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REVIEW PAPER

Quo vadis, Pep? Plant elicitor peptides at the crossroads of immunity, stress, and development

Sebastian Bartels* and Thomas Boller

Zürich-Basel Plant Science Center, University of Basel, Department of Environmental Sciences, Botany, Hebelstrasse 1, CH-4056 Basel, Switzerland

* To whom correspondence should be addressed. E-mail: sebastian.bartels@unibas.ch

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Abstract

The first line of inducible plant defence, pattern-triggered immunity (PTI), is activated by the recognition of exogenous as well as endogenous elicitors. Exogenous elicitors, also called microbe-associated molecular patterns, signal the presence of microbes. In contrast, endogenous elicitors seem to be generated and recognized under more diverse circumstances, making the evaluation of their biological relevance much more complex. Plant elicitor peptides (Peps) are one class of such endogenous elicitors, which contribute to immunity against attack by bacteria, fungi, as well as herbivores. Recent studies indicate that the Pep-triggered signalling pathways also operate during the response to a more diverse set of stresses including starvation stress. In addition, *in silico* data point to an involvement in the regulation of plant development, and a study on Pep-mediated inhibition of root growth supports this indication. Importantly, Peps are neither limited to the model plant *Arabidopsis* nor to a specific plant family like the previously intensively studied systemin peptides. On the contrary, they are present and active in angiosperms all across the phylogenetic tree, including many important crop plants. Here we summarize the progress made in research on Peps from their discovery in 2006 until now. We discuss the two main models which describe their likely function in plant immunity, highlight the studies supporting additional roles of Pep-triggered signalling and identify urgent research tasks to further uncover their biological relevance.

Key words: DAMP, danger, Pep, PEPR, plant elicitor peptide, PTI.

Plant immunity triggered by endogenous elicitors: Peps emerge as the new paradigms

Plant innate immunity is triggered by the perception of molecules of diverse chemical composition originating from organisms as disparate as bacteria, fungi and herbivores. These molecules are generally called elicitors since they have the capacity to elicit an immune response. Depending on their origin they can be subdivided into MAMPs (microbe-associated molecular patterns; also known as pathogen-associated molecular patterns; PAMPs), HAMPs (herbivore-associated molecular patterns) or VAMPs (virus-associated molecular patterns). Plants evolved the ability to perceive these patterns by using pattern recognition receptors (PRRs), which are transmembrane receptors of various classes but all are inducing, nevertheless, an astonishingly similar collection of physiological responses. This set of defence-associated responses has been termed 'PAMP-triggered immunity' (Jones and Dangl, 2006) or, more fittingly, 'pattern-triggered immunity' (PTI) (Boller and Felix, 2009). It comprises quick and transient as well as long-lasting physiological reactions, including

Abbreviations: BAK1, BRI1-ASSOCIATED KINASE 1; DAMP, damage-associated molecular pattern; ET, ethylene; GDU, GLUTAMINE DUMPER; HAMP, herbivore-associated molecular pattern; JA, jasmonic acid; LRR, leucine-rich repeat; MAMP, microbe-associated molecular pattern; MAPK, mitogen-activated protein kinase; NO, nitric oxide; Pep, plant elicitor peptide; PEPR, Pep receptor; Pst, *Pseudomonas syringae* pv *tomato*; PTI, pattern-triggered immunity; SA, salicylic acid; VAMP, virus-associated molecular pattern.

for example the production of reactive oxygen species, the induction of defence-related genes or the fortification of the cell wall.

In recent years it has become evident that endogenous patterns of the plant host also trigger PTI when perceived by the host itself. These patterns have been assigned in the literature as damage- as well as danger-associated molecular patterns (DAMPs) (Boller and Felix, 2009). The parallel use of damage and danger in the context of DAMPs points already to mechanistic as well as functional differences among DAMPs which starts with their formation. In brief, oligogalacturonides as well as cutin monomers are related to damage. They are passively released as a result of the activity of fungal enzymes trying to make way for the hyphae to enter the plant body (Boller and Felix, 2009; Ferrari *et al.*, 2013). In contrast, the production and maybe also the release of peptidic DAMPs like systemin or plant elicitor peptides (Peps) appear to be under tight control by the host (Ryan and Pearce, 2003; Yamaguchi and Huffaker, 2011). The former, especially oligogalacturonides, have been intensively studied and considerable progress has been made in understanding their generation, perception and subsequent signalling events (Ferrari *et al.*, 2013).

In case of peptidic DAMPs, to date a number of plant peptides have been described which have the ability to trigger PTI-like defence responses (reviewed in Albert, 2013). For many years systemin was the paradigm for peptidic DAMPs but due to the controversy about its potential receptor and a limitation to family Solanaceae few recent systemin studies have been published (Ryan and Pearce, 2003; Malinowski *et al.*, 2009). In 2006 a family of plant elicitor peptides from *Arabidopsis*, called AtPeps, and their receptor PEPR1 (PEP-RECEPTOR1) were reported to activate components of PTI. After identification of the second receptor for AtPeps, called PEPR2, the Pep research intensified (Huffaker *et al.*, 2006; Yamaguchi et al., 2006, 2010; Krol *et al.*, 2010). One year later the first homologue of AtPeps in maize (*Zea mays*), ZmPep1, was characterized and in 2013 it became evident that there are several active Pep homologues present in diverse plant species (Huffaker *et al.*, 2011, 2013). In the meantime perception of Peps was shown to improve the resistance of *Arabidopsis* and maize plants against bacterial or fungal infections as well as feeding herbivores (Huffaker *et al.*, 2011, 2013; Tintor *et al.*, 2013; Klauser *et al.*, 2015). These studies substantiated the initial hypothesis that Peps act as amplifiers of innate immunity. At the same time, an analysis of microarray data indicated that Peps might play an additional role in the response to stresses beside biotic stress and may even take part in the regulation of plant development (Bartels *et al.*, 2013). In this regard two studies have recently presented the first experimental evidence. Ma *et al.* reported that Pep perception might inhibit root growth via regulation of *GLUTAMINE DUMPER* (*GDUs*) genes encoding amino acid exporters (Ma *et al.*, 2014), and work from our lab uncovered an acceleration of starvation-induced senescence upon Pep perception (Gully *et al.*, 2015). While Pep research has thus far been covered only by broader reviews highlighting advances in plant immunity or the role of signalling peptides in general (Yamaguchi and Huffaker, 2011; Albert, 2013; Ferrari *et al.*, 2013), we dedicate this review exclusively to the Pep-PEPR system to give a comprehensive overview including Pep-PEPR specific features.

The molecular machinery: genesis of Peps

The first Pep to be described was AtPep1, a peptide isolated from an extract of wounded *Arabidopsis* leaves, consisting of the last 23 C-terminal amino acids of its precursor protein, called PROPEP1 (Huffaker *et al.*, 2006). PROPEPs are small proteins of ~100 amino acids and are usually encoded by small gene families. Eight PROPEP genes have been identified in *Arabidopsis* and seven in maize, of which at least five show activity (Huffaker and Ryan, 2007; Bartels *et al.*, 2013; Huffaker *et al.*, 2013). Despite their low sequence homology even within the *PROPEP* gene family of one species, a large number of *PROPEPs* has been found in numerous species within the angiosperms including important crop plants (Huffaker *et al.*, 2013; Lori *et al.*, 2015).

In terms of the transcriptional regulation of *PROPEPs* in *Arabidopsis* and maize, there are two common principles. First, Pep perception triggers the transcription of at least the corresponding *PROPEP* in a positive feedback loop. Second, most *PROPEPs* are induced by wounding and jasmonic acid (JA) (Huffaker and Ryan, 2007; Huffaker *et al.*, 2011, 2013; Bartels *et al.*, 2013; Ross *et al.*, 2014). In contrast, challenge with pathogens specifically induces individual *PROPEPs*. *AtPROPEP1* and *ZmPROPEP1* have been shown to respond to infection with fungal pathogens whereas transcription of *AtPROPEP3* and *ZmPROPEP3* rises upon detection of herbivores (Huffaker *et al.*, 2011, 2013; Liu *et al.*, 2013; Klauser *et al.*, 2015).

The *PROPEP* gene family of *Arabidopsis* has been most intensively characterized (e.g. in comparison to the *PROPEP* gene family of maize) and displays best the complex regulation of the individual *PROPEPs* within one family. Research has focused here on the first three *AtPROPEPs* due to their apparent connections to plant immunity; thus, little is known about the regulation of *AtPROPEP4* to *AtPROPEP8*. Regarding the latter, currently only wounding seems to induce the transcription of *AtPROPEP5* and *AtPROPEP8*, and this induction is restricted to the midrib of adult leaves, whereas *AtPROPEP4* and *AtPROPEP7* are not induced at all (Bartels *et al.*, 2013). Moreover, neither treatment with JA, salicylic acid (SA) nor with AtPep1 to AtPep6 led to elevated transcription of *AtPROPEP4*, *AtPROPEP5* and *AtPROPEP6* (Huffaker and Ryan, 2007). Accordingly, a biclustering analysis based on biotic stress-related microarray data did not show a clustering of these genes with genes related to defence but rather with genes involved in processes like terpenoid (gibberellin) biosynthesis, chromatin organization and reproduction. Thus, despite a PTI-inducing activity of AtPep4 to AtPep8, their precursors might be additionally involved in cellular processes unrelated to defence (Bartels *et al.*, 2013).

In contrast, regulation of *AtPROPEP1*, *AtPROPEP2* and *AtPROPEP3* has been studied in more detail. The aforementioned biclustering analysis showed a co-regulation of all

three genes with genes linked to plant defence processes, but only *AtPROPEP2* and *AtPROPEP3* appeared to be regulated similarly whereas *AtPROPEP1* was found in a different cluster of genes (Bartels *et al.*, 2013).

AtPROPEP1 transcription in leaves was shown to be induced by danger-related treatments like bacterial elicitors, wounding, fungal infection, methyl jasmonate, ethephon (which releases ethylene), and some AtPeps but not by methyl salicylate (Huffaker *et al.*, 2006; Huffaker and Ryan, 2007; Yamaguchi *et al.*, 2010; Bartels *et al.*, 2013; Liu *et al.*, 2013). Induction of *AtPROPEP1* transcription by AtPep1 was impaired in the ethylene signalling mutant *ein2-1* and the JA synthesis triple mutant *fad3,7,8*, as well as by co-application of diphenyleneiodonium chloride, an inhibitor of the NADPH oxidases involved in the formation of reactive oxygen species (Huffaker *et al.*, 2006).

Microarray data and other recent studies have shown that the transcription of *AtPROPEP2* and *AtPROPEP3* is induced upon treatment with AtPeps, bacterial elicitors, as well as fungal and bacterial pathogens (Huffaker *et al.*, 2006; Huffaker and Ryan, 2007; Tintor *et al.*, 2013; Ross *et al.*, 2014). Transcription of both genes is also induced upon wounding but, like the transcription of *AtPROPEP1*, induction is restricted to the midrib of the leaf (Bartels *et al.*, 2013). Interestingly, treatment with *Spodoptera littoralis* oral secretions or continuous darkness only induced the transcription of *AtPROPEP3* and not *AtPROPEP1* (Gully *et al.*, 2015; Klauser *et al.*, 2015). Similarly, induction of *AtPROPEP2* transcription by elf18 (the active epitope of bacterial elongation factor Tu; EF-Tu) perception was impaired in *ein2* mutants whereas *AtPROPEP3* transcription was independent of functional ethylene signalling (Tintor *et al.*, 2013). Notably, in their follow-up study the authors showed that elevated transcription of both genes based on treatments with *Pseudomonas syringae* pv *tomato* (*Pst*) *ΔhrpS* and *Pst avrRpm1* was not impaired by mutations in *ein2* as well as *dde2* or *sid2*, affecting ET, JA and SA signalling, respectively. The authors concluded that induction of both genes is especially robust to perturbations in defence hormone pathways (Ross *et al.*, 2014).

The promoters of *AtPROPEP2* and *AtPROPEP3* have been analysed in more detail than other *PROPEP* promoters. They share W boxes, cis-regulatory modules bound by WRKY transcription factors. Accordingly, the authors found *in vivo* association of WRKY33 with both promoters, and induction of *AtPROPEP2* and *AtPROPEP3* transcription by treatment with flg22 (the active epitope of bacterial flagellin) treatment was reduced in *wrky33* mutant plants (Logemann *et al.*, 2013).

Comparably little is known about *AtPROPEP* expression in the different plant tissues. Analysis of transgenic *Arabidopsis promoter::GUS* lines indicated that all *AtPROPEPs* are expressed in the root, although *AtPROPEP4* and *AtPROPEP7* are restricted to the root tips of primary and lateral roots. In leaves only the promoter activity of *AtPROPEP5* was found to be relatively strong, whereas the promoter of *AtPROPEP3* led to weak staining and the others did not produce any detectable GUS staining. Similarly, in addition to *AtPROPEP8*, *AtPROPEP3* and *AtPROPEP5* are expressed in flowers (Bartels *et al.*, 2013). To highlight the complexity of the transcriptional data, the current knowledge is summarized in Table 1.

As mentioned previously, PROPEPs are believed to be only the precursors of the active Peps since AtPep1 and AtPep5 have been isolated from *Arabidopsis* leaf extracts as PTI-inducing peptides and not the respective AtPROPEPs (Huffaker *et al.*, 2006; Yamaguchi and Huffaker, 2011). Thus PROPEPs are supposed to be cleaved or somehow processed to release their Peps. Currently, very little is known about processing or cleavage of signalling peptide precursors in plants (Tabata and Sawa, 2014). Systemin has been shown to be cleaved by treatment with intercellular wash fluid from tomato leaves or cell culture medium from tomato cell cultures but the responsible enzyme has not been determined (Dombrowski *et al.*, 1999).

Table 1. *The transcriptional landscape of the Arabidopsis PEPR and PROPEP genes*

	Tissue				Treatments							Refs
	Root	*Leaf*	*Stem*	*Flower*	*Wounding*	*MAMPs*	*Peps*	*Hormones*	*OS*	*Pathogens*	*Darkness*	
PEPR1		veins				flg22, elf18	1–6	MeJA		nd	nd	3, 4
PEPR2	stele	veins				elf18	1, 2, 4	MeJa		nd	nd	3, 4
PROPEP1			nd		midrib	flg22, elf18	1, 2, 4, 5	MeJa, ET		*Bc, Pi*		1, 3, 4, 8
PROPEP2			nd		midrib	flg22, elf18	1–6	nd		*Pst, Bc, Pi*	nd	2, 4, 5, 6, 7
PROPEP3		veins	nd		midrib	flg22, elf18	1–6	nd		*Pst, Bc, Pi*		2, 4, 5, 6, 7, 8
PROPEP4	tips		nd			flg22	1–6	MeJA, MeSA		*Bc, Pi*	nd	2, 4
PROPEP5	stele	veins	nd		midrib	flg22	1–6	MeJA, MeSA	nd	*Bc, Pi*	nd	2, 4
PROPEP6	nd	nd	nd	nd	nd	flg22	1–6	MeJA, MeSA	nd	*Bc, Pi*	nd	2, 4
PROPEP7	tips		nd			nd	nd	nd	nd	nd	nd	4
PROPEP8	stele		nd		midrib	nd	nd	nd	nd	nd	nd	4

Green represents detected promoter activity (Tissue) or induction (Treatments) whereas red marks tissues without detectable promoter activity or lack of induction after the indicated treatment.

Abbreviations: nd, not determined; OS, oral secretions of *Spodoptera littoralis*; Pst, *Pseudomonas syringae* pv. tomato; Bc, *Botrytis cinerea*; Pi, *Phytophthora infestans*.

References: 1, Huffaker *et al.*, 2006; 2, Huffaker *et al.*, 2007; 3, Yamaguchi *et al.*, 2010; 4, Bartels *et al.*, 2013; 5, Logemann *et al.*, 2013; 6, Tintor *et al.*, 2013; 7, Ross *et al.*, 2014; 8, Gully *et al.*, 2015.

Similarly, Ni and Clark (2006), by treatment with a cauliflower extract, observed the processing of recombinantly produced CLAVATA3 protein, the precursor for CLAVATA3 peptide that interacts with the CLAVATA1/CLAVATA2 receptor complex to regulate the stem cell number in the shoot apical meristem, but again no processing enzyme was identified. Only recently *Arabidopsis* type-II metacaspase METACASPASE-9 was identified to cleave the extracellular protein GRIM REAPER into the GRIM REAPER peptide that triggers cell death via binding to the extracellular domain of POLLEN-SPECIFIC RECEPTOR-LIKE KINASE 5 (PRK5) (Wrzaczek *et al.*, 2015). Since METACASPASE-9 as well as other plant metacaspases are lysine and arginine-specific proteases (Vercammen *et al.*, 2006; Tsiatsiani *et al.*, 2011) and AtPROPEP1 contains an arginine in front of the AtPep1 sequence, which appears to be conserved, it will be intriguing to investigate if metacaspases might process PROPEPs. If METACASPASE-9 would be the processing enzyme an export or release of PROPEPs into the apoplast prior to cleavage would be required. Currently PROPEPs have only been shown to localize to the cytosol with or without association with the tonoplast; thus intracellular metacaspases might be more likely targets for PROPEP processing (Tsiatsiani *et al.*, 2011; Bartels *et al.*, 2013).

Similar to METACASPASE-9 the extracellular aspartic protease CDR1 has been proposed to be a good candidate for PROPEP cleavage since CDR1 is assumed to create a mobile peptidic PTI-inducing signal which might comprise one or several Peps (Xia *et al.*, 2004; Vlot *et al.*, 2008). But also in this case, PROPEPs would first need to enter the apoplastic space.

The presence of AtPep1 and AtPep5 in the leaf protein extract might also have been an artefact of protein extraction and as a consequence uncleaved PROPEPs could be the active compounds *in planta*. The structurally and functionally closely related systemin peptide from tomato (*Solanum lycopersicum*) does not need cleavage. It has been shown that its precursor, prosystemin, is as active as the systemin peptide (Dombrowski *et al.*, 1999).

Cleavage of precursors to release active signalling peptides is a common principle in plant and animal defence and development (Khimji and Rockey, 2010; Goyette and Geczy, 2011; van de Veerdonk *et al.*, 2011; Albert, 2013; Czyzewicz *et al.*, 2013). In animals examples for both exist. Prointerleukin-1α, the precursor of interleukin-1α (IL-1α), was similarly active in inducing IL-6 release compared to its mature form IL-1α. In contrast, the proIL-1β was inactive. ProIL-1β needs to be processed e.g. by caspase-1 into the active form IL-1β (Kim *et al.*, 2013).

Taken together, PROPEPs might or might not be cleaved to be active. Detection and localization of cleavage products *in vivo* together with the identification of processing enzymes is one of the most important research tasks at the moment, since it will help to uncover the circumstances of Pep release and perception.

Perception of Peps by PEPRs

PEPRs, the receptors for Peps (and maybe PROPEPs), are transmembrane receptors belonging to the large class of leucine-rich repeat (LRR) receptor-like kinases (RLKs) (Yamaguchi *et al.*, 2010). In *Arabidopsis* promoter::GUS analysis showed that both AtPEPR genes are constitutively expressed, mainly in the root (except for the root tip), but also in the leaf veins and the stem (Table 1). Despite a restriction of *AtPEPR2* transcription to the stele of the root both show a great overlap in their tissue expression pattern (Bartels *et al.*, 2013; Ma *et al.*, 2014). Transcriptional regulation is similarly uniform. Wounding as well as treatment with methyl jasmonate led to a rapid (30 min to 1 h) but transient induction of *AtPEPR1* and *AtPEPR2* transcription (Yamaguchi *et al.*, 2010). Moreover, feeding of a range of herbivores triggered a strong induction of both promoters (Klauser *et al.*, 2015). But there are also slight differences between the transcriptional regulation of both *AtPEPRs*. *AtPEPR1* transcript levels rise after treatment with AtPep1 to AtPep6 and the bacterial elicitor derived peptides flg22 and elf18 whereas *AtPEPR2* transcription was significantly induced only by perception of AtPep1, AtPep2, AtPep4 and elf18 (Yamaguchi *et al.*, 2010). In summary, both *AtPEPRs* are transcribed in most plant organs, and they are induced by treatments linked to plant defence. Thus, they show a similar behaviour to the defence-related *AtPROPEPs*, but intriguingly, they do not overlap with the transcription and regulation of *AtPROPEP4* and *AtPROPEP7*.

Peps are detected by binding to the extracellular LRR-domain of a PEPR. In *Arabidopsis*, AtPEPR1 is able to detect all eight AtPeps, whereas AtPEPR2 detects only AtPep1 and AtPep2 (Bartels *et al.*, 2013). Recently, the crystal structure of the AtPEPR1-LRR domain in complex with AtPep1 was solved, revealing that especially the C-terminal ten residues of AtPep1 interact intensively with the AtPEPR1-LRR (Tang *et al.*, 2015). Previously an alanine-substitution approach led to the identification of three crucial and conserved amino acids within these C-terminal ten amino acids. Substitution of either serine[15] or glycine[17] to alanine or deletion of the terminal asparagine[23] resulted in a dramatically decreased sensitivity of cell cultures to these modified AtPep1 peptides (Pearce *et al.*, 2008). The importance of these amino acids was confirmed by the AtPep1/AtPEPR1-LRR crystal structure but additional amino acids also contribute to a stable Pep-PEPR interaction. Moreover, interaction of AtPEPR1 with the co-receptor BAK1 (BRI1-ASSOCIATED KINASE1) was reported to be crucial for mounting full strength defence responses upon AtPep1 perception (Roux *et al.*, 2011). Modelling of the AtPEPR1-LRR/AtPep1/AtBAK1-LRR complex revealed that proline[19] as well as glutamine[21] and histidine[22] seem to support the AtPEPR1 AtBAK1 interaction (Tang *et al.*, 2015).

However, a study on the interspecies compatibility of Peps and PEPRs suggested a high plasticity of Pep and PEPR-LRR sequences with impact on the Pep/PEPR-LRR interaction efficiency (Lori *et al.*, 2015). Generally, Peps from one plant species are not perceived by plants from distantly related families. For example AtPep1 is not recognized by maize plants and likewise ZmPep1 is not detected by *Arabidopsis*. A closer look at the amino acid sequence of these Peps revealed substantial differences and indicated that there is no common and strictly conserved Pep-motif like the aforementioned

ser[15], gly[17] and asp[23], but each plant family evolved its own rather distinct Pep-motif. This hypothesis was supported by a demonstration that Peps from distantly related plant species were recognized if the family-specific motif was introduced into the Pep amino acid sequence (Lori *et al.*, 2015).

Data mining within the growing number of sequenced plant genomes revealed that homologues of AtPEPRs are present in a large number of species throughout the angiosperms. Similar to the situation in *Arabidopsis*, most plant species contain either one or two *PEPRs* but very few of these have been characterized yet. Beside the two AtPEPRs from *Arabidopsis* ZmPEPR1 and SlPEPR1 were recently cloned, and their ability to perceive ZmPep1 as well as SlPep1 and subsequently activate PTI was shown by transient expression in *Nicotiana benthamiana* (Lori *et al.*, 2015). Based on the insensitivity of the *Arabidopsis pepr1 pepr2* double mutant to all AtPeps in all usual bioassays (Krol *et al.*, 2010; Yamaguchi *et al.*, 2010; Flury *et al.*, 2013), we can assume with confidence that these are the only receptors able to perceive Peps. Interestingly, comparison of the conservation of the LRR and the kinase domain of diverse PEPRs has revealed that the LRRs have a much lower level of conservation compared to the kinase domains (Lori *et al.*, 2015). This is another indication for a rapid evolution of the Pep-PEPR interaction, whereas the downstream signalling pathways starting from the kinase domain are highly conserved. In line with this idea is the observation that PEPRs can be transferred between plant families and still operate defence signalling pathways (Lori *et al.*, 2015). This behaviour has been noted before for the EF-Tu receptor (EFR), which is present only in Brassicaceae and triggers PTI upon detection of the bacterial protein EF-Tu. EFR was successfully transferred into plants from the Solanaceae where it improved plant resistance against bacterial pathogens (Lacombe *et al.*, 2010). Since both receptors share BAK1 as their co-receptor, it seems that BAK1-dependent defence signalling pathways are strictly conserved (Lacombe *et al.*, 2010; Schulze *et al.*, 2010; Roux *et al.*, 2011).

PEPR-triggered downstream events

The molecular events following PEPR activation have been rather well studied and are summarized in Fig. 1. Apparently PEPRs operate signalling pathways that are in part similar or even identical to the ones activated by the receptors EFR and FLS2 (FLAGELLIN SENSING2) that detect the bacterial MAMPs EF-Tu or flg22, respectively. Thus, next we chronologically list these events and highlight the similarities between Pep- and mainly flg22-triggered responses as well as the specialities of the former.

Receptor complex dynamics and phosphorylation events

Similar to FLS2, upon ligand binding AtPEPRs interact with their co-receptor BAK1 followed by the phosphorylation of both BAK1 and AtPEPRs (Schulze *et al.*, 2010). As previously mentioned this interaction is likely to be stabilized by binding of the Pep peptide (Tang *et al.*, 2015). BOTRYTIS-INDUCED KINASE 1 (BIK1) and its closest homologue PBS1-LIKE 1 (PBL1) constitutively interact with AtPEPR1 and likely AtPEPR2 (Liu *et al.*, 2013). BIK1 also gets phosphorylated at least by AtPEPR1 upon Pep perception, and might subsequently leave the complex in a similar fashion to how it leaves the FLS2 receptor complex upon flg22 perception (Zhang *et al.*, 2010). Lack of BIK1 and PBL1 compromises Pep-induced responses (Liu *et al.*, 2013; Ranf *et al.*, 2014).

Production of cyclic GMP

In contrast to FLS2, AtPEPR1 and maybe also AtPEPR2 contain a cytosolic guanylyl cyclase (GC) domain capable of producing cyclic GMP (cGMP) (Kwezi *et al.*, 2007; Qi *et al.*, 2010; Ma *et al.*, 2012). Although cGMP levels produced by recombinant AtPEPR1 *in vitro* are extraordinarily low compared to GCs from animals (Ashton, 2011), it has nevertheless been proposed that the GC activity of AtPEPR1 may form locally enough cGMP to activate the plasma membrane located CYCLIC NUCLEOTIDE GATED CATION CHANNEL 2 (CNGC2) to promote influx of extracellular Ca^{2+} and subsequent Ca^{2+}-dependent signalling (Qi *et al.*, 2010; Ma *et al.*, 2012).

Ca^{2+}-influx and signalling

Like flg22, AtPep perception leads to a rapid elevation of cytosolic Ca^{2+} levels, which is partially dependent on functional BIK1 and PBL1 (Krol *et al.*, 2010; Ranf *et al.*, 2011, 2014; Flury *et al.*, 2013). Increase of Ca^{2+} levels upon AtPep treatment (but not flg22) is also significantly reduced in the *defence no death* mutant (*dnd1*), which lacks a functional *CNGC2* coding sequence (Qi *et al.*, 2010; Ma *et al.*, 2012). Thus it has been proposed that Pep-triggered signalling involves extracellular Ca^{2+} whereas flg22 signalling rather triggers the release of Ca^{2+} from intracellular Ca^{2+}-stores (Ma *et al.*, 2012). Ca^{2+}-dependent signalling triggered upon AtPep1 or flg22 treatment requires functional CA^{2+}-DEPENDENT PROTEIN KINASES (CDPKs) since the *cpk5 cpk6 cpk11* triple mutant showed reduced ROS production, defence gene expression as well as lowered sensitivity to AtPep- or flg22-triggered resistance against infection with the virulent pathogen *Pst* DC3000 (Boudsocq *et al.*, 2010; Ma *et al.*, 2013).

Production of nitric oxide (NO) and ROS

Addition of flg22 and AtPep to leaf tissue triggers the production of NO as well as ROS (Krol *et al.*, 2010; Flury *et al.*, 2013; Ma *et al.*, 2013). Both are involved in many signalling pathways including pathogen defence signalling (Moreau *et al.*, 2010; Baxter *et al.*, 2013). Block of NO as well as ROS signalling due to the addition of specific inhibitors impairs Pep-triggered induction of defence gene expression (Huffaker *et al.*, 2006; Ma *et al.*, 2013). Whereas AtPep-triggered NO production appears to be only slightly lower compared to flg22-triggered NO, AtPep-application leads to only minor

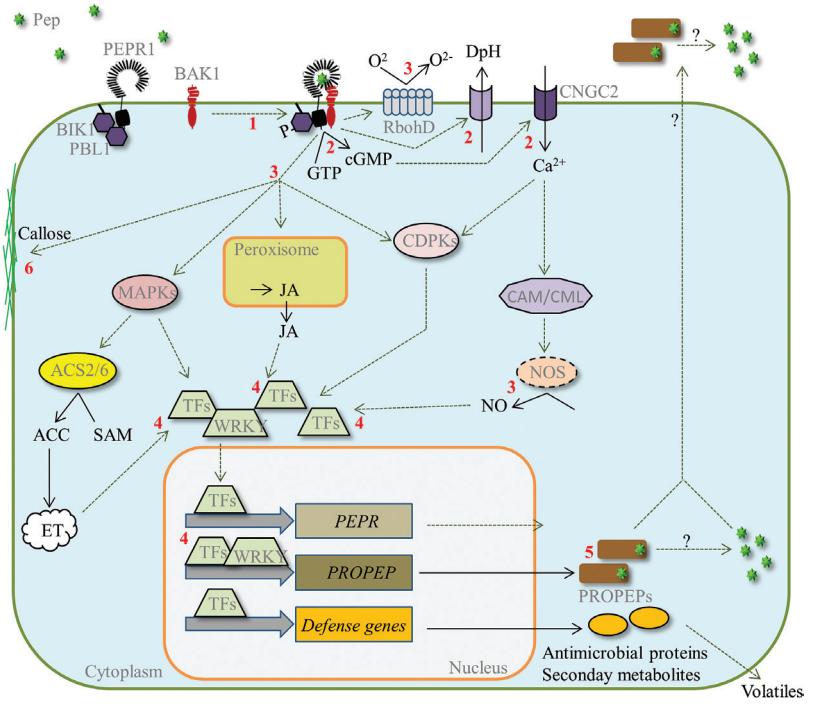

Fig. 1. Overview of the events following Pep perception. Pep perception by PEPRs leads to heteromerization with BAK1, mutual kinase phosphorylation and further to the phosphorylation and the release of BIK1 (1). Next, ion channels are opened, leading to the alkalinization of the extracellular medium and likely to influx of Ca^{2+}. In addition, PEPRs may produce cGMP, which may activate CNGC2 thereby leading to further influx of extracellular Ca^{2+} (2). The increase of Ca^{2+} plays a triple role: it supports RbohD activation leading to an oxidative burst (formation of O_2^-), it triggers NO synthesis, likely via CaM and CML Ca^{2+} sensors, and it activates CDPKs (3). In parallel MAP kinase cascades are activated and levels of the defence hormones ET and JA rise (3). All these together modulate the activity of a multitude of transcription factors (TFs) including WRKYs, which in turn induce defence gene expression as well as the transcription of *PEPRs* and *PROPEPs* (4). PROPEPs might then either accumulate or are further processed into Peps and released (5). In the long term Pep perception also leads to the formation of callose (6) and the inhibition of seedling growth.

amounts of ROS compared to the strong burst triggered by flg22 (Flury *et al.*, 2013; Ma *et al.*, 2013). However, a pretreatment of leaf tissue with flg22 led to a specific enhancement of AtPep-triggered ROS reaching ROS levels comparable to flg22 treatments (Flury *et al.*, 2013; Klauser *et al.*, 2013). This was not observed in a similar setup where the pretreatment was done with AtPeps and flg22 was used for eliciting ROS.

Phosphorylation of MAP kinases (MAPKs)

Biotic stress triggers the phosphorylation and therewith the activation of MAPKs. Perception of flg22 as well as AtPeps led to the phosphorylation of at least MPK6 and MPK3 in *Arabidopsis* (Nühse *et al.*, 2000; Ranf *et al.*, 2011; Bartels *et al.*, 2013). Activated MAPKs work in parallel and in synergy with CDPKs to induce defence genes upon flg22 perception (Boudsocq *et al.*, 2010). Since AtPep-perception induces MAPK- as well as CDPK-dependent genes it seems that this mode of action is similar for both (Flury *et al.*, 2013).

Receptor endocytosis and degradation

Minutes after flg22 treatment, FLS2-GFP fusion proteins disappear from the plasma membrane and reappear in endosomal vesicles (Robatzek *et al.*, 2006; Beck *et al.*, 2012). FLS2 degradation is facilitated by ubiquitination via two closely related PLANT U-BOX-TYPE E3 UBIQUITIN LIGASES (PUBs), PUB12 and PUB13, which are recruited to the FLS2 receptor complex after flg22 detection (Lu *et al.*, 2011). Whether similar endocytosis and degradation routes exist for PEPRs has not been determined, yet. However, other PUBs play a role in either PEPR degradation or downstream signalling. PUB22 and its close homologues PUB23 and PUB24 have been shown to act as negative regulators of PTI by targeting Exo70B2 (a subunit of the exocyst complex) for degradation. Accordingly, the *pub22 pub23 pub24* triple mutant showed increased responses to treatments with flg22, elf18, chitin and AtPep1 indicating that AtPEPRs are also regulated via PUB-mediated degradation (Stegmann *et al.*, 2012).

Production of defence-related hormones

One of the most striking differences between flg22 and Peps is in the interplay with defence-related hormones. Although both trigger the synthesis of ET in *Arabidopsis*, flg22 perception leads to elevated SA levels whereas application of Peps triggers a slight increase in JA levels (Mishina and Zeier, 2007; Flury *et al.*, 2013). Similarly, in maize perception of ZmPep1 triggers the production of ethylene as well as JA (Huffaker *et al.*, 2011). JA and PEPR-mediated signalling is particularly tightly connected. Pep-triggered responses are reduced in JA-synthesis or JA-perception mutants (Huffaker and Ryan, 2007; Flury *et al.*, 2013), and JA synthesis upon recognition of herbivore oral secretions is reduced in *pepr1 pepr2* mutant plants (Klauser *et al.*, 2015).

Changes in gene expression

As mentioned above, Peps as well as flg22 induced similar sets of defence-related genes via MAPK- and CDPK-dependent signalling pathways (Boudsocq *et al.*, 2010; Flury *et al.*, 2013). A recent study, which analysed transcriptomic changes after treatment with AtPep2 or the MAMP elf18, revealed that SA, ET and JA-inducible genes were upregulated by AtPep2 treatment whereas elf18 treatment led to an accumulation of mainly SA-responsive gene transcripts (Ross *et al.*, 2014). In addition, even if both treatments induce the same gene like *PR1* (a SA marker gene) the underlying signalling network is different since upregulation of *PR1* transcription by elf18 but not by AtPep2 was impaired in the ethylene insensitive mutant *ein2* (Tintor *et al.*, 2013).

AtPep perception was reported to induce *PDF1.2* and repress *VSP2* transcription, both marker genes for JA (Huffaker *et al.*, 2006; Tintor *et al.*, 2013). Accordingly, AtPeps seem to specifically induce the so-called ERF-branch and repress the MYC2-dependent branch of JA-responsive genes. Furthermore, *pepr1 pepr2* mutants showed reduced expression of ethylene responsive genes upon treatment with the ethylene precursor ACC indicating that AtPep-perception contributes to the transcriptional upregulation of ethylene-responsive genes (Liu *et al.*, 2013). Thus there is some support for the surprising parallel induction of SA, ET and JA responsive genes upon AtPep2 treatment.

Beside the induction of defence-related genes two studies showed an effect of AtPep perception on genes not directly linked to defence. First, AtPep1 perception led to the repression of *GLUTAMINE DUMPER* genes (*GDU*s), which encode amino acid exporters and are supposed to play a role in root development (Ma *et al.*, 2014). And second, genes related to autophagy (*APG7* and *APG8a*) and chlorophyll breakdown (*PAO*) were induced upon treatment of *Arabidopsis* leaf tissue with AtPep1 (Gully *et al.*, 2015).

Callose deposition and seedling growth inhibition

Callose deposition and seedling growth inhibition are markers of late PTI responses. AtPep as well as flg22 trigger both responses although here subtle differences exist as well (Bartels *et al.*, 2013; Liu *et al.*, 2013). Flg22 perception apparently affects the whole seedling in its development whereas the inhibitory effect of AtPep perception impairs mainly root growth (Krol *et al.*, 2010). The repression of the aforementioned *GUD* genes might explain why AtPep perception has a special impact on root growth (Ma *et al.*, 2014). Notably, the rise in cytosolic Ca^{2+} levels was reported to be equal in shoots and roots treated with AtPep1 whereas flg22 treatment triggered only a small rise in root Ca^{2+} levels (Ranf *et al.*, 2011). Thus roots might just be much less sensitive to flg22 than to AtPeps. In contrast to the *AtPEPRs*, which have shown to be well expressed in roots, *FLS2* expression is limited in roots to the stele and lateral root formation sites (Bartels *et al.*, 2013; Beck *et al.*, 2014).

Production of secondary metabolites

PEPR-mediated induction of secondary metabolite synthesis has currently only been investigated in maize. ZmPep1 treatment of maize plants triggered the production of anthranilate and indole, both precursors for benzoxazinoid hydroxamic acid-related defences. Accordingly also the amount of the derived 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside (DIMBOA-Glc), which is a strong antibiotic compound against bacterial and fungal pathogens as well as insect pests, increased significantly upon perception of ZmPep1 (Huffaker *et al.*, 2011). In the follow-up study analysing the induction of anti-herbivore defences upon ZmPep3 treatment an increase of indole as well as the highly reactive benzoxazinoid precursor 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one glucoside (HDMBOA-Glc) was reported (Huffaker *et al.*, 2013).

Plants also release volatile secondary compounds in response to herbivores; this is considered to be an anti-herbivore response (by attracting predators) as well as a conserved instrument to communicate with neighbouring plants or tissues. Perception of ZmPep3 in maize was shown to trigger the release of sesquiterpenes. The amount released was comparable to the one released upon detection of N-linolenoyl-L-glutamine (Gln-18:3), a strong elicitor present in the oral secretions of many lepidopteran species (Huffaker *et al.*, 2013).

The Pep-PEPR system contributes to local and systemic immunity

There is a growing body of evidence that the Pep-PEPR system is involved in local as well as systemic immunity, and that it contributes to plant resistance against diverse pathogens including bacteria, fungi and herbivores. In *Arabidopsis*, AtPep pretreatment or overexpression of *AtPROPEP1* or *AtPROPEP2* has been reported to increase resistance to the bacterial pathogen *Pst* DC3000 and the oomycete root pathogen *Pythium irregulare*, respectively (Huffaker *et al.*, 2006; Yamaguchi *et al.*, 2010). But pretreatment approaches are likely to create a rather artificial response, which might not be present under natural conditions. However, further pathogen studies were performed with the *pepr1 pepr2* double mutant, which is insensitive to all AtPeps and better suited to uncover the contribution of the Pep-PEPR system to plant immunity.

Spray inoculation of *Arabidopsis pepr1 pepr2* plants with *Pst* DC3000 revealed a slightly increased susceptibility towards this pathogen (Tintor *et al.*, 2013). Notably, infiltration of *Pst* DC3000 and other less virulent *P. syringae* strains did not show any increased susceptibility indicating that the Pep-PEPR system might play a role in stomatal immunity although neither *AtPEPRs* nor *AtPROPEPs* seem to be significantly expressed in guard cells (Bartels *et al.*, 2013; Tintor *et al.*, 2013; Ross *et al.*, 2014).

The involvement of the Pep-PEPR system in fungal resistance also was confirmed. JA and ethylene are key hormones to orchestrate fungal resistance. Treatment of *Arabidopsis pepr1 pepr2* plants with the ethylene precursor ACC revealed a reduced induction of defence-related genes. The protective

effect of an ACC pretreatment against infection with the fungal pathogen *Botrytis cinerea* was also impaired in *pepr1 pepr2* plants (Liu *et al.*, 2013).

Recently, the contribution to resistance against herbivores, first noted in ZmPep-pretreated maize plants (Huffaker *et al.*, 2013), was confirmed in *Arabidopsis* by a challenge of *pepr1 pepr2* plants with *Spodoptera littoralis*. Larvae of this generalist herbivore performed much better on *pepr1 pepr2* plants compared to wild-type *Arabidopsis* plants (Klauser *et al.*, 2015).

In maize, resistance against fungi as well as herbivores has been studied with respect to the Pep-PEPR system (Huffaker *et al.*, 2011, 2013). Due to the lack of receptor mutants in maize, current data are based on ZmPep-treatment studies only. The response patterns triggered by ZmPep1 and ZmPep3 show great similarity with those in *Arabidopsis* triggered by the perception of AtPeps. Both induce the production of JA and ET and activate the transcription of defence-related genes (Huffaker *et al.*, 2011, 2013). Pretreatment of maize plants with ZmPep1 leads to increased resistance against the fungal pathogens *Cochliobolis heterostrophus* and *Colletotrichum graminicola* (Huffaker *et al.*, 2011) whereas ZmPep3 pretreatment strengthens the resistance to the herbivore *Spodoptera exigua* including the release of anti-herbivore volatiles (Huffaker *et al.*, 2013).

Recently, the first Pep-related study in tomato was performed. Silencing of a putative tomato *SlPROPEP1* by virus-induced gene silencing led to a reduced expression of defence-related genes compared to the expression of these genes in control-treated plants. Moreover, silenced plants showed a reduced resistance towards the necrotrophic fungus *Pythium dissotocum* (Trivilin *et al.*, 2014).

Taken together there are numerous studies supporting the contribution of the Pep-PEPR system to plant resistance against a surprising diversity of pathogens. Notably, the induction of JA, SA as well as ethylene-specific genes, revealed by microarray-based determination of the AtPep2-triggered transcriptional changes (Ross *et al.*, 2014), appears to be one special feature of the Pep-PEPR system that enables this broad contribution to the plant's defence system.

Intriguingly the Pep-PEPR system takes part in systemic immunity as well. Similar to flg22, local AtPep2 application is sufficient to induce systemic immunity (Ross *et al.*, 2014; Mishina and Zeier, 2007). Also induction of systemic immunity by local infection with *Pst* DC3000 *avrRpm1* is impaired in *pepr1 pepr2* double mutants (Ross *et al.*, 2014). Although it has been hypothesized that Peps might travel over long distances and contribute to systemic immunity, this seems not to be the case since Pep-responsive genes are not induced in systemic leaves of AtPep2-treated plants. Thus the Pep-PEPR system rather contributes to or amplifies the generation of an unknown systemic signal.

Peps are regarded as damage- or danger-associated molecular patterns: the two models

Researchers have long wondered about the role of the Pep-PEPR system in plant biology but due to the lack of experimental data analysing the molecular circumstances that enable and promote a release of Peps into the extracellular space (and therewith to the potential activation of PEPRs), a clear picture has not yet emerged. Currently, two models are debated (Fig. 2).

(i) The damage model is based on the idea that PROPEPs and Peps reside in the cytosol and are released upon loss of cellular integrity due to damage. Detection of Peps by cells close to the site of damage induces their defence program and thus forms a barrier for pathogens to enter the plant body via the wounded tissue (Fig. 2A). This model would require a constitutive presence of PROPEPs in most cells of the plant body to develop a broad protective effect but sufficient protein data for PROPEPs is lacking. Furthermore, a rapid processing of PROPEPs into Peps would be crucial unless PROPEPs

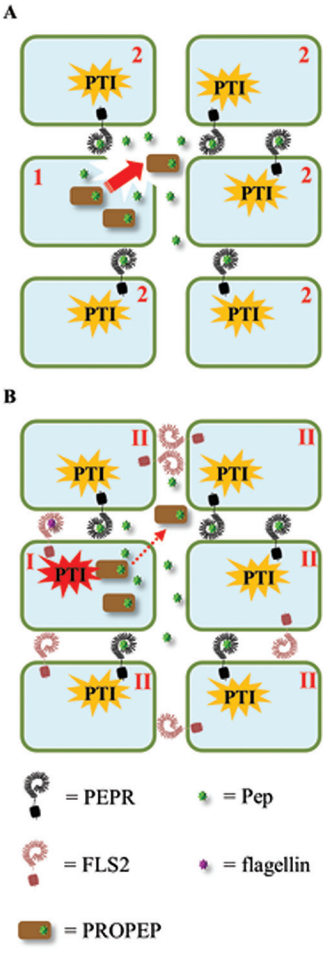

Fig. 2. The damage and the danger model for activation of the Pep-PEPR system. (A) The damage model: upon cellular damage (1), PROPEPs and Peps are passively released into the extracellular space (red arrow) and diffuse to neighbouring cells. Subsequently surrounding cells (2) detect the presence of PROPEPs and Peps in the extracellular space and induce a PTI-like response (orange). (B) The danger (or amplifier) model: after detection of a MAMP (here flagellin) the cell (I) triggers PTI (red). This cell then produces and actively releases PROPEPs and Peps into the extracellular space (red dotted arrow). As in model A, neighbouring cells (II) will subsequently detect the presence of PROPEPs and Peps in the extracellular space and induce a PTI-like response (orange) and therewith amplify the original danger signal.

are as 'active' as Peps. Here experimental data is needed to clarify if and how PROPEPs are processed.

(ii) The amplification model postulates a release of the peptides into the extracellular space in the situation of danger. Thereby the Peps might either prolong the immune response in the active cell (autocrine pathway) or spread the information locally to neighbouring cells to additionally induce their defence response (paracrine pathway) (Fig. 2B).

PROPEPs seem to lack a classical signal sequence to enter the secretory pathway and PROPEP-YFP fusion proteins did not localize to the secretory pathway (Huffaker *et al.*, 2006; Bartels *et al.*, 2013). Thus, PROPEPs or Peps would need to be exported as leaderless secretory proteins (LSPs) via unconventional routes similar to animal interleukin-1β or the yeast mating factor Matα (Ding *et al.*, 2012; Piccioli and Rubartelli, 2013). In brief, release of LSPs can either work via non-vesicular direct crossing of proteins through the plasma membrane or via fusion of membrane-bound structures with the plasma membrane (Ding *et al.*, 2012). Intriguingly two studies showed that after pathogen attack or treatment with SA a large number of LSPs are released into the apoplast but as yet the release of PROPEPs has not been shown (Cheng *et al.*, 2009; Agrawal *et al.*, 2010).

Ultimately both models might be correct, depending on the specific PROPEP. In *Arabidopsis*, the expression patterns differ strongly between the PROPEPs and their overall amino acid sequence shows little similarity. Moreover they also differ in their subcellular localization; thus it is possible that some are constitutively expressed and released upon damage, whereas others are induced upon danger detection and released in a strictly controlled manner. We should keep in mind that both models are based on the assumption that PROPEPs or Peps enter the extracellular space to bind to the PEPR-LRR domain and activate the PEPRs. If only one of the many PROPEPs is secreted via the secretory pathway it could bind already within the cell to PEPRs and trigger PEPR signalling.

Emerging roles of the Pep-PEPR system in the regulation of plant stress and development

Compared to the amount of data connecting PROPEPs and PEPRs to plant immunity there is still only a small number of studies supporting their roles in abiotic stress and plant development. This seems rather surprising since the authors who identified AtPep1 in 2006 already noted that overexpression of *AtPROPEP1* or *AtPROPEP2* led to increased root biomass production (Huffaker *et al.*, 2006). Remarkably, this observation is counterintuitive since perception of MAMPs and DAMPs often inhibit plant growth. Indeed, addition of AtPeps to *Arabidopsis* seedlings strongly inhibits root growth (Krol *et al.*, 2010). However, since the roots of *Arabidopsis pepr1* and *pepr2* single mutant plants were found to be significantly shorter than wild-type roots (Qi *et al.*, 2010; Ma *et al.*, 2014) it has been hypothesized that cell-type-specific expression of PROPEPs and PEPRs might be responsible for a coordinated regulation of root growth (Krol *et al.*, 2010; Ma *et al.*, 2014). Beside development a study on 69 root-expressed LRR-RLKs reported *Arabidopsis pepr1* to be more resistant to osmotic stress and auxin but more sensitive to darkness. Similarly, *Arabidopsis pepr2* mutants were found to be more resistant to elevated NaCl concentrations and again more sensitive to darkness (ten Hove *et al.*, 2011). Intriguingly, in *Arabidopsis* continuous darkness induced *AtPROPEP3* transcription (Gully *et al.*, 2015). In the same study we showed that a combination of continuous darkness and treatment with AtPeps accelerated dark/starvation-induced senescence. Due to the observation that AtPep perception triggered the transcription of genes encoding central enzymes of the autophagy machinery we tend to speculate that the Pep-PEPR system might be involved in the regulation of nutrient remobilization. Whether an enhanced nutrient remobilization is meant to be part of the Pep-induced defence response or if the Pep-PEPR system plays a role in starvation resistance needs to be investigated in more detail. However, it is not a side-effect of PTI activation upon Pep perception since the bacterial elicitors flg22 and elf18 had no effect on the dark/starvation-induced senescence response (Gully *et al.*, 2015).

Further support for roles of the Pep-PEPR system beside plant immunity comes from *in silico* analyses. First, based on a phylogenetic approach, both AtPEPRs cluster together in the leucine-rich repeat receptor-like kinase subfamily XI, which comprises receptors involved in plant development and differentiation, and not in subfamily XII with pattern recognition receptors like FLS2 or EFR (Yamaguchi *et al.*, 2010). This could be an indication of their evolutionary background and thus they might still operate in signalling pathways involved in plant development in addition to the PTI-inducing pathway. Second, an evaluation of microarray data revealed a co-expression of some *AtPROPEPs* with genes linked to reproduction (Bartels *et al.*, 2013). Also experimental data showed that only some of the *AtPROPEP* promoters are responsive to biotic stress whereas others are insensitive to this type of stress suggesting that they might respond to abiotic stress or developmental signals (Huffaker *et al.*, 2006; Bartels *et al.*, 2013).

Important targets for Pep research

Without doubt Peps and PEPRs contribute to plant immunity. Compared to flg22 and elf18, Peps induce a distinct defence response pattern, despite large commonalities of their signalling pathways. One of their hallmarks is the simultaneous induction of JA, ET and SA-dependent defence responses and the respective full spectrum resistance against bacterial, fungal and herbivorous pathogens. Understanding the likely processing and release mechanism will reveal if Peps are damage signals or if they amplify signals of danger or even both. The identification of Metacaspase-9 as the processing enzyme for GRIM REAPER points here to a new direction (Wrzaczek *et al.*, 2015). A closer look at the PROPEP sequences reveals a conserved arginine in front of the Pep sequences. Since metacaspases tend to cleave their substrates after arginine and lysine (Vercammen *et al.*, 2004), they appear to be interesting candidates for PROPEP cleavage. For the investigation of the release of PROPEPs and Peps two approaches

might be fruitful. First, the ongoing proteomics approaches investigating the *Arabidopsis* secretome could be combined with immunity-inducing treatments to promote the possible (unconventional) release of PROPEPs or Peps. Alternatively, PROPEPs could be fused to fluorescent proteins known to be detectable in the extracellular milieu like mCherry. Therewith the real-time behaviour of PROPEPs upon damage or danger could be monitored.

Small signalling peptides are widely used by the plant to coordinate its development. Clustering of AtPEPRs with LRR-RLKs involved in plant development, and coregulation of some *AtPROPEP*s with genes linked to developmental processes, fosters the idea that the PROPEP-PEPR system is derived from systems regulating plant development (Yamaguchi *et al.*, 2010; Bartels *et al.*, 2013). The aberrant root development of *Arabidopsis pepr1* and *pepr2* noted by Ma *et al.* (2014) may provide a first hint in this direction. In this regard, the exclusive expression of *AtPROPEP4* and *AtPROPEP7* in root tips might also be an indication for an involvement in root development (Bartels *et al.*, 2013). In the future, the *Arabidopsis pepr1 pepr2* double mutant should also be carefully investigated with respect to plant development. This mutant certainly has no obvious phenotype, since it has been studied intensively already by many scientists. However, experts in plant development may have the trained eye and the suitable tools to discover more subtle phenotypes. Thus it is important not to ignore these first fine connections between the Pep-PEPR system and the regulation of plant development.

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