The Arabidopsis Pep-PEPR system is induced by herbivore feeding and contributes to JA-mediated plant defence against herbivory

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Abstract

A number of plant endogenous elicitors have been identified that induce pattern-triggered immunity upon perception. In Arabidopsis thaliana eight small precursor proteins, called PROPEPs, are thought to be cleaved upon danger to release eight peptides known as the plant elicitor peptides Peps. As the expression of some PROPEPs is induced upon biotic stress and perception of any of the eight Peps triggers a defence response, they are regarded as amplifiers of immunity. Besides the induction of defences directed against microbial colonization Peps have also been connected with herbivore deterrence as they share certain similarities to systemins, known mediators of defence signalling against herbivores in solanaceous plants, and they positively interact with the phytohormone jasmonic acid. A recent study using maize indicated that the application of ZmPep3, a maize AtPep-orthologue, elicits anti-herbivore responses. However, as this study only assessed the responses triggered by the exogenous application of Peps, the biological significance of these findings remained open. By using Arabidopsis GUS-reporter lines, it is now shown that the promoters of both Pep-receptors, PEPR1 and PEPR2, as well as PROPEP3 are strongly activated upon herbivore attack. Moreover, pepr1 pepr2 double mutant plants, which are insensitive to Peps, display a reduced resistance to feeding Spodoptera littoralis larvae and a reduced accumulation of jasmonic acid upon exposure to herbivore oral secretions. Taken together, these lines of evidence extend the role of the AtPep-PEPR system as a danger detection mechanism from microbial pathogens to herbivores and further underline its strong interaction with jasmonic acid signalling.

Key words: DAMP, herbivory, jasmonic acid, oral secretions, Pep, PEPR, Spodoptera littoralis.

Introduction

Plants use sophisticated perception and signalling systems to detect biotic dangers, such as microbial pathogens or feeding herbivores and, subsequently, to induce an efficient defence response against these threats. In the case of microbial pathogens, several membrane-bound pattern recognition receptors (PRRs) have been characterized that specifically detect conserved microbial structures (referred to as microbe-association molecular patterns—MAMPs) and eventually trigger a set of defence responses. This mechanism is commonly referred to as pattern-triggered immunity (PTI) (Boller and
Felix, 2009; Schwessinger and Ronald, 2012; Macho and Zipfel, 2014).

In the case of herbivorous insects, plants rely on similar detection systems to induce defence signalling and, eventually, herbivore deterrence. The initial recognition of herbivore attack is at least partially achieved by the detection of elicitor compounds in insect oral secretions (Howe and Jander, 2008; Xue et al., 2015) and is potentially mediated by a set of membrane-bound receptors similar to MAMP recognition (Schmelz et al., 2009).

In addition to mechanisms for the detection of exogenous danger, plants also rely on endogenous signalling molecules that are capable of eliciting defence responses (Boller and Felix, 2009; Albert, 2013). Whereas some of these so-called danger-associated molecular patterns (DAMPs), such as cell wall fragments and cutin monomers, are derived from the degradation of the plant cell wall caused by invading pathogens (Sieber et al., 2000; D’Ovidio et al., 2004), others, like the systemin of solanaceous plants, are actively produced by the plant upon the detection of danger (Ryan and Pearce, 2003). Interestingly, systemins have been postulated to be involved in both the deterrence of microbes and herbivores as they have been shown not only to trigger PTI-like responses but also specific defence responses against herbivory. The latter include the biosynthesis of proteinase inhibitors (PI) and the emission of volatile compounds to attract herbivore predators (Ryan and Pearce, 2003; Degenhardt et al., 2010; Sun et al., 2011). However, since the systemin receptor(s) have yet to be fully identified or are under dispute (Holton et al., 2008; Lanfermeijer et al., 2008; Malinowski et al., 2009), the assessment of the biological relevance of systemins to defence signalling has remained difficult.

More recently, a family of endogenous elicitor peptides has been discovered in Arabidopsis thaliana, referred to as AtPeps (Huffaker and Ryan, 2007; Bartels et al., 2013). Like systemins, AtPeps are small peptides (23–29 amino acids long) derived from the C-terminal ends of larger precursor proteins, the PROPEPs (Yamaguchi and Huffaker, 2011). In contrast to the still contested perception mechanism of systemins, AtPeps have been shown to be perceived by two membrane-based receptors referred to as PEPR-Receptor 1 (PEPR1) and PEPR2 (Yamaguchi et al., 2006, 2010; Krol et al., 2010). Upon AtPep perception, both PEPRs trigger PTI-like defence responses reminiscent of the ones elicited by well-known MAMPs, such as flg22 or elf18 (Yamaguchi et al., 2010; Bartels et al., 2013; Flury et al., 2013). Given this similarity between MAMP and AtPep-induced responses and the fact that PROPEP/AtPep expression is induced upon biotic stress, AtPeps are believed to function as amplifiers of the initial defence response. In addition they might also be involved in spreading the signal of danger from the damaged or infected area to distal, not yet infected parts of the plant (Boller and Felix, 2009; Yamaguchi and Huffaker, 2011; Ross et al., 2014). A variety modes of amplification of defence signalling by AtPeps have recently been proposed, either by interacting with defence-related plant hormones (Liu et al., 2013; Tintor et al., 2013; Ross et al., 2014) or by amplifying the production of reactive oxygen species upon previous MAMP detection (Flury et al., 2013; Klauser et al., 2013).

Further support for a role of AtPeps as amplifiers of defence responses came from the fact that the exogenous application of AtPeps has been shown to enhance immunity against the hemibiotrophic pathogens Pseudomonas syringae (Yamaguchi et al., 2010) and the necrotrophic pathogen Botrytis cinerea (Liu et al., 2013). Moreover, the application of ZmPep1, an AtPep homologue in Zea mays has been shown to induce resistance against Cochliobolus heterostrophus and Colletotrichum graminicola (Huffaker et al., 2011). However, despite the apparent similarities to systemins, it was only very recently that the exogenous application of ZmPep3 has been shown to induce herbivore defence signalling, including the production of plant volatile emissions, insect deterrent metabolites, and defence-mediating phytohormones, rendering treated maize plants more resistant to the generalist herbivore Spodoptera exigua (Huffaker et al., 2013).

However, since only the exogenous application of Peps has so far been shown to induce an increased resistance against herbivore feeding, the contribution of endogenous Pep-signalling to herbivore deterrence has largely remained elusive. Using promPROPEP and promPEPR reporter lines driving a β-glucuronidase (GUS) reporter gene, the expression patterns of both ProPEPs, as well as PROPEPs, were investigated here upon feeding by caterpillars of the noctuid moth Spodoptera littoralis. Using mutant plants insensitive to AtPeps, the contribution of endogenous AtPep-signalling to herbivore deterrence was also investigated and our observations were linked to specific hormone signalling cascades involved in mediating defence responses against herbivores.

Materials and methods

Plant material

Arabidopsis plants of the indicated phenotypes were grown individually in small pots at 21 °C with a 10 h photoperiod for 4–5 weeks. T-DNA insertion mutants for the pep1 pep2 mutants are in a Col-0 background and were obtained from Birgit Kemmerling (University of Tübingen). The promPEPR::GUS and promPROPEP::GUS reporter lines used are described in Bartels et al. (2013).

Elicitor peptides and insect oral secretions

Peptides of flg22 (QRLSTGSRINSAKDDAAGLQIA) and AtPep1 (ATKVKAKQRGKEKVSSGRPGQHN) obtained from EZBiolabs were dissolved in a solution containing 1 mM 5-bromo-4-chloro-3-indolyl-β-glucuronidase (GUS) reporter gene, the expression patterns of both ProPEPs, as well as PROPEPs, were investigated here upon feeding by caterpillars of the noctuid moth Spodoptera littoralis. Using mutant plants insensitive to AtPeps, the contribution of endogenous AtPep-signalling to herbivore deterrence was also investigated and our observations were linked to specific hormone signalling cascades involved in mediating defence responses against herbivores.

GUS staining

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

Gene expression analysis
Total RNA was extracted from Arabidopsis leaves using the NucleoSpin RNA plant extraction kit (Macherey-Nagel) and treated with RNase according to the manufacturer’s specifications. AMV reverse transcriptase together with oligo(dT) primers were used to synthesize cDNA. Quantitative RT-PCR was performed in a 96-well format using a LightCycler® 480 Instrument (Roche). Based on the obtained CT values, normalized expression to the reference gene UBQ10 (AT4G05320) was calculated using the qGene protocol (Muller et al., 2002). The gene-specific primers used were as follows: UBQ10 (AT4G05320) with UBQ_fw (5'-GGCCCTTGTAATCCCTTGATGATAAG) and UBQ_rev (5'-AAAGAGATAACACAGGGACGGAAACATAG), PEPR1 (AT1G73080) with PEPR1_qRT-fw (5'-ATCCCTATTGAGATAAGGAG) and PEPR1_qRT_rv (5'-CCTCTTCTAAGCTGCAGTTCTC), PEPR2 (AT1G17750) with PEPR2_qRT_fw (5'-ACCAATAATCCACCGCACATC) and PEPR2_qRT_rv (5'-CGCATTTCTCAGACCATGTTGTC), PROPEP1 (AT5G64900) with PROPEP1_qRT_fw (5'-ATCCAGATAGCAAGCGCGAAG) and PROPEP1_qRT_rv (5'-CATATGTGGGCGGACGAC), and PROPEP3 (AT5G64900) with PROPEP3_qRT_fw (5'-CAACAGTTGGAGAATTTCAGA) and PROPEP3_qRT_rv (5'-CTAATTGTGTGTGCTCCTT).

Microarray data analysis
Data from two recent microarrays depicting gene expression patterns after either Spodoptera littoralis feeding for 8 d (Schweizer et al., 2013) or the exogenous application of AtPep2 (Ross et al., 2014) were compared to identify similarly induced genes. This was done by cross-referencing the 50 most strongly up-regulated genes after herbivore feeding to the 1000 most strongly induced genes 2 h after the application of 1 μM AtPep2.

Herbivore feeding assays
Adult plants in the vegetative stage were separately exposed to Spodoptera littoralis first instar larvae (10 per plant) for 10 d. Larvae were weighed at the beginning and at the end of the experiment to assess the mass gain, and live larvae were counted to assess survival at the end of the assay. Differences between treatments were then analysed using one-way ANOVA (α=0.05, JMP9). Weight data were square root transformed to meet the assumptions of the model. A total of 15 plants of each of the two Arabidopsis lines tested were used.

Plant hormone analysis
Several leaf discs (90 mg fresh weight) were cut from leaves treated by applying 1 μl of insect oral secretions on to the upper leaf surface. Leaf tissue samples were then flash-frozen in liquid nitrogen and stored at –80 °C until hormone level quantification. Hormone extraction and analysis was performed as described by Glauser et al. (2013).

Measurement of ethylene production
For the measurement of ethylene accumulation, three leaf discs of 4.5-week-old plants were harvested using a 5 mm cork borer and placed into a 6 ml glass vial containing 0.5 ml of ddH2O, then put back into the growth chamber and left overnight (~16 h). Elicitor peptides (1 μM final concentration) and Spodoptera oral secretions (0.5% v/v final concentration) were added and vials were closed with air-tight rubber septa. After 4 h of incubation at room temperature, ethylene accumulating in the free air space was measured by gas chromatography (GC-14A Shimadzu).

Results
The expression of PROPEP3 as well as both PEPRs is induced upon perception of Spodoptera littoralis oral secretions as well as herbivore feeding
In maize, individual PROPEPs and Peps were shown to have individual functions. Treatment with ZmPep1 led to an improved resistance against fungal pathogens whereas ZmPep3 application boosted plant defense against herbivores. Similarly, ZmPROPEP1 and ZmPROPEP3 transcription was induced upon treatment with a fungal pathogen or herbivore oral secretions, respectively (Huffaker et al., 2011, 2013). The involvement of the Pep-PEPR system in fungal resistance has also been shown in Arabidopsis and tomato (Huffaker et al., 2006; Liu et al., 2013; Trivilin et al., 2014) but the biological relevance of the observation that a ZmPep3 pretreatment induces anti-herbivore resistance has not been shown due to the lack of PEPR mutants in maize. Thus a switch was made to the model plant Arabidopsis thaliana and the generalist herbivore Spodoptera littoralis to answer this question.

Transgenic Arabidopsis plant lines expressing a β-glucuronidase (GUS) reporter gene under the control of the promoter regions of either PROPEP1, PROPEP3, PEPR1, or PEPR2 were used as described by Bartels et al. (2013) and Spodoptera littoralis oral secretions (OS) were applied as two small droplets on to the upper leaf surface of unharmed leaves. In agreement with the up-regulation of ZmPROPEP3 upon OS detection (Huffaker et al., 2013) AtPROPEP3 is also induced locally at the site of OS application as detected by GUS staining (Fig. 1A) and via transcript quantification by real-time PCR (Fig. 1B). By contrast, AtPROPEP1 showed neither a detectable GUS-response (Fig. 1A) nor an increase in transcript abundance after OS application (Fig. 1B). The response of both PEPR promoters upon OS perception was also assessed. Similar to PROPEP3, both genes are induced upon OS application as shown by local GUS staining (Fig. 1A) as well as by real-time PCR (Fig. 1B). Notably, in contrast to the OS application procedure performed by Huffaker et al. which involved scratch-wounding, OS was just pipetted on to the leaf surface and so avoiding wounding and therefore any potential pleiotropic effects of the treatment procedure on our gene expression analysis (Huffaker et al., 2013).

To assess directly PROPEP and PEPR gene expression upon herbivore attack, the response of PROPEP3 and both PEPRs to feeding S. littoralis larvae were analysed. Similar to the OS application, feeding of S. littoralis also strongly activated all three promoters (Fig. 2). The increased activity of the PEPR promoters is located directly around areas of herbivore attack and does not extend to unharmed parts of the

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Fig. 1. *Spodoptera* oral secretions are sufficient to activate both PEPR and PROPEP3 promoters. (A) 1 μl of *Spodoptera littoralis* oral secretions were pipetted as two small droplets (red circles) onto the leaves of transgenic *Arabidopsis* plants expressing pPEPR::GUS, pPROPEP1::GUS, and pPROPEP3::GUS reporter constructs. After 12 h, leaves were detached from the plant, fixed, and stained. For each construct, two independent lines were assessed with similar results. (B) Leaves of *Arabidopsis* Col-0 wild-type plants were treated with 1 μl of *Spodoptera littoralis* oral secretions as described above. After 0, 1, 2, and 16 h they were detached from the plant and transcript levels of the respective genes were assessed by qRT PCR. Error bars show ±1 SE of three independent replicates, asterisks indicate significant differences in transcript accumulation compared with untreated plants (t test, P < 0.05).

Fig. 2. *Spodoptera* feeding strongly induces the promoters of PEPR1, PEPR2, and PROPEP3. Leaves of transgenic *Arabidopsis* plants expressing pPEPR::GUS and pPROPEP3::GUS reporter constructs were either wounded using cork borers or exposed to feeding *Spodoptera littoralis* (S.l) larvae. After 12 h, the leaves were detached from the plant, fixed, and stained. For each construct, two independent lines were assessed with very similar results.
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leaves. In the case of the promoter of PROPEP3 the detected GUS staining was not limited to the actual feeding sites, but also spread into the leaf veins (Fig. 2). No GUS signal was detected upon wounding the plants by cutting out small leaf pieces using a sterile cork borer (Fig. 2).

It was notable that the activation of PEPR promoters was not limited to feeding of *S. littoralis*. A variety of herbivores were tested on our promPEPR-GUS lines and GUS staining was found in all cases, whereas sterile wounding did not lead to detectable GUS staining (Fig 3). This was independent of the herbivores mode of attack as sucking herbivores such as thrips (*T. tabaci*) were also included, as well as whether the attackers were displaying a generalist (*S. littoralis*) or specialist feeding behaviour (e.g. *P. coehleariae* or *P. brassicae*). Overall, these findings further underline the importance of the Pep-PEPR system for herbivore resistance.

**Spodoptera littoralis larvae perform better on pepr1 pepr2 double mutant plants**

To assess further the indicated importance of the Pep-PEPR system during herbivore challenge, the feeding performance

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**Fig. 3.** The promoters of PEPR1, PEPR2, and PROPEP3 are activated independently of feeding behaviour and specification of the feeding herbivore. Leaves of transgenic Arabidopsis plants expressing pPEPR::GUS and pPROPEP3::GUS reporter constructs were either wounded using cork borer or exposed to feeding insects. After 12h, they were detached from the plant, fixed, and stained. The following insects were assessed (from the top): *Spodoptera littoralis* (generalist, chewing), *Pieris brassicae* (specialist, chewing), *Phaedon coehleariae* (specialist, chewing), and *Thrips tabaci* (generalist, sucking).
of *Spodoptera littoralis* on *pepr1 pepr2* mutant plants, fully impaired in *AtPep*-signalling (Krol et al., 2010; Yamaguchi et al., 2010), was compared to Col-0 wild-type plants. Ten first instar larvae were placed on each plant for feeding. Ten days later, the larvae were removed and their performance was determined by weight gain. A remarkable difference was found in growth. Larvae feeding on Col-0 wild-type plants reached an average weight of 2.86 mg whereas the ones feeding on *pepr1 pepr2* plants showed an average weight of 5.37 mg (Fig. 4). Comparing the performance, it was found that *pepr1 pepr2* feeding larvae grew a significant 87% larger than their counterparts on Col-0 wild-type plants (*F*<sub>1,13</sub>=4.82, *P*=0.047). Therefore, the biological relevance of the Pep-PEPR system for herbivore resistance could be proved.

The response to *AtPep* perception and *S. littoralis* feeding overlaps in the induction of genes related to jasmonic acid signalling and herbivore resistance

Investigating the mechanism behind the contribution of an activated *AtPep*-signalling system to herbivore recognition and, potentially, deterrence, recently published gene expression data from *Arabidopsis* plants treated either with exogenously applied *AtPep2* (Ross et al., 2014) or exposed to feeding *Spodoptera littoralis* larvae (Schweizer et al., 2013) were compared. This analysis revealed several genes which were similarly up-regulated under both circumstances (Table 1). The identified genes encode proteins potentially contributing to direct herbivore deterrence, such as proteinase inhibitors like LTP and TI1 and peroxidases (*PRX52*), transcription factors in defence signalling (FAD-binding proteins) as well as several genes involved in jasmonic acid (JA) biosynthesis and signalling pathways (*JAZ10*, *LOX3*, *AOC1*). Intriguingly, similar categories of genes were found to be induced upon the application of *ZmPep3* in maize by Huffaker et al. (2013), namely proteinase inhibitors (*WPI1*, *SerPIN*) and genes involved in JA signalling (*AOC*, *AOS*).

**PEPR signalling contributes to JA signalling upon herbivore detection**

The induction of JA-related genes upon *AtPep* perception indicates a central role of JA to mediate the induction of herbivore resistance upon PEPR activation. However, the *AtPep-PEPR* system has been shown to interact positively with several hormonal pathways, enhancing defence responses against a variety of pathogens. These include the salicylic acid (SA) (Huffaker et al., 2006; Huffaker and Ryan, 2007; Ross et al., 2014), the ethylene (Huffaker and Ryan, 2007; Liu et al., 2013; Tintor et al., 2013; Ross et al., 2014), and the JA pathways (Huffaker et al., 2006; Huffaker and Ryan, 2007; Ross et al., 2014). To dissect this network further in the specific context of herbivory, the levels of the respective plant hormones were compared between Col-0 wild-type plants and the *pepr1 pepr2* mutant plants before and after the application of herbivore OS (Fig. 5). Upon the perception of OS, the levels of SA did not increase at the time points assessed, with generally no difference being observed between wild-type and mutant plants (Fig. 5A). By contrast, the application of herbivore OS triggered the production of ethylene, with again no detectable difference between Col-0 wild-type and *pepr1 pepr2* mutant plants (Fig. 5B). This, however, was

Table 1. Genes induced by both the exogenous application of *AtPep2* and exposure to feeding *Spodoptera littoralis* larvae

<table>
<thead>
<tr>
<th>Gene annotation</th>
<th>Description</th>
<th>Expression ratio (log&lt;sub&gt;2&lt;/sub&gt;) <em>Pep</em></th>
<th>Expression ratio (log&lt;sub&gt;2&lt;/sub&gt;) <em>Spodoptera</em></th>
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<td>LOX3, lipoxigenase</td>
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</tr>
<tr>
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<td>Oxidereductase, 2OG-Fe(II) oxygenase</td>
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<tr>
<td>AT2G43510</td>
<td>TI1, trypsin inhibitor</td>
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</table>

Fig. 4. Generalist herbivores perform better on plants impaired in AtPep-signalling. Mass of *Spodoptera littoralis* larvae (mean ±1 SE) at the beginning of the experiment (left) and after 10 d of feeding (right) on *Arabidopsis* Col-0 wild-type and *pepr1 pepr2* mutant plants. Letters indicate significant differences between the means (α=0.05, one-way ANOVA, JMP9).
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5333 different for the accumulation of JA and its active derivate JA-isoleucine (JA-Ile), where a greater increase of both JA and JA-Ile levels upon OS perception was observed in wild-type Col-0 plants compared with the pepr1 pepr2 mutant plants (Fig. 5C, D). This difference was most visible 4h after OS application and disappeared when JA levels flattened 12h after treatment, indicating an additional attenuation of the JA response in the mutant. Taken together, the lack of functional PEPR signalling during herbivore perception leads to reduced and/or slower production of JA which probably results in reduced herbivore resistance.

Discussion

Recently, it was shown that the exogenous application of ZmPep3, an AtPep orthologue in maize, induced defence responses against herbivore feeding (Huffaker et al., 2013). Although these findings already suggest a role for Pep-signalling in the plant’s response against herbivores, the biological relevance remained unclear.

With this work, the biological relevance of the Pep-PEPR system in the context of herbivore resistance can now be ascertained by showing that, first, the Pep-PEPR system is induced upon herbivore recognition and, second, that plants lacking a functional Pep-PEPR system are indeed more susceptible to herbivore feeding.

Herbivore feeding activates the promoters of Arabidopsis PROPEP3, but also PEPR1 and PEPR2

In maize, the application of herbivore OS was shown to trigger transcript accumulation of ZmPROPEP3 (Huffaker et al., 2013). Using quantitative real-time PCR as well as transgenic plants expressing a GUS-reporter gene under the control of the AtPROPEP3 promoter sequence, these findings could now be confirmed for the model organism Arabidopsis thaliana. Intriguingly, a similar expression pattern was also observed for AtPROPEP1 and ZmPROPEP1, both of which are not induced by herbivore oral secretions (Huffaker et al., 2013), but respond to the detection of fungal pathogens (Huffaker et al., 2011; Liu et al., 2013). In addition to AtPROPEP3, the promoters of AtPEPR1 and AtPEPR2, the two receptors for AtPeps (Krol et al., 2010; Yamaguchi et al., 2010) also showed rapid activation upon exposure to herbivore OS. This activation was stronger for the promoter of AtPEPR1 than for AtPEPR2, supporting the assumption that AtPEPR1

![Fig. 5.](https://academic.oup.com/jxb/article-abstract/66/17/5327/541064/175235415a64)
is the more important Pep-receptor and reflecting the generally more pronounced expression of *AtPEPR1* as well as the fact that *AtPEPR1* is able to detect all *AtPeps* whereas *AtPEPR2* can only detect *AtPep1* and *AtPep2* (Krol et al., 2010; Yamaguchi et al., 2010; Bartels et al., 2013). Moreover, based on the report that *AtPEPR2* is important for the repression of *Glutamine Dumper* (*GUD*) genes and the inhibition of root growth, this receptor might play a more dominant role in the root (Ma et al., 2014). However, in addition to the already mentioned similarities between the regulation of maize *PROPEPs* and *Arabidopsis* *PROPEPs*, the activation of, specifically, the promoter of *AtPROPEP3* seems to be in line with other recent expression studies, which have shown that, in particular, the expression patterns of *AtPROPEP2* and *AtPROPEP3* are linked to defence signalling (Logemann et al., 2013; Ross et al., 2014). However, despite the apparent central role of *PROPEP3* regarding herbivore resistance other *PROPEPs* and *Peps* are likely to contribute as well. In *Arabidopsis*, *PROPEP5* is constitutively expressed in leaves and *Pep1* has been isolated from unharmed leaves indicating that these *PROPEPs* and *Peps* might be released upon damage due to herbivore feeding (Huffaker et al., 2006; Bartels et al., 2013). This would again activate PEPR-triggered defence responses probably contributing to herbivore resistance. Thus, an analysis of *PROPEP* knock-out mutants could help in understanding the specific contribution of each *PROPEP* to plant immunity in general and herbivore resistance in particular.

The application of herbivore OS alone, however, constitutes a slightly artificial system as it does not involve the mechanical damage generally occurring upon herbivore feeding (Howe and Jander, 2008). Wounding is known to induce JA accumulation rapidly which would activate anti-herbivore responses and so herbivores make use of elicitors of, for example, microbial origin present in their oral secretions to activate SA signalling and therewith counteract JA signalling (Glauser et al., 2009; Chung et al., 2013). This strategy was shown to be effective in suppressing the induction of wounding-responsive genes upon herbivore feeding using the same model system (*Arabidopsis* and *S. littoralis*) used here (Consales et al., 2012). It was found here that the Pep-PEPR system is induced upon OS perception. This is in line with the robustness of *PROPEP3* induction upon microbial challenges which is not impaired by the dysfunction of either the JA, the ethylene or the SA signalling pathways (Ross et al., 2014). Thus the Pep-PEPR system seems to be immune to a potential perturbation of anti-herbivore signalling by OS elicitors.

Similar to OS application, *Spodoptera* feeding also led to a very strong and local induction of the promoters of both *PEPRs* and *PROPEP3*, whereas sterile wounding alone did not induce the promoters of both *PEPRs* nor *PROPEP3*. It is notable that, previously, activation of the *PROPEP3* promoter was found upon mechanical damage applied with a forceps but this activation was limited to the damaged section of the central vasculature of the leaf and was not detectable in the areas which were treated in this study and where the *Spodoptera* larvae were feeding (Bartels et al., 2013). This indicates a distinct pattern of the Pep-PEPR system activation depending on the danger signal perceived.

Herbivores attack plants with different feeding strategies. Apart from chewing herbivores, such as *S. littoralis*, others, such as aphids and thrips, can nourish themselves from the plant tissue by using stylets either to attack single cells or to suck phloem juice from the plant’s vascular tissue (Howe and Jander, 2008). Most herbivore-derived elicitors have so far been identified in the regurgitant of chewing herbivores (Mithofer and Boland, 2008). However, the activation of the Pep-PEPR system upon feeding of thrips (lacking the production of regurgitant) indicates additional or different modes of herbivore detection, potentially through substances and/or microbes in the attacker’s saliva (Delphia et al., 2007; Chung et al., 2013).

Taken together, the observed local induction of the Pep-PEPR system is not a general response to mechanical damage but a specific and robust response to the perception of herbivore oral secretions and elicitors therein. Unfortunately, given this plethora of potential sources for the HAMP(s) triggering an activation of the Pep-PEPR system, it remains a challenge eventually to identify actual compound(s). Still, the combination of these findings with the fact that the Pep-PEPR signalling system seems to be abundant in all higher plants (Huffaker et al., 2013; Lori et al., 2015) suggests that the Pep-PEPR system is a conserved signalling mechanism for herbivore defence.

**An intact AtPep-signalling system is required for full defence responses against herbivores**

Several sources have proposed Peps to be considered as endogenous amplifiers of defence responses against a variety of biotic dangers, based on their ability to trigger defence responses and to interact positively with other defence signalling pathways (Boller and Felix, 2009; Yamaguchi and Huffaker, 2011; Macho and Zipfel, 2014). Our comparative analysis of recent transcription profile studies of plants either exposed to exogenously applied *AtPep2* (Ross et al., 2014) or to feeding herbivores (Schweizer et al., 2013) has led to the identification of a set of similarly induced genes under both conditions, indicating that transcriptional changes upon Pep-signalling include a set of herbivory responsive genes. When combining these findings with the fact that the expression of *PROPEP3* as well as both *PEPRs* is induced by feeding herbivores, it is tempting to expand the aforementioned amplifier theory for AtPep-signalling to herbivore deterrence. In agreement with this, feeding *Spodoptera littoralis* larvae perform significantly better on mutant plants lacking a functional *AtPep*-PEPR-signalling system.

**The AtPep-system contributes to JA-mediated defence responses**

*AtPeps* have been suggested to interact with several plant hormone pathways involved in responses to abiotic stress. These pathways include SA (Huffaker et al., 2006), ethylene (Liu et al., 2013; Tintor et al., 2013), and JA (Huffaker and...
Ryan, 2007; Flury et al., 2013), with ethylene and JA being particularly strongly and positively intertwined with Pep-signalling (Huffaker and Ryan, 2007; Flury et al., 2013; Ross et al., 2014). Furthermore, JA is particularly known to be a major mediator of plant defence responses upon herbivore attack (Hoeve and Jander, 2008). Aligned with this, our studies revealed that, upon OS detection, both ethylene as well as JA biosynthesis was strongly induced whereas SA levels did not increase.

Moreover, this JA and JA-Ile accumulation was significantly reduced in mutant plants lacking a functional Pep-signalling system. These findings are also aligned with the aforementioned transcriptome analysis, which led to the identification of several genes involved in JA biosynthesis pathways being induced upon both herbivore challenge and treatment with AtPep2 (Schweizer et al., 2013; Ross et al., 2014).

Interestingly, recent studies have shown a positive feedback loop between AtPep- and JA-signalling with the application of AtPeps leading to increased JA accumulation and a functional JA signalling system being required for full-strength Pep-signalling (Flury et al., 2013; Huffaker et al., 2013). In this context, our findings provide additional lines of evidence that support a potential role of the AtPep system as an amplifier of JA-mediated defence responses, as shown here in the case of herbivore deterrence.

Both the temporal as well as the spatial resolution of this positive interaction between AtPep- and JA-signalling in the context of herbivore deterrence remain at least partially elusive: First, since PROPEPs are induced by JA and Peps trigger JA accumulation, it needs to be investigated whether the detection of herbivory leads first to an activation of JA signalling, which then induces the Pep-system, or vice-versa. The use of a JA-insensitive mutant might give further insights here but will be complex to analyse due to the positive feedback between both, with not only JA signalling being impaired in JA mutants but also PEPR signalling being reduced which also feeds back on the induction of PROPEP and PEPR expression. Second, as the transcription of both AtPEPRs as well as AtPROPEP3 is induced locally around the site of herbivore detection, it is tempting to speculate that the AtPep-PEPR system is mainly involved in local defence responses. However, Ross et al. (2014) showed that, in addition to triggering local defence responses, Pep-signalling is also required for the full activation of systemic defence responses, although as yet only in the context of microbial pathogens. Therefore, apart from the temporal, the spatial resolution of Pep-signalling in the context of herbivore deterrence also requires further investigation and the assays and reporter lines described here could prove helpful tools to investigate these processes further.

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