels. First the direct perception of the herbivore via HAMPs that can be present in oral secretions (OS), the saliva, and oviposition fluids of the herbivore, or second, the perception of the damage caused by the herbivore via the perception of specific damage-associated molecular patterns (DAMPs) (Heil et al., 2012; Klauser et al., 2015). The latter will be discussed in more detail in the following chapter. Several herbivore derived elicitors have been identified so far (Fürstenberg-Hägg et al., 2013). The very first one which was identified is β-glucosidase found in regurgitate of *Pieris brassicae* caterpillars (Mattiacci et al., 1995). Further HAMPs found in OS of insects are the *Spodoptera exigua* fatty acid-amino acid conjugate

volicitin, inceptin found in OS of *Spodoptera frugiperda,* and caeliferins produced by Caelifera species (Turlings et al., 2000; Alborn et al., 2007). Inceptins are actually endogenous plant peptides derived from the highly conserved γ-subunit of chloroplastic ATP synthase (cATPase) (Schmelz et al., 2003; Schmelz et al., 2006). During insect feeding cATPase is degraded by herbivore digestive enzymes and breakdown products (inceptins of 11-13 aa length) contained in the regurgitate of the herbivore are perceived as strong indicators for actual feeding damage. Their perception induces amongst others the production of VOCs including terpenes, indole and methyl salicylate.

As a precaution against damage by, not yet hatched caterpillars plants are able to perceive molecules that characterize oviposition on leaves. Bruchins derived from *Bruchus pisorum* and benzyl cyanide found in *P. brassicae* oviposition fluids are both strong elicitors perceived by various plants (Doss et al., 2000; Huigens et al., 2011). In addition to the induction of PTI and other specific HAMP responses, the responses to egg deposition include hypersensitive or necrotic responses, production of ovicidal chemicals and the development of neoplasm (Howe and Jander, 2008). Neoplasm is hardened tissue that is formed underneath an insect egg to hamper penetration or feeding of the hatched insect (Hilker and Meiners, 2011).

Besides the knowledge about membrane-bound receptors that enable the perception of volicitin the identification of a specific HAMP receptor is still missing (Truitt et al., 2004; Mithofer and Boland, 2008).

Nematode-associated Molecular Patterns

Nematodes cause tremendous damage annually to many agricultural plants (Singh et al., 2015). Ascarosides are glycosides that carry a fatty acid side chain and have been identified exclusively from nematodes, in which they regulate development and social behavior (Choe et al., 2012). Only recently the first nematode specific elicitor ascr#18, the major ascaroside in plant-parasitic nematodes, has been described as a potent elicitor in several plant species (Manosalva et al., 2015). Further induction of defense responses by nematodes have been found to be dependent on plant PRRs, but no specific elicitor was identified in this context (Cai et al., 1997; Lozano-Torres et al., 2012).

1.2.3 Damage-associated Molecular Patterns (DAMPs)

Direct contact with herbivores or pathogens can not only be detected via exogenous molecular signatures, but also indirectly via the changes that happen to the plant. The most common changes in the host plant physiology to which pathogen infections and herbivore feeding are inevitably linked is the emergence of damaged or wounded tissue (Heil et al., 2012; Heil and Land, 2014). Damaged tissue can be a direct consequence of feeding, or it occurs indirectly from HR or other forms of passive or induced cell death (Mur et al., 2008). Mechanical wounding alone has been shown to be sufficient to elicit plant defense responses and thus to be independent of an external stimulus but being triggered somehow by endogenous signaling molecules (Hilker and Meiners, 2010). A further specific set of PRRs has been found responsible for the detection of endogenous damage-associated molecular patterns (DAMPs) that get released from damaged cells. DAMPs induce a PTI comparable to the PTI initiated upon MAMP and HAMP perception (Macho and Zipfel, 2014; Zipfel, 2014). DAMPs can either be actively synthesized, or passively produced upon the damage of plant tissue (Ferrari et al., 2013; Tanaka et al., 2014; Minibayeva et al., 2015).

Wounding is a strong predictor of infection as well, and therefore DAMP perception is a key element of innate immunity since it enables plants to detect damage independently of its origin from biotic or abiotic causes (Heil and Land, 2014). Consequently DAMP perception should in best cases fulfill three key mechanisms. First their perception should induce defenses against the potential perpetrators, like herbivores. The DAMP plant elicitor peptide 3 of *Zea mayze* (ZmPEP3) for example has been shown to trigger the biosynthesis of VOCs and protease inhibitors in a similar way as herbivore oral secretions so that pretreatment of maize plants with ZmPEP3 successfully conferred defense against the herbivore *S. exigua* (Huffaker et al., 2013). Second DAMPs should also induce PTI like responses to prevent the wounding site from turning into an infection site. The production of reactive oxygen species (ROS) is a key element of PTI, and ROS are also actively produced by damaged cells and have been shown to have direct antimicrobial as well as signaling functions (Jabs et al., 1996; Lamb and Dixon, 1997; Minibayeva et al., 2015). Additionally, wound-induced methanol, and the plant hormone derivatives methyl jasmonate and methyl salicylate were tested positively for antimicrobial activity and are all signaling components either in the systemic tissue or to

neighboring plants (Goodrichtanrikulu et al., 1995; Zhang et al., 2006; Dorokhov et al., 2012; Komarova et al., 2014). And finally the detection of DAMPs and therefore damage should induce healing responses. DAMP perception induces relocalisation of hydroxyproline-rich glycoproteins (HRGP) to the apoplast, where they undergo oxidative cross-linking and thereby play a central role in cell wall strengthening and wound healing. The ROS H_2O_2 is a required co-substrate during cell wall maturation at all stages (Tisi et al., 2008; Sujeeth et al., 2010; Delaunois et al., 2014).

Peptidic DAMPs

Peptides that are produced by the plant itself but trigger defense responses when perceived by the same plant are generally considered as DAMPs, even if not all peptide DAMPs are expressed during defense responses (Schmelz et al., 2006). Systemin was the very first peptide with signaling functions identified in plants; up to this point the known plant signaling molecules had nothing to do with peptides (Pearce et al., 1991). Meanwhile plenty of peptides have been identified in plants that are involved in all kinds of developmental and signal transduction processes (Tavormina et al., 2015). Systemin is an 18-amino acid peptide which is released from the 200-amino acid precursor ProSystemin upon cell damage or herbivore and pathogen attack by a so far unknown mechanism (Ryan and Pearce, 2003). Systemin was shown to induce various defense reactions and especially the expression of protease inhibitors which are crucial components in defense against herbivores (Zavala et al., 2004; Zhu-Salzman et al., 2008; Hartl et al., 2011). The development of the alkalinization assay, by which the pH shift of suspension cultured plant cells during a defense response is measured, enabled the identification of Systemin homologues in tobacco leaf extracts. The two Hydoxyproline-rich glycoproteins *Nicotiana tabaccum* Hydroxyproline-rich Systemin I and II (NtHypSysI/II) have been identified in tobacco due to their induction of defense reponses in a way similar to Systemin, but they do not share any sequence homology to tomato ProSystemin (Pearce et al., 2001b; Pearce, 2011). Both are derived from a single precursor protein NtPreProHypSys, NtHypSysI from the N-terminus and NtHypSysII from the C-terminus. The precursor protein is, like many Hydroxyproline-rich proteins, localized to the cell wall.

The use of the alkalinization assay also helped to identify three further DAMP peptides. The PLANT ELICITOR PEPTIDE 1 (PEP1) was identified in Arabidopsis, termed *At*PEP1, and found to have homologues in basically all plant species (they will be addressed in detail in a separate chapter) (Huffaker et al., 2006).

Two peptide DAMPs found in the soybean *Glycine max* are the 8 aa long *Gm*PEP914 and the 12 aa long subtilase peptide (*Gm*SubPEP).The first is derived from the N-terminus of a 52 aa precursor termed GmPROPEP914. A second homolog *Gm*PEP890, formed from *Gm*PROPEP890, was identified by in silico analysis and differs from GmPEP914 in only one amino acid at the C-terminus (Yamaguchi et al., 2011). The two precursors share 85% sequence similarity but like the PEP precursors PROPEPs and Prosystemin they lack any known signal sequence for secretion. Also similar to PROPEPs, *Gm*PROPEPs get upregulated in their expression upon perception of the mature peptides why they are assumed to serve as amplifiers of defense signaling responses (Heil et al., 2012). Just like PROPEPs they are furthermore upregulated by jasmonate, salicylate and ethylene signaling and they are mostly constitutively expressed in the roots (Yamaguchi et al., 2011).

*Gm*SubPEP, in contrast to GmPEPs, is a cryptic peptide since it is derived from a precursor with different primary function. *Gm*SubPEP is formed from the S8 peptidase region of a Subtilisin-like protease (Pearce et al., 2010b; Pearce et al., 2010a). That region is specific to leguminose subtilases and therefore *Gm*SubPEP is specific to only few plant species. The subtilase contains a signal sequence for secretion and is therefore believed to be secreted into the apoplast where the small signaling peptide is released from the subtilase by proteolytic cleavage either by itself or by another protease that is localized in the apoplast. In contrast to other peptide DAMP precursors the subtilase is constitutively expressed and not induced by any stress, defense or other immunity related processes (Pearce et al., 2010a).

Systemin and *PEP*s are also believed to be activated by proteolytic cleavage of the precursor during stress responses or after the direct perception of other elicitors. Nevertheless no specific mechanism is known yet in any of these cases, and Prosystemin has furthermore been shown to be a functional elicitor in its Pro-form without further processing (Dombrowski et al., 1999).

Further DAMPs

Inceptins and *Gm*SubPEP are not the only molecules that are released from functional components of plant cells and act as DAMPs during cell damage. Many other building blocks of an intact cell are released during cell damage and can be sensed by neighboring cells.

Around 7% of the plants` primary cell wall is built up by the pectic polymer Rhamnogalacturonan I whose backbone is composed predominantly of D-galacturonosyl units (McNeil et al., 1980). Microbial pathogens secrete polygalacturonases (PG) to destabilize the host cell wall whereas endogenous PGs are induced by wounding and mechanical damage (Strand et al., 1976; Bergey et al., 1999; Orozco-Cardenas and Ryan, 1999; Jorge et al., 2006). Cell wall degradation by PGs leads to the release of galacturonosyl oligomers, so called oligogalacturonides (OGAs) (Ferrari et al., 2013). The exogenous application of OGAs induces PTI-like defense responses, such as the production of ROS, callose deposition (Galletti et al., 2008), the induction of defense genes and the activation of MAP Kinase cascades (Galletti et al., 2011). OGAs are perceived through WALL-ASSOCIATED KINASE 1 (WAK1), an RLK that belongs to the WAK family which has only five members encoded in Arabidopsis (Verica et al., 2003).

Extracellular ATP (eATP) is another example for a DAMP that is also sensed by mammalian cells. In plants it binds to the cell surface lectin-domain RK DOES NOT RESPOND TO NUCLEOTIDES 1 (DORN1) which thereby senses nearby damage (Choi et al., 2014).

1.3 PATTERN RECOGNITION RECEPTORS (PRR)

Pattern recognition receptors are key components of the plants´ innate immunity. They reside in the plasma membrane and transduce the presence of MAMPs or DAMPs into intracellular signals (Macho and Zipfel, 2014; Zipfel, 2014). More than 600 RLKs that are associated with all kinds of signaling pathways and responses have been identified in the Arabidopsis genome (Shiu and Bleecker, 2001; Shiu et al., 2004). RLKs contain a divergent extracellular domain, formed by the N-terminus of the protein, that enables ligand binding, followed by a membrane anchor, an intracellular protein kinase domain,

and in some cases a juxtamembrane domain in between (Toer et al., 2009; Greeff et al., 2012; Zipfel, 2014). The extracellular domain of RLKs determines their ligand specificity and their classification into further classes like leucine-rich repeat (LRR), lysine-motif (LysM), lectin motif, or epidermal growth factor (EGF)-like RLKs. LRR-RLKs are typical receptors for peptide ligands, like the MAMPs and DAMPs flg22, elf 18 and PEP peptides, respectively, and form the largest subgroup of RLKs in Arabidopsis (Chinchilla et al., 2006; Yamaguchi et al., 2006; Zipfel et al., 2006). But also other ectodomains are involved in MAMP perception, like the lectin RLK DORN1 that senses eATP (Choi et al., 2014). The RLKs can also be further separated into the two classes of RD and non-RD kinases (Shiu and Bleecker, 2003; Tor et al., 2009). RD kinases are serine/threonine kinases with a conserved arginine (R) residue in front of the catalytically active aspartate (D) which is important for its function as a kinase (Schwessinger et al., 2011). Non-RD kinases lack these amino acids , and may require the association with a RD-RLK as a co-receptor for the initiation and amplification of phosphorylation signals, just like RLPs (Dardick et al., 2012).

A second class of PRRs binds their ligands at the ectodomain but lacks the intracellular kinase domain for further signal transduction, why they are termed RECEPTOR-LIKE PROTEINS (RLPs) and thus require the assembly with a co-receptor upon ligand binding to transduce the signal (Zipfel, 2014). Carbohydrates, like bacterial PGN and fungal chitin, are bound by LysM-RLPs. RLPs as well as RLKs have been shown to have functions in various physiological, developmental or defense-associated processes (Shiu and Bleecker, 2001; ten Hove et al., 2011; Araya et al., 2014).

The most prominent PRR in plants is the receptor of bacterial flagellin FLS2 (Chinchilla et al., 2006). Also due to its similarity to mammalian TLR5, FLS2 has been intensively studied already. Orthologues of FLS2 have been identified in tomato, rice, grapevine and tobacco (Hann and Rathjen, 2007; Robatzek et al., 2007; Takai et al., 2008; Trdá et al., 2014). FLS2 is a LRR-RLK, with an ectodomain consisting of 28 LRRs with each LRR 23-25 aa in length followed by a single plasma membrane spanning domain and a non-RD serine/threonine kinase domain (Robatzek and Wirthmueller, 2012). Other peptide binding PRRs share similar structural patterns. The elf18-binding LRR-RLK EFR carries only 21 LRRs in its ectodomain, but it has been demonstrated that swaps of

the kinase domains between EFR and FLS2 resulted in a still functional EFR-like receptor (Albert et al., 2010).

In contrast to FLS2 and EFR, which are both non-RD kinases there are important examples of RD kinases involved in MAMP perception. The LysM-RLK CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) can assemble in different ways with co-receptors or with itself (Petutschnig et al., 2010; Willmann et al., 2011). *At*CERK1 has been shown to sense bacterial PGN in a complex with the PGN binding RLPs *At*LYM1/3 in Arabidopsis and *At*CERK1 can also directly serve as a sensor for fungal chitin whilst it forms a homodimer.

Some PRR with a non-RD kinase domain have been shown to assemble with a RD receptor kinas, which functions as a co-receptor after ligand binding. The best characterized example is the LRR-RLK BRI1-ASSOCIATED KINASE 1 (BAK1). BAK1 is a member of the SERK family and has initially been found to dimerize with the brassinosteroid receptor BRI1 to positively regulate brassinosteroid signaling. *At*BAK1 has then been found to form ligand-dependent heteromeric complexes with several MAMP and DAMP receptors such as *At*FLS2, *At*EFR, and *At*PEPR1 and *At*PEPR2 (Chinchilla et al., 2007; Roux et al., 2011b; Schwessinger and Ronald, 2012). Therefore BAK1 mutants are strongly impaired in the perception of MAMPs and DAMPs.

1.4 DEFENSE RESPONSES INDUCED DURING PTI

The perception of elicitors triggers a variety of defense responses with different patterns and kinetics depending on the perceived elicitor. The induction of protease inhibitors for example is specifically induced after perception of HAMPs and some DAMPs but not MAMPs (Zebelo and Maffei, 2015; Zhu-Salzman and Zeng, 2015). Nonetheless there is a remarkable overlap by the induced responses and their kinetics. The most common PTI responses will be discussed in the following paragraphs, and an overview of the chronological induction and endurance of PTI responses is given in figure 1.3.

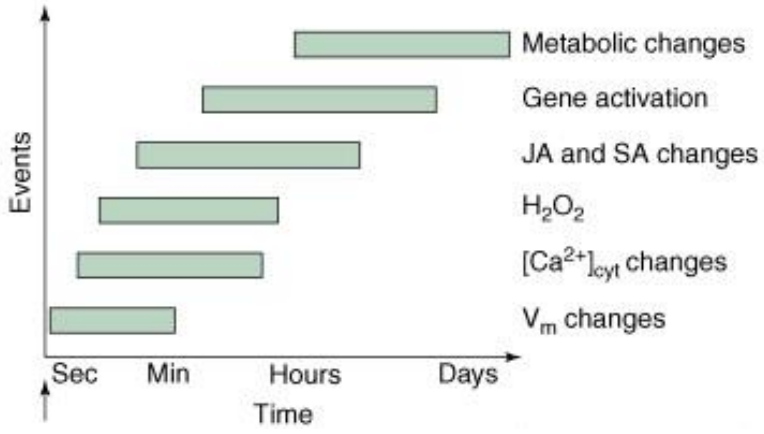

Figure 1.3. The chronology of PTI responses. Consecutive events can be measured after elicitation of plant cells. Altered membrane potentials (V_m) together with Ion fluxes are the earliest events, followed by production of messenger and defense molecules (JA, SA and ROS/H_2O_2). Ultimately gene activation and metabolic changes can lead to lasting adaptations and are induced at about one hour after elicitation. (adapted from Maffei et al. 2007)

1.4.1 Electrical Signaling

Biotic and abiotic stresses lead to an immediate change in the cell membrane potential by modulation of the ion flux at the plasma membrane level (Fürstenberg-Hägg et al., 2013). The *altered membrane potential* can travel through the plant in an action potential-like way and thereby serve as a systemic defense signal. *Membrane* depolarization and ion flux have been measured after OS treatment but not after volicitin and inceptin treatments alone (Maffei et al., 2004; Maischak et al., 2007). The MAMPs elf18 and flg22 have been shown to induce strong membrane depolarization within 1-5 minutes after elicitor treatment and depolarization lasted for around 1 to 1.5 hours (Jeworutzki et al., 2010). The measureable depolarization of the plasma membrane mostly depended on the opening of different ion channels upon biotic stress for example, but some of the produced molecules like H_2O_2 are strong depolarizing molecules (Maffei et al., 2007).

1.4.2 Ion fluxes

Elicitor perception induces the opening of plasma membrane channels and therefore within 1-2 min after a treatment the intracellular concentration of Ca^{2+} strongly increases (Lecourieux et al., 2002). This ion flux is accompanied by the influx of H^+, together with the efflux of K^+ and other anions like Cl^- (Boller and Felix, 2009). That on one

hand leads to depolarization at the plasma membrane and also the influx of H^+ results in alkalinization of the surrounding medium of suspension cultured plant cells (Mithofer et al., 2005). In healthy cells the Ca^{2+} concentration is lowest in cytoplasm, around 10`000 times higher in the apoplast, and up to 100`000 times higher in cellular organelles like the vacuole. This gradient forces the ions to flow quickly into the cytoplasm once ion channels are opened, and once Ca^{2+} gets released into the cytoplasm it serves as a second messenger by activating calcium-sensing proteins, such as calmodulin, calmodulin-like proteins, calcineurin B-like proteins, and Ca^{2+}-dependent protein kinases (CDPKs). As a result a cascade of downstream effects, like altered protein phosphorylation and gene expression patterns, can be detected (Ludwig et al., 2005; Boudsocq et al., 2010)

1.4.3 Oxidative burst

Also within only a few minutes, the enhanced production of ROS by the RESPIRATORY-BURST HOMOLOGUES (RBOHs), that are plasma membrane-bound, can be measured (Torres et al., 2006; Miller et al., 2009). Tremendous amounts of ROS can be produced by the plant (O'Brien et al., 2012), which directly inhibit pathogen or herbivore growth like in the case of toxic H_2O_2 (Peng and Kuc, 1992; Apel and Hirt, 2004), or indirectly by cell wall cross linking (O'Brien et al., 2012). During defense reactions ROS is mainly produced by RBOHD, which has been shown to form a complex with EFR and FLS2 and to be phosphorylated by BIK1 upon elicitor treatments (Li et al 2014). ROS production is also accompanied by down-regulation of ROS scavengers that are normally produced in the cell to protect against toxic ROS produced during photosynthesis or other physiological processes (Apel and Hirt, 2004; Torres et al., 2006; Miller et al., 2009). Furthermore ROS serves as a second messenger by triggering the synthesis of SA and MAPK activation (O'Brien et al., 2012).

1.4.4 Changes in protein phosphorylation and MAPK activation

Cross-phosphorylation between FLS2 and its co-receptor BAK1 is observed immediately after the two receptors associate during flg22 perception and this might be a common feature for LRR-RLKs that assemble with a co-receptor (Chinchilla et al., 2007; Schulze et al., 2010).

The intracellular MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) cascade is a cascade of phosphorylation events that starts with the phosphorylation of a MAP KINASE KINASE KINASE (MAPKKK), which in turn phosphorylates a MAP KINASE KINASE (MAPKK) that then phosphorylates a MAP KINASE (MAPK). MAPKs can activate a variety of transcription factors to alter gene expression, and activate or inactive different proteins by transferring a phosphate group (Meng and Zhang, 2013). Hence MAPK cascades are not only activated after MAMP or DAMP perception but are also involved in signaling during developmental processes (Rodriguez et al., 2010). Phosphorylation and thereby activation of MPK3 and MPK6 for example is used as an assay for defense signaling (Asai et al., 2008; Rodriguez et al., 2010; Galletti et al., 2011). MAPK cascades are also conserved in mammals were they play a role in stress perception too and are generally seen as signal transducers of external stimuli into a cellular response (Kyriakis and Avruch, 2012).

1.4.5 Changes in plant hormone concentrations

Ethylene is an important gaseous plant hormone that is produced during fruit ripening, flowering, or the detection of abiotic and biotic stresses like salt or water stress (Wang et al., 2013). The initiation of PTI responses also comprises the increased production of ethylene that can be measured already after 15 min but lasts up to several hours after elicitor treatments. Perception of elicitors induces the activation of the rate limiting enzyme in the production of the ethylene precursor molecule 1-aminocyclopropane-1-carboxylate (ACC), the ACC synthase. The enzyme gets activated within 10 min and ACC is then later converted by the enzyme ACC oxidase to ethylene (C_2H_4), cyanide (HCN) and CO_2 (Spanu et al., 1994; Ben-Amor et al., 1999). The production of ethylene serves as an important messenger molecule during defense responses and the ETHYLENE RESPONSE FACTOR (ERF) class of transcription factors play a very important role during PTI (Núñez-Pastrana et al., 2013).

Other plant hormones that undergo changes during defense responses are JA, SA, abscisic acid (ABA) and auxin (Denance et al., 2013). Ethylene has been shown to cooperate in various cases with the expression of JA and thereby especially targets defense against nectrotrophic pathogens and herbivore pests (Rojo et al., 2003; Lorenzo and Solano, 2005). JA gets upregulated upon perception of HAMPs and DAMPs

(Klauser et al., 2015). In contrast the hormone SA is rather related to defense against biotrophic pathogens, and it gets upregulated after the perception of various MAMPs (Glazebrook, 2005).

The important growth regulator auxin has been shown to indirectly promote infections, most likely by weakening of the cell wall during tissue expansion, and auxin treatment has been shown to downregulate SA and JA signaling genes (Ding et al., 2008). It seems not surprising that the reversed effect of suppression of auxin signalling during defense responses has also been observed. The perception of flg22 induces the microRNA 393 (miR393) in Arabidopsis, which negatively regulates the RNA levels of the auxin receptor TRANSPORT INHIBITOR RESPONSE 1 (TIR1) (Navarro et al., 2006). Furthermore flg22 induces SA accumulation, which is known to stabilize the Aux/IAA protein and thereby also downregulates auxin-triggered responses (Wang et al., 2007). Moreover also JA has been shown to block growth by interfering with gibberellin signaling. JA stabilizes inhibitory DELLA proteins, and gibberellic acid (GA) responses like cell elongation and seed germination are inhibited (Yang et al., 2012).

The prolonged treatment with MAMPs/DAMPs/HAMPs by supplementing them to liquid growth medium has an inhibitory effect on seedling growth (Boller and Felix, 2009). The so called seedling growth inhibition is also used as a bioassay in plant defense responses and it is believed to depend on the trade-off effect from resource allocation away from growth stimulation to increased defense (Walters and Heil, 2007).

In conclusion most plant hormones have been shown to be either influenced by, or to influence themselves, the defense responses in plants. Especially with regard to physiological adaptations against biotic and abiotic stresses a complex network of plant hormones seems to interact with each other (Denance et al., 2013; Nováková et al., 2014).

1.4.6 Receptor endocytosis

The PRR FLS2 is localized in the plasma membrane but within 20-30 minutes after flg22 binding the receptor gets translocated into endosomes inside the cell (Robatzek et al., 2006). The mechanism of receptor internalization seems to be a common phenomenon and it is a requirement for full signaling strength (Robatzek, 2007; Irani

and Russinova, 2009). In tomato, it has been shown that the internalization of the PRR LeEIX2 upon EIX binding is dependent on a specific Yxxϕ motif in its C-terminus, which is important for clathrin-dependent endocytosis (Bar et al., 2010). Most interestingly mutation of this motif and therefore blocking of endocytosis abolished HR induction after EIX treatments (Ron and Avni, 2004). FLS2 does not contain the same motif but a PEST-like motif which mediates endocytosis after its mono-ubiquitination, and alteration of the motif again abolishes endocytosis (Robatzek et al., 2006).

1.4.7 Transcriptional changes

By altering the gene transcription every cell can control the strength of different cellular pathways, or the production of specific secondary metabolites for example, and short as well as long term responses against abiotic and biotic stresses can be regulated. Around 30 minutes after flg22 and elf26 treatment, about 1000 genes have been found to be up- and further 200 to be downregulated (Zipfel et al., 2004; Zipfel et al., 2006). In the field of gene expression analysis, the fast progress in whole transcriptome/proteome technology highly facilitated data collection and comparison of different treatments on the whole transcriptome level. An important example is the comparison of gene expression patterns between resistant and susceptible plant cultivars. Comparison in the gene expression patterns of two susceptible and resistant wheat (*Triticum aestivum*) cultivars upon *Blumeria graminis* infection showed overlapping expression patterns for only around 50% of the 2978 identified genes; all other genes were significantly differently down, or upregulated (Xin et al., 2012). Similar work done with *S. lycopersicum* infected with tomato leaf curl virus showed that the resistant cultivar in comparison to the susceptible one had less downregulated WRKY genes (only 9 out of 16), a four times higher upregulation of RLKs in the resistant cultivar and also more genes upregulated that are involved in the production of phenolic compounds (Chen et al., 2013). Such experiments show the important role of proper gene regulation during defense responses.

1.4.8 Callose deposition

After MAMP treatment, for example with flg22 or chitosan, accumulation of callose ((1,3)-β-glucan) deposits can be observed within Arabidopsis leaves (Gomez-Gomez et al., 1999). Callose is located in so called papillae, which stretch from the plasma membrane to the cell wall. Whilst the role of callose is yet unclear its deposition goes hand in hand with that of phenolic compounds, ROS, cell wall proteins and polymers which have a clear antimicrobial or cell wall reinforcing function (Voigt, 2014).

1.6 THE PROPEP-PEP-PEPR SYSTEM

1.6.1 PLANT ELICITOR PEPTIDEs (PEPs)

In 2006, Alisa Huffaker and co-workers found that a specific fraction of extracts prepared from Arabidopsis leaves induces medium alkalinization when added to cultured Arabidopsis cells (Huffaker 2006). A 23 aa peptide termed PLANT ELICITOR PEPTIDE 1 (PEP) was identified as the active compound of the fraction. *At*PEP1 is derived from the C-terminus of the 96 aa precursor protein *At*PROPEP1 (which will be described in more detail in the following chapter, figure 1.4). Synthetic *At*PEP1 activates many PTI-associated responses when added to seedlings or plant parts, like medium alkalinization, ethylene, nitric oxide (NO) and ROS production, calcium influx, MAPK activation, the production of cGMP, increased JA levels, and expression changes of numerous genes already at low nanomolar concentrations (Huffaker et al., 2006; Krol et al., 2010; Flury et al., 2013; Ma et al., 2013; Gully et al., 2015; Klauser et al., 2015). Soon after the discovery of *At*PEP1 two receptors that bind AtPEP1 were identified, the LRR-RLKs *At*PEPR1 and *At*PEPR2 (which will be described in more details below) (Yamaguchi et al., 2006; Krol et al., 2010; Yamaguchi et al., 2010). *At*PEP1 and *At*PEP5 have been found in tissue extracts but in total seven *At*PEPs and their associated PROPEPs (figure 1.4) have been identified in the genome of Arabidopsis by *in silico* analysis, and all of them have been shown to induce similar responses when added as synthetic peptides (Huffaker et al., 2006; Yamaguchi et al., 2010).

The field of PEP research intensified when PEP orthologues have been identified first in *Zea mays* and subsequently in many other plant species including important crop species

(Huffaker et al., 2011; Huffaker et al., 2013). Elicitor activity of PEPs has then been shown in various species together with the induction of plant resistance against the bacterial pathogen *Pst* DC3000, and the fungal pathogens *Cochliobolis heterostrophus* and *Colletotrichum graminicola* (Yamaguchi et al., 2010; Huffaker et al., 2013). But not only microbial defenses were efficiently induced, *Zm*PEP3 treatment has been shown to mediate defense against *S. exigua* larval feeding (Huffaker et al., 2013). The importance of the PEP-PEPR system has been shown in Arabidopsis by the decreased resistance against *S. littoralis* feeding on *pepr1 pepr2* mutant plants which are fully insensitive to PEPs (Klauser et al., 2015). Lastly the induction of JA synthesis is a trait more exclusive to HAMPs and some DAMPs to induce herbivore specific responses; MAMPs mostly induce SA rather than JA to mediate resistance against pathogens and to mediate SAR (Zhang et al., 2010). Herbivore specific defense responses but also microbial defenses often involve the production of secondary metabolites which have been analysed in more detail in maize. *Zm*PEP1 treatment induced the production of anthranilate and indole, precursors in the production of compounds in the benzoxazinoid hydroxamic acid defense, including for example DIMBOA-Glc (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside), whose amount was also increased in *Zm*PEP1 treated plants (Huffaker et al., 2011). DIMBOA-Glc is an antibiotic substance that protects plants against bacterial and fungal pathogens, and insect pests. In addition also *Zm*PEP3 has been found to induce indole and HDMBOA-Glc (2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one glcoside) a substance similar to DIMBOa-Glc (Huffaker et al., 2013). Both *Zm*PEPs furthermore induced the production of VOC, that serve as an anti-herbivore response by attracting herbivore predators and indeed a highly significant increase of parasitoids on *Zm*PEP3 treated plants has been shown (Huffaker 2013). Together with the induction of sesquiterpenes the role of PEPs involves in contrast to most MAMPs, the induction of several herbivore specific defense responses. This hypothesis is supported by the finding that PEPs not only of maize but of various species induce VOCs and that PROPEP expression is strongly induced by application of oral secretions of *S. exigua* and *S. littoralis* (Huffaker et al., 2013; Klauser et al., 2015).

1.6.2 PROPEPs

*At*PROPEP1 includes the sequence of *At*PEP1 within its C-terminus (figure 1.4) and was thus identified as the precursor protein of the *At*PEP1 peptide (Huffaker et al., 2006). *At*PROPEP1 is 96 aa in size and carries a large number of positively charged aa residues but lacks any known signal sequences. As mentioned, seven genes encoding *At*PROPEPs have been identified in the Arabidopsis genome. The individual *At*PROPEPs share rather low aa sequence homology; thus in silico searches were performed based on the conserved PEP motif in the C-terminus in order to find additional *At*PROPEPs. Overexpression of *At*PROPEP1 in Arabidopsis led to increased root branching and enhanced resistance against the oomycete *Pythium irregulare*. Like the PEPs identified in other species, also the corresponding PROPEPs gained attention in the respective species (Huffaker et al., 2011; Huffaker et al., 2013). Silencing of *SlPROPEP* was the first study performed on *Solanum lycopersicum* PROPEP (Trivilin et al., 2014). As a consequence of *SlPROPEP* silencing the plants showed weaker expression of defense genes and were highly attenuated in their resistance against the fungus *Pythium dissotocum.*

With regard to the expression of the different *PROPEP* genes, most of them seem to be inducible in a positive feedback by PEP perception as well as by wounding and JA treatment (Huffaker et al., 2006; Huffaker et al., 2011; Huffaker et al., 2013; Ross et al., 2014). Whilst some PROPEPs like AtPROPEP4, 5 and 6 seem to be generally very weakly expressed and are not inducible by JA and PEPs, other PROPEPs like *At*PROPEP1, 2 and 3 and *Zm*PROPEP3 are highly responsive to treatments inducing defense responses. *Sm*PROPEP, *Gm*PROPEP, *Zm*PROPEP3 and *At*PROPEP3 for example were highly induced upon OS treatments, whereas *At*PROPEP1 and *Zm*PROPEP1 responded strongest to fungal pathogen treatments (Liu et al., 2013; Klauser et al., 2015).

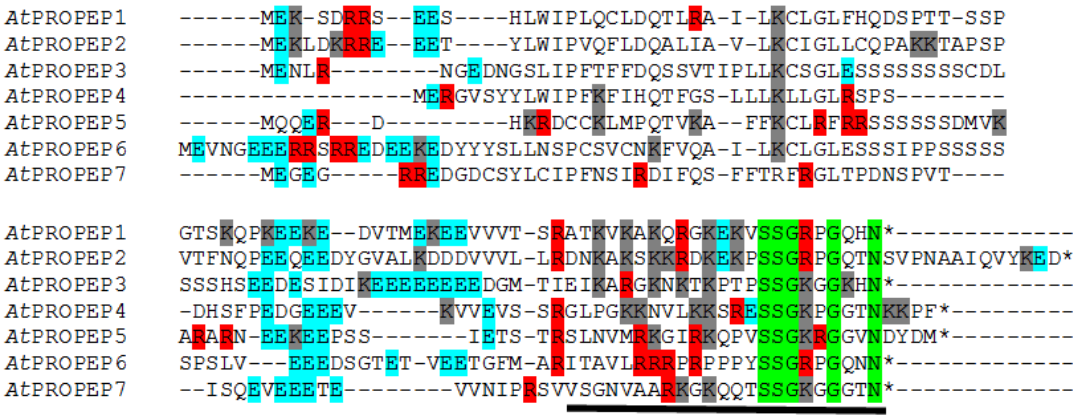

Figure 1.4. Clustal Omega alignment of the amino acid sequence of *At*PROPEPs 1-7. *At*PEP1 and 5 were initially identified by mass spectrometry and found to reside in the C-terminal region of their respective PROPEPs (underlined in black). The putative conserved SSGR/KxGxxN motif (highlighted in green) was used to identify further paralogues in the genome.

1.6.3 PEPRs

*At*PEPR1 was initially identified by its affinity to labeled *At*PEP1, and it has been confirmed as a functional receptor by enabling the perception of PEP in PEP-insensitive *N. tabaccum* (Yamaguchi et al., 2006). Since Arabidopsis mutants in PEPR1 (*pepr1*) only partially lost their responsiveness to PEP treatments, a second PEP receptor was hypothesized. A few years later *At*PEPR2, which shares 76% sequence homology to *At*PEPR1, was identified, the *pepr1 pepr2* double mutant is completely insensitive to PEPs (Krol et al., 2010; Yamaguchi et al., 2010). *AtPEPR1* and *AtPEPR2* are induced by wounding, JA application, *At*PEPs, MAMPs, OS and herbivore feeding but *AtPEPR2* induction is mostly weaker than that of *AtPEPR1* (Klauser et al., 2015). None of the two is induced by SA as it is the case for their ligand precursor *AtPROPEP1* (Huffaker et al., 2006).

AtPEPR1 and 2 are transmembrane LRR-RLKs of around 170 kDa in size, but in contrast to FLS2 and EFR they belong to the RD-kinases. Both *At*PEPRs belong to the class LRR-XI RLKs which also includes various receptors associated with plant development, like for example CLAVATA1 (CLV1), the receptor of the CLE peptides (Wang and Fiers, 2010). *At*PEPR1 carries 26 LRR and *At*PEPR2 25 LRR-motifs in their extracellular ligand binding domain (Yamaguchi et al., 2006; Yamaguchi et al., 2010). Both receptors carry a guanyl cyclase (GC) domain which leads to the production of cGMP, an important messenger molecule (Qi et al., 2010; Ma et al., 2012). cGMP

activates Ca^{2+} channels, and the calcium influx normally observed after PEP perception was blocked in *At*PEPR1-GC mutants. Surprisingly it has been found that FLS2 mediated calcium influx, that is not cGMP mediated and originates from the vacuolar pool of calcium, whilst activation of PEPRs leads to influx of extracellular calcium due to cGMP activation of plasma membrane bound Ca^{2+} channels (Ma et al., 2012).

Both *At*PEPRs have a functional RD-kinase domain, but nevertheless they assemble with the co-receptor kinase BAK1, after PEP binding (Schulze et al., 2010; Tang et al., 2015). Previous studies with alanine substitutions in the *At*PEP1 aa sequence have shown that serine 15 and glycine 17 are crucial for *At*PEP1 activity and that the glycine 17 substituted *At*PEP1 is unable to compete for PEPR binding with *At*PEP1 (Pearce et al. 2008) . The determination of the crystal structure of the *At*PEP1-*At*PEPR1 complex confirmed that Serine 15, Glycine 17 and Arginine 23 (figure 1.4) are crucial for *At*PEP1 binding to the *At*PEPR1 LRRs, whilst in a model proline 19, glutamine 21 and histidine 22 support the complex formation of *At*PEPR1 and *At*BAK1 upon *At*PEP1 binding (Tang et al., 2015).

Surprisingly, instead of BAK1 being important for signal transduction in the PEPR/BAK1 complex, BAK1 seems to be rather some sort of a regulator of PEPRs. BKK1 and BAK1 are known as important negative regulators of cell death, and it has been shown that whilst the phenotype of *bak1-4* or *bkk1-1* single mutants looked similar to wildtype plants, the double mutant *bak1-4/bkk1-1* develops a lethal phenotype (He et al., 2007). This phenotype has been found to be partially restored in the *pepr1 pepr2* double mutant, indicating that PEPRs might play a role in cell death regulation (Yamada et al., 2016). That assumption is further supported by the fact that the lack of BAK1 leads to increased cell death in plants after *At*PEP2 treatments instead of an attenuation and also other defense responses were increased.

1.7 FURTHER SIGNALING PEPTIDES

A growing number of small signaling peptides have been identified in recent years also thanks to increasing technical advances, for example in quicker mass spectrometry analysis with much higher throughput. Since the discovery of Systemin, the very first signaling peptide in plants, in 1991 by Pearce and colleagues, plenty of peptides with a

plethora of functions were described in plants and are shown in a schematic overview in figure 1.6 (Tavormina et al., 2015).

Peptides, defined as proteins smaller than 100 aa, they execute important signaling functions in all areas of physiological processes including all aspects of plant development as well as reactions to abiotic and biotic stresses. In the field of plant defense, peptides do not exclusively serve as signaling peptides but can also have direct antimicrobial or protease inhibitor functions and are best categorized based on their

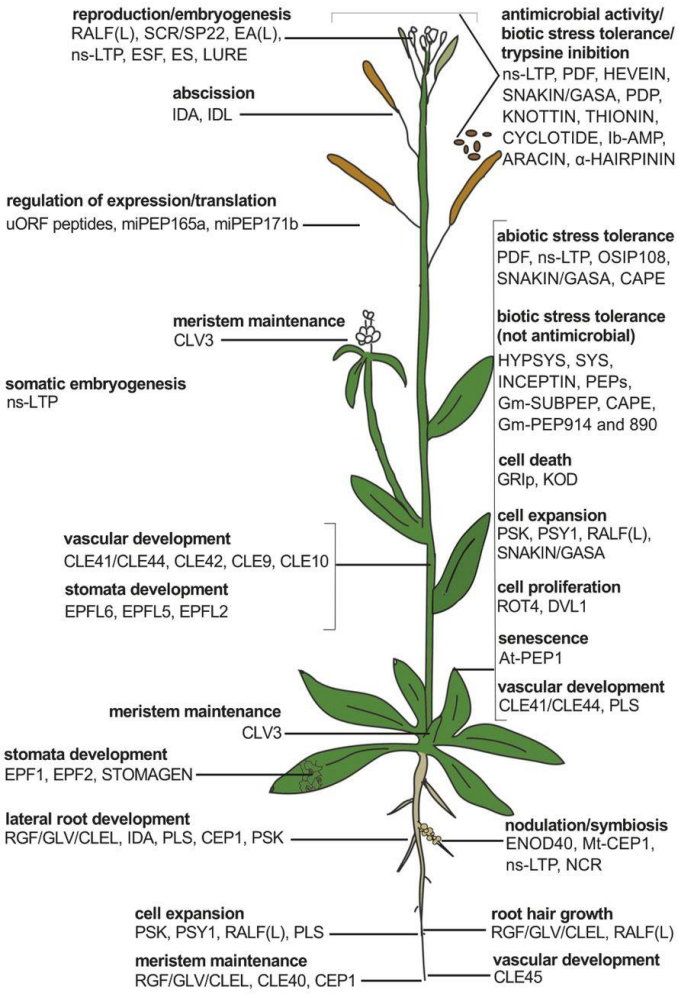

Figure 1.6. Diversity of Plant Peptides. Numerous peptides are produced by plants with biological functions as signaling peptides or with secondary functions in defense for example. (adapted from Tavormina et al. 2015)

origin (Tavormina et al., 2015). Most peptides are expressed as larger proteins without any additional function and get further processed for the generation of the mature peptide. *Gm*SubPEP and Inceptin peptides are exceptions, since they derive from proteins with different primary functions. Protease inhibitor peptides like

CYCLOTIDES, peptides with antimicrobial function like PLANT DEFENSINs (PDF), or some developmental regulators like CLE peptides are mostly expressed as preproteins or preproproteins (Jun et al., 2008; Craik and Malik, 2013; De Coninck et al., 2013; Lay et al., 2014). Preproteins carry an N-terminal secretion signal (NSS) that guides the peptide to the secretory pathway where it is active without further processing, whereas preproproteins undergo a further processing to release the peptide from the secreted proprotein (Tavormina et al., 2015). Proproteins, to which also the PROPEPs belong, as the name suggests, lack an NSS but get processed to release the peptide from the precursor and are potentially secreted by an unconvential secretory pathway either before or after processing (Ding et al., 2012).

The first fully described pathway from secretion to processing and downstream signaling was just recently described for the Arabidopsis GRIM REAPER peptide (GRIp) (Wrzaczek et al., 2009; Wrzaczek et al., 2015). The preproprotein GRIM REAPER (GRI) is 169 aa in size and aa 1-30 serve as an NSS. In the extracellular space it co-localizes with the type II metacaspase 9 that cleaves GRI at arginine (R) 67 in the motif serine-lysine-threonine-arginine (SKTR), at lysine (K) 97 in the motif lysine-alanine-asparagine-lysine (KANK), and at lysine 78 in the motif lysine-lysine-isoleucine-lysine-lysine (KKIKK) leading to the release of the 11 aa GRIM REAPER peptide (GRIp). Whilst the precursor GRI is unable to bind to the LRR-RLK POLLEN SPECIFIC RECEPTOR-LIKE KINASE 5 (PRK5) GRIp binds PRK5 and thereby induces ROS-dependent cell death (Wrzaczek et al., 2015).

For the precursor ProSystemin it has been shown, that activity depends only on the amino acids motif and therefore the precursor showed full activity already without further processing to the Systemin peptide (Dombrowski et al., 1999). Other signaling peptides like the CLE peptide CLAVATA3 have been shown to be processed in vitro, but no protease or inducing conditions were specified (Ni and Clark, 2006).

1.8 PLANT PROTEASES

Proteases are enzymes that are able to hydrolyze peptide bonds either within a protein, as endoproteases, or at the ends of proteins as exoproteases (Pesquet, 2012). In plants, five classes of endoproteases have been described: serine, cysteine, metallo, aspartic,

and threonine protease (Rawlings et al., 2014). Exoproteases are classified based on their specificity as aminopeptidases, that cleave proteins at the N-terminus, or carboxypeptidases, that cleave proteins at the C-terminus. In the MEROPS protease database 764 putative protease are currently listed to be present in the Arabidopsis genome (Rawlings et al., 2014). The cysteine protease class metacaspases have been shown to play important roles in cell death regulation and to be crucial for HR induction during pathogen infections (Watanabe and Lam, 2011a) . Overexpression of the aspartic protease CDR1 has been shown to be involved in a so far unknown upregulation of defense responses and many proteases have been found to be upregulated during infections or defense responses (van der Hoorn and Jones, 2004; Simoes et al., 2007; Hatsugai et al., 2015). The way in which a protease contributes to resistance is mostly unknown. Current hypotheses propose three different ways in which proteases could correspond to defense mechanisms (van der Hoorn and Jones, 2004). First, proteases could facilitate activation of PTI by releasing elicitors from pathogens. Second, they might act as positive regulators by releasing signaling peptides like Systemin or PEPs, and third, they might directly act on pathogen-derived proteins like crucial effectors and degrade them (discussed in more detail in van der Hoorn 2004).

1.9 AIMS OF THE THESIS

In the field of PEP research numerous responses to perception of synthetic PEP peptides have been described. Moreover, qPCR based expression data of some of the *PROPEP*s and both *PEPR*s have been collected but an enormous gap remains between PROPEP transcription in the beginning and eventually the perception of mature PEP peptides. The quite high number of seven identified PROPEPs in Arabidopsis might point either to genetic and functional redundancy or to independent functions of these genes and their encoded proteins due to specific expression and localization patterns. To gain understanding in the potential involvement of PROPEPs in different physiological processes, a first aim was to study the expression patterns and localization of Arabidopsis PROPEP proteins *in planta*. In addition, *in silico* analysis of available expression data was used to analyze potential biotic and abiotic influences on the expression of various *PROPEP*s, whilst the use of GUS reporter lines should lead to the identification of tissue-specific expression patterns. Furthermore the overexpression of

some PROPEPs translationally fused to YFP were used for determination of its subcellular localization. The overall aim of this part is to identify differences between individual PROPEPs to either prove or disprove a potential redundancy of the seven identified PROPEPs in Arabidopsis.

To further fill the gap between *PROPEP* transcription and PEP perception, the circumstances as well as the mechanism of PEP genesis are addressed. The isolation of *At*PEP1 and 5 from tissue extracts indicated a processing of PROPEPs but if PROPEPs are processed continuously or only upon damage during the extract preparation has not been revealed. Furthermore, the enhancer theory suggests PEPs to serve as amplifiers of previous defense responses like the perception of MAMPs, thus mature PEPs might be generated during defense responses as well. However, data supporting this hypothesis is lacking. Here, the circumstances of PROPEP processing was experimentally addressed using a variety of danger-associated treatments. In addition the application of various protease inhibitors might point to specific proteases that perform PROPEP processing and thus play a role in innate immunity as well.

Finally PEP research focused on Arabidopsis PEPs although sequence analysis indicated the presence of orthologues in other plant species. Recently elicitor activity of *Zea mays* PEP1 has been demonstrated but preliminary data from our group indicated a missing interspecies compatibility of PEPs. Thus the identification of PEPs within the plant kingdom based upon comprehensive sequence analysis was performed. Intra- and interspecies elicitor activity of novel PEPs was determined and species specific amino acid motifs were deduced. Eventually corresponding PEPRs, which have not been described yet in other species, will be cloned and characterized.

Taken together this work aims to characterize the PROPEP-PEP-PEPR system in more detail, to fill the gap between PROPEP transcription and PEP perception and to underline undermine the biological relevance of this system by demonstrating the presence and activity of this system in many plant species across the plant kingdom.

2. THE FAMILY OF *AT*PEPS AND THEIR PRECURSORS IN ARABIDOPSIS: DIFFERENTIAL EXPRESSION AND LOCALIZATION BUT SIMILAR INDUCTION OF PATTERN-TRIGGERED IMMUNE RESPONSES

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This work was prepared in collaboration with the team of Silke Robatzek. The aim was to analyze the potential redundancy of PROPEPs in Arabidopsis. Various PROPEPs were investigated for their specific expression patterns under various conditions as well as the tissue specific expression and subcellular localization were determined. I contributed on the experimental setup, prepared most of the transgenic lines used in this study and contributed in the evaluation of the results.

2.1 ABSTRACT

In *Arabidopsis thaliana*, the endogenous danger peptides *At*Peps have been associated with plant defenses reminiscent to those induced in pattern-triggered immunity. *At*Peps are perceived by two homologous receptor kinases, PEPR1 and PEPR2, and are encoded in the C-termini of the PROPEP precursors. Here we report that, contrary to the seemingly redundant *At*Peps, the PROPEPs fall at least into two distinct groups. As revealed by promoter-GUS studies, expression patterns of *PROPEPs 1-3, 5* and *8* partially overlapped and correlated with those of the *PEPR1* and *PEPR2* receptors, whereas the ones of *PROPEPs 4* and *7* do not share any similarities with the former. Moreover, bi-clustering analysis indicates an association of *PROPEPs 1*, *2*, and *3* with plant defense, whereas *PROPEP5* expression was related to patterns of plant reproduction. Also at the protein level, PROPEPs appeared to be distinct. PROPEP3::YFP was present in the cytosol, but in contrast to previous predictions PROPEP1::YFP and PROPEP6::YFP localized to the tonoplast. Together with the expression patterns, this could point to potentially non-redundant roles between the members of the PROPEP family. By contrast, their derived *At*Peps including a newly reported *At*Pep8, when exogenously applied, provoked activation of defense-related responses in a similar manner suggesting a high level of functional redundancy between the *At*Peps. Taken together, our findings reveal an apparent antagonism between *At*Pep-redundancy and PROPEP variability and indicate new roles for PROPEPs beside plant immunity.

2.2 INTRODUCTION

Danger- or damage-associated molecular patterns (DAMPs) are diverse molecules, which trigger the immune system upon perception (Scaffidi et al., 2002; Seong and Matzinger, 2004; Ahrens et al., 2012). Unlike microbe/pathogen-associated molecular patterns (MAMPs/PAMPs), which originate from microorganisms, DAMPs are endogenous molecules of the host (Boller and Felix, 2009). In animals, DAMPs can be produced in the context of damage as degradation products of proteins, DNA or the

cytoskeleton (Ahrens et al., 2012; Pisetsky, 2012), or they are signals associated with danger and thus are actively released (Wang et al., 1999). The latter DAMPs are reminiscent of cytokines such as interleukins, which are processed and released upon an imminent threat, for example the detection of MAMPs (van de Veerdonk et al., 2011). In plants, much less is known about potential DAMPs or cytokine-like proteins. Paradigms of plant DAMPs are cell wall degradation products such as oligogalacturonides (OGs), which trigger PTI (pattern-triggered immunity) upon detection (Rasul et al., 2012). They are released by the activity of microbe-secreted cell wall degrading enzymes and perceived by transmembrane PRRs (pattern recognition receptors) (D'Ovidio et al., 2004). Beside these prototype DAMPs, endogenous peptides have been identified that trigger a PTI-like response as well.

The Systemins from the Solanoideae, a subfamily of the Solanaceae that comprises e. g. tomato (*Solanum lycopersicum*), pepper (*Capsicum annuum*) and potato (*Solanum tuberosum*), were first identified to induce the accumulation of proteinase-inhibitors, a typical anti-herbivore response, and later connected to the regulation of diverse defense responses (Pearce et al., 1991; McGurl et al., 1992; Ryan and Pearce, 2003). Tomato Systemin is an 18 amino acid (aa) long peptide processed from the 200 aa precursor protein called Prosystemin (PS). Despite many years of Systemin research the Systemin receptor is still a matter of debate (Holton et al., 2008; Lanfermeijer et al., 2008; Malinowski et al., 2009). Recently, the *PS* gene from tomato was shown to be expressed mainly in floral tissues, especially pistils, anthers and sepals, and only at lower levels in leaves. Treatment of leaves with methyl jasmonate (MeJA) led to a strong induction of *PS* expression (Avilés-Arnaut and Délano-Frier, 2012). Similar to the expression patterns, PS protein was constitutively found in floral organs including sepals, petals and anthers as well as in the vascular phloem parenchyma cells of leaves and stems, where it localizes to the cytosol and the nucleus (Narváez-Vásquez and Ryan, 2004).

Endogenous DAMP- or cytokine-like peptides have also been found in *Arabidopsis thaliana*. Two of these 23 aa peptides, *At*Pep1 and *At*Pep5, have been purified from Arabidopsis leaf protein extracts (Huffaker et al., 2006; Yamaguchi and Huffaker, 2011). They belong to a small family of seven homologous peptides, which comprise the C-terminal part of seven small precursor proteins called PROPEPs (Huffaker and Ryan, 2007). It is believed that the PROPEPs are cleaved to release the *At*Peps which in turn are perceived by the two homologous receptor-like kinases PEPR1 and PEPR2

(Krol et al., 2010; Yamaguchi et al., 2010). Upon detection, the PEPRs trigger a set of responses reminiscent of PTI including induced resistance against subsequent infections with virulent *Pseudomonas syringae* bacteria (Huffaker and Ryan, 2007; Krol et al., 2010; Yamaguchi et al., 2010). In addition to the classical PTI-associated responses, recent data show that treatment with *At*Pep3 led to an increase in cytosolic cyclic GMP (cGMP) suggesting that *At*Peps activate cGMP-dependent signaling pathways (Qi et al., 2010).

To date, little is known about the expression, localization and function of the PROPEPs. The expression of a number of *PROPEPs* is induced upon treatment of Arabidopsis leaves with methyl jasmonate (MeJA), methyl salicylate (MeSA) as well as MAMPs and *At*Peps (Huffaker and Ryan, 2007). At the cellular level, PROPEPs are thought to reside in the cytosol and to be exported to the extracellular space via an unconventional secretion system as the PROPEPs carry no known secretion or subcellular localization signals (Yamaguchi and Huffaker, 2011; Ding et al., 2012).

In this study, we focused on the PROPEPs including an additional, eighth member of the PROPEP family in Arabidopsis, reported here for the first time. Our data demonstrate that all eight *At*Peps elicit PTI-type responses in a similar manner and depend on the PEPR1/2 receptor pair revealing greatly functional redundancy. By contrast, bi-clustering analysis, promoter-GUS expression and PROPEP::YFP localization studies identified significant tissue-specific differences and subcellular patterns that highlight potentially non-redundant properties of the precursors. Furthermore, our data led to the idea that some PROPEPs might play a role in plant development and reproduction, in addition to their described function in plant immunity.

2.3 RESULTS

AT5G09976 is a novel member of the Arabidopsis *PROPEP* family

In order to gain insights into the sequence homology of PROPEPs compared to other precursors of plant signaling peptides, we searched the Arabidopsis genome and identified AT5G09976 as a new member of the PROPEP family. It clusters with the other seven PROPEPs despite an overall low sequence homology, and its C-terminus contains the conserved *At*Pep motif SSG-x_2-G-x_2-N (Fig. 2.1A). According to sequence

similarity PROPEP4 is the closest homolog of AT5G09976. Moreover, addition of a synthetic peptide based on the last 23 aa of AT5G09976 (Fig. 2.1A, highlighted with red bar) triggers similar responses in Arabidopsis plants like the other *At*Peps (see below). Thus, we designate AT5G09976 as *PROPEP8*. Further searches for non-annotated sequences with similarity to the PROPEPs did not reveal any further PROPEP in Arabidopsis.

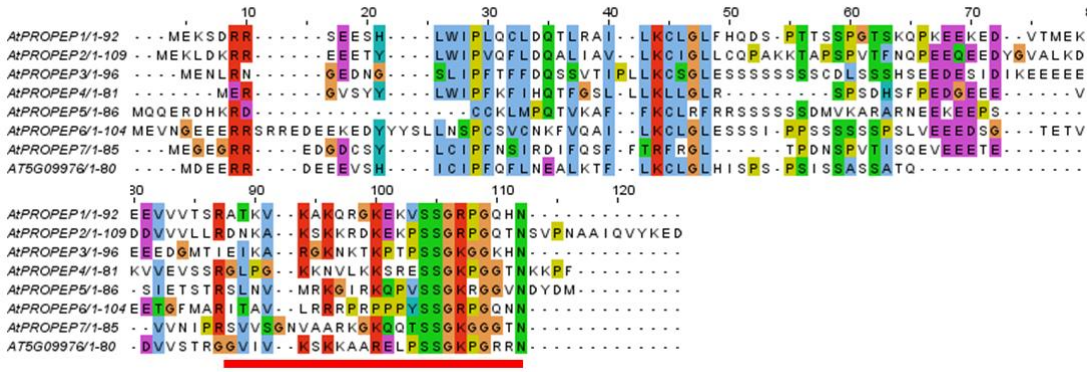

Figure 2.1: Alignment of the eight Arabidopsis PROPEPs. ClustalW alignment of the amino acid sequences of all identified Arabidopsis PROPEPs including AT05G09976. Coloring is based in the Clustal color scheme.

Bi-clustering expression analysis points at distinct roles for individual *PROPEPs*

PROPEPs are thought to assist via the release of *At*Peps in biotic stress resistance, but their individual roles have not been investigated in detail (Huffaker and Ryan, 2007; Boller and Felix, 2009). Whereas *At*Peps are assumed to act rather redundantly, little is known about the spatial and temporal expression of *PROPEPs*. It has been shown that *PROPEPs* respond with slight differences to treatments with MeJA, MeSA and *At*Peps (Huffaker and Ryan, 2007). In order to get a better idea about potential redundant as well as specific expression patterns of the *PROPEPs* in the context of biotic stress resistance, we performed a bi-clustering analysis focusing on 278 biotic stress-related microarrays that were downloaded from the TAIR website (ftp.arabidopsis.org/Microarrays/analyzed_data). Hereby the 22810 probes (representing genes) present on the used Affymetrix 25K arrays are grouped based on their expression patterns over the various biotic stress treatments. Genes with similar expression patterns group more closely together, as indicated by the dendrogram, and become part of a sub-group (sub-clusters). Enrichment of GO-terms within one sub-cluster can be used to get

indications about the function of the genes in this sub-cluster. Moreover, the relative distance of genes within the main cluster shows the diversity of regulation of these genes.

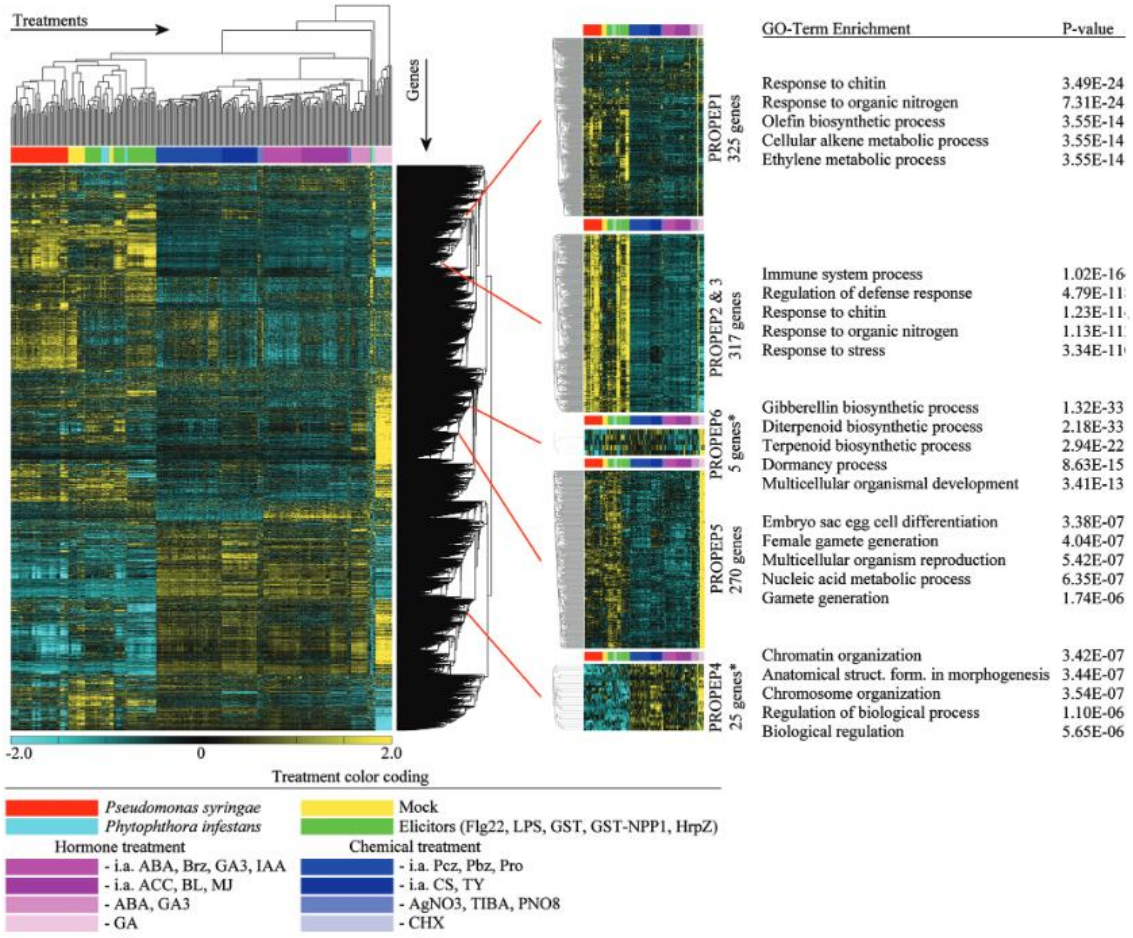

Figure 2.2: Bi-clustering analysis of *PROPEPs* based on expression profiles of biotic stress treatments. The similarity in expression pattern of 22810 different probes (representing genes) was assessed by performing a bi-clustering analysis of the Log2 transformed expression values from 278 biotic stress related microarrays (upregulated genes are represented in yellow whereas downregulated genes are colored blue). The different types of treatments within this bi-clustering analysis are color coded above the clusters, with their details at the bottom of the figure. For multiple treatments typical examples are given as not each individual treatment could be color coded clearly. A full list of all treatments, including the dendrogram and the same color coding, is given in Supplemental Table 2. Genes that cluster relatively close are expressed similarly under various biotic stresses and vice versa. Only *PROPEP* 2 and *3* cluster very close together, suggesting that only those two *PROPEPs* are involved in similar processes under biotic stress. To obtain an indication in which processes each *PROPEP* is involved, GO-Term enrichment was performed on each sub-cluster containing a *PROPEP* (represented as separate clusters). The top five enriched GO-Terms of the sub-cluster, indicating the related processes, is shown to the right of each sub-cluster. Asterisks denote sub-clusters that showed no enriched GO-terms, therefore co-expressed genes with the *PROPEP* having a Pearson correlation coefficient > 0.6 were used for GO-term enrichment analysis.

PROPEP containing clusters were selected by setting an individual cutoff within the dendrogram for each *PROPEP* to yield a cluster with less than 500 genes (Fig. 2.2).

PROPEP7 and *PROPEP8* are not spotted on the used arrays, and therefore no bi-cluster analysis could be performed for these precursors. Of the family members *PROPEP1-6*, only *PROPEP2* and *PROPEP3* clustered together indicating that most of the *PROPEPs* are expressed in different ways upon treatment with various biotic stimuli. To get an indication in which processes the individual *PROPEPs* are involved in, a GO-term enrichment analysis was performed on the obtained clusters of which the top 5 terms are shown in Figure 2.2. Most enriched GO-terms within the top 5 of each cluster represent a relatively broad description of a process. To also provide data on the more specific processes that are underlying the expression of these clusters a full overview of all major and minor enriched GO-terms for each *PROPEP* is provided in Supplemental Table S1. As most *PROPEPs* appear to be involved in very different processes besides biotic stress resistance, also a co-expression analysis followed by a GO-term analysis on a set of abiotic or development related microarrays was performed (Supplemental Table S2). These results further support the idea that *PROPEP* transcription seems to be regulated individually and does not follow a general pattern valid for all *PROPEPs*.

The *PROPEP* that has the most similar global expression pattern compared to *PROPEP2* and *3* is *PROPEP1*, but beside the shared enriched defense associated GO-terms (Fig. 2.2), they also have some characteristically different enriched GO-terms. The *PROPEP1* cluster revealed an additional enrichment of GO-terms related to abiotic stress, hypoxia and ABA-signaling, whereas the *PROPEP2* and *3* cluster is also associated with SA-signaling, (programmed) cell death and (trans-membrane) ion transport (Supplemental Table S1 and S2). Distinct from these more defense-associated *PROPEPs* is the cluster of *PROPEP5* that is enriched for processes related to reproduction and shares the enrichment for gibberellin/terpenoid biosynthesis and lipid-signaling with *PROPEP6.* The most directly noticeable different *PROPEP* in Figure 2.2 is *PROPEP4* which expression is induced in conditions where all the other *PROPEPs* are repressed and vice versa. As the cluster of *PROPEP4* contained only 25 genes it was too small to result in any enriched GO-terms. To circumvent this, the genes that are co-expressed with *PROPEP4* given a Pearson correlation coefficient cutoff > 0.60 were used to perform a GO-term enrichment analysis. This resulted in an enrichment of organismal development, developmental processes and chromosome/chromatin organization associated GO-terms.

To further diversify the view on *PROPEP* regulation, we also analyzed the type of treatments and or conditions that have the strongest influence on the expression of each *PROPEP*, a full overview of treatments, conditions and their influence on expression is given in Supplemental Table S3. Here we found in agreement with the bi-clustering analysis *PROPEP1* to be highly induced by abiotic stress treatments like salt, drought and osmotic stress whereas e. g. *PROPEP5* transcription is highest in certain developmental stages of seeds and flowers.

Overall our analysis indicates that, in contrast to *At*Peps, the transcriptional regulation of *PROPEPs* is most likely non-redundant. Moreover, based on these findings we suggest that individual PROPEPs could play a role in very distinct functions in *Arabidopsis* as they appear to be not only associated with defense but also with processes ranging from abiotic stress resistance to development and reproduction.

Analysis of *PROPEP* promoters reveals diverse spatial and temporal expression patterns

To further investigate the potential difference in *PROPEP* expression at the tissue level, we generated transgenic Arabidopsis lines containing the putative promoter sequences of the *PROPEP* genes fused to β-glucuronidase (GUS). As shown in Figure 2.3, the promoters of *PROPEP1*, *PROPEP2* and *PROPEP3* exhibit similar expression patterns. These promoters confer expression mainly in in the root excluding the root tip. In adult leaves even after 24 h of staining nearly no blue precipitate is visible indicating very low activity of these promoters without stimuli. In contrast, wounding of leafs using a forceps led to a clear induction of these *PROPEP* promoters, which was restricted to the vasculature (Fig. 2.3, yellow arrows). Beside the great overlap between the expression patterns of the promoters of *PROPEP1*, *PROPEP2* and *PROPEP3*, the latter produced also a GUS staining in the anthers of flowers.

The promoters of *PROPEP5* and *PROPEP8* are also active in the root but restricted to the vascular tissue, reminiscent of the promoter of *PEPR2* (see below). They share with the promoters of *PROPEP1-3* the wound inducibility in the central vasculature of adult leaves. But whereas the promoter of *PROPEP5* shows strong activity in the leaf-veins, the promoter of *PROPEP8* did not produce any GUS staining in untreated leaves. In addition, they produced distinct stainings in adult flowers. The promoter of *PROPEP5* is highly active in the filaments of flowers (Fig. 2.3, white arrow) whereas the one of

PROPEP8 is active in all flower tissues except for the petals. Thus, the promoters of *PROPEP5* and *PROPEP8* partially share their expression patterns with the ones of the promoters of *PROPEP1-3* but show also differences to them and among each other.
Intriguingly, the activity of the promoters of *PROPEP4* and *PROPEP7* are restricted to the tips of primary and lateral roots (Fig. 2.3, red arrows), whereas neither the other *PROPEP* promoters nor the promoters of *PEPR1* and *PEPR2* (see below) conferred any obvious GUS expression. Moreover, the expression of the promoters of *PROPEP4* and *PROPEP7* was not detected in flowers and not induced by wounding.
Taken together, the promoter-mediated expression patterns of the *PROPEP*s fall clearly into two distinct groups. Group one, which comprises the promoters of *PROPEP1*, *2, 3, 5* and *8*, shows expression in the roots and slightly in the leaf vasculature. They are inducible by wounding. Group two, containing the promoters of *PROPEP4 and 7*, is not inducible by wounding and the basal expression is restricted to the root tips.

PROPEP::YFP fusions identify localization to distinct subcellular compartments

Next we generated transgenic Arabidopsis plants constitutively expressing PROPEP::YFP fusion proteins to assess their subcellular localization. It has been hypothesized that all PROPEPs localize to the cytoplasm based on the predicted function and the lack of an identifiable localization signal (Huffaker et al., 2006). In line with this hypothesis, PROPEP3::YFP localized to the cytoplasm (Fig. 2.4A). However, our findings with PROPEP1::YFP and PROPEP6::YFP were rather surprising and showed that these precursor proteins were associated with the tonoplast. To clearly distinguish the tonoplast from the plasmamembrane we performed a brief FM4-64 staining (Fig. 2.4A, red) that is often used to image the plasmamembrane. The overlay confirms that the YFP fluorescence and the one emitted from FM4-64 do not overlap. To exclude the possibility that subcellular localization is dependent on the cell-type, we imaged both, epidermal cells of cotelydons as well as root epidermal cells and observed that the localization patterns were the same. In contrast, a fusion protein of just the C-terminal part of PROPEP1, which represents *At*Pep1, with YFP produced a cytosolic localization indicating that the association of PROPEP1 with the tonoplast seems to depend on the N-terminal part of PROPEP1 and is not due to a binding of *At*Pep1 to a yet unidentified tonoplast-localized protein (Fig. 2.4B).

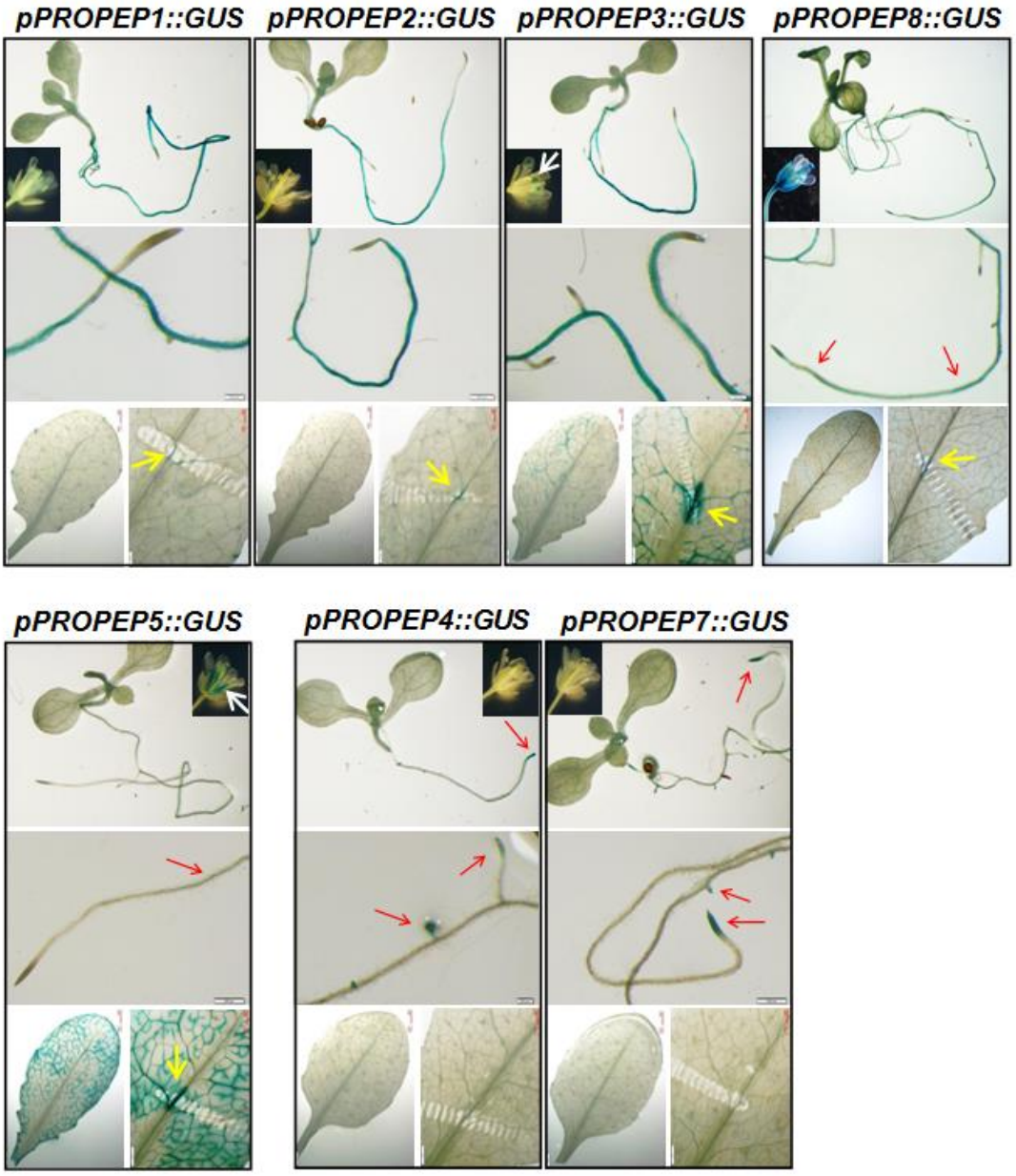

Figure 2.3: Spatial and temporal expression patterns of *PROPEP* promoter-GUS lines. Fusion of putative promoter sequences of indicated *PROPEPs* to a β-d-glucuronidase (GUS) reporter reveals distinct expression patterns. Pictures show staining (2 h) of untreated 10 d old seedlings grown in sterile conditions on MS plates and adult leaves and flowers of soil grown plants (24 h staining). Wounding of adult leaves was done with a forceps and incubated for 2 h before staining. Red arrows indicate expression in root tips of the primary and lateral roots. Yellow arrows point to GUS staining in the vasculature after wounding. White arrows highlight flowers with GUS expression. Three independent transgenic lines have been analyzed for each construct showing similar results.

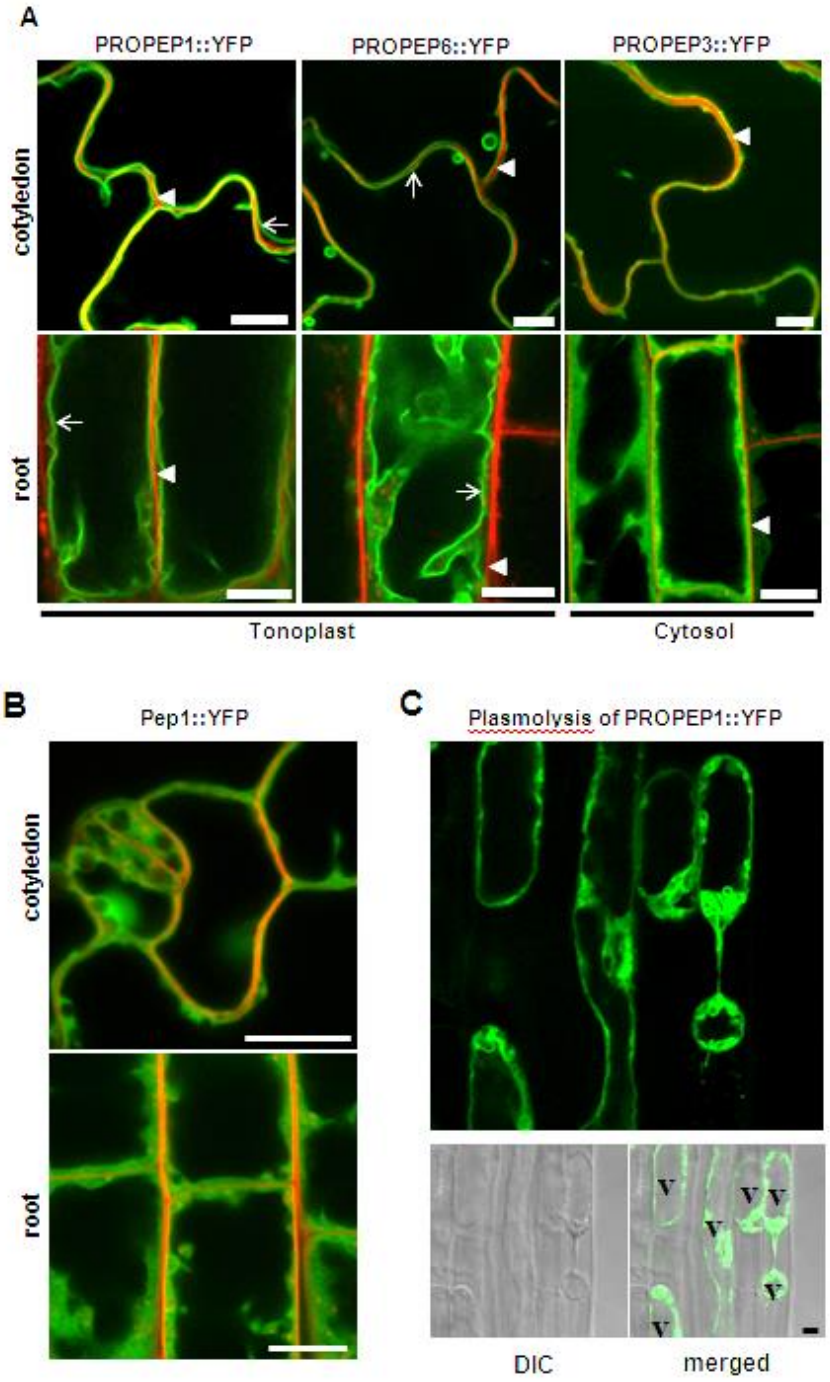

Figure 2.4: **Subcellular localization of PROPEP::YFP fusion proteins.** Confocal micrographs of *Arabidopsis* transgenic lines, expressing PROPEP::YFP (A) and Pep1::YFP (B) fusion proteins as indicated under the control of the CaMV35S promoter show single optical sections of cotyledon epidermal cells (top panel) or root epidermal cells (bottom panel). Co-staining with FM4-64 (red channel) highlights the plasma membrane (arrowheads). PROPEP1 and PROPEP6::YFP fusions localize to the tonoplast in both tissues (right panels - arrows) while PROPEP3::YFP (left panel) and Pep1::YFP (B) fusion protein localizes to the cytosol in both tissues. Similar results were obtained in two independent transgenic lines for each construct. Bars = 10 µm. C) Plasmolysis of root cells after 2 min of 500 mM NaCl treatment. DIC = Differential interference contrast.

In order to further test the association of PROPEP1::YFP with the tonoplast we performed a plasmolysis triggered by a brief treatment with 500 mM NaCl. As shown in Figure 2.4C the YFP fluorescence remains at the tonoplast of the shrunken vacuoles.
These findings demonstrate that members of the PROPEP family are present at two different subcellular compartments, the cytosol and the tonoplast. This might indicate non-redundant functions between the PROPEPs at the protein level or a yet not understood level of complexity of their involvement in cellular immunity. Moreover, it provides evidence for a potential role of PROPEP1 and 6 associated with the vacuole.

The promoters of *PEPR1* and *PEPR2* confer overlapping patterns of expression, which resemble those of some but not all *PROPEP* promoters

*At*Peps are known to be detected by two homologous receptors, PEPR1 and PEPR2 (Krol et al., 2010; Yamaguchi et al., 2010). To investigate the potential overlap of the expression patterns between the two PEPRs and the PROPEPs, we generated transgenic Arabidopsis lines containing the putative promoter sequences of the *PEPR* promoters fused to GUS. As shown in Figure 2.5, both promoters conferred expression in the vascular tissue of roots and leaves. No *PEPR1/2* promoter-mediated GUS expression was observed in root tips. Focusing on the expression in roots, the activity of *PEPR2* promoter was more restricted to the central cylinder of the root whereas GUS expression of the *PEPR1* promoter was present in most root tissues. Additionally, GUS expression mediated by the *PEPR1/2* promoters was detected in stems but was almost absent in flowers.

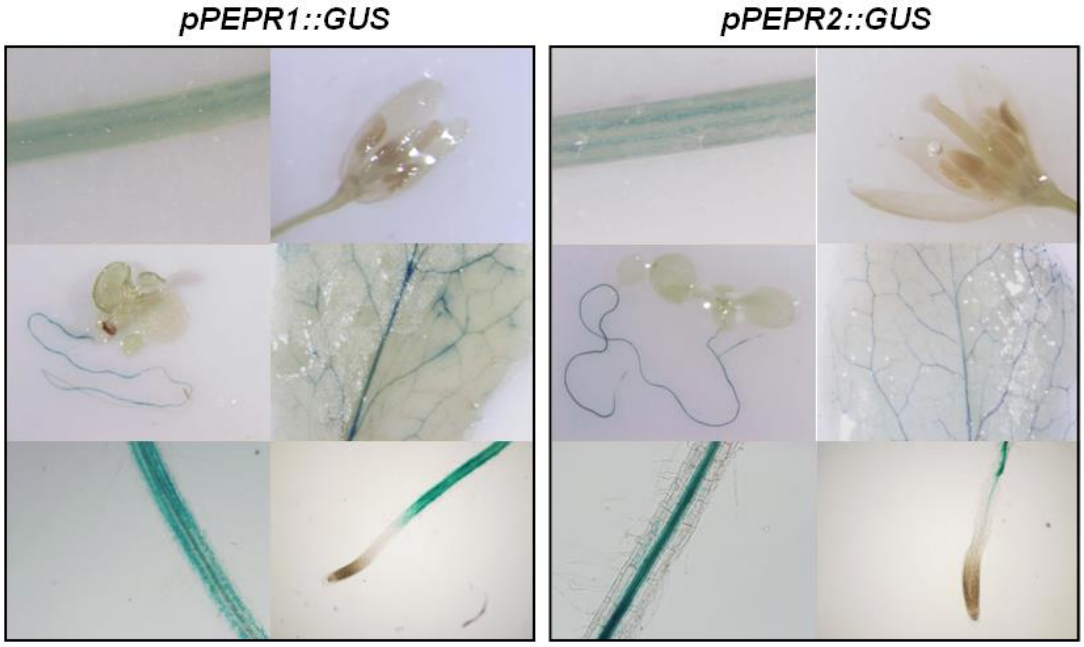

Figure 2.5: Overlapping expression patterns of *PEPR1* and *PEPR2* promoter-GUS lines. Putative promoter sequences of *PEPR1* and *PEPR2* were fused to GUS and stably introduced in Arabidopsis plants. Tissues of transgenic plants were stained for 2 h (roots and seedlings) and 24 h (adult leaves, stems and flowers). GUS staining reveals a great overlap in the tissue-dependent expression of *PEPR1* and *PEPR2*. Three independent transgenic lines were analyzed for each construct. Pictures show representative samples.

When comparing the expression patterns between the receptors and the precursors, the *PEPR1/2* promoter-mediated expression showed partially overlapping patterns with *PROPEP1*, *2*, *3, 5* and *8*. By contrast, *PROPEP4* and *7* promoter-mediated expression was exclusive to the root tip, a tissue where the *PEPRs* were not expressed. These results show that, whereas the promoters of *PEPR1* and *PEPR2* highly overlap in their conferred expression patterns, they share only little overlap with the expression patterns generated by *PROPEP* promoters. This indicates potential new, unknown roles for at least PROPEP4 and PROPEP7 independent of PEPRs.

PEPR1 and PEPR2 as well as all eight *At*Peps trigger similar defense responses

Previous studies showing that *At*Peps triggered alkalinization in cell cultures and induced resistance to *Pseudomonas syringae* infection in plants provided evidence that some *At*Peps are functionally redundant (Huffaker and Ryan, 2007; Yamaguchi et al., 2010). To address the extent of functional redundancy between all known *At*Peps, we monitored the activation of MAP kinases, the release of ethylene and the inhibition of seedling growth stimulated by the eight *At*Peps in the single and double *pepr1 pepr2*

receptor mutants. As shown in Figure 2.6, all eight *At*Peps activated the stress-related MAP kinases MPK3 and MPK6, induced the production of ethylene and inhibited seedling growth in a PEPR1- and partially PEPR2-dependent manner. Notably *At*Pep3 to *At*Pep8 were not perceived in the *pepr1* mutant, indicating that PEPR2, which is active in this mutant, does not perceive these peptides and, thus, is specific for *At*Pep1 and *At*Pep2, whereas the *pepr2* mutant responded to all peptides, indicating that PEPR1 recognizes all eight *At*Peps in a similar way (Fig. 2.6). Taken together, all eight *At*Peps trigger a similar set of defense responses reminiscent of PTI in a PEPR1 and partially PEPR2 dependent manner. Thus, in contrast to the PROPEPs, the *At*Peps as well as PEPRs appear to be highly redundant.

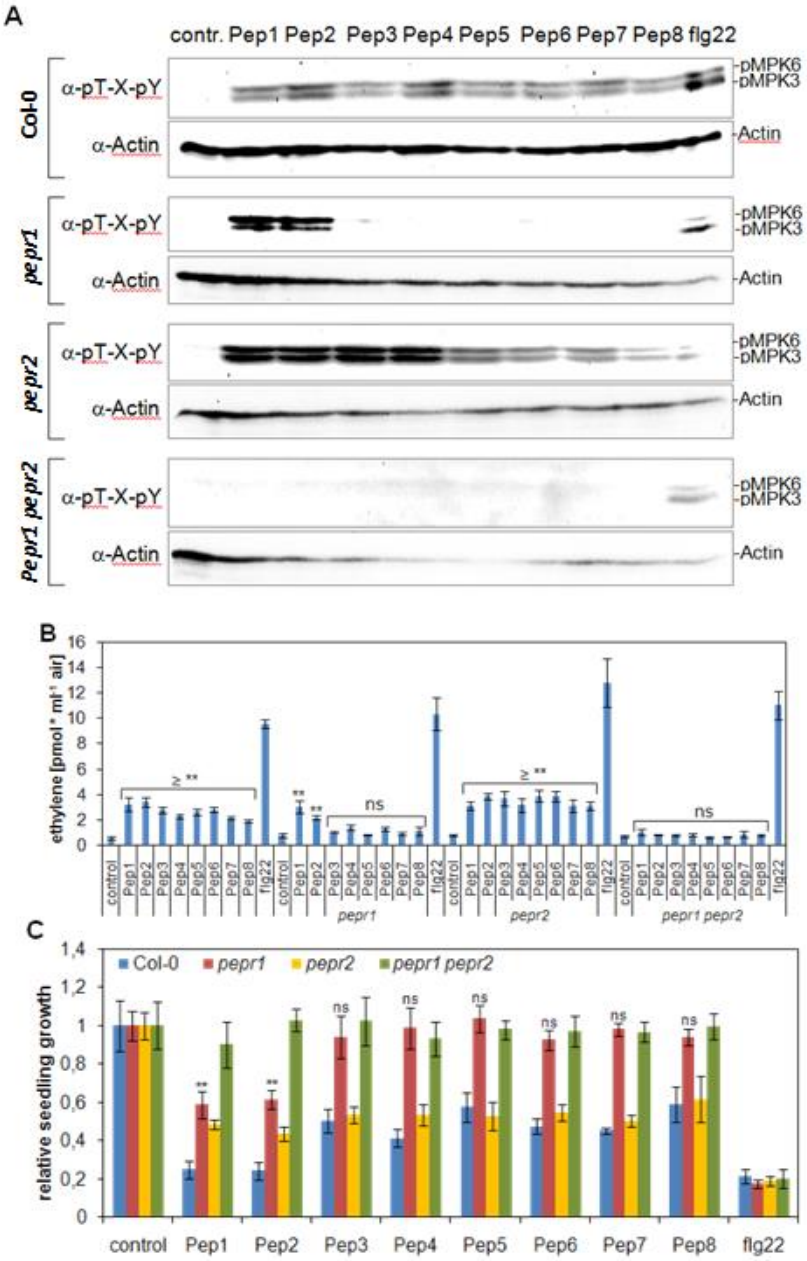

Figure 2.6: Defense responses activated by all eight *At*Peps and both PEPRs. A) MAPK phosphorylation. Seedlings of indicated genotypes were treated for 15 min with 1 µM of the indicated elicitor peptide or without any peptide (contr.). MAPK phosphorylation was detected by immunoblotting using an anti-phospo-p44/42-MAP kinase antibody detecting the pTE-pY motif of MPK6 and MPK3. The immunoblot was reprobed with anti-Actin antibody to determine equal loading. B) Ethylene production. Seedlings of indicated genotypes were treated for 5 h with 1 µM of the indicated elicitor peptides or without any peptide (control). Columns represent averages of detected ethylene values of 5 biological replicates. Error bars indicate standard error of the mean. C) Seedling growth inhibition. 5 day old seedlings of the indicated genotypes were treated for 5 d with 1 µM of the indicated elicitor or without any peptide. Columns represent the mean weight of 12 seedlings out of 6 biological replicates. Error bars indicate standard error of the mean. Asterisks represent t-test results generated by comparing the labeled value to the respective control (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$; ns = not significant).

2.4 DISCUSSION

Current models discuss PROPEPs and *At*Peps as i) enhancers of immunity, ii) damage-signaling peptides or iii) elicitors of systemic defense responses, but based on published data reliable support for each model is scarce (Boller and Felix, 2009; Yamaguchi and Huffaker, 2011). Previous studies primarily focused on plant responses triggered by the addition of the synthetically produced peptides *At*Pep1 or *At*Pep3 and firmly established, that a treatment with these peptides enhances plant immunity via PEPRs (Yamaguchi *et al.*, 2010, Krol *et al.*, 2010). Likewise, the constitutive, ubiquitous expression of *PROPEP1* or *PROPEP2* improved plant resistance to an oomycete pathogen (Huffaker et al., 2006). But these studies did not adress the question of the presence or the underlying mechanism of the PROPEP/*At*Pep/PEPR system and thus cannot fully answer which (if any) of the current models are valid.

Recently, two studies involving the *pepr1 pepr2* double mutant suggested an interaction of *At*Pep-signaling with the defense hormone ethylene to maintain PTI responses (Liu et al., 2013; Tintor et al., 2013). Thus the "enhancer of immunity" model appears now as the most likely one. Here, we investigated the presence and regulation of PROPEPs to either further substantiate the "enhancer model" or to deduce new biological role(s) of the PROPEP/*At*Pep/PEPR system.

PROPEP1, 2, 3 and maybe *5* and *8* play a role in immunity

In agreement with previous works (Huffaker and Ryan, 2007; Yamaguchi et al., 2010) our bi-clustering showed that *PROPEP1*, *2* and *3* clustered together with genes implicated in plant defense. Moreover, the almost exclusive expression of these *PROPEPs* in the roots revealed by promoter::GUS fusions partially overlap with the ones of *PEPR1* and *PEPR2*. Thus *PROPEP1*, *2* and *3* might play specific roles in the immune response of the root, which is supported by the report that constitutive expression of *PROPEP1* led to an induced resistance against the oomycete root pathogen *Pythium irregulare* (Huffaker and Ryan, 2007). In contrast, these *PROPEPs* are not or only weakly expressed in adult leaves but rapidly induced in wounded leaf-veins. Recently we showed that a pretreatment of leaf tissue with bacterial MAMPs led to an enhanced output of reactive oxygen species triggered by *At*Pep perception (Flury et

al., 2013). Since a progressive wave of reactive oxygen species has been discussed as a potential systemic signal, the enhanced expression of PROPEPs in wounded vasculature might contribute to the robustness of this system (Miller et al., 2009; Mittler et al., 2011).

PROPEP5 and *PROPEP8* display expression patterns that partially overlap with the ones of *PEPR1* and *PEPR2* but in contrast to *PROPEP1*, *2* and *3*, *PROPEP5* and *8* are restricted to the root vasculature but are more expressed in the leaf-veins (*PROPEP5*) and the flowers (*PROPEP8*). However, *PROPEP1*, *2*, *3, 5* and *8* together cover most plant tissues and since all eight *At*Peps trigger redundant responses PROPEP5 and PROPEP8 could play in leaves and flowers, respectively, similar roles like PROPEP1, 2 and 3 in roots.

It has been hypothesized that PROPEPs are located to the cytoplasm and could be released to the extracellular space in a situation of danger using unconventional protein secretion mechanisms (Ding et al., 2012). We found indeed that PROPEP3::YFP was localized in the cytoplasm, but surprisingly PROPEP1::YFP as well as PROPEP6::YFP were detected at the tonoplast. Due to the acidic environment of the vacuole negatively impacting on YFP fluorescence, we assume that PROPEP1::YFP and PROPEP6::YFP are associated with the cytoplasmic side of the vacuolar membrane. Notably, the localization signal that directs the PROPEP to the tonoplast or a hitherto unidentified interaction domain, that could attach the PROPEP to a tonoplast-localized protein, resides in the N-terminus of the PROPEP since a fusion protein of only *At*Pep1 and YFP localized in the cytoplasm. Therefore it can be excluded that the *At*Pep itself binds to a tonoplast-localized receptor-like protein. Recently, it was shown that infection of Arabidopsis with the compatible oomycete *Hyaloperonospora arabidopsidis* (*Hpa*), the causal agent of the downy mildew, triggered a rearrangement of intracellular membranes leading to a relocation of the tonoplast close to the extra-haustorial membrane (Caillaud et al., 2012). However, neither the involvement of PROPEPs in resistance to *Hpa* nor the necessity of a tonoplast localization of PROPEP1 in the context of resistance to *Pythium* infection has been shown yet, but it will be interesting to study this potential connection.

Root tip expressed *PROPEP4* and *7* are distinct from the other PROPEPs and might have dual functions

PROPEP4 and PROPEP7 are located on chromosome 5 within a ~3.5 kb stretch. Both share the specific expression in the tips of primary and lateral roots, which does not overlap with the ones of *PEPR1* and *PEPR2*. Moreover, they are currently the only *PROPEPs,* which are not induced by wounding. Therefore they are less likely to enhance plant immune responses locally. However, PROPEP4 and 7 could still be part of a systemic defense response. It has been reported that the Systemin peptide is transported via the phloem sap (Narváez-Vásquez et al., 1995). Moreover, a plethora of peptide transporters are encoded in the Arabidopsis genome which might facilitate the transport of *At*Peps for systemic signaling (Stacey et al., 2002). Thus PROPEP4 and 7 could be ideal candidates to study, if PROPEPs or *At*Peps are transported systemically.

The Affymetrix 25K microarrays do not represent *PROPEP7.* Our bi-clustering analysis produced only a small cluster of 25 genes that contained *PROPEP4*. Intriguingly, this cluster showed an expression pattern opposite to the ones of the other *PROPEP* containing clusters meaning that whenever biotic stress treatments lead to an induction of *PROPEP4* expression, other *PROPEPs* are downregulated and vice versa. GO-term enrichment points at biological processes including chromatin and chromosome organization. However, this does not exclude a function in immunity. The mammalian DAMP high-mobility group protein B1 (HMGB1) binds to DNA, modifies the shape and regulates transcription. In case of danger it can be secreted by activated monocytes and macrophages, or it is passively released by necrotic or damaged cells. Detection of extracellular HMGB1 by RAGE (receptor for advanced glycation end products) of adjacent cells triggers inflammation (Scaffidi et al., 2002; Sims et al., 2010).

The small *PROPEP4* including gene cluster also shows the limitations of the bi-clustering. Most of the used arrays were probed with samples based on seedlings or adult leaves. Genes with tissue restricted expression patterns like *PROPEP4* might only be weakly detected on some of the biotic stress arrays leading to erroneous expression patterns.

Taken together, PROPEP4 (and PROPEP7) are clearly distinct from the other PROPEPs in terms of tissue expression pattern as well as regulation within the biotic stress treatments. A more detailed analysis is needed to uncover their biological roles.

PROPEPs might play roles in plant reproduction and development

Most plant signaling peptides originate from small (around 100 aa) proteins, which are processed at the C-terminus to release the active signaling peptide. These peptides have various functions especially in developmental processes such as apical meristem development as well as root growth (Matsubayashi and Sakagami, 2006; Katsir et al., 2011). PROPEPs have been associated with plant innate immunity but share structural similarities (size and presence of signaling peptide in the C-terminus) with Arabidopsis signaling peptide precursors like RGF1, TDIF, CLV3, PSK1, CEP1 or PSY1. Remarkably, there might be also a functional overlap. In contrast to *PROPEP1*, *2* and *3* bi-clustering showed that *PROPEP5* clusters with genes associated with plant reproduction. Although we did not find this for *PROPEP3*, GUS analysis revealed an expression of both in the stamen. Thus, beside the proposed role in plant immunity, *PROPEP5* and maybe also *PROPEP3* could be involved in the development of the stamen and therewith in the regulation of reproduction. The involvement of small signaling peptides in this process has been demonstrated just recently. RALF (rapid alkalinization factor) signaling peptides regulate pollen tube elongation and the development of the female gametophyte in *Solanaceous* species (Covey et al., 2010; Chevalier et al., 2012). Thus, as a next step a detailed analysis of *PROPEP3* and *PROPEP5* knock-out mutants would be needed, to investigate a potential role of these *PROPEPs* in plant reproduction.

Beside the impact of constitutive expression of *PROPEP1* on resistance against *Pythium* infection it also promoted an increase in root biomass production (Huffaker et al., 2006). It was assumed that *PROPEP1* expression somehow generated an advantage for Arabidopsis roots to grow in soil. In contrast, exogenous application of *At*Peps blocked root growth and biomass production similarly to seedling growth inhibition triggered by MAMPs. Notably, application of *At*Peps has a more pronounced negative effect on root growth compared to MAMPs (Krol et al., 2010).

If root growth was also enhanced in sterile conditions by constitutive expression of *PROPEP1* was not assessed (Huffaker et al., 2006), thus this advantage might or might not be based on an increased pathogen resistance of the root. A detailed analysis of *propep1* knock-out mutants is needed to clarify if PROPEP1 takes part in additional processes like root development.

2.5 CONCLUSIONS

Previous studies and our new data reported here show that all eight *At*Peps trigger a PTI-like response by binding to either PEPR1 or both PEPR1/2 receptors (Huffaker and Ryan, 2007; Yamaguchi et al., 2010). Interestingly, PEPR2 is specific for *At*Pep1 and *At*Pep2 whereas PEPR1 is unspecific and recognizes all eight *At*Peps.

In contrast to the *At*Peps and the PEPRs we provide data indicating that PROPEPs are probably not redundant. They show individual spatial and temporal expression patterns and localize to distinct subcellular compartments. Beside their potentially diverse roles in innate immunity they might additionally be involved in plant development and reproduction. A detailed characterization of each PROPEP together with an analysis of their processing and release will be necessary to uncover the full array of functions of PROPEPs in plant biology.

2.6 MATERIAL AND METHODS

Plant material

Mature Arabidopsis plants were grown in individual pots at 21° C and an 10 h photoperiod for 4-5 weeks. For induction of flowering, plants were moved to a 16 h photoperiod. For preparation of sterile seedlings, *Arabidopsis thaliana* seeds were surface-sterilized with 70 % ethanol and plated on half-strength Murashige and Skoog medium supplemented with 1% sucrose and 0.5% Phytagel (SigmaAldrich), stratified for at least 2 d at 4 °C and then germinated at 21 °C in continuous light (MLR-350; Sanyo). The T-DNA insertion lines SALK_059281 (*pepr1*) and SALK_098161 (*pepr2*) were obtained from by the Nottingham Arabidopsis Stock Centre (Nottingham, United Kingdom) and are in the Col-0 accession background.

Generation of transgenic Arabidopsis lines

The PROPEP and PEPR putative promoter sequences were amplified by PCR from genomic Col-0 DNA with specific primers (see Supplemental Table S4 for primers and

promoter sequences). Obtained sequences were introduced into the binary destination vector pBGWFS7 (Karimi et al., 2002) using Gateway-based cloning. *PROPEPs* were cloned from Col-0 cDNA using gene specific primers (Supplemental Table S4). Introducing PROPEP sequences into the binary destination vector pEarley101 by Gateway-based recombination led to the in frame fusion of YFP to their c-terminal ends (Earley et al., 2006). Arabidopsis plants were transformed by *Agrobacterium tumefaciens* using the floral dip method (Clough and Bent, 1998).

Peptides

Peptides of flg22 (QRLSTGSRINSAKDDAAGLQIA), *At*Pep1 (ATKVKAKQRGKEKVSSGRPGQHN), *At*Pep2 (DNKAKSKKRDKEKPSSGRPGQTNSVPNAAIQVYKED), *At*Pep3 (EIKARGKNKTKPTPSSGKGGKHN), *At*Pep4 (GLPGKKNVLKKSRESSGKPGGTNKKPF), *At*Pep5 (SLNVMRKGIRKQPVSSGKRGGVNDYDM), *At*Pep6 (ITAVLRRRPRPPPYSSGRPGQNN), *At*Pep7 (VSGNVAARKGKQQTSSGKGGGTN), *At*Pep8 (GGVIVKSKKAARELPSSGKPGRRN) obtained from EZBiolabs were dissolved in a solution containing 1 mg/mL bovine serum albumin and 0.1 M NaCl to get peptide stocks of 100 µM concentration. Further dilutions were done with water.

Microarray and data analysis

Bi-clustering and co-expression analysis was performed as described by van Verk et al. (2011), with the following minor modifications: For bi-clustering, the Euclidean distance measure was used. To obtain separate clusters containing the *PROPEPs*, the first cluster within the dendogram containing less than 500 genes was selected. For gene annotations into biological categories, the AmiGO Term Enrichment software was employed (Carbon et al., 2009). For categorization of enriched GO-terms, the CateGOrizer tool (Hu et al., 2008) using Plant GO-Slim terms, applying the consolidated single occurrences count option. Supplemental Table S2 provides a list of the Affymetrix 25K microarrays from NASCArrays and AtGenExpress (downloaded from ftp.arabidopsis.org).

GUS staining

Plant tissue was fixed in ice-cold 90 % acetone for 20 min, washed with water and then placed in GUS staining buffer (1 mM 5-bromo-4-chloro-3-indolyl β-d-glucuronide (Gold BioTechnology, St. Louis, Missouri, USA), 100 mM sodium phosphate (pH 7.5), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA, and 0.1% (v/v) Triton X-100) at 37 °C for 2 h (seedlings) and 24 h (adult leaves). Plant tissue was cleared with 70% (v/v) ethanol and photographed using an Olympus SZX12 binocular in combination with an Olympus DP72 camera and the CellSens imaging software (Olympus America, Pennsylvania, USA).

Fluorescence microscopy

7 days old seedlings expressing the PROPEP::YFP and Pep1::YFP fusions were stained for 5 min in an aqueous solution containing FM4-64 (SynaptoRed, Sigma-Aldrich) diluted at 5 µg/mL and washed for 5 min in water prior imaging using an SP5 Leica Confocal Microscope. YFP (500 to 560 nm) and FM4-64 (620 to 650 nm) fluorescence was recorded simultaneously after excitation at 488 nm using a 63x water immersion objective. Plasmolysis was achieved by mounting roots in 500mM NaCl solution 2 min prior imaging.

Measurement of ethylene production

For measurement of ethylene accumulation, 5 seedlings (5 days after germination (dag)) were harvested into a 6 ml glass vial containing 0.1 ml of ddH_2O, placed back into the growth chamber and left overnight (~ 16 h). Peptides were added to 1 µM final concentration and vials were closed air-tight with rubber septa. After 5 h of incubation on a shaker (100 rpm) at room temperature, ethylene accumulating in the free air space was measured by gas chromatography (GC-14A Shimadzu).

MAPK phosphorylation

10 seedlings (10 dag) were placed into 0.5 ml sterile water and left over night (16 h) floating. Peptides were added to a final concentration of 1 µM. After 15 min seedlings were shock frozen and ground to fine powder before addition of 80 µl extraction buffer (0.35 M Tris-HCl pH 6.8, 30 % (v/v) glycerol, 10 % SDS, 0.6 M DTT, 0.012 % (w/v) bromphenol blue). After boiling for 5 min, 10 µl of the total cellular protein extract was

separated by electrophoresis in 12 % SDS-polyacrylamide gel and electrophoretically transferred to a polyvinylidene fluoride membrane according to the manufacturer's instructions (Milipore). We used monoclonal primary antibodies against phospho-p44/42 MAP kinase (Cell Signaling Technologies) and actin (Sigma-Aldrich), with alkaline phosphatase-conjugated anti-rabbit and anti-mouse immunoglobulins (Sigma-Aldrich) as secondary antibodies, as required. Signal detection was performed using CDPstar (Roche).

Growth inhibition assays

5 days after germination, sterile seedlings were transferred to liquid MS medium supplied with the peptides at 1 μM final concentration (one seedling per 500 μl of medium in 24-well plates). The effect of treatment with different peptides on seedling growth was analyzed after 10 days by weighing fresh weight.

2.6 SUPPLEMENTARY DATA

Supplementary data and tables are accessible via: http://jxb.oxfordjournals.org/content/64/17/5309/suppl/DC1

3. EVOLUTIONARY DIVERGENCE OF THE PLANT ELICITOR PEPTIDES (PEPS) AND THEIR RECEPTORS: INTERFAMILY INCOMPATIBILITY OF PERCEPTION BUT COMPATIBILITY OF DOWNSTREAM SIGNALLING

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This work aimed to identify family specific patterns in the perception of Peps by PEPRs of various species. I designed some of the performed experiments and contributed the work done on the PEPRs (fig. 5) and wrote that part of the manuscript.

3.1 ABSTRACT

Plant elicitor peptides (Peps) are potent inducers of pattern-triggered immunity and amplify the immune response against diverse pathogens. Peps have been discovered and studied extensively in Arabidopsis and only recently orthologs in maize were also identified and characterized in more detail.

Here we investigated the presence of PROPEPs, the Pep precursors, and PEPRs, the Pep receptors, within the plant kingdom and identified PROPEPs and PEPRs in most sequenced species of the angiosperms. We analysed the conservation and compatibility of the Pep-PEPR-system by using plants of the two distantly related dicot families, Brassicaceae and Solanaceae and a representative family of monocot plants, the Poaceae. All three plant families contain important crop plants including maize, rice, tomato, potato and canola. We found that Peps are not recognized by species outside of their plant family of origin, apparently because of a divergence of the Pep sequences. We define three family-specific Pep motifs and show that the integration of such a motif into the Pep sequence of an unrelated Pep enables its perception. We also observed transient transformation of *Nicotiana benthamiana* with the coding sequences of the AtPEPR1 and ZmPEPR1a led to the recognition of Pep peptides of Brassicaceae or Poaceae origin, respectively, and to the proper activation of downstream signalling. We conclude that the signalling machinery downstream of the PEPRs is highly conserved whereas the LRR domains of the PEPRs coevolved with the Peps leading to distinct motifs and with it interfamily incompatibility.

3.2 INTRODUCTION

Plant immunity is triggered by the recognition of exogenous as well as endogenous elicitors. MAMPs (microbe-associated molecular patterns) are well known representatives of the former whereas the latter are often classified as DAMPs (danger- or damage-associated molecular patterns) (Boller and Felix, 2009; Albert, 2013; Ferrari et al., 2013). Plant elicitor peptides (Peps) are emerging as paradigms for DAMPs due to their presence in dicot as well as monocot model plants and their supposed release upon damage (Huffaker et al., 2011; Yamaguchi and Huffaker, 2011; Bartels et al., 2013). In brief, Peps mature from larger precursor proteins called PROPEPs and are recognized

by LRR-RLKs (leucine-rich repeat receptor-like kinases) known as PEPRs (PEP RECEPTORs). Pre-treatment of Arabidopsis or maize plants with Peps triggers defence responses and significantly improves their resistance against diverse pathogens including bacteria, fungi as well as herbivores (Huffaker et al., 2006; Yamaguchi et al., 2010; Huffaker et al., 2011; Huffaker et al., 2013; Liu et al., 2013; Tintor et al., 2013).

In Arabidopsis eight PROPEPs (PROPEP1-8) and two PEPRs (PEPR1 and PEPR2) have been identified (Krol et al., 2010; Yamaguchi et al., 2010; Bartels et al., 2013). Current models suggest a cleavage or processing of PROPEPs to produce Peps, which represent roughly the last 23 amino acids of the C-terminal part of the PROPEPs (Yamaguchi and Huffaker, 2011). Individual PROPEPs have been shown to localize to either the cytoplasm or to be associated with the tonoplast contributing to the assumption that Peps are released into the apoplast either actively as a response to danger signals or passively during damage and loss of cell integrity (Bartels et al., 2013). Once in the apoplast they can reach PEPRs of adjacent cells and trigger and/or amplify immunity.

Little is known about Peps and PROPEPs and even less about PEPRs in other dicot plants. A small member of Peps from Solanaceae (Eggplant SmPep1, Pepper CaPep1 and Potato StPep1) and Fabaceae (Soybean GmPep3, Medicago MtPep1, Peanut AhPep1) was shown to induce the release of volatiles, a typical defence response against herbivore attack (Huffaker et al., 2013). In addition a very recent study reported the reduced expression of defence-related genes as well as a reduced resistance towards the necrotrophic fungus *Pythium dissotocum* in tomato plants upon silencing of a putative tomato *PROPEP* (Trivilin et al., 2014).

In maize (*Zea mays*) two PROPEPs have been studied in more detail, ZmPROPEP1 and ZmPROPEP3 (Huffaker et al., 2011; Huffaker et al., 2013). The former is induced upon fungal infections whereas the latter is induced upon application of *Spodoptera exigua* oral secretions. Accordingly, treatments with ZmPep1 and ZmPep3 led to an upregulation of defence-related genes and improved resistance against fungal infections as well as herbivore feeding (Huffaker et al., 2011; Huffaker et al., 2013).

In Arabidopsis an alanine-substitution approach has been used to identify the crucial amino acids for Pep perception by PEPRs (Pearce et al., 2008). In this study a minimum core of the last 15 amino acids of AtPep1 [AtPep1(9-23)] was described to show a comparably similar activity as the unmodified AtPep1. Moreover, exchange of serine[15]

or glycine[17] to alanine as well as the deletion of the terminal asparagine[23] produced a dramatic decrease in AtPep1 activity (Pearce et al., 2008). Recently the crystal structure of the AtPEPR1-LRR domain in complex with AtPep1 was released (Tang et al., 2014). The authors report that especially the C-terminal ten residues of AtPep1 interact closely with the AtPEPR1-LRR and they include the previously described and conserved ser[15], gly[17] and asp[23]. In addition, modelling of the PEPR1-LRR/AtPep1/BAK1-LRR complex indicated that proline[19] as well as glutamine[21] and histidine[22] are important for the PEPR1 BAK1 (BRI1-ASSOCIATED KINASE1) interaction. This interaction has been shown before to be crucial for mounting full strength defence responses upon AtPep1 perception (Roux et al., 2011b).

Despite the apparent common defence-amplifying action of PROPEPs from plant species as diverse as Arabidopsis and maize their amino acid-based homology is very low (Huffaker et al., 2011). Even among the PROPEPs from Arabidopsis there is only an overall amino acid sequence identity between 12% and 47% (Yamaguchi et al., 2006). Moreover, already published Pep sequences show alterations in the conserved key amino acids, for example ZmPep1 has a C-terminal his[23] instead of the asp[23] whereas Peps of the Solanaceae show a gly[15] instead of a ser[15] (Huffaker et al., 2013). In contrast, ZmPep3 is neither recognized by Eggplant (Solanaceae) nor by Soybean (Fabaceae) despite the presence of ser[15], gly[17] and asp[23] (Huffaker et al., 2013).

Here, we performed a comprehensive search for PROPEPs and PEPRs throughout the plant kingdom taking into account the many recently sequenced plant genomes. We used the elicitor-triggered release of ethylene as a robust and widespread read-out to investigate the interspecies recognition of known and newly identified Peps from the many dicot crop plants in the Brassicaceae and Solanaceae and the monocot crops in the Poaceae. Indeed, Peps from one plant family are generally not perceived by plants belonging to another plant family despite the presence of PEPRs. Individual sequence alignment of all tested Peps from one family revealed family-specific Pep motifs. Inclusion of family-specific motifs into the sequence of incompatible Peps enabled their recognition. Further we cloned functional PEPRs from tomato and maize. Transient expression of AtPEPR1 and ZmPEPR1a in *Nicotiana benthamiana* led to AtPep1 and ZmPep1 sensitivity indicating that in contrast to Peps, PEPRs are interspecies compatible.

3.3 RESULTS

Identification of PROPEP and PEPR homologs in multiple plant species within the angiosperms

The structure and function of the Pep-PEPR system has been studied mainly in the model plants *Arabidopsis thaliana* and *Zea mays* (Huffaker et al., 2006; Krol et al., 2010; Yamaguchi et al., 2010; Huffaker et al., 2011; Bartels et al., 2013). However, already the initial publication suggested that PROPEPs might be present in a couple of plant species and not limited to Arabidopsis (Huffaker et al., 2006). We performed an extensive sequence search in public databases using the few previously described PROPEP sequences as well as the sequences of the hitherto only known PEPRs, AtPEPR1 and AtPEPR2, and identified a large number of novel PROPEPs and PEPRs (Supplementary Table S2). In Figure 3.1, we present the phylogenetic trees of all PROPEPs (Fig. 3.1A) and PEPRs (Fig. 3.1B). PROPEPs form plant family specific clusters, for example AtPROPEP1 clusters primarily with most Brassicaceae PROPEPs and not with PROPEP1 orthologs of distantly related plant species. We also performed a sequence comparison of all identified PROPEPs and found an astonishingly small sequence identity between PROPEPs (Supplementary Table S3). For example the orthologs AtPROPEP1/ ZmPROPEP1 and AtPROPEP3/ZmPROPEP3, which have been linked to fungal and herbivore resistance, respectively (Huffaker et al., 2006; Huffaker et al., 2011; Huffaker et al., 2013; Liu et al., 2013; Klauser et al., 2015), show as little as 5.5 % and 5.3 % identical amino acids, respectively. In general, a large number of PROPEPs show less than 10 % identical amino acids compared to other PROPEPs. Only within family-specific clusters and subclusters inside the Brassicaceae sequence identity ranges above 50 % (Supplementary Table S3). It has been proposed that the PROPEP C-terminal end To date there are two exceptions from the apparent rule that PROPEPs form family-specific clusters: AtPROPEP5 and AtPROPEP6. Especially the latter seems to be closely related to the PROPEPs of the Solanaceae and groups within their family-cluster.

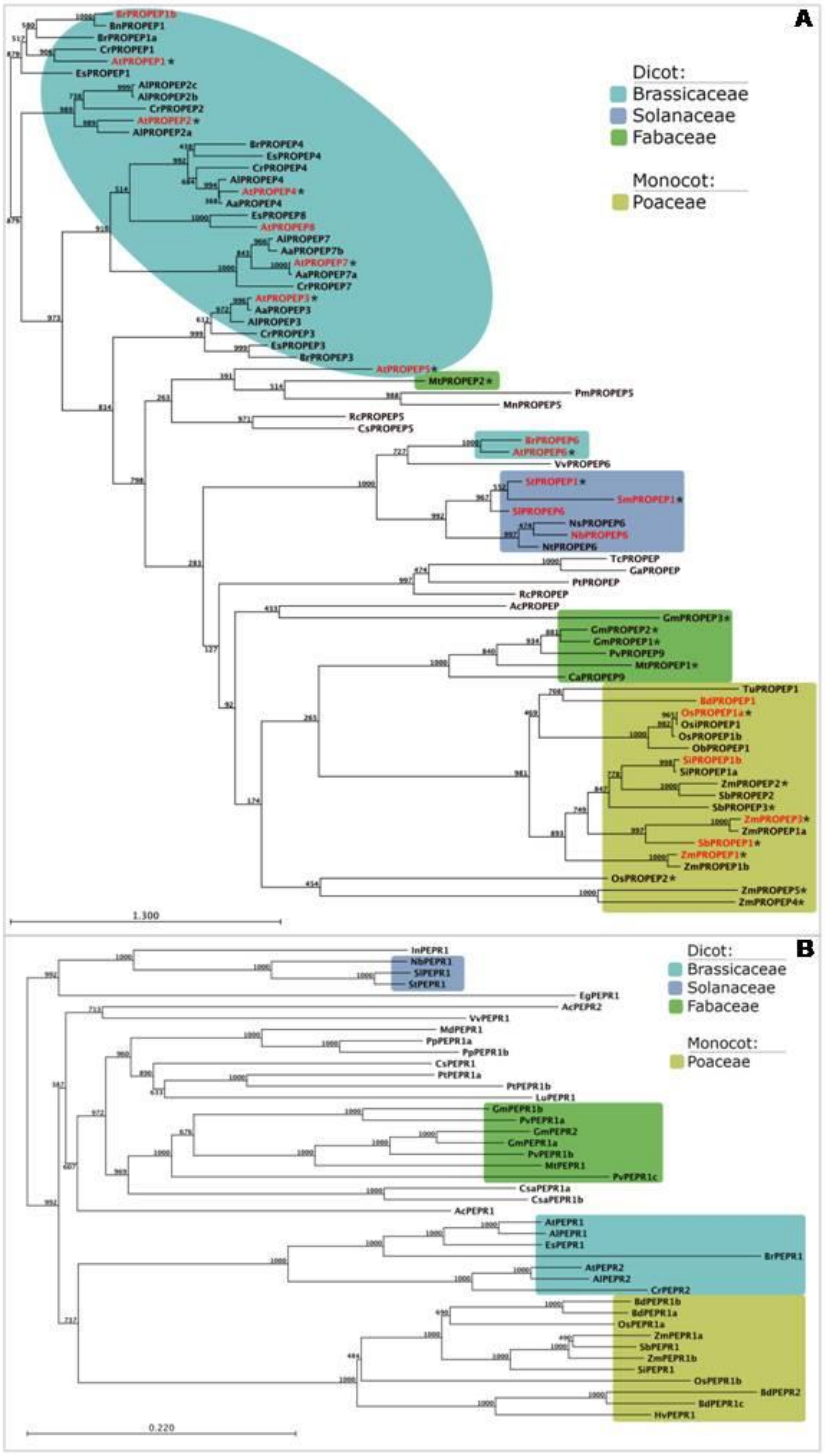

Figure 3.**1: Bootstrapped neighbour-joining tree of PROPEP and PEPR sequences.**
A Full-length amino-acid sequences of published and novel HMMER identified PROPEP sequences were used to build a bootstrapped neighbour-joining tree. PROPEPs in red highlight PROPEPs of which the respective Pep was shown to be an active elicitor in this study. (*) mark PROPEPs of which the respective Pep was shown in previous studies to be an active elicitor. Major families are highlighted with colours according to the legend. Scale-bar: amino-acid substitutions per site. **B** Full-length amino-acid sequences of PEPR sequences were used to build a bootstrapped neighbour-joining tree. Major families are highlighted with colours according to the legend. Scale-bar: amino-acid substitutions per site.

Therefore we propose to reclassify the two already described *St*PROPEP1 and *Sm*PROPEP1, as *St*PROPEP6 and *Sm*PROPEP6, respectively.

In comparison to the limited number of PROPEPs in other plant families there is a clear overrepresentation of PROPEPs from species belonging to the Brassicaceae. Provided that other plant genomes are as well annotated as the ones from Arabidopsis and its relatives it seems that there was a recent multiplication of *PROPEPs* in the genome of a Brassicaceae ancestor and not in dicot species. A similar number of PROPEPs within one species has only been found in the monocot species maize (*Zea mays*, 7 PROPEPs) and rice (*Oriza sativa* Japonica group, 3 PROPEPs) (Fig. 3.1A and Supplementary Table S2).

Regarding PEPRs it seems that to date most species contain only one PEPR (Fig. 3.1B) although two have been characterized in Arabidopsis (Krol et al., 2010; Yamaguchi et al., 2010). Similar to PROPEPs PEPR sequences form family-specific clusters (Fig. 3.1B) with sequence identities ranging from 60 % to 90 % within a family cluster (Supplementary Table S5). Contrary to the low overall conservation of the PROPEPs the overall level of conservation of the PEPRs is around 40 % sequence identity much higher.

So far no PROPEPs or PEPRs have been identified outside the angiosperms.

Interfamily incompatibility of Peps

Given the aforementioned variability of the PROPEP as well as Pep sequences the question arises, what is the structural basis of Pep perception and specificity. A first report in 2013 indicated that Eggplant and Soybean do not perceive Peps originating from species outside the Solanaceae and Fabaceae, respectively (Huffaker et al., 2013). However, the authors used only these two species together with the monitoring of volatile production to characterize the perception of Peps and it is currently not certain if volatile emission is a typical response triggered by Pep binding to PEPRs. Here we used the production of ethylene as a robust and reliable output that has been used by multiple studies characterising Pep responses in conjunction with additional PTI-related responses like the production of reactive oxygen species or the phosphorylation of MAP kinases to monitor the perception of Peps in a number of different species (Krol et al., 2010; Roux et al., 2011b; Bartels et al., 2013; Flury et al., 2013). We selected two species each of the distantly related plant families Brassicaceae (*Arabidopsis thaliana*

and *Brassica rapa*), Solanaceae (*Solanum lycopersicum* and *Nicotiana benthamiana*) and Poaceae (*Zea mays* and *Lolium perenne*) together with a representative Peptide (AtPep1, SlPep6 and ZmPep1, respectively) to determine interspecies and interfamily perception of Peps. As shown in Fig. 3.2 AtPep1 is only perceived by Arabidopsis and its close relative *Brassica rapa* causing a highly significant release of ethylene absent in the more distantly related species. The same is true for the perception of SlPep6 and ZmPep1 that are only perceived by the species belonging to the same plant family (Fig. 3.2). Taken together, there seems to be an interfamily but not an interspecies incompatibility of Pep perception.

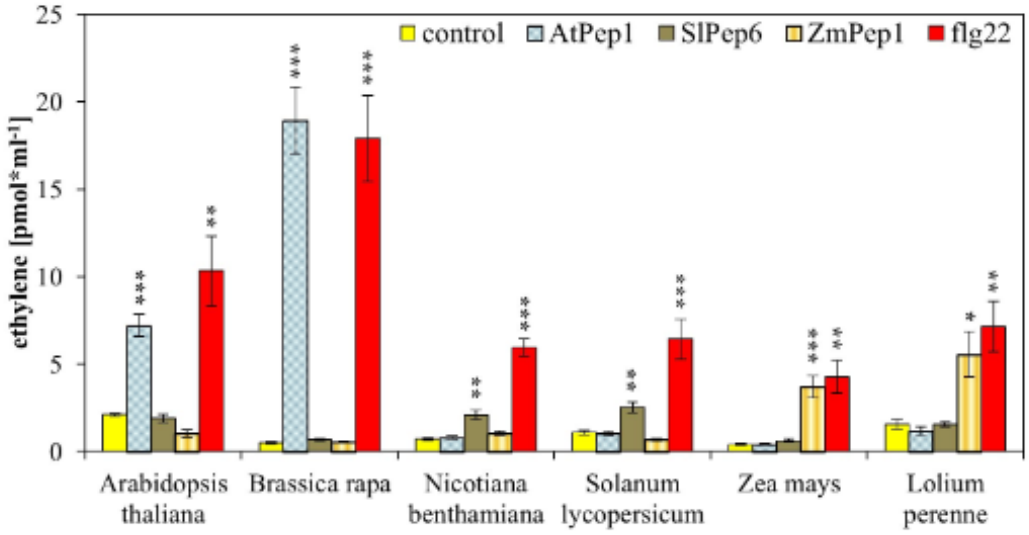

Figure 3.2: Interfamily incompatibility of Peps.
Eight to ten leaf discs of indicated plant species were treated for 5 h with 1 µM of the indicated elicitor peptides or without any peptide (control). Columns represent averages of detected ethylene values of 5 biological replicates. Error bars show the standard error of the mean. Asterisks indicate significant differences of the labelled column to the control based on t-test results (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$).

Determination of novel family-specific Pep motifs

The molecular characteristics of Pep recognition has been exclusively studied in Arabidopsis. An alanine-substitution approach using AtPep1 in combination with monitoring the medium alkalinisation response led to two major findings: i) A minimum core of the last 15 amino acids of AtPep1 [AtPep1(9-23)] is sufficient to cause activity comparable to that of full length AtPep1 whereas ii) exchange of serine[15] or glycine[17] to alanine or deletion of the terminal asparagine[23] almost completely abolishes elicitation of the alkalinization response (Pearce et al., 2008). Thus it seemed that the motif

SxGxxxxxN, which is strictly conserved within all eight AtPeps, is critical for Pep activity.

The peptides SlPep6 and ZmPep1 used in Fig. 3.2 do not conform to this rule. SlPep6 contains a glycine at position 15 instead of a serine and ZmPep1 shows a histidine at the terminal position 23 instead of an asparagine and this might explain why they are not recognized by the Brassicaceae. In order to identify plant family-specific motifs we tested a larger number of family-specific peptides and derived consensus sequence. Fig. 3.3A shows the recognition of all eight AtPeps and two BrPeps from *Brassica rapa* by Arabidopsis and *B. rapa*. In Fig. 3.3B a similar experiment is shown using four peptides from Solanaceae together with *S. lycopersicum* and *N. benthamiana* and in Fig. 3.3C six peptides from Poaceae are tested using *Z. mays* and *L. perenne*. Consistently, the collection of family-specific peptides triggered a significant induction of ethylene production indicating that these peptides were perceived by the respective species. This indicates that the Peps derived from the newly identified PROPEPs are indeed active Peps and that all peptides related to a plant family are recognized by (at least two) species from this plant family. Given these findings, we used the Pep sequences to build a weblogo for the visualization of the consensus sequence of each peptide group (Fig. 3.3D). It shows that each family has evolved distinct and specific Pep motifs. For example in the Brassicaceae-specific sequence there is only one partially conserved proline whereas proline residues seem to play an important role in the sequence of Peps from Solanaceae whereas the Poaceae-specific consensus sequence is rich in glycine residues and conserved histidine residues at the terminal end of the peptides.

Validation of novel Pep-motifs

Are family-specific Pep-motifs sufficient for Pep recognition? In order to address this question, we mutated the sequences of AtPep1, SlPep6 and ZmPep1 to introduce the family-specific motif of non-origin plant families resulting in AtPep1-SOL and AtPep1-MONO (containing the motifs of the Solanaceae (SOL) and the Poaceae (MONO, Monocots)), SlPep6-BRA and SlPep6-MONO (containing the Brassiceae (BRA) and Poaceae motifs, respectively) and ZmPep1-BRA and ZmPep1-SOL (see Supplementary Table S1). As demonstrated by the ethylene production of leaf tissue taken from the Brassicaceae representatives (Arabidopsis and *B. rapa*) these modified peptides containing the BRA-Pep-motifs are recognized and likewise the Solanaceae as well as

the Poaceae species respond to the “SOL” and the “MONO” peptides, respectively (Fig. 3.4A, B and C, respectively). However, despite a significant response to all peptides, the ZmPep1-BRA and ZmPep1-SOL peptides do not trigger an ethylene production comparable to the one triggered by perception of the species-specific control peptide indicating that additional residues outside the motifs contribute to Pep-PEPR interaction.

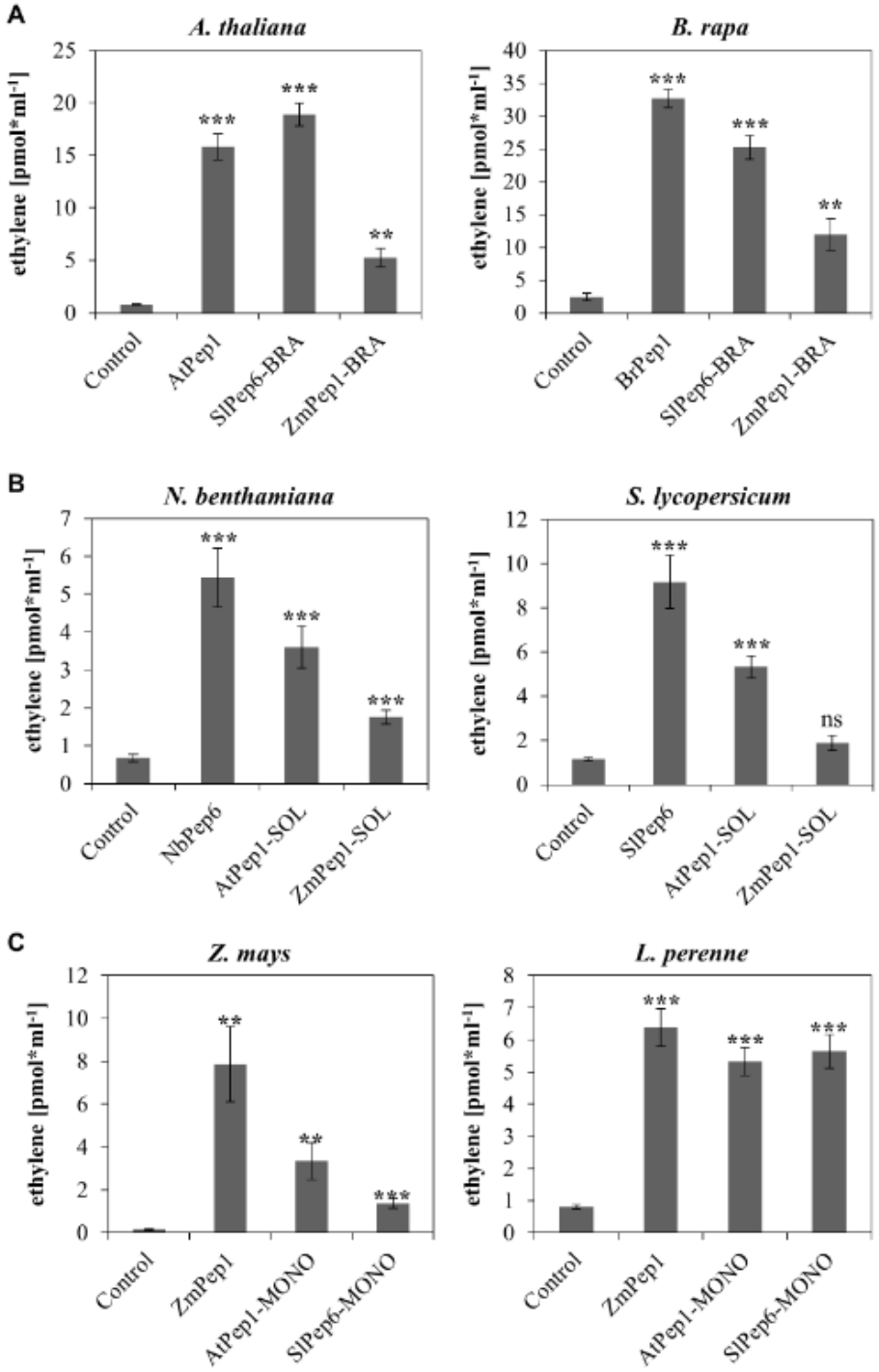

Figure 3.4: Validation of family-specific Pep motifs with mutated Peps
Ten leaf discs of indicated plant species were treated for 5 h with 1 µM of the indicated elicitor peptides or without any peptide (control). BRA indicates the introduction of the Brassicaceae-specific motif into the Pep sequence, SOL indicates the introduction of the Solanaceae-specific motif into the Pep sequence and MONO marks mutated peptides containing the Poaceae (MONOcot)-specific motif in their sequence. Columns represent averages of detected ethylene values of 5 biological replicates. Error bars show the standard error of the mean. Asterisks indicate significant differences of the labelled column to the control based on t-test results (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$).

PEPRs show interfamily compatibility

Furthermore, we investigated if PEPR sequence divergence correlated with an interfamily incompatibility of the system. In the first study describing AtPEPR1 the authors used the alkalinisation response of transgenic tobacco cells expressing AtPEPR1 to show that AtPEPR1 recognises AtPep1 (Yamaguchi et al., 2006). Thus, at least AtPEPR1 functions also in tobacco cells and not just in Brassicaceae. Here we cloned and studied additional PEPRs by introducing the coding sequences of AtPEPR1, the tomato PEPR SlPEPR1 and the maize PEPR ZmPEPR1a into the expression vector pGWB517 and transiently expressing them in *N. benthamiana* leaves. Again we used the elevated production of ethylene as a read-out for the activation of PEPR signalling upon perception of Peps. Leaf tissue of *N. benthamiana* is naturally insensitive to AtPep1 and ZmPep1, however, when transformed with AtPEPR1 or ZmPEPR1 it responded with a strong production of ethylene (Fig. 3.5). Remarkably, in this assay we did not detect a significant ethylene production in SlPep6-treated leaf discs despite the previously noted sensitivity of wild type *N. benthamiana* leaves to SlPep6 (Fig. 3.2 and Fig. 3.3B). In contrast, leaf discs transiently expressing SlPEPR1 again responded with a strong ethylene production upon addition of SlPep6 (Fig. 3.5). This apparent discrepancy is based on the use of only three discs per replicate harvested from the transiently transformed leaves in this experiment compared to ten discs per replicate used in the assays based on wild type leaves. Three discs are not enough to detect the little ethylene production elicited in wild type discs upon SlPep6 treatment but are sufficient to show the strong SlPep6-dependent production of ethylene in leaf discs transiently transformed with SlPEPR1. Thus as reported before (Flury *et al.*, 2013)Thus as reported before the overexpression of PEPRs boosts Pep-triggered responses.

Like SlPEPR1, also AtPEPR1 and ZmPEPR1a are able to activate downstream signalling pathways despite their transfer into the unrelated species *N. benthamiana*. This prompted us to further analyse PEPR-LRR domain, that detects Peps, and the PEPR-kinase domain which is crucial for downstream signalling. As shown in Supplementary Table S6 and Supplementary Table S7 the PEPR-LRR domains show a distinctly lower sequence identity than the PEPR-kinase domains. For example the sequence identity of AtPEPR1-LRR and SlPEPR1-LRR is 47.6 % whereas the one of AtPEPR1-kinase and SlPEPR1-kinase is 55.9 %. Within the large cluster of Poaceae-

PEPRs the sequence identity of these PEPR-LRRs ranges between 52.4 % and 89.3 % whereas the sequence identity of the kinase domains ranges from 66.4 % to 95.3 %. This data supports the idea that the kinase domain is more strictly conserved since it has a catalytic role and interacts with the complex defence signalling network whereas the LRR domain is not subjected to catalytic constraints but has evolved necessary plasticity to recognise specific ligands as has done so with the Peps (PROPEPs). Based on the AtPEPR1-LRR-AtPep1 crystal structure a number of amino acids within the LRRs LRR4 to LRR18 of the AtPEPR1-LRR domain were identified to interact with AtPep1 (Tang et al., 2015). Thus we further analysed the plasticity of the Pep-PEPR interaction by determining the conservation of the interaction site within the PEPR-LRR domain. As shown in Supplementary Figure S1 we aligned the LRR-domain sequences of all identified PEPRs and highlighted the Pep-interacting amino acids based on the AtPEPR1-LRR in magenta. Only seven of the 25 amino acids interacting with Pep show a considerable degree of conservation whereas the other 18 appear not to be conserved.

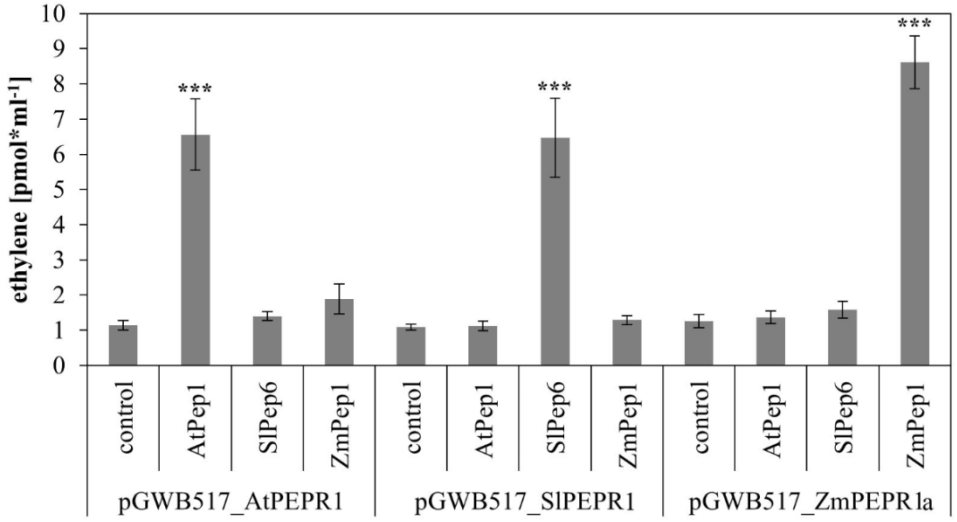

Figure 3.5: Detection of Peps by transiently expressed PEPRs.
Nicotiana benthamiana plants were transiently transformed with *Agrobacteria* containing pGWB517 plasmids harbouring the coding sequences of either AtPEPR1, SlPEPR1 or ZmPEPR1a (as indicated). Leaf discs were harvested one day past transformation. Columns represent averages of detected ethylene values of 6 biological replicates (containing three leaf discs each) 5 h after treatment with the indicated peptides or without any peptide (control). Error bars show the standard error of the mean. Asterisks indicate significant differences of the labelled column to the control based on t-test results (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$).

Taken together, contrary to the PROPEPs PEPRs are interfamily compatible. Their kinase domains are more strictly conserved than their LRR including the Pep interaction site arguably reflecting the coevolution of the LRRs and the PROPEPs.

3.4 DISCUSSION

In recent years a couple of distinct endogenous signalling peptides involved in plant defence processes were reported (Albert, 2013). They were identified from different species but most of them, notably systemins, appear to be restricted to specific plant families (Ryan and Pearce, 2003; Pearce et al., 2010b). Two exceptions are PSKs (phytosulfokines) and RALFs (Rapid Alkalinization Factors) which have been shown to be present in a broad range of species but only a small number of reports link them to plant defence (Pearce et al., 2001a; Igarashi et al., 2012; Albert, 2013; Mosher et al., 2013; Sauter, 2015). PSKs were classified as growth factors with additional functions in diverse developmental processes and PSK-triggered signalling was shown to negatively affect PTI (Igarashi et al., 2012; Hartmann et al., 2014; Sauter, 2015). Likewise, RALFs also regulate plant growth as well as other developmental processes including pollen tube elongation (Covey et al., 2010; Murphy and De Smet, 2014). Their association with plant defence is based only on the induction of physiological responses which have been linked to PTI (Pearce et al., 2001a; Albert, 2013). Thus, even though these peptides and their dependent signalling network are currently discussed as integrators of plant growth and defence they are regarded as growth factors rather than DAMPs (Murphy and De Smet, 2014; Sauter, 2015).

In contrast, Peps have been tightly linked to plant defence and are regarded as DAMPs (Albert, 2013; Bartels and Boller, 2015). Despite their discovery in Arabidopsis already previous studies indicated that the Pep-PEPR system is not an invention made by the Brassicaceae but that at least PROPEPs are present in multiple species (Huffaker et al., 2006; Huffaker et al., 2011; Huffaker et al., 2013; Trivilin et al., 2014). However, the first identified and characterized ortholog of AtPeps, ZmPep1, showed extensive differences in its amino acid sequence raising some doubts about its homology to AtPep1 (Huffaker et al., 2011). But their functional similarity has been shown in a number of studies thus the sequence diversity seems to be rather a sign for a strong

divergence of the system (Huffaker et al., 2006; Huffaker et al., 2011; Liu et al., 2013). Since a detailed analysis of the presence and activity of the Pep-PEPR system including the PEPRs has hitherto not been undertaken we took this opportunity to analyse the interspecies and interfamily compatibility of the system.

Based on our data it is now clear that the Pep-PEPR system is widely present within the angiosperms. We could neither identify potential PROPEPs or PEPRs in the gymnosperms nor in lower plants. This does not necessarily mean that there are no PROPEPs or PEPRs since most sequenced plant genomes belong to species of the angiosperms and the seemingly high plasticity of the PROPEPs could mask their identification. Moreover, PEPRs likely evolved from the numerous receptors regulating plant development which additionally exacerbates their conclusive identification in the more primordial plant species (Yamaguchi et al., 2010).

Consistent with previously reported data from Huffaker et al. (2013) we uncovered an interfamily incompatibility of Peps. Although a considerable number of Peps contain the previously identified ser^{15}, gly^{17} and asp^{23} (Pearce et al., 2008), it appears that contrary to the previous assumptions these residues may not be a prerequisite of Pep activity in general. The novel conserved motifs described in this study (Fig. 3.3D) rather point to the fact that a larger number of Pep residues are important for Pep-PEPR interaction and with it for Pep "activity". The recently resolved crystal structure of AtPEPR1-LRR in conjunction with AtPep1 supports this idea since multiple Pep residues were found to be in close contact with the PEPR1-LRR (Tang et al., 2014). In addition, Proline^{19} as well as Glutamine^{21} and Histidine^{22} seem to be crucial for the interaction of PEPR1 with its co-receptor BAK1 (Tang et al., 2014). Notably, the lack of BAK1 together with its closest relative BKK1 (BAK1-LIKE1) completely impairs PEPR signalling (Roux et al., 2011b). In summary, we found that there is no typical strictly conserved Pep motif and thus we propose that Peps and their precursors PROPEPs as well as the ligand-binding (LRR) domain of the PEPRs rapidly diverged producing distinct Pep motifs and as a consequence the interfamily incompatibility. However, it is also possible that some Peps retained a rather more generic sequence and structure that is still binding loosely to LRRs from more distantly related species.

Contrary to the incompatibility of Peps the PEPRs appear to be interfamily compatible. Transient expression of AtPEPR1 and ZmPEPR1a in *N. benthamiana* enabled AtPep1 and ZmPep1 sensitivity. In light of the higher level of conservation of the PEPR kinase

domain compared to the rather variable sequence of the PEPR LRR domain including the Pep interaction site it seems that only the Pep detection via the LRR domain features a substantial plasticity whereas the intracellular part of the PEPR operates a strictly conserved defence signalling system. In support of this view is the involvement of BAK1 and BKK1 as co-receptors of PEPRs (Schulze et al., 2010; Roux et al., 2011b). BAK1 in particular has been linked to numerous receptors involved in plant defence signalling and is thus regarded as a signalling hub (Chinchilla et al., 2009; Roux et al., 2011b). In addition an observation similar to the PEPR interfamily transfer has been made with the interfamily transfer of EFR (EF-Tu RECEPTOR), that has evolved in the Brassicaceae to detect the presence of the bacterial protein EF-Tu (Zipfel et al., 2006; Lacombe et al., 2010). Expression of EFR in *N. benthamiana* or *S. lycopersicum* enabled the detection of EF-Tu in both species and improved the resistance against a number of pathogenic bacteria (Lacombe et al., 2010). Finally, PEPR signalling has been reported to induce jasmonic acid, salicylic acid as well as ethylene-dependent genes (Ross et al., 2014). Since plant immunity is constructed as a robust network where jasmonic acid, salicylic acid and ethylene signalling significantly overlap to compensate for the loss of individual signals (Tsuda et al., 2009) PEPRs seem to occupy a central and/or flexible role here. Thus there is most likely no room for plasticity of the intracellular part of the PEPRs. However, if the plasticity of the Pep/PEPR-LRR interaction is of advantage for PEPR signalling (e.g. by evading inhibitory action of bacterial peptides) still needs to be determined.

3.5 CONCLUSION

Contrary to the detection of conserved MAMPs that requires an equally conserved detector domain of the MAMP receptor the sequences of Peps and PEPR-LRRs appear to evolve more dynamically resulting in a considerable divergence of the Pep-PEPR system. The identification of the variable plant family-specific Pep motifs will probably help to uncover more PROPEPs with the advancing number of sequenced plant genomes and the improved gene annotation. Moreover, activation of the Pep-PEPR system has been shown to effectively improve resistance against a broad spectrum of pathogens including bacteria, fungi as well as herbivores. Having learnt that the Pep-

PEPR system is common among angiosperms, two approaches could be valuable for improving cultivation of crop plants. Firstly, marker assisted breeding should be implemented to track and conserve the Pep-PEPR system during crop plant breeding, and secondly, rationally designed synthetic Peps could be used to boost plant resistance of especially valuable crops when pathogen attack is imminent. Thus it is no surprise that integral parts of the Pep-PEPR system have already been patented.

3.6 MATERIAL AND METHODS

Plant material

Arabidopsis thaliana Col-0 and *Brassica rapa* plants were grown individually in small pots at 21° C and a 10 h photoperiod for 4-5 weeks. Plants of the species *Solanum lycopersicum*, *Nicotiana benthamiana*, *Zea mays* and *Lolium perenne* were grown as single plants per pot at 24 °C and a 16 h photoperiod for 3-5 weeks.

Peptides

Peptides were obtained from Selleckchem (Houston, Texas, USA) and dissolved in a solution containing 1 mg/mL bovine serum albumin and 0.1 M NaCl to reach stock concentrations of 100 µM. Further dilutions were done in ddH_2O. The list of peptides and their sequences can be found in Supplementary Table S1.

Bioinformatics:

Novel PEPR sequences were identified using NCBI blastp as well as tblastn on phytozome.com using the AtPEPR1 and AtPEPR2 sequences. For the identification of novel PROPEP sequences, all sequences from Bartels *et al*. (2013) and Huffaker *et al*. (2013) were aligned per plant family and used as input for an hmmsearch search (HMMER v1.9; (Finn et al., 2011)) against the NR, RefSeq and UniProtKB databases with standard settings. Identified sequences were manually curated for the presence of a Pep motif at the C-terminal end of protein. Newly identified PROPEPs were used as additional input for a new hmmsearch. All identified PEPRs and PROPEPs are listed in Supplementary Table S2.

Identification of the kinase and LRR domain within the PEPR sequences was done by scanning for full Pfam domains (Finn et al., 2014) with default settings using CLC Main Workbench 6.7.1 (CLC bio, Aarhus, Denmark). For building the trees and identity graphs, all sequences were first aligned using CLC Main Workbench 6.7.1 (CLC bio, Aarhus, Denmark) and subsequently identities were called or the trees were built using neighbour-joining with 1,000 bootstraps.

Visualization of Pep consensus sequences was done using WebLogo 2.8.2 (http://weblogo.berkeley.edu/logo.cgi) (Crooks et al., 2004).

Ethylene Measurement

For wild type plants eighte to ten leaf discs (5 mm diameter cork borer) or equal leaf squares (cut with scissors) were harvested from fully expanded leaves and placed into a 6 ml glass vial containing 0.5 ml of ddH_2O. In case of transiently transformed *N. benthamiana* leaves, discs were harvested from at least three independently transformed leaves, mixed and distributed into the vials (3 each). After a 16 h incubation period in the growth chamber elicitor peptides (1 µM final concentration) were added and vials were closed with air-tight rubber septa. Vials were incubated for 5 h at room temperature before ethylene accumulating in the free air space was measured by gas chromatography (GC-14A Shimadzu).

Cloning of ZmPEPR1a and SlPEPR1

Total RNA of *Zea mays* and *Solanum lycopersicum* was extracted from a 1:1 mix of root and leaf material of three week old plants using Nucleospin RNA plant spin columns (Macherey-Nagel). Reverse transcription of mRNA into cDNA was performed using AMV-RT enzyme kit (Promega) together with a 21nt oligo dT primer. ZmPEPR1a coding sequence was amplified from *Z. mays* cDNA using forward (5`-GGGACAAGTTTGTACAAAAAAGCAGGCTTGATGAAGCTGGTTTTCTGGCATTGGATTTTTCTATTCTTC-3`) and reverse primer (5`-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTGCCGGTAGGCGCTGCTGTTGGATTGCGATCCTG-3`) in a PCR reaction with Phusion polymerase (New England Bio Labs) in GC reaction buffer and 3% DMSO for amplification of GC-rich targets to generate a 3429-bp product. SlPEPR1 coding sequence was amplified from *S. lycopersicum* cDNA using forward (5`-

GGGACAAGTTTGTACAAAAAAGCAGGCTTGATGAAGATAGCTGTTCATAAT TTGATCTTTTTCTACTGC-3`) and reverse primer (5`-GGGGACCACTTTGTACAAGAAAGCTGGGTCGTACTTGCTTCGTATACTCGA ACTTGACCTTGTTAATAG-3`) in a standard PCR reaction with Phusion polymerase to generate a 3372-bp product. Correct PCR products were cloned into pDONR207, sequenced and subcloned into pGWB517 using the Gateway cloning technique according to the manufacturers protocol (Invitrogen).

Transient Expression of PEPRs in *Nicotiana benthamiana*

Agrobacterium tumefaciens GV3101 strains harbouring pGWB517 plasmids with either the coding sequence of AtPEPR1, SlPEPR1 or ZmPEPR1a were grown for 24 h in liquid YEB medium supplemented with appropriate antibiotics. Harvested cultures were resuspended in a solution containing 10 mM MES (pH 5.6) and 10 mM $MgCl_2$ to reach OD_{600} = 0.1 and syringe infiltrated into 3-week-old *N. benthamiana* leaves. Infiltrated leaf areas were harvested 24 h after infiltration and used for the measurement of ethylene production upon peptide treatment as described above.

3.7 SUPPLEMENTARY DATA

Supplementary data and tables are accessible via: http://jxb.oxfordjournals.org/content/early/2015/05/22/jxb.erv236/suppl/DC1

4. THE DAMP PRECURSOR *AT*PROPEP1 IS PROCESSED BY THE CYSTEINE PROTEASE *AT*METACASPASE4 DURING THE WOUND RESPONSE

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This manuscript represents the initial and main work of my thesis and was performed in collaboration with Simon Stael and his colleagues of the University in Ghent, Belgium. The work aimed to identify the key players and specific circumstance for the formation of *At*PEP1 from the precursor protein *At*PROPEP1. I performed the initial experiments showing that *At*PROPEP1 is indeed cleaved in vivo that led to the collaboration with Simon Stael. Sebastian Bartels and me outlined the project and planned the individual experiments. I performed the experiments presented in figures 4.1 to 4.3 and S 4.1, and prepared cloning vectors and transgenic lines that were used in the other experiments. The manuscript was written by Simon Stael, Sebastian Bartels and me and is about to be submitted to Nature Cell Biology.

4.1 ABSTRACT

In animals the immunological response to trauma comprises the production and release of cytokines such as interleukins. Although, the wound response has been intensively studied in plants, little is known about proteins with cytokine-like function. Here we report that mechanical damage triggers the instantaneous activation of *Arabidopsis thaliana At*METACASPASE4 and the subsequent cleavage of *At*PROPEP1 to release the mature *At*PEP1 peptide that induces basal immunity against a broad diversity of plant pathogens. Furthermore, confocal microscopy of targeted cell damage by laser wounding revealed that i) the inactive zymogen of *At*MC4 and *At*PROPEP1 both reside in the cytosol until a constitutive increase in calcium concentration upon cell membrane integrity loss triggers *At*MC4 activation and thus *At*PROPEP1 cleavage and ii) *At*PROPEP1 cleavage is restricted to few cells surrounding the damage site. Our results favour a model in which tissue damage is a prerequisite for *At*PEP1 signalling and highlight conserved spatiotemporal dynamics and control mechanisms of the wound response.

4.2 INTRODUCTION

Plants as sessile organisms are frequent victims of tissue and cellular damage. A multitude of animals feed on plants or cause collateral damage due to their movement. Whereas herbivore feeding is an immediate threat to the life of a plant, through the loss of tissue and cellular integrity, the wounds generated are also potential entry sites for microbial pathogens with often even more dramatic consequences for plant survival and reproduction (Heil and Land, 2014). Thus, plants need to respond rapidly to cellular damage with defense responses and tissue regeneration by recognizing it on the molecular level. The release of damage-associated molecular patterns (DAMPs) by pattern recognition receptors (PRRs) is one important danger signal originating in the wounded area, which may alert the surrounding still intact tissue. DAMP perception has been shown to induce plenty of responses in the local and systemic tissue (Heil et al., 2012). The so far described molecules that serve as DAMPs are characterized by their relocalisation from specific cellular compartments (in intact cells) to the extracellular

space upon cellular damage, combined with their potential to elicit defense responses upon perception (Hernandez-Onate and Herrera-Estrella, 2015).

A number of diverse DAMPs have been identified in plants including ATP, oligogalacturonides and peptides (Norman et al., 1999; Huffaker et al., 2006; Tanaka et al., 2014). The plant elicitor peptides (PEPs) have been recognized as peptidic DAMPs based on their endogenous origin and their ability to elicit pattern-triggered immunity (PTI) (Bartels and Boller, 2015). In Arabidopsis, the *PROPEP* gene family encodes eight PEP precursor proteins (PROPEP1 to PROPEP8) which each contain a PEP (PEP1 to PEP8) at its C-terminus (Huffaker et al., 2006; Bartels et al., 2013). PEPs are perceived by the extracellular LEUCINE-RICH REPEAT (LRR) domain of transmembrane RECEPTOR-LIKE KINASEs (LRR-RLKs) named PEP RECEPTORs (PEPRs) and trigger PTI in conjunction with the their co-receptor BRI1-ASSOCIATED KINASE1 (BAK1) (Yamaguchi et al., 2006; Krol et al., 2010; Yamaguchi et al., 2010).

The ORFs of all Arabidopsis PROPEPs lack a signal sequence, and they were reported to either localize to the cytoplasm (PROPEP3) or to be associated with the cytoplasmic side of the tonoplast (PROPEP1 and PROPEP6) (Bartels et al., 2013). Provided that they are not exported via hitherto unknown unconventional routes they might enter the extracellular space only in a situation of compromised cellular integrity like mechanical damage, but experimental data are lacking. Moreover, the isolation of PEP1 and PEP5 from leaf-protein extracts is currently the only indication for the release of PEPs from their respective PROPEP, but here as well the circumstances and the mechanism is unknown (Huffaker et al., 2006; Yamaguchi and Huffaker, 2011). In mammals, proinflammatory cytokines like INTERLEUKIN-1α (IL-1α, classified as DAMP as well) or IL-1β are released during trauma and cell death (Gabay et al., 2010). Both are expressed as precursors (ProINTERLEUKIN-1α and ProIL-1β, respectively) which are proteolytically processed by the proteases calpain and CASPASE-1 to generate IL-1α and IL-1β, respectively (Watanabe and Kobayashi, 1994; Brough and Rothwell, 2007).

In plants, metacaspases (MCs) have been previously suggested as homologs of the mammalian caspases based on structural similarities and their role in initiating and executing apoptosis (Uren et al., 2000; Fuchs and Steller, 2011; Tsiatsiani et al., 2011). Metacaspases strictly cleave C-terminally of the basic amino acids, arginine and lysine, whereas caspases prefer the acidic amino acid aspartate (Vercammen et al., 2004). The

Arabidopsis thaliana genome contains nine metacaspases that are classified based on their structure. Type I MCs, *At*MC1-3, have an N-terminal prodomain containing a proline - glutamin rich repeat motif and a zinc finger motif, whereas type II MCs, AtMC4-9, have a larger linker region separating the large and small catalytic domains, p20 and p10, respectively (Tsiatsiani et al., 2011). *At*MC4-9 are produced as inactive zymogens and are activated by autocatalytic cleavage of the linker region. Furthermore, most type II metacaspases require calcium for their activation and have a pH optimum of 7.5, except *At*MC9 that is not calcium dependent and functions optimally at pH 5.5 (Vercammen et al., 2004; Watanabe and Lam, 2011b). Several metacaspases from fungi and plants were found to be involved in programmed cell death (PCD) (Tsiatsiani et al., 2011; Lam and Zhang, 2012), although the paradigms from metazoan PCD do not apply to plants (van Doorn et al., 2011) and lateral thinking has possibly even stalled the research of plant PCD and metacaspase function (Stael et al., 2014).

Interestingly, increasing evidence is gained that caspases are involved in other processes than apoptosis, including development and wound healing (Fuchs and Steller, 2011).

4.3 RESULTS

*At*PROPEP1 is instantaneously cleaved upon tissue damage

To investigate if and how the plant elicitor peptides PEPs are released from their PROPEP precursors, we focused our effort on studying the conversion *At*PROPEP1 to *At*PEP1. *At*PEP1 has been detected by mass spectrometry in an Arabidopsis leaf protein extract indicating a preceding endogenous cleavage or processing of *At*PROPEP1 (Huffaker et al., 2006). We used our previously described transgenic *A. thaliana* lines (Bartels et al., 2013) constitutively expressing either an *At*PROPEP1-YFP fusion protein, an *At*PEP1-YFP fusion protein or just YFP. With an anti-YFP antibody, we observed the composition of PROPEP1-YFP and PEP1-YFP in extracts prepared from frozen, homogenized whole seedlings in analogy to the initial publications. PROPEP1-YFP, PEP1-YFP and YFP were clearly distinguishable by their size on a western blot (Figure 4.1A) and we realized that PROPEP1-YFP is rapidly cleaved during protein extraction. As little as 30 seconds of incubation after thawing of the frozen tissue powder, obtained by grinding under liquid nitrogen resulted in detectable accumulation

of PEP1-YFP. The levels of PEP1-YFP increased and peaked at around 5 minutes (Figure 4.1A). Free YFP generated in parallel, perhaps by the further cleavage of the *At*PEP1-YFp (Figure 4.1A) Thus, proper and rapid handling of the samples is crucial to prevent PROPEP1-YFP protein cleavage during extraction in order to determine the background level of PEP1-YFP cleavage product in either unharmed or specifically treated leaf tissue. Besides the rapid accumulation of PEP1-YFP the amount of PROPEP1-YFP significantly decreased over time and also unspecific protein degradation occurred at later timepoints (Figure 4.1A). Next, we wondered if cleavage also occurs *in vivo* after wounding. We applied wounding by bruising of leaves and roots of PROPEP1-YFP expressing seedlings with a serrated forceps. We were able detect accumulation of PEP1-YFP but in a more uniform fashion over time compared to grinding of the seedlings (Figure 4.1B).

Cleavage of AtPROPEP1 depends on the conservation of R^{69}, the availability of Ca^{2+} ions, and is inhibited by a metacaspase-specific inhibitor

Aligning the amino acids sequences of all eight Arabidopsis PROPEPs (Figure 4.2A) revealed a conservation of the arginine (R) residue N-terminal to the cleavage site in PROPEP1, indicating that the processing enzyme might be an R-specific protease (Huffaker et al., 2006). To investigate this, we mutated the arginine R^{69} residue in PROPEP1-YFP (positively charged at physiological pH) to the negatively charged glutamate (E). As shown in Figure 4.2B the negatively charged glutamate residue in PROPEP1^{R69E}-YFP fully impaired the formation of PEP1-YFP and also blocked the degradation of the PROPEP1-YFP protein in both treatments (wounding of seedlings, and thawing of ground tissue).

To further narrow down the group of enzymes that might cleave PROPEP1-YFP to release PEP1-YFP, we infiltrated seedlings with protease inhibitors and ion-chelators prior to grinding and determined PEP1-YFP formation after thawing. Most tested protease inhibitors had a slight attenuating effect on the accumulation of PEP1-YFP but only EGTA, EDTA and the metacaspase-specific protease inhibitor Z-VRPR-fmk were

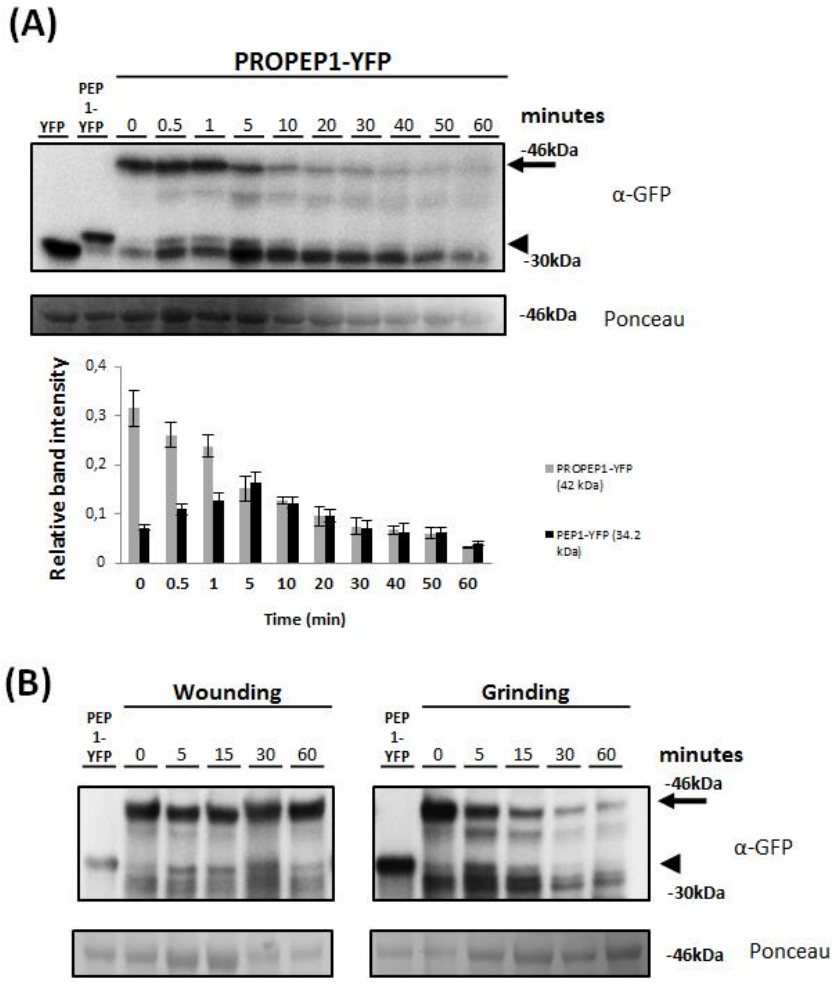


Figure 4.1: *At*PROPEP1 gets rapidly processed to PEP1 *in vitro* and in wounded tissue *in vivo*. A PROPEP1-YFP (arrow) degradation and appearance of PEP1-YFP (arrowhead) after incubation of tissue powder of whole seedlings at room temperature for the indicated time. YFP was loaded as a size control to distinguish the accumulation of free YFP in contrast to PEP1-YFP. Quantified band intensities (using ImageJ) are shown below the blot. Each bar represents mean of 5 replicates ± SE. **B** Comparison of PEP1-YFP (arrowhead) accumulation following wounding or incubation after thawing (Grinding).

able to fully abolish the formation of PEP1-YFP (Figure 4.2C). Surprisingly a commercially available protease inhibitor cocktail for plant cells (Sigma Aldrich P9599) was not sufficient to block PEP1-YFP formation thus we suggest that EGTA or EDTA should be added in future formulations.

In summary, the evidence points to the cleavage of PROPEP1-YFP by an arginine-specific and Ca^{2+}-dependent plant protease sensitive to a metacaspase-specific inhibitor. Thus, we further focused on plant type II metacaspases.

_At_METACASPASE4 is instantaneously activated upon damage and cleaves AtPROPEP1

Since *MC4* is the most ubiquitously expressed metacaspase gene in Arabidopsis, which is a fitting prerequisite for a general damage response regulator. Therefore, we focused on MC4 (Watanabe and Lam, 2011a; Bartels et al., 2013; Kwon and Hwang, 2013). The inactive zymogen MC4 requires autocatalytic cleavage into lower molecular weight species, including the p10 and p20 (active) subunits (Watanabe and Lam, 2011b). We used an *At*MC4-specific antibody (Watanabe and Lam, 2011b) to determine the activation of MC4 in ground leaf tissue (Figure 4.3A) as well as in wounded seedlings (Figure 4.3B). Indeed, in both experiments MC4 zymogen is rapidly cleaved to generate active MC4 with kinetics comparable to the cleavage of PROPEP1, and the addition of ion chelators blocked the autocatalytic activation of MC4 (Figure 4.3C). The presence of p20 subunit on western blot is not necessarily an indication of proteolytic activity, i.e. the p20 subunit that is present at t0 might be a long-lived proteolytically inactive cleavage product (Watanabe and Lam, 2011b) (Figure 4.3A-B). However, the correlation between minute-scale PROPEP1 cleavage with appearance of MC4 lower molecular weight species, including a presumed active 26 kDa species (Watanabe and Lam, 2011b) (Figure 4.3A-C) prompted us to investigate if MC4 is indeed capable of cleaving PROPEP1. To test this, we used an *in vitro* transcription translation approach combined with recombinant protease treatment (TNT-protease assay). Increasing concentrations of recombinant MC4 (rMC4) led to the efficient cleavage of PROPEP1 fused C-terminally to glutathione s-transferase (GST) already at low rMC4 concentrations (Figure 4.3D). Furthermore in accordance to the *in vivo* assay PROPEP1E^{R69E} was not processed to PEP1 but a band slightly smaller in size appeared that potentially derives from an alternate cleavage site in the C-terminal region, indicating that the cleavage and not the binding site of the protease was altered by this mutation (Figure 4.3D and 2A).

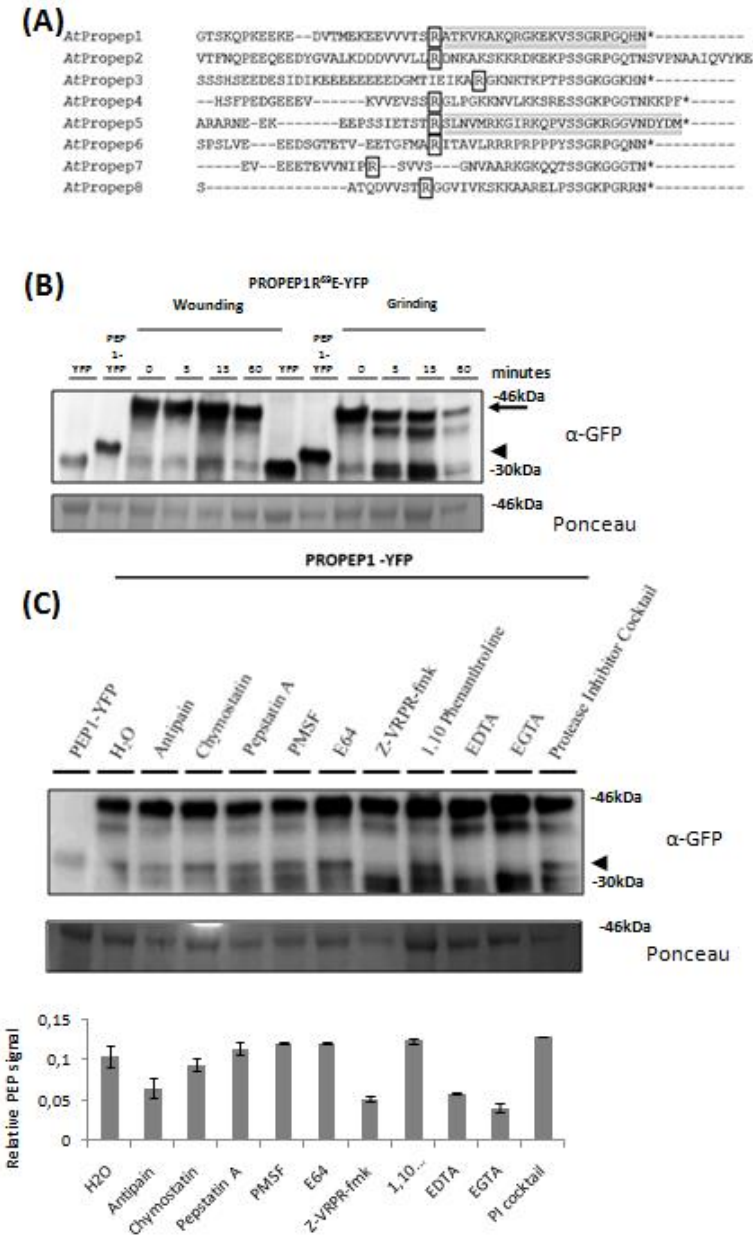


Figure 4.2: Arginine 69 is the critical amino acid for *At*PROPEP1 cleavage *in vivo*. A PROPEP1 arginine 69 lies N-terminal to the PEP1 cleavage site and is conserved within the *A. thaliana* PROPEPs (framed). *At*PEP1 and *At*PEP5 that have been identified by mass spectrometry in protein extracts are highlighted in grey. **B** Mutation of arginine 69 (R69) in PROPEP1 with glutamate (E) abolished wounding-induced accumulation of PEP1-YFP (arrowhead). **C** Whole seedlings overexpressing PROPEP-YFP were vacuum infiltrated with, ddH_2O as a control or the protease inhibitors Antipain (100µM), Chymostatin (100µM), Pepstatin A (1µM), PMSF (1mM), E64 (10µM), 1,10-Phenanthroline (20mM), Z-VRPR-fmk (50µM), EDTA (1mM), EGTA (1mM), and Protease inhibitor cocktail (1:100, Sigma Aldrich) respectively and assayed 5 minutes after tissue grinding and subsequent thawing.

Finally, we transformed the PROPEP1-YFP construct into an *atmc4* knock-out background to study if and to which extent the lack of MC4 impairs PROPEP1-YFP cleavage *in vivo*. As shown in Figure 4.3E lack of MC4 strongly impairs cleavage of PROPEP1-YFP in seedlings treated with our forceps-based wounding scheme.

Altogether, these results support the critical role of MC4 in the proteolytic cleavage of PROPEP1 into mature PEP1.

_At_PROPEP1-YFP fluorescent signal delocalizes from tonoplast to cytosol as revealed by laser ablation confocal scanning microscopy life cell imaging

*At*PROPEP1-YFP localizes to the cytoplasmic side of the tonoplast by an as yet unexplained mechanism whereas *At*PEP1-YFP was detected in the cytosol (Bartels et al., 2013). Tonoplast localization of *At*PROPEP1-YFP may present an obstacle to its export to the extracellular space via plasma membrane-localized peptide transporters or other means of unconventional secretion routes (Bartels et al., 2013). To gain a detailed view of the potential spatiotemporal behaviour of PROPEP1-YFP and PEP1-YFP localization in conjunction with its damage-induced cleavage we performed life cell imaging of *At*PROPEP1-YFP transgenic *A. thaliana* roots by confocal scanning microscopy together with laser ablation (Figure 4.4A). Laser ablation has been utilized in plants to study the molecular mechanisms of tissue repair, embryo development, graviperception and microtubule remodelling (Blancaflor et al., 1998; Xu et al., 2006; Sampathkumar et al., 2014; Liu et al., 2015). The use of multi-photon laser ablation allowed us to afflict highly localized damage to root cells and immediate imaging of minute-scale subcellular changes in the cells surrounding the laser ablation site as well as the targeted cell(s). Initially, we targeted the epidermal cells of the root "transition zone" (Figure 4.4A) as these are known to have a weakly formed cell wall and the cells are highly interconnected. As previously reported in the transition zone of resting cells PROPEP1-YFP localization appears as a dynamically moving cluster of enlarged membrane vesicles (first two panels of Figure 4.4B) (Bartels et al., 2013). Propidium iodide (PI) is frequently used

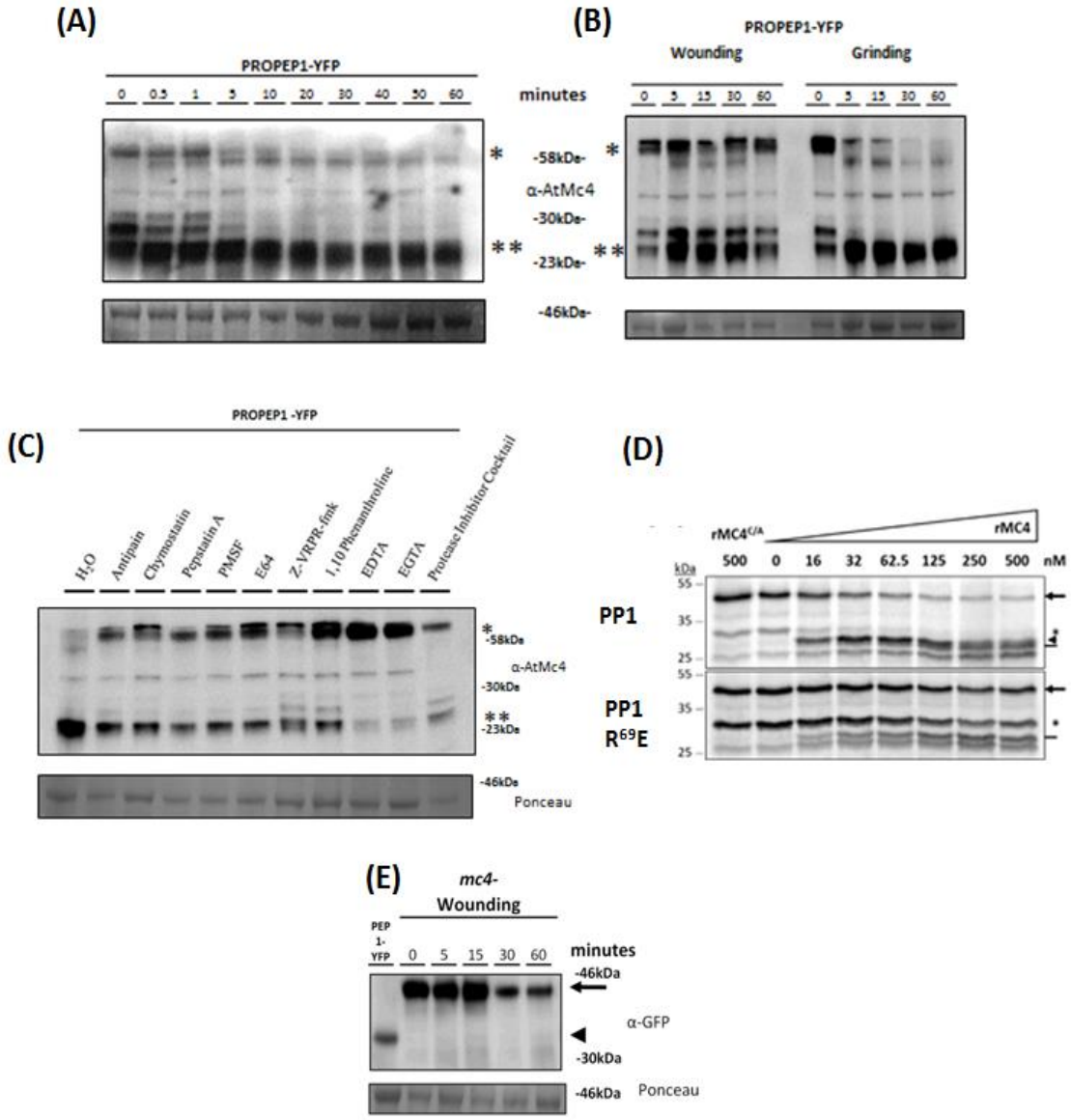


Figure 4.3: The cysteine protease *At*Metacaspase 4 gets activated during cell damage and cleaves PROPEP1 to release PEP1 *in vitro* and *in vivo* **A** Conversion of zymogenic *At*Metacaspase 4 (*) to ist active p20 subunit (**) in tissue powder incubated at RT for the indicated time. **B** Comparison of *At*Metacaspase 4 inactive (*) and active (**) forms after tissue wounding and grinding shows different strength of autocatalytic protease activation. **C** Addition of various protease inhibitors hinders *At*Metacaspase 4 activation (**) whilst ion chelation blocks the accumulation of the active subunit. **D** TNT®-protease assay of PROPEP1 (PP1) and mutant variants fused to glutathione S-transferase (GST) with increasing amount of recombinant AtMC4 (rMC4). Full-length PROPEP1-GST (arrow) was observed as wild type or mutated at Arg 69 (R) to Glu (E) The initial cleavage product occurring at lowest rMC4 concentrations is indicated with an arrowhead. At higher rMC4 concentration extended cleavage occurs at a site downstream resulting in a lower band indicated with a dash (-).**E** The wounding induced appearance of the PEP1-YFP band (arrowhead) was not observed in the metacaspase 4 T-DNA insertion mutant *mc4-*. Representative blots out of at least four replicates are shown.

used as a cell wall stain in plants and is not permeant to intact plasma membrane (bottom panels of Figure 4.4B). Entry of PI to the cytoplasm and nucleus was observed

as a proxy for loss of plasma membrane integrity due to the laser ablation treatment. In the three cells hit by the laser, this happened immediately (time 00:00). Concomitant with PI entry, the YFP fluorescence (PROPEP1-YFP or PEP1-YFP) delocalized from tonoplast to cytosol, observed as a diffuse signal overlapping with the PI signal (Figure 4.4B). Interestingly, the cells surrounding the initially targeted cell(s) would similarly react with a delocalization of the YFP signal from tonoplast to cytosol. This occurred within minutes after laser ablation (Figure 4.4B and Figure 4.5A). Usually, YFP signal dispersal from tonoplast to cytosol occurred concomitantly with PI entry.

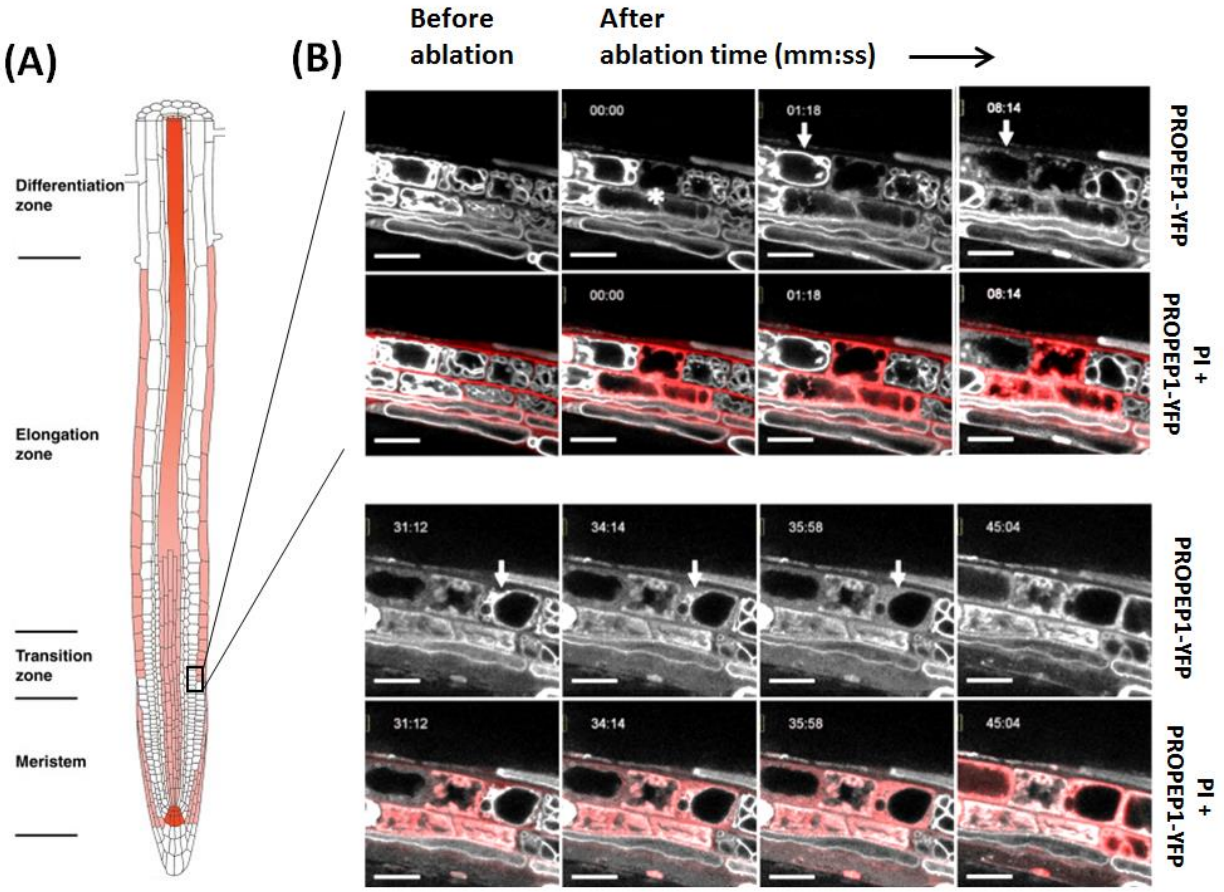


Figure 4.4 PEP1-YFP relocalizes from tonoplast to cytoplasm upon laser ablation in damaged and surrounding cells. A Region of the Arabidopsis root with the targeted zone indicated. **B** Epidermal cell layer of the root meristem transition zone of PROPEP1-YFP expressing seedlings was imaged before (top left panels) and after laser ablation (rest of the panels) by confocal microscopy. The wound site was continuously imaged up to 45 min after laser ablation over multiple confocal planes in Z dimension and the confocal plane shown here is midway the wounded cells. Laser target is indicated with an asterisk (* in timeframe 00:00). Notice the dispersal of YFP signal from tonoplast (PROPEP1-YFP) to the cytosol (PEP1-YFP) at time 00:00 in the cells surrounding the asterisk and the concomitant entry of PI (red signal) into these cells. In the next timepoints (01:18 to 08:14 min), YFP signal in the cell on the left of the wounded cells (indicated with an arrow) likewise undergoes dispersal. However, this occurs before PI entry. In the later timepoints (31:12 – 35:58 min), YFP signal in the cell on the right (indicated with an arrow) underwent dispersal, concomitant with PI entry. Scalebar is 20 µm.

To test if the same phenomenon could be observed in older cells, we targeted the more expanded epidermal cells of the elongation and differentiation zone (Figure 4.4A). In agreement with our previous findings, incubation of the Arabidopsis roots with 1 mM EGTA inhibited YFP signal dispersal after laser ablation induced wounding suggesting that the relocalized YFP signal refers to PEP1-YFP (Figure 4.5B). *At*PEP1-YFP again localizes to the tonoplast, which, due to turgor pressure in these cells, resembles a uniform inflated balloon (Figure 4.6A).

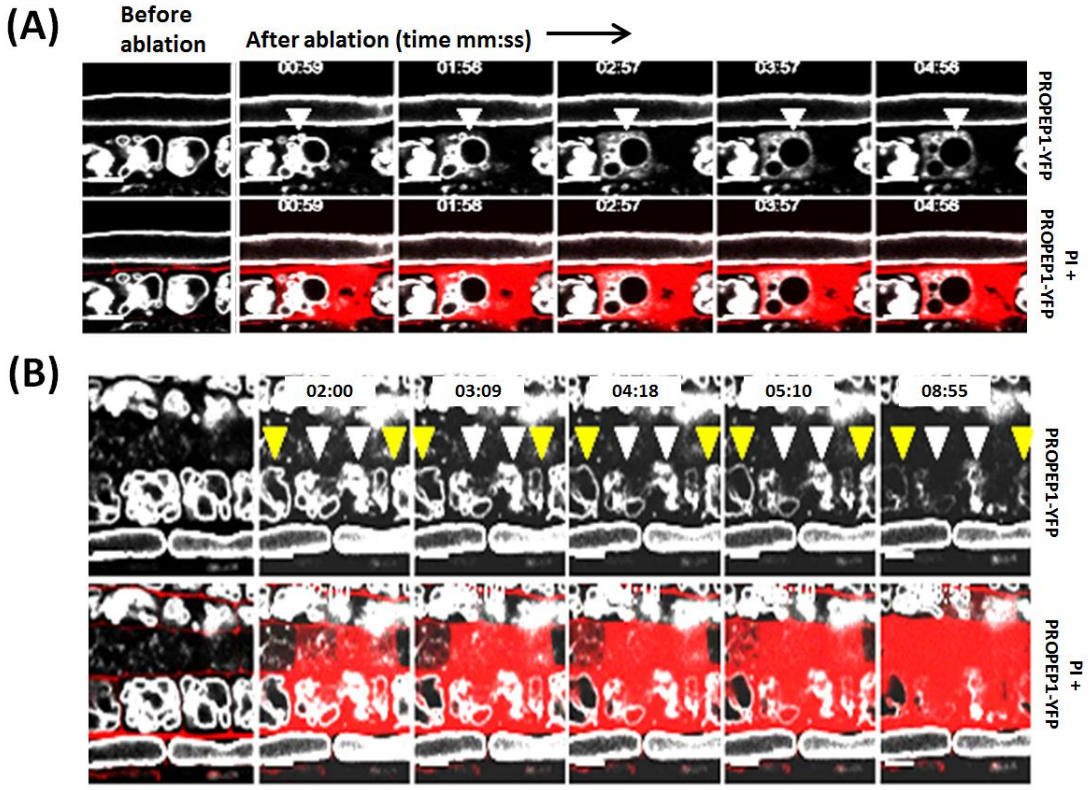


Figure 4.5 EGTA inhibits PROPEP1-YFP relocalisation from tonoplast to cytosol after wounding. **A** Laser ablation of Ler overexpressing PROPEP1-YFP (line 9358-2) in control condition (MES buffer pH 5.5). Note the transition of the sharp PROPEP1-YFP signal from tonoplast to the diffuse signal in the cytoplasm (indicated with white arrowhead) in the cell adjacent to the ablated cell. **B** Laser ablation of Ler overexpressing PROPEP1-YFP (line 9358-2) in presence of 1 mM EGTA (in MES buffer pH 5.5). The transition of the sharp PROPEP1-YFP signal from tonoplast to the diffuse signal in the cytoplasm in the ablated cell and the adjacent cell does not take place (indicated with white arrowhead). The surrounding cells start accumulating PI slightly later (indicated with yellow arrowheads). Also in these cells the transition is absent. Scalebar is 20 µm.

Due to the larger size of the cells as compared to those of the transition zone (Figure 4.4B), it was possible to damage and subsequently view the dispersal of PROPEP1-YFP signal in the targeted cell (Figure 4.6A). Incubation in 50 µM VRPR-fmk or 1 mM EGTA inhibited PROPEP1-YFP signal dispersal (Figure 4.6B and 4.6C, respectively).

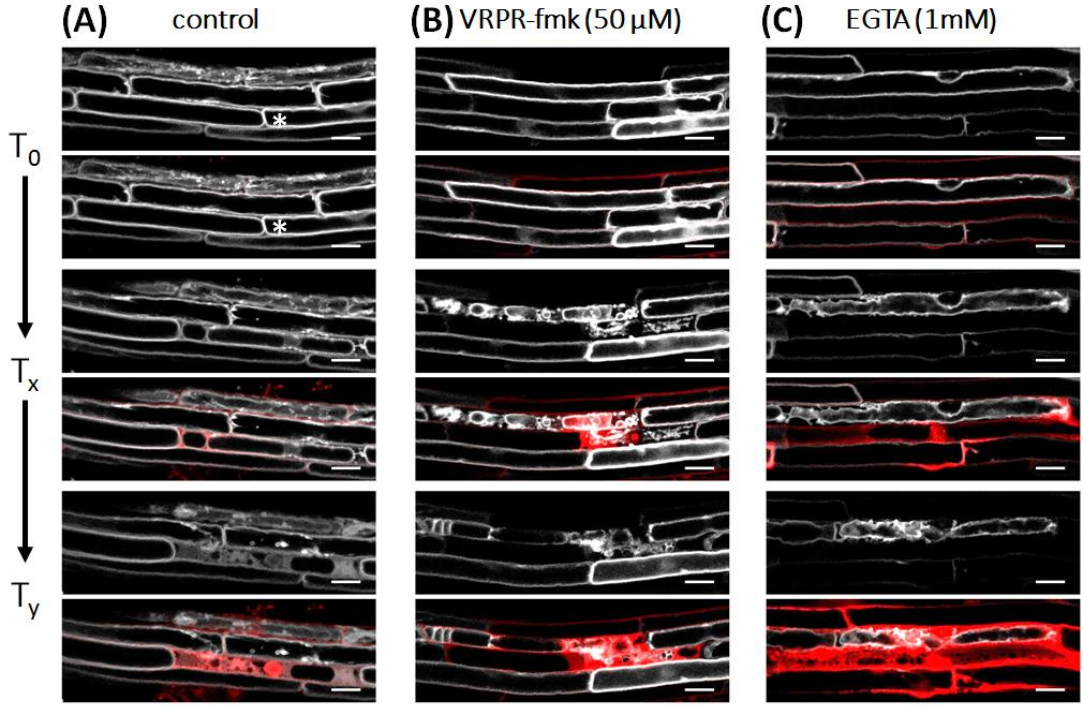


Figure 4.6 VRPR-fmk and EGTA inhibit PROPEP1-YFP delocalization from tonoplast to cytosol in epidermal cells of the transition and maturation zone. A Laser ablation of Ler overexpressing PROPEP1-YFP (line 9358-2) in control conditions (MES buffer pH 5.5). The targeted cell is indicated (*) on the image before ablation (time point 0, T_0). Note that in the subsequent images at time points T_x and T_y, the center of imaging was moved to the right to focus more on the targeted cell. At each time point, the upper image shows the PROPEP1-YFP signal and the lower image shows the overlap with PI signal. Treatment with 50 µM VRPR-fmk (in MES buffer pH 5.5) **B** and 1 mM EGTA (in MES buffer pH 5.5) **C** inhibits PROPEP1-YFP dispersal, indicated by an incomplete overlap of PROPEP1-YFP and PI signals. Scale bar is 20 µm.

Wounding by laser ablation induces PEPR1 internalization in adjacent cells

As wounding triggers cleavage of PROPEP1-YFP to release PEP1-YFP we wondered if sufficient amounts of PEP1 also enter the extracellular space and bind to PEPRs of adjacent cells. We therefore repeated the laser ablation experiment during confocal microscopy but used lines expressing PEPR1-GFP under its native promoter to observe ligand-induced receptor endocytosis. Endocytosis of receptors occurs upon ligand binding and leads to an accumulation of the receptor molecules in intracellular vesicles that can be observed as clustering of the labelled receptor (Irani and Russinova, 2009). Around 5 minutes after laser ablation clustering of PEPR1-GFP could be observed in the close area around the wounding site indicating the binding of PEP peptides by the receptor (Figure 4.7A). The receptor clustering spread away from the wounded area until the maximal observation time of 40 minutes. An alignment of several images

shows the spread of the signal over several rows of cells from the initial wounding site (Figure 4.7B).

The triggering of PEPR1-GFP internalization around the wounded area points to a release of active PROPEPs or PEPs from these cells what then led to activation of the receptor.

Laser ablation leads to increased calcium concentrations in the surrounding cells

In this study, we demonstrate the necessity of calcium signalling for cleavage of PROPEP1-YFP, however the threshold concentration of calcium ions in the cytosol needed to induce cleavage remains unknown. Calcium spikes are abundant during normal plant development and stress responses (Dodd et al., 2010; Zhang et al., 2014a), nevertheless, it would seem counter-productive to have PROPEP cleavage after every spike. To determine the precise role of calcium regulation in this process, we have imaged calcium fluxes after laser ablation in epidermal cells of the transition and maturation zone of the root tip with a nuclear localized Yellow Cameleon (YC3.6) probe (Figure 4.8). Calcium spikes of short duration are elicited immediately after laser ablation in the cells in close vicinity to the wounded area. While most cells return to calcium concentrations observed during resting state (Figure 4.8E,F,G), targeted cells and neighbouring cells that start accumulating PI keep a high calcium concentration (Figure 4.8E,F) or slowly accumulate high levels of calcium ions (Suppl. Fig. 4.1). It follows that a calcium spike per se is not sufficient to activate metacaspase and cleavage of PROPEP, but a more sustained elevation of calcium concentration is needed.

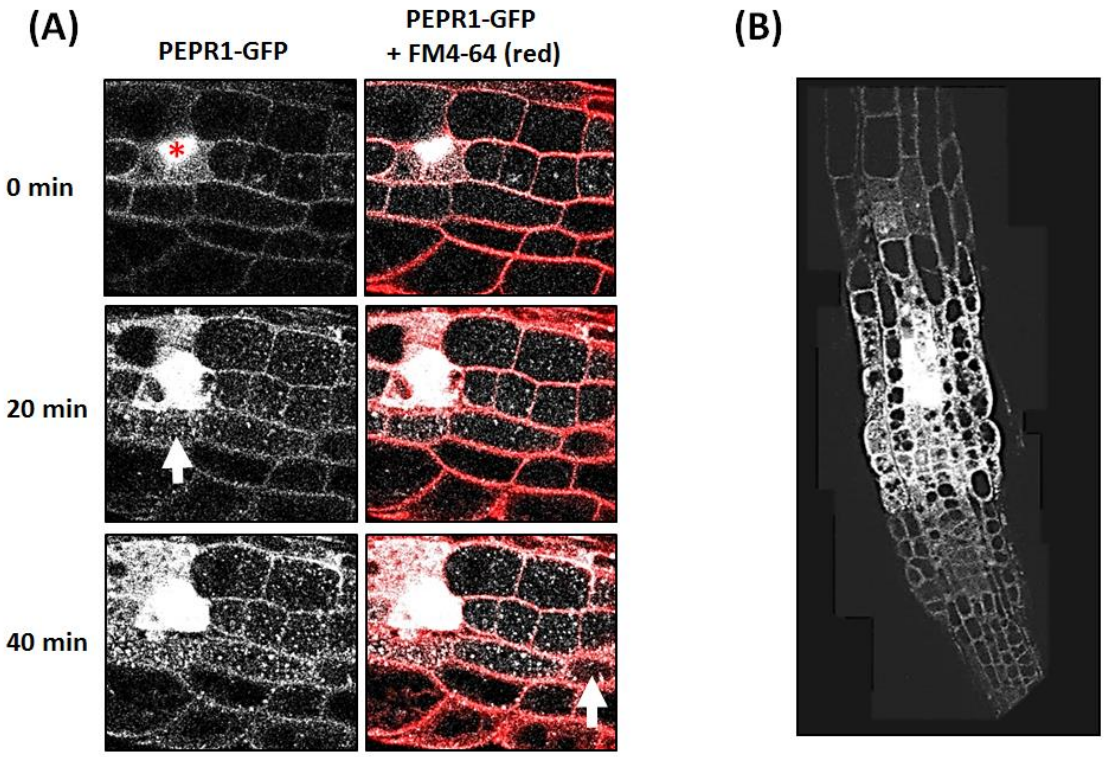


Figure 4.7 Laser ablation reveals the spatiotemporal behavior of PEPR1-GFP in wounded and surrounding cells. A The epidermal cell layer of the root meristem transition zone was imaged immediately after laser ablation (top panel) continuously until 40 min after ablation (bottom panel) by confocal microscopy. Images were taken in Z dimension and the maximum intensity projection is shown here. Laser target is indicated with an asterisk (*). Notice the overall increase in PEPR1-GFP signal strength in the surrounding cells with time. The neighboring cells located closest to the wounded cells respond first (appearance of endosomal bodies?) (indicated with arrows) with a lag period for the more distal cells indicated with arrows . **B** Distribution of PEPR1-GFP signal in a stitched composite image of the surrounding region approximately 1 hour after laser ablation treatment (bright white region in the middle). Stitching performed with ImageJ MosaicJ plugin.

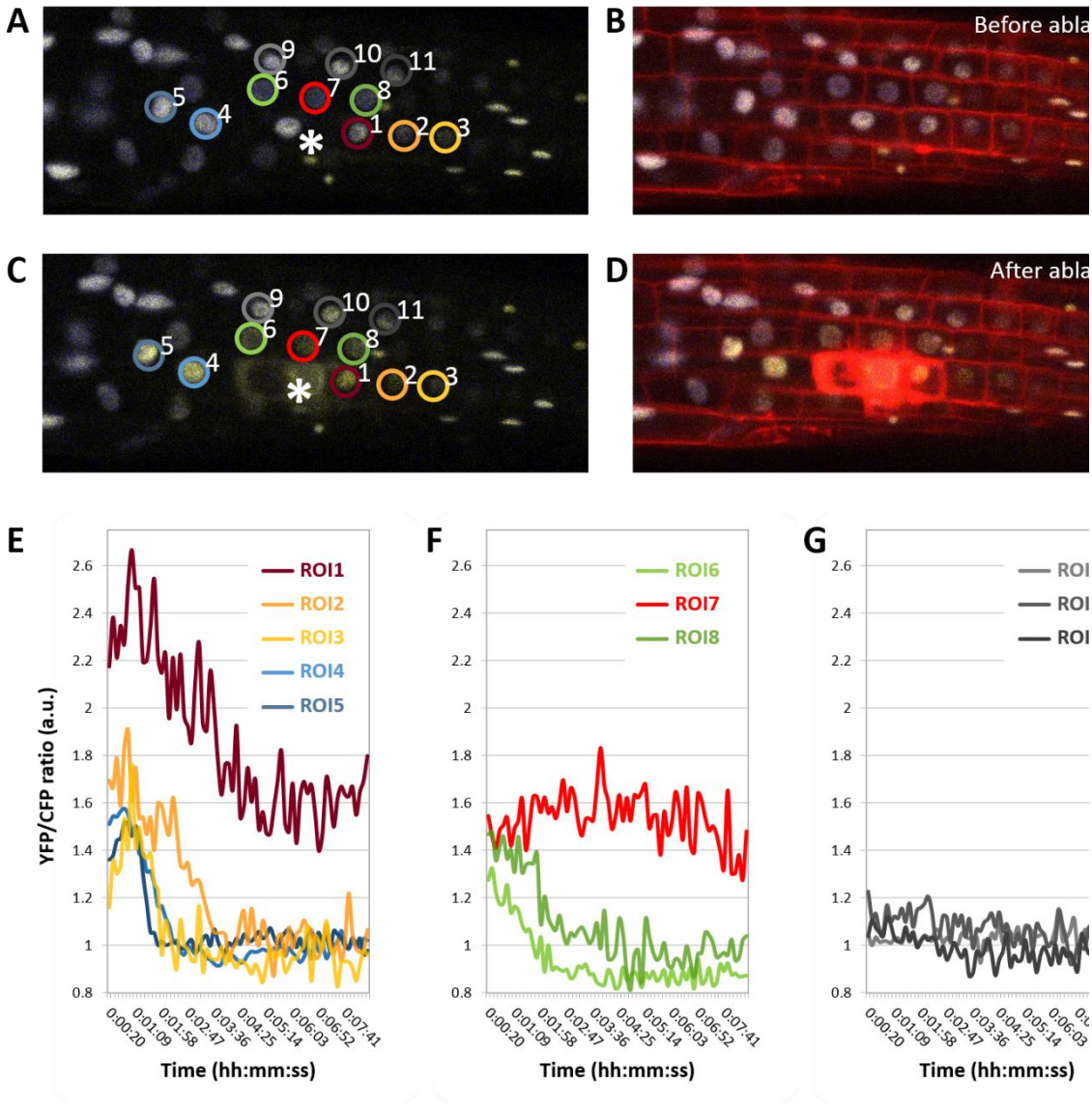


Figure 4.8 Varying calcium fluxes are elicited in the cells in close proximity of a laser ablation wound site. A 3D confocal image stack of the epidermal transistion zone cells of a nuclear localized YC3.6 calcium measurement construct (YC3.6-NLS) before laser ablation. Nuclei of interest are encircled and numbered. **C** Image immediately after laser ablation treatment (*). Notice the shift in color of white to yellow between **A** and **C** images for the encircled nuclei. **B** and **D** Overlay of propidium iodide (PI) stain image on **A** and **C**, respectively. **E,F,G** Quantitation of the YC3.6-NLS probe YFP/CFP ratio for the nuclei of interest as a readout for calcium flux in time. ROI = region of interest

4.4 DISCUSSION

Numerous studies on the downstream effects upon application of synthetic PEPs have been performed within the last ten years after the first isolation of *At*PEP1 from tissue extracts (Bartels and Boller, 2015). As an endogenous molecule with elicitor activity *At*PEP1 has been categorized as a DAMP without further prove of its specific genesis during damage or stress treatments. Here we showed the proteolytic release of *At*PEP1 from its precursor *At*PROPEP1 by the cysteine protease *At*MC4 upon wounding. Both the activation of the protease as well as the processing of PROPEP1 happened immediately after the wounding stimulus. Mutation of the critical arginine 69 in front of the cleavage site as well as chelation of Ca^{2+} ions impaired PROPEP1 cleavage. Both are necessary requirements for MC4 to be functional and thus provide further evidence that MC4 is the natural processing enzyme of PROPEP1 (Vercammen et al., 2004; Watanabe and Lam, 2011b). Moreover, MC4 is localized in the cytosol with PROPEP1 being attached to the tonoplast but likely accessible to MC4 since laser ablation induced relocalization of PROPEP1-YFP is tightly connected to MC4 activity. Addition of metacaspase-specific inhibitors or chelation of Ca^{2+} ions both impaired relocalization of the YFP signal strongly indicating that PEP1-YFP relocalizes to the cytosol and not PROPEP1-YFP. Finally PROPEP1 was not processed in a *mc4* mutant background indicating that MC4 is the only or at least the main processing enzyme for PEP1 release from PROPEP1 in vivo.

Arabidopsis MC4 has been characterized as a positive mediator of PCD (Watanabe and Lam, 2011a). PCD-inducing treatments led to an increasing MC4 activation starting after 6 h up to 96 h. In contrast, MC4 activation upon wounding is already detectable within the first 5 minutes to rapidly release PEP1. PEP1 perception does not trigger cell death in wild type plants but very recently PEPR1 PEPR2 activation was shown to induce extensive cell death in case of the depletion of their co-receptor BAK1 (Yamada et al., 2016). BAK1 depletion is a frequent strategy of microbial pathogens to deactivate MAMP-triggered immunity. Thus depending on additional signals and/or circumstances PEP1 release by MC4 activation might either contribute to PTI or to the formation of cell death. Notably, previous work already demonstrated that PEP1 treatment triggers a different outcome depending on additional signals. In that case the additional signal was a preceding treatment with the MAMP flg22 which exclusively induced a much stronger

oxidative burst upon subsequent treatment with PEP1 (Flury et al., 2013). These are interesting parallels to the comprehensive work on mammalian cytokines which can trigger very different responses depending on the surrounding milieu (Hanada and Yoshimura, 2002; Zhang and An, 2007).

Another still unsettled question remains whether the mature PEP gets passively released upon loss of tissue integrity or actively secreted to the extracellular space to bind to PEPRs? Our data slightly favours the former, because PEP1-YFP dispersal usually occurs concomitant with intracellular accumulation of PI indicating loss of membrane integrity. Only on very few occasions, PEP1-YFP dispersal was seen before the entry of PI. Therefore, it is not unreasonable that if PI is able to enter the cell, PEP1 might be able to escape. Again, there are striking parallels with the mammalian cytokine system. The pro-inflammatory cytokine IL-1ß gets processed by the cell death cysteine protease caspase-1 (Brough and Rothwell, 2007). The release of mature IL-1ß as well as that of IL-1α depends on the activity of caspase-1 which is believed to lead to increased membrane permeability as it happens during cell death (Gross et al., 2012). PI has a molecular weight of 668.4 Da, whereas AtPEP1 (ATKVKAKQRGKEKVSSGRPGQHN), has a theoretical molecular weight of 2491.84 Da, respectively, so roughly 4 times the size of PI. However, our data do not disprove active secretion, neither prove passive release due to technical limitations, and await further experimentation for example the use of fluorescently labelled dextrans in the MW range of AtPEP1 to probe membrane permeabilization. Wound repair dynamics in single cell wounding assays reveal that the membrane can be open for minutes before complete resealing of the plasma membrane (Abreu-Blanco et al., 2011). This is well in the timeframe of PROPEP1 cleavage by MC4.

On the basis of our results, we propose a working hypothesis presented in figure 4.9, in which PROPEP1 is spatially separated from the PEPRs and co-localized with the inactive protease MC4. Upon wounding MC4 gets activated in a calcium dependent way, what lead to the immediate release of the PEP1 peptide from PROPEP1. The release of PEP1 into the extracellular space for binding to the PEPRs of nighboring cells remains to be shown in this context. A similar mechanism could apply to other organisms or other PROPEPs as well. Our data also implies the presence of other proteases that get activated especially during tissue grinding and lead to the degradation

of PROPEP1 at other positions than arginine 69 as observed for PROPEP1^{R69E} that displayed degradation but without accumulation of PEP1-YFP (Figure 4.1A and 4.2B).

*At*Metacaspase 9 has recently been shown to activate the cell death mediator GRIM REAPER by proteolytic cleavage and the protease is also involved in the initiation of autophagy (Wrzaczek et al., 2015; Escamez et al., 2016). Autophagy is a process that often precedes the later apoptotic cell death, and *At*PROPEP3 was shown to be strongly upregulated during autophagy-like senescence and might thereby serve as an additional DAMP in the pathway of self-inflicted cell damage potentially activated by metacaspase 9 (Minina et al., 2014a; Gully et al., 2015). PROPEP3-YFP localized freely in the cytoplasm and metacaspase 9 has a functional optimum at pH5.5 (Bartels et al., 2013; Kwon and Hwang, 2013). To meet MC9 PROPEP3 would either be present in intracellular vesicles which are formed during autophagy or enter the extracellular space. The latter has just been demonstrated (Yamada et al., 2016). Another potential pair might be formed by metacaspase 8 and PROPEP2. PROPEP2 gets upregulated by numerous stress responses, patterns similar to metacaspase 8 which was shown to be involved in and being upregulated by numerous stress related responses (He et al., 2008; Bartels and Boller, 2015).

Notwithstanding our evidence of a novel co-localization system between the inactive protease metacaspase 4 and its target substrate PROPEP1, further investigations will likely lead to the identification of further PROPEP and protease pairs which, however, may differ in their way of interaction from the mechanism of PROPEP1 and metacaspase 4 reported in this study.

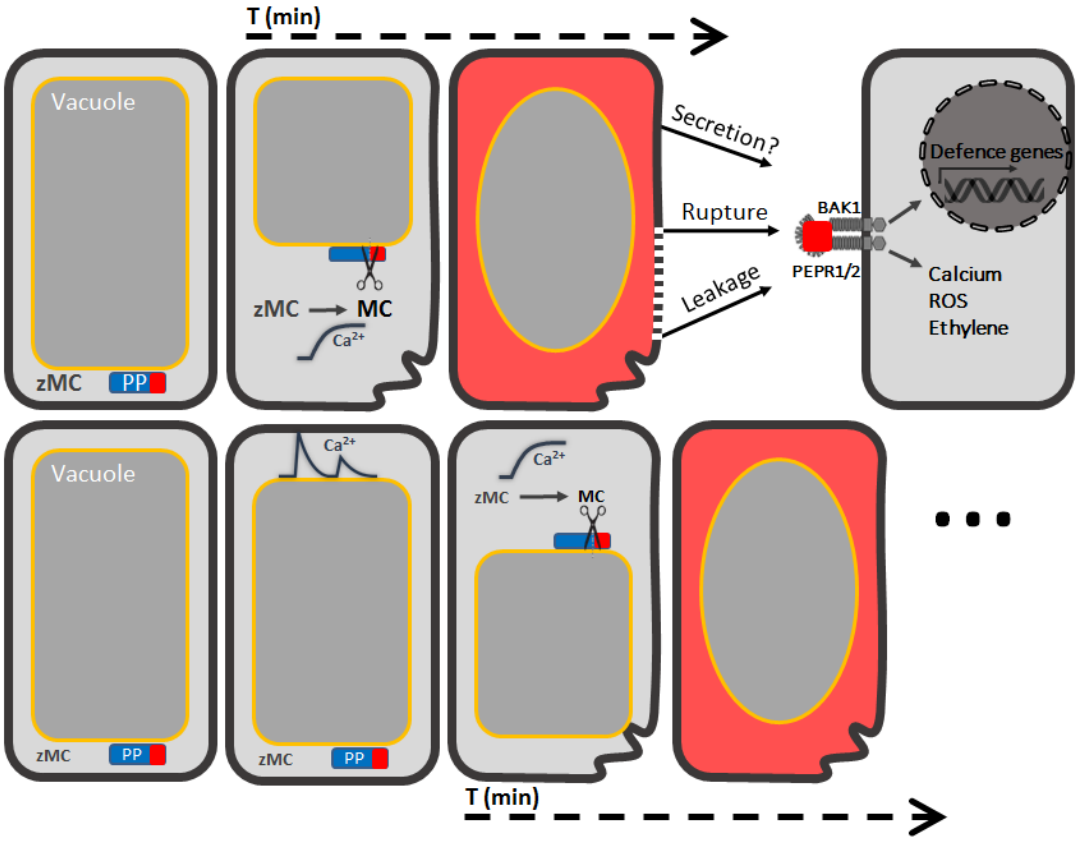


Figure 4.9 Model of Pep generation and downstream signalling. Cells in resting state (at left) express the zymogen form of metacaspase (zMC) with the PEP precursor PROPEP (PP) together in the cytosol with PP attached to the tonoplast. After cell damage and loss of membrane integrity, a steady influx of calcium to the cytoplasm converts zMC to its active form (MC) to cleave the PEP peptide. In a matter of seconds to minutes PEP is released from the tonoplast to the cytosol from where it can passively diffuse through the compromised plasma membrane, or alternatively is actively secreted to the extracellular space, to signal the surrounding intact cells through the BAK1-PEPR1/2 receptor kinase complex. Potentially, PEP is actively secreted. Surrounding cells can sense the initial perturbation through the generation of calcium spikes (bottom row second cell from left). Surrounding cells can lose their plasma membrane integrity and undergo similar fate as the initially damaged cells (bottom right cells).

4.5 MATERIAL AND METHODS

In vitro TNT-protease assay

Unmodified and mutated PROPEP coding sequences (CDS) were cloned by gateway method to destination vectors pDEST15 (N-terminal glutathione S-transferase (GST)

tag) and pDEST24 (C-terminal GST tag). TNT-protease assays were performed as described in (Minina et al., 2014b). Recombinant AtMC4 (rMC4) and mutated AtMC4^{C139A} (alanine substitution of active site cysteine at position 139) fused to a His-tag were expressed and purified from *E. coli* as previously described (Vercammen et al., 2004).

Laser wounding and microscopy

Seedlings were grown upright on ½ MS plates (0.7% agarose, no sucrose) with 48 h of stratification and seven to ten days of growth at 16 h light, 8 h dark conditions. Seedlings were transferred to microscopy slides in 150 µl of tap water containing propidium iodide (PI; 0.01 mg/ml) and 10 mM 2-(N-morpholino)ethanesulfonic acid (MES-KOH) buffer pH 5.7, and when indicated, 50 µM Z-Val-Arg-Pro-DL-Arg-fluoromethylketone trifluoroacetate (Z-VRPR-fmk; Bachem) or 1 mM ethylene glycol tetra-acetic acid (EGTA). Microscopy slides were taped at one end as a spacer to avoid squeezing and damaging the root tip after transfer and roots were carefully covered with standard glass slips. Laser wounding was carried out with a Ti:Sa laser (MaiTai DeepSee multiphoton laser from SpectraPhysics) at an excitation wavelength of 900 nm at 70% power and for variable durations of 300 -7000 ms, for multiphoton laser ablation of precise focal regions. Confocal images were acquired on a Zeiss LSM780 confocal microscope (Zeiss, Jena, Germany) with a Plan-Apochromat 63x/1.4 Oil objective or 40x water objective, argon laser at excitation wavelength 514 nm and respective regions of emission for yellow fluorescent protein (YFP) and PI. Ratiometric measurement of intracellular calcium concentrations was performed with a Yellow Cameleon 3.6 probe fused to a nuclear localization signal (YC3.6-NLS) according to (Costa et al., 2013). PI stain was excited at wavelength 561 nm, as not to excite the YFP moiety of YC3.6-NLS. Z-stacks were taken repeatedly over time following laser wounding. Ratiometric signal was quantified using ImageJ software for regions of interest (ROI) encircling the nuclei of cells surrounding the wound site.

Plant material and treatments

For preparation of sterile seedlings, *A. thaliana* seeds were surface sterilized with 70% ethanol and plated on ½ Murashige and Skoog (MS) medium supplemented with 1% sucrose and 0.5% Phytagel (Sigma-Aldrich), stratified for at least 2 d at 4 °C, and then

germinated at 21 °C in continuous light (MLR-350; Sanyo). After 5 days indivual seedlings were transferred into liquid ½ MS 1% sucrose medium and grown for additional 9 days. Seedlings treated with protease inhibitors [Antipain 100µM, Chymostatin 100µM, Pepstatin A 1µM, PMSF 1mM, E64 10µM, 1,10-Phenanthroline 20mM, Z-VRPR-fmk 50µM, EDTA 1mM, EGTA 1mM, Protease Inhibitor Cocktail Sigma Aldrich 1:100 (MFCD00677817) (all PIs: Sigma Aldrich).] were vacuum infiltrated with the individual solution for 3x 2minutes and incubated at RT for additional 10 minutes before freezing. For the wounding treatment, 8 seedlings were pooled and squeezed with serrated forceps 5 times and incubated at room temperature (RT) for the indicated time before freezing in liquid nitrogen and subsequent analysis by western blot. Treated and untreated seedlings were ground to powder with mortar and pestle under constant supply of liquid nitrogen since the use of automated homogenizers lead to thawing and immediate PEP detection in the western blot. Tissue powder was stored at -80°C and defrozen at RT. PROPEP1-YFP, PEP1-YFP and YFP in *Col-0* were described in Bartels et al. 2013.

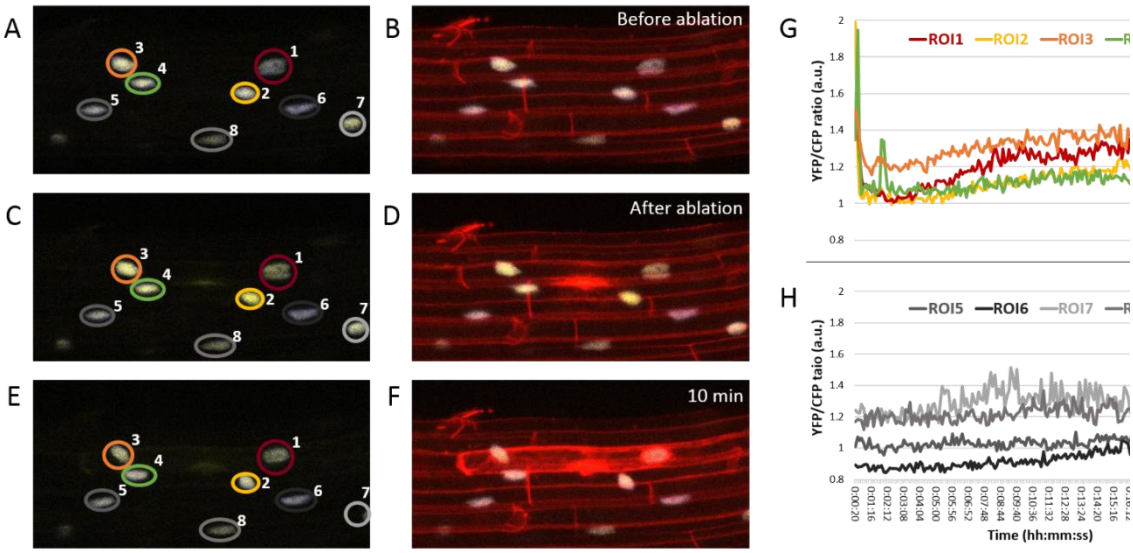
Generation of transgenic Arabidopsis lines

Mutated PROPEP sequences were prepared by site specific mutagenesis of the original coding sequence in the plasmid pearley101 (Bartels 2013). Arabidopsis plants were transformed by *Agrobacterium tumefaciens* using the floral dip method (Clough and Bent, 1998)

Western blotting

Ground tissue was immediately supplemented with preheated 3xSDS loading buffer (w/v; 0.5M Tris pH 6.8, 15% glycerol, 0.3M DTT, 5% SDS, Bromophenol blue) boiled for 5 minutes and centrigued for 5 minutes at 16´300g to remove cellular debris. Proteins were separated in 10% precasted SDS polyacrylamide gels (Genscript) for PROPEP-YFP and PEP-YFP separation or in 4-20% precast gradient SDS polyacrylamide gels (Genscript) for MC4 subunit separation. Analysis was done by semi dry western blotting using anti-GFP antibodies (mouse, 1:1000 Roche) or anti-*At*Metacaspase 4 (rabbit, 1:15000 (Watanabe and Lam, 2011a)).

4.6 SUPPLEMENTARY DATA



Supplementary Figure 4.1 Varying calcium fluxes are elicited in the nearby cells of a laser ablation wound site. (A) 3D confocal image stack of epidermal maturation zone cells of a nuclear localized YC3.6 calcium measurement construct (YC3.6-NLS) before laser ablation. Nuclei of interest are encircled and numbered. **(C)** Image immediately after laser ablation treatment. Notice the shift in color of white to yellow between **(A)** and **(C)** images for the encircled nuclei. **(B)** and **(D)** Overlay of propidium iodide (PI) stain image on **(A)** and **(C)**, respectively. **(E)(F)** Images ten minutes after ablation show the increased accumulation of PI in nuclei 1 and the cell. **(G)(H)** Quantitation of the YC3.6-NLS probe YFP/CFP ratio for the nuclei of interest as a readout for calcium flux in time. ROI = region of interest

5. GENERAL DISCUSSION

Since the discovery of PEPs and PEPRs in 2006, PEP research has mainly revolved around its impact on plant immunity (Bartels and Boller, 2015). Hypotheses were postulated about PEPs being enhancers of PTI or about the DAMP nature of PEPs. Both hypotheses are well supported by numerous studies and more recently, to increase the complexity of the picture, first studies associate PEPs and PEPR signaling with plant development. The work presented here contributes important pieces to this puzzle and opens up new routes to fully understand the possibly many roles of the PROPEP-PEP-PEPR system.

The links between plant development and the PROPEP-PEP-PEPR system

The idea of the PROPEP-PEP-PEPR system being involved in plant development was born already with the discovery of the Arabidopsis PEP receptors PEPR1 and PEPR2. Unlike other PTI-activating PRRs like FLS2, both cluster in LRR-RLK subfamily XI which mostly contains receptors involved in plant development and differentiation processes (Krol et al., 2010; Yamaguchi et al., 2010). Thus the PROPEP-PEP-PEPR system might have evolved from a previous developmental system to a system with a new function in innate immunity. In Drosophila the prominent Toll system, which is conserved in mammals, has evolved from a development regulation system to a system with dual function in development and innate immunity (Valanne et al., 2011). In Drosophila nine Toll receptors have been identified of which Toll is crucial for innate immunity whilst the others are associated with developmental processes. The inactive precursor peptide Spätzle resides in the haemolymph and gets processed either upon MAMP detection by the endogenous serine protease Spätzle Processing Enzyme (SPE) or by pathogen derived proteases (Jang et al., 2006; Hetru and Hoffmann, 2009; Yamamoto-Hino and Goto, 2016). The binding of Spätzle to Toll induces the expression of antimicrobial molecules and mutants in this system are highly compromised in their defense against pathogens (Aggarwal and Silverman, 2008; Ming et al., 2014).

Conversion from development to immunity might have happened within the LRR-RLK class XI in the PROPEP-PEP-PEPR system in similar way. In light of this idea the observation of a bushy root phenotype in *At*PROPEP1 overexpression lines and a more bushy growth of the aerial parts in *At*PROPEP2 overexpression lines gets a new quality (Huffaker et al., 2006). Notably, a continuous exposure to synthetic AtPEP1 or AtPEP2 does not induce a bushy growth but triggers SGI indicating that PROPEPs might have retained some impact on plant development. SGI is accredited to the trade-off between resource allocation to either defense or growth, but in contrast to flg22, that leads to an overall reduction of seedling growth, *At*PEP1 most strongly affects root growth with an overall level of SGI being weaker compared to SGI elicited by MAMPs (Krol et al., 2010). Ma and coworkers linked this effect to the regulation of amino acid exporters, the GLUTAMIN DUMPERS, via PEPR2 signaling raising doubts about PEP-triggered SGI being a sign for activated PTI (Ma et al., 2014).

Heil et al. (2014) proposed, that a "real" DAMP should be present in all tissues that might be exposed to damage. Some classic DAMPs like ATP and OGAs fulfill this criteria, whereas most *At*PROPEPs and both *At*PEPRs were found to be expressed exclusively in the roots (Ferrari et al., 2013; Tanaka et al., 2014). The PEP system might thus have developed from a system with regulatory function in the roots and indeed other closely related receptors of the LRR-RLK class XI have been already characterized in more detail regarding developmental regulation of root growth. For example, treatments with CLE peptides or overexpression of their receptor the, LRR-RLK CLV1 leads to inhibition of lateral root formation but does not alter growth of the primary root and thereby serves as negative regulators of lateral root formation, similar to their function in the shoot (Araya et al., 2014). GSO1 and GSO2 are the closest phylogenetic relatives of PEPR1 and PEPR2 in the class XI LRR-RLKs (Yamaguchi et al., 2010). They are important regulators of cuticle formation in the embryo and mutants in this pathway are (as a secondary effect) strongly affected in root growth (Tsuwamoto et al., 2008; Racolta et al., 2014). Based on the observation that the extracellular protease (subtilase) ABNORMAL LEAF-SHAPE 1 (ALE1) is crucial for GSO1/GSO2-

dependent cuticle formation, GSO1/GSO2 seem to be activated by a so far unknown peptide ligand (Xing et al., 2013).

Just recently we linked PEP signaling to the induction of dark-induced senescence, a mechanism, which might either be induced as some sort of defense response by restricting available nutrients to impair pathogen growth or it might again point to the involvement of PEP signaling in other regulatory processes (Gully et al., 2015). Interestingly, GSO1 functions in the formation of the casparian strip and thereby has a strong influence on the proper homeostasis of nutrients (Pfister et al., 2014).

PEPRs have been shown to interact with the co-receptors BAK1 and BKK1, and in a structural modeling approach three amino acids in *At*PEP1 have been hypothesized to stabilize the bond between *At*PEPR1 and *At*BAK1 (Roux et al., 2011a; Tang et al., 2015). This motif of proline 19, glutamine 21 and histidine 22 (figure 1.4) found in *At*PEP1 is not conserved within the Arabidopsis PEPs and did not play a role in the activity of *At*PEP1 when mutated to alanine (Pearce et al., 2008). Surprisingly, PEPR activation in a BAK1-deficient background triggered extensive cell death whereas *bak1-5 bkk1* mutants were insensitive to PEP1 treatments (Yamada et al., 2016). The former was linked to PROPEP3 thus PEPRs might recruit different SERKs via different binding affinities depending on the PEPR-bound PEP and its amino acid motif at position 19-21-23.

Such mechanism might point to potentially different roles of different PROPEPs, which is further supported by the work presented here since tissue-specific expression patterns of PROPEPs supports their non-redundant functions. We found PROPEP4 and PROPEP7 promoters activated in the root tip up to the elongation zone and Bicluster analysis showed association of *PROPEP*4 with morphogenesis and other developmental processes. In addition we showed that PEPR promoters are not or only weakly expressed in this region, so that either PROPEP4 and 7 might signal on a weaker intensity than other PROPEPs due to lower expression of the receptor in this region, or they might signal not directly to neighboring cells but a bit further from the root tip to the elongation zone or further on the root. If we assume PROPEPs to serve as sensors for damage or other unsuitable conditions, PROPEPs 4 and 7 might detect growth of the

root in unsuitable ground and then signal to distant PEPRs that might induce root branching as observed after PROPEP overexpression (Huffaker et al., 2006).

Lastly since the measurement of defense responses are mostly performed with synthetic peptides that are known to activate the receptor in a strong way and the peptides are also applied mostly in high nano- to μ-molar concentrations, the measured responses are somewhat artificial with regard to their strength since PEPs are rather unlikely to be released in μ-molar concentrations. The TDIF peptide, a CLE family peptide hormone, has been shown to have a dose-dependent effect on shoot and root morphology, and also intracellular PLETHORA transcription factors have been shown to regulate root development in a dose dependent way by expression regulation of PIN proteins in the root tip (Galinha et al., 2007; Hirakawa and Bowman, 2015). Furthermore, Stahl and Simon mentioned the importance of posttranslational modifications on signal peptides for their specificity and activity which is ignored when working with synthetic peptides (Stahl and Simon, 2012). Additionally studies on FLS2 showed that responses were of different strength depending on the tissue in which the receptor was activated, so that also a tissue-specific effect might apply to the PROPEP-PEP-PEPR system (Wyrsch et al., 2015).

The role(s) of the PROPEP-PEP-PEPR system in innate immunity

The involvement of the PROPEP-PEP-PEPR system in innate immunity has doubtlessly been shown (Bartels and Boller, 2015). Our findings in this context prove on the one hand previous assumptions like the assignment of PROPEPs (or at least *At*PROPEP1) as DAMPs but on the other hand raise new questions due to the predominant localization of *At*PROPEP1 in roots. We observed immediate processing of Arabidopsis PROPEP1 after cell damage, and based on our explanation, PEP1 serves as a sensor of cell damage rather than as an amplifier of for example MAMP-triggered defense responses. However, preceding MAMP perception seems to induce an increase in PROPEP1 abundance that could enable a more pronounced damage response in case of a subsequent cellular damage (Bartels and Boller, 2015). This might allow discrimination

between benign microbes and damage-inflicting pathogens and thus the elicitation of an adequate response. Due to the lack of specific antibodies, PROPEP abundance in tissues and cells can only be deduced from transcriptional and promoter-GUS-based data which currently favors a constitutive presence of PROPEP1 at least in the roots. Beside PROPEP1 also PROPEP2 and 3 were clearly linked to plant immunity (Bartels et al., 2013; Logemann et al., 2013). A recent study by Yamada and coworkers introduced a PROPEP3-specific antibody and revealed that *At*PROPEP3 protein is not present under normal conditions but is expressed only upon previous elicitation for example due to bacterial infection (Ross et al., 2014). But in contrast to PROPEP1, that is proteolytically cleaved, PROPEP3 seems not to be processed prior to secretion into the medium after *At*PEP perception (Yamada et al., 2016). Such behavior would indicate that PROPEP3 is not involved in direct damage perception but serves as an actual amplifier of the PEP response. Such a role of amplification of previous defense responses has also been assigned to the PEP-PEPR system as it has been shown that MAMP perception prior to PEP treatments led to an increased oxidative burst (Flury et al., 2013; Klauser et al., 2013). Moreover, the PROPEP-PEP-PEPR system seems to be involved in the regulation of defense responses in general, since it has been shown that the system enhances ethylene-induced defense responses and is crucial for proper calcium signaling during defense responses as well as for the induction of systemic responses (Qi et al., 2010; Ma et al., 2012; Liu et al., 2013; Tintor et al., 2013; Ross et al., 2014).

In addition to an amplifying effect, the PROPEP-PEP-PEPR system also enhances the robustness of plant innate immune against pathogen-mediated perturbations. PROPEP2 and PROPEP3 expression has been tested in the defense signaling mutants *sid2*, *ein2* and *dde2*. Whilst several defense responses are strongly affected in these mutants PROPEP2 and PROPEP3 expression induced by pathogen infection was highly robust and thereby also coupled locally to systemic defense responses (Ross et al., 2014). The robustness of the plants innate immune system has been suggested to be the main benefit over an adaptive immune system since this ensures perception of non-self while it

lowers the risk of aberrant self-perception, the predominant cause of autoimmune diseases and hybrid necrosis (Janeway and Medzhitov, 2002; Chae et al., 2014).

In this thesis also evolutionary aspects of the PROPEP-PEP-PEPR system have been investigated which further support the view of PROPEP-PEP-PEPR being important for the robustness of plant innate immunity. Based on our observed interspecies compatibility of PEPRs they appear to be well integrated into the invariant core of the plants` immune system, whereas PEPs and PEPR LRRs exhibited extensive changes in their sequences resulting in interspecies incompatibility that is, however, irrelevant for effective PEPR signaling within one species.

The PROPEP-PEP-PEPR system can also serve as a backup of the immune system. Several microbial effectors have been identified to target Arabidopsis BAK1 and therewith impair PTI (Zhou et al., 2014). Although BAK1 serves as a co-receptor for PEPRs as well, PEPR activation in *bak1-3* and *bak1-4* backgrounds led to much stronger defense responses and even induced a hypersensitive response in leaves (Yamada et al., 2016). That effect might be enabled by other members of the SERK family that interact with PEPRs and again points to the robustness of the PROPEP-PEP-PEPR system in plant immunity compared to other signaling pathways operated by PRRs like FLS2 and EFR.

An important question concerns the activation of the PROPEP-PEP-PEPR system after expression of the PROPEP protein. As described above, PROPEP3 seems to be secreted in its full length without any further processing. We found PROPEP3-YFP to be localized to the cytoplasm, and secretion via unconventional pathways from the cytoplasm to the extracellular space might be a possible mechanism (Ding et al., 2012). In the case of the Systemin peptide, it has been shown that albeit it is cleaved off from its precursor Pro-Systemin, the cleavage is not a requirement for its activity, and the precursor holds full elicitor activity (Dombrowski et al., 1999). The same might be true for at least some PROPEPs, and since PROPEP3 secretion has been observed in its full length, it might have full elicitor activity without further processing (Yamada et al., 2016). PROPEP1-YFP was found to be localized to the tonoplast membrane and even though it might have elicitor activity in its precursor form, its tonoplast localization

prevents interaction with PEPRs. PEP1-triggered PEPR signaling first requires the activation of MC4 to cleave PROPEP1; this leads to relocalization of the mature PEP1 peptide into the cytoplasm, which seems to enable its subsequent entry into the extracellular space. In other similar systems a precursor molecule and its activating enzyme are located in different cellular compartments. Cellular damage disrupts this separation, brings together the two components and therewith enables the processing of the precursor into the mature signaling compound (Borlinghaus et al., 2014). Notably, the processing of PROPEP1 by metacaspase 4 describes a new mechanism since the protease is already co-localized with its substrate but as an inactive zymogen that requires first an activating stimulus. Wounding (or wounding-induced Ca^{2+} influx) as a robust stimulus and the subsequent rapid generation of PEP1 again points to the robustness of the PROPEP-PEP-PEPR system. Wounding and/or cellular damage is a process that inevitably occurs during feeding but also during pathogen infection due to lytic enzymes or as a consequence of the hypersensitive response (Heil, 2012; Heil et al., 2012). Although pathogens might also carry protease inhibitors to block the activity of metacaspases, the release of unprocessed PROPEP3 from the cytoplasm might in this context serve as a backup mechanism (Song et al., 2009).

The activity of full length PROPEPs has not been investigated yet, but since *At*PROPEP1 localizes to the tonoplast membrane it should anyway be unable to bind to the extracellular membrane. KEKE motifs have been shown to be involved in protein-protein interactions in a calcium-dependent way and to target proteins to the proteasome (Rani et al., 2016). *At*PROPEP1 carries a KEKE motif in its N-terminus and in the C-terminal region flanking the cleavage site at arginine 69 (figure 1.4). The N-terminal motif might mediate the localization to the tonoplast by interacting with another protein embedded into the membrane and the second KEKE motif close to the cleavage site might be important for binding of MC4. Alteration of the motif would be a suitable tool to investigate this.

Parallels of the PROPEP-PEP-PEPR system with mammalian cytokines

PEPs have been assigned a role as plant DAMPs, and our observation of Arabidopsis PEP1 formation upon cell damage is another strong support for this view (Huffaker et al., 2006; Bartels and Boller, 2015). However, a closer look at the suggested criteria defining a DAMP raises doubts about PEPs being just DAMPs (Heil and Land, 2014):

(1) A DAMP should not be present or be inactive in an intact cell and should be present in exposed cells. On the one hand we found PROPEP1-YFP to be bound to the tonoplast membrane and the mature PEP to be released during cell damage but on the other hand the expression of PROPEP1 is restricted mainly to the roots and is thus, in contrast to other DAMPs like ATP and OGAs, not present in all cells.

(2) Since damage is often followed by infections a DAMP should effectively induce defense or resistance mechanisms. This criterion seems to be sufficiently fulfilled by the PROPEP-PEP-PEPR system as described by various examples above.

(3) A DAMP should induce wound healing. Currently not much is known about the induction of wound healing processes by PEP treatments but this is an interesting point for future studies on PEP-triggered responses.

Nevertheless most DAMPs described in plants and especially mammals are characterized by their inactive nature and a distinct primary role in unwounded cells. This is the case especially for structural molecules like actin, OGAs, cholesterol or strictly intracellular molecules like mtDNA, RNA, ATP that get exclusively released from damaged cells (Heil, 2012; Heil and Land, 2014). Peptides with signaling function, especially in the well-studied mammalian field, are mostly not classified exclusively as DAMPs but often as cytokines due to their multiple functions. Cytokines are small secreted peptides with signal functions either on the cells that secreted them, nearby cells or on distant cells (Zhang and An, 2007). Cytokines mostly act within the mammalian adaptive and innate immune system and activate or inactivate different cell types and thereby modulate various layers of the immune system. The most prominent cytokines are the Interleukins that have crucial functions in activation and regulation of the immune system and together with Toll-like receptors (TLRs) form a sophisticated machinery for MAMP perception and response induction (Akdis et al., 2011).

The Toll system of Drosophila introduced before is conserved in mammals, in which Toll-like receptors (TLR) and Interleukin receptors (IL-R) serve as the key receptors in innate immunity (Kawai and Akira, 2011). While Toll signaling controls both immunity and development in Drosophila in mammals TLRs and IL-Rs are exclusively involved in immunity but with much broader spectrum than in Drosophila (Valanne et al., 2011). In mammals thirteen TLRs have been identified that, in contrast to Toll, directly bind MAMPs and therefore serve as PRRs directly. IL-Rs, in contrast, only bind endogenous cytokines such as the interleukins (Dinarello, 2011). Tolls, IL-Rs and TLRs share intracellular TIR domains that interact with the conserved downstream signaling component MyD88 (Valanne et al., 2011; O'Neill et al., 2013). A similar observation has been made in plants as it has been shown that developmental regulators like the hormone receptor BRI1 as well as several PRRs interact with BAK1 for downstream signaling (Chinchilla et al., 2009).

In mammals the Interleukins describe a comparably large class of mostly pro-inflammatory signaling peptides (Akdis et al., 2011). Since the specific immune cells in mammals are very diverse different Interleukins have been found to ultimately stimulate different cells via specific receptors (Akdis et al., 2011). But already the initiation of IL signaling can be very diverse even within one family. In the IL-1 family (IL-1F) the individual peptides can have very different effects and become activated in different ways. IL-1α and IL-1β have been found to be like the initial activation machinery of pro-inflammatory reactions (Garlanda et al., 2013). Both bind to the same receptor (IL-1R1) but have different expression and activation patterns. Il-1α is expressed constantly in various epithelial and endothelial cells, gets released upon cell death and is directly active in its full-length form (Kim et al., 2013). IL-1α can be processed by the extracellular cysteine protease calpain in a calcium-dependent way but the cleavage does not affect its activity (Watanabe and Kobayashi, 1994; Gross et al., 2012). Since IL-1α serves as a primary inducer of immune responses in a DAMP like fashion, the character of its upregulation by various immune responses was discussed. As a conclusion IL-1α upregulation is seen as an enhancer for subsequent wounding by increased IL-1α release (Carta et al., 2013). In contrast, IL-1β is only expressed in hematopoietic cells after a

stimulus, for example from IL-1 perception, or after TLR mediated activation of the innate immune system (Dinarello, 2011). IL-1β is inactive in its full length and requires processing by the endogenous cysteine protease caspase-1 or by external proteases to release the active IL-1β peptide (Netea et al., 2010). Caspase 1 processing of IL-1β can occur in the cytoplasm and peaks around 20 minutes after a stimulus (Brough and Rothwell, 2007). IL-1α, and IL-1β both induce the expression of further interleukins and thereby trigger enhanced immune responses (Ben-Sasson et al., 2013). IL-1α and IL-1β lack a known secretion signal, but their secretion has been shown to depend on caspase-1 activity (Keller et al., 2008; Sollberger et al., 2014). The underlying mechanism remains unclear so far but might be dependent on increased permeability of the plasma membrane through activation of the cell death protease caspase-1. A strong link can be drawn here to the plant PROPEPs. Just like IL-1α, Arabidopsis PROPEP1 serves as a rapid and primary inducer of further defense responses via the perception of wounding. IL-1β, just like PROPEP3, gets upregulated as a second layer of defense and has to be induced by a previous stimulus (Garlanda et al., 2013). We recently described the role of the PROPEP-PEP-PEPR system in autophagy and especially the strong upregulation of *AtPROPEP3* that was observed during autophagy (Gully et al., 2015). Autophagy is a catabolic recycling process which leads to the turnover of proteins and cellular organelles in a way similar to apoptosis (Wu et al., 2014). Autophagy has been shown to be also a positive mediator of IL-1β, but in contrast to TLR mediated IL-1β upregulation, IL-1β does not get secreted but accumulates in vesicles during autophagy and leads to the formation of lysosomes (Carta et al., 2013). So called autophagosomes can also be observed during autophagy in plants. It would be interesting to investigate a potential accumulation of PROPEP3 therein (Bassham, 2015; Michaeli et al., 2016). Interestingly different caspases and also calpain have been shown to be strongly involved in the initiation and regulation of autophagy, for example via the cleavage of Autophagy-related Gene 5 (Atg5) by calpain (Yousefi et al., 2006; Wu et al., 2014). In this tightly regulated process, caspases can also serve like a switch and convert pro-autophagy factors into pro-apoptotic mediators (Cho et al., 2009). Again also for plant metacaspase an important regulatory role in the induction and modulation of autophagy

has been described (Minina et al., 2013; Minina et al., 2014a). Metacaspase 9 for example has been shown to be crucial for the conversion of an autophagy to an apoptotic state in a way that involved the upregulation of ATG2 (Escamez et al., 2016). When we investigated the effect of PEP-mediated darkness-induced senescence we surprisingly made the observation that the effect was lost in the metacaspase 9 (*atmc9*) and in the metacaspase 4 and 8 (*atmc4 atmc8*) double mutant but not in the metacaspe 4 single mutant (Kay Gully personal communication, Gully et al., 2015). Since exclusively *PROPEP3* was upregulated during darkness-induced senescence it might be speculated that PROPEP3 gets processed by metacaspase 8 or 9 during autophagy to have a so far unknown signaling function.

Furthermore, just like some cytokines lead to the expression of further cytokines in nearby or distal cells, a positive feedback loop has been shown for the PROPEPs, and also the role in the induction of other defense pathways like the systemic resistance has been shown (Ross et al., 2014; Bartels and Boller, 2015).

A very interesting pathway has been described for the release of IL-1α. First, the secretion of IL-1α and other leaderless cytokines does not happen via conventional pathway. But IL-1α has been shown to interact with caspase-1 (although it does not get processed by it) and is then secreted via an unknown pathway that requires caspase-1 activity (Keller et al., 2008). Just like caspase-1, metacaspase 4 has been associated with cell death in various cases, and since caspase-1 dependent secretion is believed to be mediated by increased cell permeability during cell death, metacaspase 4 might also not only be required for PROPEP1 processing but also for its subsequent release from the cell interior (Keller et al., 2008; Bergsbaken et al., 2011; Watanabe and Lam, 2011a).

Second and most interesting, motif analysis of the PROPEP amino acid sequences predicts nuclear localization motifs in *At*PROPEPs 5 and 7 (http://myhits.isb-sib.ch/cgi-bin/motif_scan). We indeed observed fluorescence in the nucleus in transgenic seedlings expressing *At*PROPEP4 and/or 5 fused to YFP (data by Hendrik Schatowitz unpublished). IL-1F interleukins have been shown to localize to the nucleus during inflammation and directly influence transcription thereby (Sharma et al., 2008; Luheshi et al., 2009). Most strikingly the mechanism of nuclear localisation of IL-1α does not

only alter transcription but also prevents it from getting released into the extracellular space (Cohen et al., 2010). During necrotic cell death IL-1α gets released as described above and serves as a pro-inflammatory factor. But upon apoptosis IL-1α localizes to the nucleus and is not released for the induction of downstream responses. Such a possible function in Arabidopsis PROPEPs remains to be investigated, but the nuclear localization of PROPEP4 and its role in chromatin remodeling and regulation of developmental processes, as yielded by the Biclustering analysis, might point to completely novel roles of the PROPEPs independent of PEPR signaling, again in analogy to cytokines Figure 5.7 presents an overview of the signaling pathways within the IL-1 family side by side with that of PROPEPs.

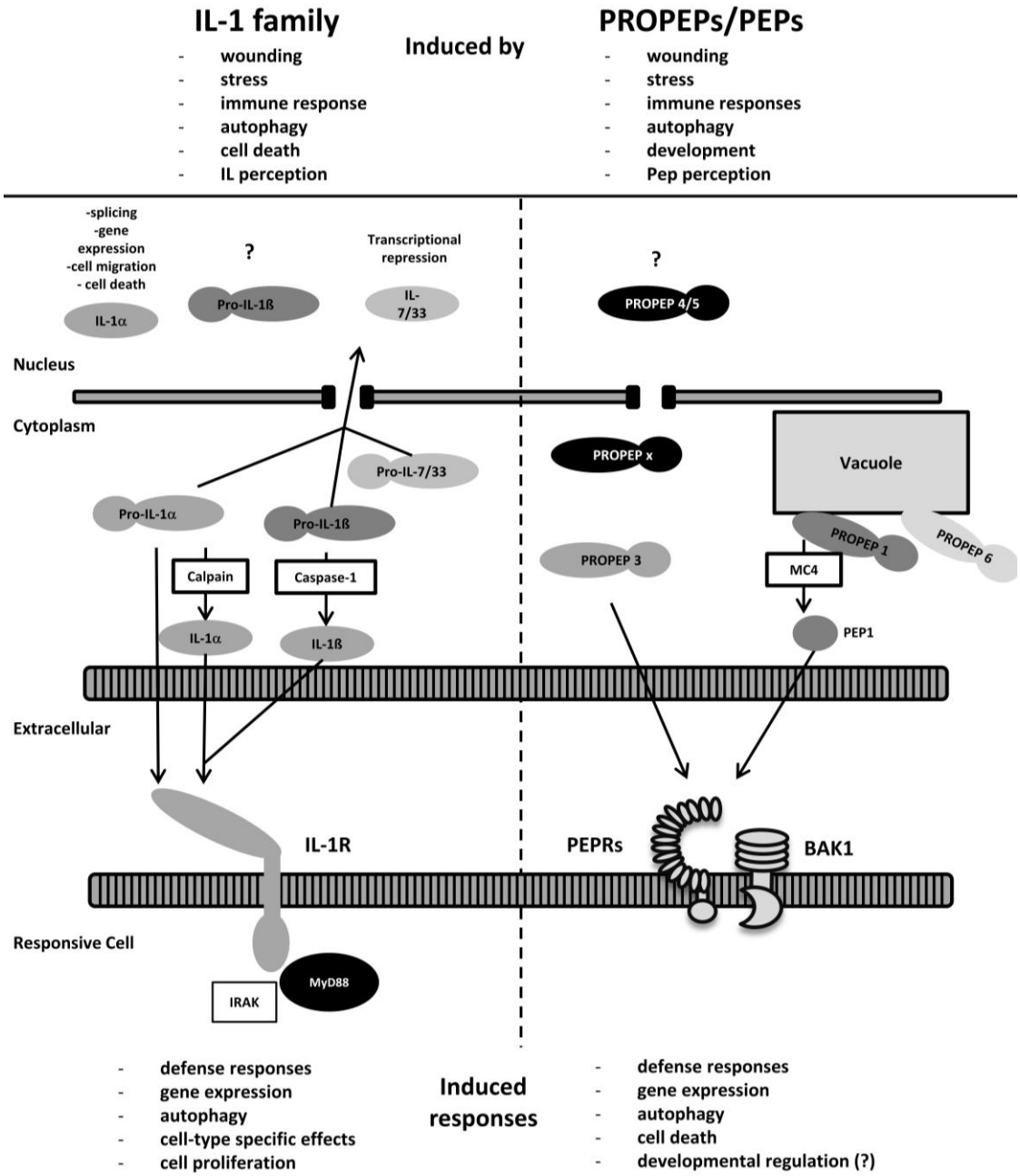


Figure 5.7. Overview of the IL-1F and PROPEP signaling principles and their downstream responses. Both systems get induced by innate immune responses (top). IL-1s influence gene expression either directly by nuclear localization or by binding to the extracellular domain of the IL-1 receptor, whilst some IL-1s require proteolytic cleavage to be activated. A similar mechanism with dual-function remains to be observed in PROPEPs/PEPs. Activation of the individual receptors triggers other cells to induce further defense and cell death-associated responses (bottom).

6. CONCLUSION AND OUTLOOK

The PROPEP-PEP-PEPR system seems to play multiple roles in plant innate immunity and likely also in plant development, which might explain the need for numerous PROPEPs present in several of the plant genomes. Clearly, there is a striking analogy to the mammalian cytokines although mammalian cytokines are a very large group with plenty of functions that do not easily allow a foreign peptide with signaling function to be classified as a cytokine (Zhang and An, 2007).

The mode of action of cytokines as well as of many other developmental processes in plants happens in a dose-dependent manner (Grienenberger and Fletcher, 2015; Guo et al., 2015). Currently this has not been well addressed within PEP research but could be crucial to further understand PEPR signaling. Moreover, two approaches might be needed to shed some light behind the regulatory role of the PROPEP-PEP-PEPR system.

(1) Ross and colleagues used a PROPEP3 antibody to show that there is no protein present in untreated tissue but a stimulus like bacterial infection is needed to boost PROPEP3 abundance (Ross et al., 2014). In contrast, our promoter-GUS studies and qPCR data suggested a constitutive transcription of *PROPEP3*. Thus specific antibodies are needed to investigate the abundance of PROPEPs. This could be coupled to in situ hybridization approaches to generate a tissue-specific map of PROPEP presence. Potential cross-reactivity could be overcome by low sequence similarity between the individual PROPEPs, and iTRAQ-based labelling of N-termini coupled to mass spectrometry would not only enable distinguishing PROPEPs but also detection of PEPs.

(2) Besides the Solanum PROPEP, that is an orphan PROPEP in its genome, mutants of the precursor proteins did not receive any attention so far (Trivilin et al., 2014). Initially PROPEPs/PEPs were assumed to act redundantly and therefore *pepr1 pepr2* mutants were used to indirectly study the lack of PROPEPs and PEPs (Bartels and Boller, 2015). Furthermore mutants in multiple *PROPEP*s would be rather impossible to generate due to the clustering of several *PROPEP* genes on the chromosomes. Nevertheless it might

be interesting to study the impact of a lack of the potential PTI amplifier PROPEP3, or a lack of PROPEP4 and/or 5 due to their potential nuclear localization, which might indicate their impact on transcriptional regulation. This could lead to the discovery of so far unidentified roles of individual PROPEPs.

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