RESEARCH PAPER

PAMP-INDUCED SECRETED PEPTIDE 3 modulates immunity in Arabidopsis

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Abstract

Small post-translationally modified peptides are important signalling components of plant defence responses against phytopathogens, acting as both positive and negative modulators. PAMP-INDUCED SECRETED PEPTIDE (PIP) 1 and 2 have been shown to amplify plant immunity. Here we investigate the role of the related peptide PIP3 in the regulation of immune response in Arabidopsis. Treatment with synthetic PIP peptides led to similar transcriptome reprogramming, indicating an effect on innate immunity-related processes and phytohormones, including jasmonic acid (JA) biosynthesis and signalling. PIP3 overexpressing (OX) plants showed enhanced growth inhibition in response to flg22 exposure. In addition, flg22-induced production of reactive oxygen species and callose deposition was significantly reduced in PIP3-OX plants. Interestingly, PIP3-OX plants showed increased susceptibility toward both Botrytis cinerea and the biotrophic pathogen Pseudomonas syringae. Expression of both JA and salicylic acid (SA) biosynthesis and signalling genes was more induced during B. cinerea infection in PIP3-OX plants compared with wild-type plants. Promoter and ChIP-seq analyses indicated that the transcription factors WRKY18, WRKY33, and WRKY40 cooperatively act as repressors for PIP3. The results point to a fine-tuning role for PIP3 in modulation of immunity through the regulation of SA and JA biosynthesis and signalling pathways in Arabidopsis.

Keywords: Arabidopsis, biotic stress, Botrytis cinerea, PAMP-induced secreted peptide, peptide ligand, transcriptome.

Introduction

As sessile organisms, plants have developed sophisticated communication systems between cells and tissues, especially when dealing with a constantly challenging environment. They are continuously attacked by diverse phytopathogens including viruses, bacteria, fungi, and nematodes. Resistance or susceptibility is determined by successful strategies employed by the plant or the invading microbe. Pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) acts as the first active response to microbial perception and is thought to be an ancient form of immunity (Chisholm et al., 2006; Jones and Dangl, 2006). PTI is activated upon perception and recognition of PAMPs by specific pattern-recognition receptors (PRRs) on the cell membrane. PRR activation is followed by a burst of Ca²⁺ and reactive oxygen species (ROS), activation of mitogen-activated protein (MAP) kinase signalling cascades, transcriptional reprogramming, deposition of callose at the site...
of infection to reinforce the cell wall against pathogen penetration, and stomatal closure (Ligerink et al., 1997; Gómez-Gómez and Boller, 2000; Nürnberger et al., 2004; Melotto et al., 2006; Luna et al., 2011). Plant responses to pathogens are also associated with the transcriptional reprogramming of a large number of host genes after pathogen attack, including genes encoding transcription factors (TFs) involved in regulation of plant defence responses. The WRKY family of TFs have been comprehensively investigated with regard to plant defence responses. WRKY18, WRKY40, and WRKY33 have been shown to actively regulate the expression of numerous genes in response to pathogens and flg22 treatment. WRKY33 has been found to have an enhanced activation in the salicylic acid (SA)-related host response as well as reduced activation in the jasmonic acid (JA)-associated responses when infected with B. cinerea. (Qiu et al., 2008; Pandey et al., 2010; Birkenbihl et al., 2012, 2017; Liu et al., 2015).

ROS generation is one of the very early responses to biotic and abiotic stimuli including PAMPs (Felix et al., 1999; Baxter et al., 2014). The ROS produced autopropagates as a wave, travelling rapidly through the apoplast of neighbouring cells and activating a systemic response to the stimuli (Karpinski et al., 1999; Miller et al., 2009). In order to act as a signalling molecule, a non-toxic level of ROS between production and scavenging should be maintained (Mittler et al., 2004). Signalling pathways initiated by ROS have many intersections with other signalling components, including calcium, mitogen-activated protein kinases, phytohormones, and TFs, and play a vital role in fine-tuning of plant responses to developmental programmes and stress conditions (Gilroy et al., 2014; Xia et al., 2015; Sewelam et al., 2016).

Immune responses can also be induced by endogenous molecules produced upon pathogen attack. Among several classes of endogenous elicitors, active peptides have attracted attention for their role in regulation of plant immunity (Boller and Flury, 2012). AtPEP1 was the first peptide with damage-associated molecular pattern (DAMP) activity purified from Arabidopsis (Huffaker et al., 2006). The Arabidopsis genome encodes eight peptides with similar structure to PROPEP1 that are perceived by two receptor-like kinases, PEP-RECEPTOR 1 (PEPR1) and PEP-RECEPTOR 2 (PEPR2) (Krol et al., 2010; Yamaguchi et al., 2010). Another example is phytosulfokine (PSK), originally identified as a regulator of plant growth and development (Matsubayashi and Sakagami, 1996; Hanai et al., 2000). PSKs can also regulate plant immunity against biotrophic and necrotrophic pathogens in an antagonistic manner through their receptor PSKR1 and are proposed to act as part of a fine-tuning system in growth–defence trade-offs (Igarashi et al., 2012; Mosher et al., 2013).

Recently, two members of a new peptide family termed PAMP-INDUCED SECRETED PEPTIDES (PIPS). PIP1 and PIP2, were shown to amplify immunity through RECEPTOR-LIKE KINASE 7 (RLK7) in Arabidopsis (Hou et al., 2014). Plants overexpressing prePIP1 and prePIP2 showed increased resistance against Pseudomonas syringae and Fusarium oxysporum. The PIP family consists of eleven members (PIP1 to 3 and PIP-LIKE (PIPL) 1 to 8); several of the family members are transcriptionally induced by biotic and/or abiotic stress (Hou et al., 2014; Vie et al., 2015). PIP and PIPL propeptides are short (<115 amino acids), with N-terminal aliphatic residues predicted as signal peptide and a C-terminal conserved motif (SGPS) believed to act as a part of biologically active peptides. PIP2 and PIP3 possess two SGPS motifs at the C-termini of the encoded prepropeptides (Hou et al., 2014; Vie et al., 2015).

Here, we present a functional study of PIP3 (At2g23270), another member of this family with high sequence similarity to PIP2. Ectopic application of the conserved C-terminal region of PIP1, PIP2, and PIP3 followed by gene expression analysis by microarray and real-time quantitative reverse transcription–PCR (qRT-PCR) revealed that many marker genes involved in immunity responses were differentially regulated by this treatment. PIP3 loss-of-function plants challenged by P. syringae and Botrytis cinerea did not exhibit an altered phenotype, but plants overexpressing prePIP3 were susceptible to both pathogens compared with wild-type (WT) plants. Production of ROS and callose deposition in response to flg22 were also impaired in PIP3:OX plants. Gene expression analysis of B. cinerea-infected plants showed that key genes involved in JA and SA biosynthesis and signalling pathways were up-regulated in overexpressing plants. These findings suggest that signalling initiated by PIP3 is involved in fine-tuning of plant immune responses to pathogens with different lifestyles.

**Materials and methods**

**Plant material**

All experiments were conducted with Arabidopsis Columbia-0 (Col-0) WT and mutants selected in Col-0 background. A Δspm transposon insertion mutant from the JIC_SM collection (Tissier et al., 1999), pip3 (SM_3_22412), fls2 (SALK_026801), nrk7 (SALK_094492H), and a T-DNA insertion mutant, pip2-1 (SAIL_127S_B11; Sessions et al., 2002), were obtained from Nottingham Arabidopsis Stock Centre, wrlk18, wrlk40, and wrlk18/40 lines were described by Pandey et al. (2010) and wrlk33 plants were described by Birkenbihl et al. (2012). Homozygous plants were screened based on growth on selectable medium and PCR using a combination of gene-specific and transposon or T-DNA-specific primers (Supplementary Table S1 at JXB online). Due to the very low expression of PIP genes under normal conditions, we induced their expression with flg22 treatment (see below), and null functionality was confirmed at the mRNA level. Overexpression lines of PIP3 (At2g23270) were generated by PCR amplification of coding sequences from Col-0 plants and subsequent cloning into the destination vector pEG100 (Earley et al., 2006) under control of the 35S promoter using Gateway technology. The construct was introduced to Agrobacterium tumefaciens strain CS8C1 pGV2260 and transformed into Col-0 WT plants using the floral dip method (Clough and Bent, 1998). Independent transgenic T3 lines with a single copy of T-DNA and constitutive expression of PIP3 were screened for further analysis.

**Peptide treatments**

Col-0 seeds were surface sterilized and sown on half-strength Murashige and Skoog (MS) agar plates (0.6% w/v) supplemented with 2% sucrose at a density of 15 seeds per Petri dish. Seeds were stratified at 4°C for 2 d and transferred to growth room under 16 h (70 μmol m−2 s−1)–8 h light–dark photoperiod at 22°C for 2 weeks. Seedlings were treated by spraying with an aqueous peptide solution (100 nM) supplemented with 0.02% (w/v) Silwet L-77 (Lehle Seeds, Round Rock, TX, USA). The peptide sequences were selected from the C-terminus of PIP propeptides consisting of the conserved SGPS motif as follows: PIP1:
Expression analyses

RNA isolation, cDNA synthesis and qRT-PCR, microarray experiments, statistical analysis, and Gene Ontology (GO) analysis were performed as described in Vie et al. (2015). Sequences of primers used in this study are listed in Supplementary Table S1. Genome-wide expression analysis was performed using the Arabidopsis (V4) Gene Expression Microarray 4×44K (Agilent Technology). The study is minimum information about a microarray experiment (MIAME) compliant. Raw data have been deposited in GEO, NCBI (accession number GSE79025). Gene ontology analysis and enrichment were performed after network integration and predicted gene functions using the GeneMANIA server (Mostafavi et al., 2008).

Genome-wide chromatin immunoprecipitation sequencing (ChIP-seq) experiments were described and performed by Birkenbihl et al. (2012, 2017). The same datasets were used to retrieve WRKY33 and WRKY18/40 binding to PIP1-3 promoter regions after B. cinerea and flg22 treatments, respectively.

Expression analysis, growth inhibitory, ROS production and callose deposition assays in response to flg22

For temporal expression of PIP genes in response to flg22, seeds were grown for 2 weeks as described above for peptide treatment. Seedlings were sprayed with 100 nM flg22, and tissue was harvested at OD600=0.02. Co-cultivated seedlings were washed with 70% ethanol and rinsed twice (for 10 s) to remove surface-attached bacteria. Three seedlings per replicate were placed into each of three 1.5 ml tubes containing 100 μl of water and ground with a pestle. Extraction was achieved in serial diluted samples as colony forming units (CFU) in WT, flg22, PIP3, and PIP3:OX lines at 1, 2 and 3 d post-infection (dpi). A P. syringae assay was also conducted on 5-week-old plants grown in short day (10 h light–14 h dark) conditions. Leaf disks from fully grown leaves were used for susceptibility assessment as CFU described above.

A fungal pathogen assay was conducted as described by Birkenbihl et al. (2012). Briefly, plants were grown for 5 weeks under short day conditions (8 h light–16 h dark) in a growth chamber set to 20 °C and 80% humidity. Spores of B. cinerea isolate 2100 (CECT2; Spanish type) were diluted in Vogel buffer (43.86 mM sucrose, 11.63 mM Na-citrate, 28.7 mM KH2PO4, 0.81 mM MgSO4, 0.9 mM CaCl2, 24.98 mM NaH2PO4) to a density of 2.5×105 spores ml−1. For droplet inoculation and phenotype assay, 2 μl was applied to each side of fully developed leaves from 3-week-old plants. For mock treatment, only Vogel buffer was applied. For gene expression analysis higher concentrations of spores (5×105 spores ml−1) were sprayed on plants, and leaf tissue was harvested for RNA isolation at 6, 12, and 24 h post-infection (hpi). Mock treatment was done only for 6 h, and changes of gene expression were measured relative to mock-treated plants at this time point. In planta pathogen growth assays were performed as described by Gachon and Saindremain (2004). Briefly, droplet-infected leaves of plants were harvested 3 d after inoculation for DNA isolation. The relative ratio of B. cinerea and Arabidopsis DNA was determined by qPCR using pathogen (Bc CutA) and plant specific (AtCUTA) primers listed in Supplementary Table S1.

Results

Synthetic PIP1 and PIP2 C-terminal peptides alters the expression of stress responsive genes

PAMP-INDUCED SECRETED PEPTIDE 1 (PIP1) (At4g28460), PIP2 (At4g37290), and PIP3 (At2g23270) belong to a recently identified gene family with 13 members in the Arabidopsis genome, encoding peptides with similarity to INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) and IDA-LIKE (IDL) peptides (Hou et al., 2014; Vie et al., 2015). An in silico co-expression analysis (ATTED-II; atted.jp, Obayashi et al., 2018) showed that PIP1, PIP2, and PIP3 are highly co-regulated under different experimental conditions (Supplementary Fig. S1A). The top 100 co-expressed genes for PIP1, PIP2, and PIP3 were downloaded from ATTED-II, and a Venn diagram was made to represent the common sets of co-expressed genes (Supplementary Dataset S1). Our analysis revealed a high degree of overlap between the three gene lists, with 40 genes represented in the intersection category, further supporting a potential functional redundancy (Supplementary Fig. S1B). By comparison, only 18 genes were co-expressed with PROPEP1, PROPEP2, and PROPEP3 (Supplementary Fig. S1C).

To improve our understanding of the downstream signalling pathways affected by these peptides, we designed synthetic peptides containing the C-terminal conserved domain of PIP1 (last 25 amino acids) and PIP2 (last 39 amino acids, including both conserved SGPS motives). Two-week-old WT plants were treated with 100 nM synthetic peptides for 3 h, and tissue was harvested for global transcriptome analysis. One hundred and ten genes (89 genes up-regulated, 21 genes down-regulated)
were significantly regulated (2-fold or more compared with control treatment, \( P \leq 0.05 \)) by PIP1 peptide treatment. The transcriptome response to PIP2 treatment was stronger; 513 genes were differentially expressed (326 up-regulated and 187 down-regulated) using the same cut-off (Supplementary Dataset S2). Sixty-eight genes were similarly regulated by both peptides (Fig. 1A). Gene Ontology enrichment of regulated genes revealed that both peptides regulate similar gene sets related to SA signalling were regulated. In addition, a number of other components of JA biosynthesis and signalling were differentially regulated in response to at least one of the peptides (Table 1). In contrast to the JA signalling pathway, few genes related to SA signalling were regulated. In addition, a number of ethylene-responsive TFs were also differentially regulated, including JAZ7, JAZ8, and JAZ9, were among the most up-regulated genes by ectopic application of both peptides. Furthermore, other components of JA biosynthesis and signalling were differentially regulated in response to at least one of the peptides (Table 1). The expression of nine genes involved in redox homeostasis, including RRTF1, GRX480, GRXS13, GRXC11, and NTRB was also up-regulated by PIP2 peptide treatment.

Of 21 genes down-regulated by PIP1 treatment, 19 were also repressed by PIP2. Among these were genes involved in plant immune regulation, including GLK1, CRK5, CRK15, CRK22, and WRKY60 TFs. More CRKs as well as other receptor-like kinases, especially LRR-RLKs, were also down-regulated only by PIP2 treatment (Supplementary Dataset S1). Based on functional categorization of up- and down-regulated genes, we speculated that PIP1 and PIP2, together with PIP3, might have a role in regulation of plant immunity. To evaluate the effect of PIP3, we repeated the experiment using PIP1, PIP2, and PIP3 C-terminal conserved domain peptides for analysis of innate immunity-related genes in response to these peptides. qRT-PCR analyses confirmed the differential regulation observed in the microarray experiments (Fig. 2A).

It has been reported that PIP1 amplifies plant immune response through RLK7 (Hou et al., 2014). To test whether RLK7 also involved in PIP3 perception, we analysed expression regulation of selected genes in \( rlk7 \) and WT backgrounds in response to exogenous application of the synthetic PIP3 peptide. The majority of the selected genes responded similarly to PIP3 treatment in both WT and \( rlk7 \) backgrounds (Fig. 2B). In the case of JAZ9, RRTF1, and TAT3, differential regulations were observed. TAT3 regulation was found independent from PIP3 peptide treatment and similar deviations were also observed in control \( rlk7 \) plants without treatment. These observations suggest that RLK7 is not a major receptor for PIP3.

**PIP genes are induced by flg22 and pathogen treatment**

The bacterial-derived PAMP flg22 is known as a strong inducer of PTI-related genes in Arabidopsis (Felix et al., 1999). Previous transcriptome studies of flg22 responses in Arabidopsis indicate that PIPs are induced by endogenous and exogenous elicitors in WT plants but repressed in \( flg22 \) mutants lacking the flg22 receptor (Denoux et al., 2008; Lyons et al., 2013; Supplementary Table S2). To get a better understanding of the dynamics of the response, a time course experiment on Arabidopsis seedlings treated by flg22 (100 nM) was performed. qRT-PCR analysis showed that the expression of all three genes started to increase significantly 15 min after treatment and reached a maximum at 30 min (Fig. 3A). PIP1, PIP2, and PIP3 expression was induced 244-, 28- and 88-fold, respectively, at this time point. The expression levels of the analysed genes were still high after 1 h, but dropped to basal levels at later time points. flg22-induced expression of PIPs was completely abolished in \( flg22 \) background (Fig. 3B).

The expression patterns of PIP genes in response to *B. cinerea* infection at 6, 12 and 24 h post-infection (hpi) were also studied. As shown in Fig. 3C, PIP1 expression increased throughout the experiment, whereas PIP2 and PIP3 expression reached maximum levels at 12 hpi and declined at 24 hpi, with stronger induction observed for PIP3.

**Identification of mutant and overexpression lines**

To assign a biological function to the PIP peptides, we characterized putative knockout lines for PIP2 (SAIL_1275_B11; \( pip2 \)) and PIP3 (SM_3_22412; \( pip3 \)). The T-DNA insertion in \( pip2 \) was placed 80 bp upstream of the start codon, whereas the En/Spm transposon insertion in \( pip3 \) resulted in loss of the eight C-terminal amino acids, including a part of the last SGPS motif (Fig. 4A). Loss of PIP3 expression in the \( pip3 \) knockout line was confirmed (Fig. 4B). However, the \( pip2 \) line showed increased PIP2 expression both under normal growth conditions and after flg22 treatment (Fig. 4B). No T-DNA or transposon insertion lines for PIP1 were found in available seed stocks. Due to the lack of proper null mutant lines for PIP1 and PIP2, we focused on functional characterization of PIP3. Transgenic plants expressing PIP3 coding sequence under control of the constitutive CaMV35S promoter were generated. Two independent T3 lines with constitutive expression of PIP3 were chosen for further analysis. Under normal growth conditions, no significant growth or developmental abnormalities were observed in any of the lines (Supplementary Fig. S2).

**PIP3 overexpression alters responses to flg22 and pathogens**

flg22 is known to impede Arabidopsis seedling growth (Gómez-Gómez and Boller, 2000). We therefore examined the phenotype of the \( pip3 \) mutant and constitutive PIP3 overexpression lines by measuring the fresh weight (FW) of seedlings grown in the presence or absence of flg22. As shown in Fig. 5A, there were no significant differences between the examined lines grown in medium without flg22. In contrast, flg22 caused significant growth inhibition of PIP3:OX seedlings compared with WT seedlings, with an average FW reduction of 69% (PIP3:OX6) and 77% (PIP3:OX7) (Fig. 5A). Growth inhibition of \( pip3 \) (53%) was comparable to WT (55%).
Perception of flg22 is accompanied by a rapid and transient oxidative burst and ROS production followed by callose deposition that is diminished in fls2 background (Felix et al., 1999; Gómez-Gómez and Boller, 2000; Luna et al., 2011). We used a luminol-based assay to evaluate ROS production in response to flg22 and PIP3 synthetic peptides in WT, pip3 and
Table 1. Differentially regulated genes after PIP1 or PIP2 peptide treatment

<table>
<thead>
<tr>
<th>Locus</th>
<th>Description</th>
<th>PIP1 Fold change</th>
<th>Adjusted P-value</th>
<th>PIP2 Fold change</th>
<th>Adjusted P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jasmonic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>At2g34600</td>
<td>JA27 (jasmonate ZIM domain-containing protein 7)</td>
<td>7.78</td>
<td>0.0432</td>
<td>3.63</td>
<td>0.0329</td>
</tr>
<tr>
<td>At1g30135</td>
<td>JA28 (jasmonate ZIM domain-containing protein 8)</td>
<td>3.94</td>
<td>0.0241</td>
<td>2.20</td>
<td>0.0163</td>
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<td>At1g70700</td>
<td>JA29 (jasmonate ZIM domain-containing protein 9)</td>
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<td>0.0198</td>
<td>2.64</td>
<td>0.0086</td>
</tr>
<tr>
<td>At5g13220</td>
<td>JA20 (jasmonate ZIM domain-containing protein 10)</td>
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<td>0.0198</td>
<td>1.79</td>
<td>0.0236</td>
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<tr>
<td>At1g17380</td>
<td>JA25 (jasmonate ZIM domain-containing protein 5)</td>
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<td>0.0359</td>
<td>1.88</td>
<td>0.0238</td>
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<tr>
<td>At3g22275</td>
<td>JA23 (jasmonate ZIM domain-containing protein 13)</td>
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<td>0.0284</td>
<td>NS</td>
<td></td>
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<tr>
<td>At1g74950</td>
<td>JA22 (jasmonate ZIM domain-containing protein 2)</td>
<td>1.92</td>
<td>0.0385</td>
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<tr>
<td>At3g25760</td>
<td>AOC1 (allene oxide cyclase 1)</td>
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<td>0.0475</td>
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<td>0.0116</td>
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<td>LOX3 (lipoxygenase 3)</td>
<td>1.70</td>
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<td>0.0243</td>
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<td>At2g06050</td>
<td>OPP3 (OPDA-reductase 3)</td>
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<td>2.04</td>
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<td>At1g18020</td>
<td>OPRIII (OPDA-reductase 5)</td>
<td>NS</td>
<td></td>
<td>2.14</td>
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<tr>
<td>At1g34160</td>
<td>RAP2.6 (RELATED TO APETALA2 6)</td>
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<td>0.0290</td>
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<td>JIN1/MYC2 (JASMONATE INSENSITIVE 1)</td>
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<td>0.0189</td>
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<td>At3g15500</td>
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<td>0.0176</td>
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<td>At1g06160</td>
<td>ORA59 (octadecanoid-respoNSive AP2/ERF 59)</td>
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<tr>
<td>At3g36650</td>
<td>CYP94B3 (jasmonoyl-isoleucine-12-hydroxylase)</td>
<td>3.94</td>
<td>0.0437</td>
<td>3.58</td>
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<td>Salicylic acid</td>
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<td>At1g09415</td>
<td>NIMIN3 (NIM1-interacting 3)</td>
<td>NS</td>
<td></td>
<td>1.75</td>
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<td></td>
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<tr>
<td>At4g39030</td>
<td>SID1 (SALICYLIC ACID INDUCTION DEFICIENT 1)</td>
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<td></td>
<td>−1.56</td>
<td>0.017</td>
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<tr>
<td>At1g37380</td>
<td>SAR1 (SAR DEFICIENT 1)</td>
<td>−1.57</td>
<td>0.0432</td>
<td>−1.59</td>
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<td>At1g19250</td>
<td>FM01 (flavin-dependent monooxygenase 1)</td>
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<td></td>
<td>−2.55</td>
<td>0.0291</td>
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<td>At5g13320</td>
<td>PBS3 (avrPphB susceptible 3)</td>
<td>−1.81</td>
<td>0.0479</td>
<td>−2.74</td>
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<td>Redox homeostasis</td>
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<td>At4g34410</td>
<td>ERF109/RRTF1 (redox responsive transcription factor 1)</td>
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<td>0.0176</td>
<td>7.26</td>
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<td>At1g28480</td>
<td>GRX9 (glutaredoxin-C9)</td>
<td>NS</td>
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<td>2.79</td>
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<td>At3g62950</td>
<td>GRXS11 (glutaredoxin-C11)</td>
<td>NS</td>
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<td>At1g03850</td>
<td>GRXS13 (monothiol glutaredoxin-S13)</td>
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<td>At2g29450</td>
<td>ATGSTU5 (glutathione S-transferase U5)</td>
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<td>0.0479</td>
<td>3.01</td>
<td>0.0089</td>
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<td>At1g10360</td>
<td>ATGSTU18 (glutathione S-transferase U18)</td>
<td>NS</td>
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<td>0.0176</td>
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<tr>
<td>At2g29420</td>
<td>ATGSTU7 (glutathione S-transferase U5)</td>
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<tr>
<td>At2g25080</td>
<td>ATGPE1 (phospholipid hydroperoxide glutathione peroxidase 1)</td>
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Fold change in transcript abundance represented if significant regulation was found at P<0.05. NS, not significant.
Najafi et al. PIP3:OX plants, fls2 was used as a negative control. Our assays showed a decrease in ROS production in PIP3:OX plants while a marginal increase was observed in pip3 plants (Fig. 5B). Co-treatment of WT plants with flg22 and PIP3 peptides, but not flg22 and mock peptide, caused a marginal but not significant decrease in ROS production (Supplementary Fig. S3). Callose deposition in response to flg22 treatment was also quantified. Our data showed that PIP3:OX plants produce significantly (*P* ≤ 0.05) less callose 24 h after flg22 treatment (Fig. 5C).

The enhanced susceptibility of plants overexpressing PIP3 to flg22 prompted us to investigate whether PIP3 is involved in pathogen responses by infecting pip3 and PIP3:OX seedlings with the hemibiotrophic bacterium *P. syringae* pv. *tomato* DC3000 (*Pst*). Plants overexpressing PIP3 displayed enhanced susceptibility to *P. syringae* (Fig. 6A). In contrast, pip3 plants showed a marginal but significant reduction in CFU at day 2 compared with WT. *Pseudomonas syringae* assays were also performed on 5-week-old soil-grown plants, with similar results (Supplementary Fig. S4).

We also investigated the response to the necrotrophic fungal *B. cinerea* isolate 2100 (Spanish type). Leaves of 5-week-old WT, pip3, and PIP3:OX lines were inoculated with a fungal spore suspension. WT Col-0 and *wrky33* mutant plants have been reported to be resistant and susceptible to this strain, respectively (Birkenbihl et al., 2012). To evaluate the degree of susceptibility, we used *wrky33* as a susceptible control in our experiments. In WT and pip3 plants inoculation caused lesions at the site of infection 2 d after treatment, but development of necrotic lesions halted after day 3 (Fig. 6B). In contrast, the
symptoms developed much faster in *wrky33* plants and caused complete destruction of infected plants 5 d post-infection. The observed lesion sizes and symptoms in *PIP3:OX6* and *PIP3:OX7* lines were stronger compared with WT and *pip3* plants after day 2, and caused severe chlorosis in infected leaves after 5 d. However, in both evaluated *PIP3* overexpression lines, the symptom development rate and severity were much lower than in *wrky33* plants (Supplementary Fig. S5).

Successful colonization of host plants by *B. cinerea* and the degree of susceptibility was quantified as *in planta* fungal growth using qPCR analysis. Three days after inoculation, DNA from whole inoculated leaves were isolated and subjected to qPCR using *Bc CutA* and *At SKII* as pathogen and plant specific primers, respectively. The qPCR analysis showed a significantly higher ratio of *Bc CutA*/*At SKII* in *wrky33* in comparison with all other genotypes (Fig. 6C). However, *PIP3* overexpression lines also exhibited significantly elevated susceptibility compared with WT and *pip3* plants.

Together, the bioassay results showed that ectopic expression of *PIP3* negatively regulates the plant immune response when challenged by pathogens with different lifestyles.

**Disease-related genes are differentially regulated in WT and PIP3 overexpression plants**

To understand the altered disease phenotype in *PIP3* overexpression plants and to link it to known pathways, the temporal expression of disease-related genes was assessed. WT, *pip3*, and the *PIP3* overexpression line *PIP3:OX7* were inoculated with *B. cinerea* and leaf tissue was harvested for gene expression analysis 6, 12 and 24 hpi. The basal transcript level of the SA-inducible gene *PATHOGENESIS RELATED1* (*PR1*) was similar in all analysed genotypes; no strong induction was observed before 12 hpi (Fig. 7A). *PR1* transcript levels increased strongly in WT and *PIP3:OX7* plants after 12 hpi. At 24 hpi, *PR1* expression was significantly higher in *PIP3:OX7* plants compared with WT. Interestingly, no *PR1* induction was observed in *pip3* plants (Fig. 7A).
Based on the enhanced induction of PR1 in PIP3:OX plants, we examined a set of genes involved in SA biosynthesis and signalling in B. cinerea challenged plants, including ISOCHORISMAE SYNTHASE 1 (ICS1), the main gene in SA biosynthesis, ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1), NON-EXPRESSOR OF PR GENES 1 (NPR1), a master regulator of SA signalling, EDS5, and PHYTOALEXIN DEFICIENT 4 (PAD4). Some common properties of the transcript responses were observed (Fig. 7B–F). For all genes, transcript induction at 24 hpi was strongest in PIP3:OX7 plants (with the exception of PAD4).

We also evaluated the effect of altered PIP3 expression on JA signalling upon B. cinerea infection. The JA-inducible gene PLANT DEFENSIN1.2 (PDF1.2), as well as two key genes in JA biosynthesis (AOA3 and OPR3) and three JAZ genes, were chosen for this analysis. The expression profile of PDF1.2 showed the same pattern as for PR1 up to 12 hpi, and the transcript levels increased by 24 hpi (Fig. 8A), with the expression levels of PDF1.2 significantly higher in PIP3:OX7 plants at 24 hpi compared with the other genotypes (P>0.05). OPR3 was also upregulated in PIP3:OX7 24 hpi compared with WT and pip3 (Fig. 8C). Interestingly, the expression levels of AOC3 (Fig. 8D), JAZ1 (Fig. 8D), JAZ7 (Fig. 8E), and JAZ8 (Fig. 8F) were significantly induced in PIP3:OX7 plants at both 12 hpi and 24 hpi, but unchanged or moderately induced in WT as well as in pip3.

PIPs are regulated by WRKY transcription factors

Sequence analysis of PIP1, PIP2, and PIP3 revealed that the promoter regions of these genes are enriched in W-boxes, which are target sites for binding of WRKY TFs. In silico data suggest that the expression of PIPs is affected in wrky33 and wrky18/40 plants (Birkenbihl et al., 2017). To further investigate this, we analysed the expression levels of PIP1-3 in wrky18, 40 and 33 knockout mutants. PIP1-3 expression was not affected in wrky18 nor wrky40 single knockout plants (Fig. 9). In contrast, PIP1 and PIP3 expression increased significantly in the wrky18/40 double mutant. Similar analysis revealed that PIP2 and PIP3 expression was significantly induced in wrky33 background. These results indicate that WRKY18, WRKY40, and WRKY33 function cooperatively as repressors of PIP1-3. This observation is consistent with previous studies, which have shown that WRKY18 and WRKY40 are able to bind DNA as heterodimers (Xu et al., 2006; Chen et al., 2010; Pandey et al., 2010). To further support these observations, genome-wide ChiP-seq data revealed that the promoter regions of PIP1, PIP2, and PIP3 are occupied by WRKY18, WRKY40, and WRKY33 2 h after flg22 treatment (Supplementary Fig. S6), while WRKY33 actively binds to the promoter regions of PIP1 and PIP3 14 h after infection by B. cinerea (Supplementary Fig. S7).

**Discussion**

Plant peptides are derived from different genomic sources and are engaged in a plethora of functions in plant growth, development, and stress responses (Tavormina et al., 2015). However, only a fraction of them, including IDA, have been assigned to a biological function (Butenko et al., 2003). We have previously studied the phylogeny and expression of the IDA/IDL and PIP/PIPL gene families showing, that several PIP genes are highly inducible by different biotic and abiotic stresses (Vie et al., 2015). Hou et al. (2014) reported that PIP1 amplifies plant immune responses against biotrophic and necrotrophic pathogens through RLK7. The present study aimed to further
elucidate the role of the related PIP3 peptide in regulation of the plant immune response. The PIP3 prepropeptide contains two conserved SGPS motifs (Fig. 4). These two motifs might result in two distinguished mature peptides with the ability to activate independent pathways under different conditions or to be incorporated in a single pathway by binding to different receptor partners. Such a phenomenon has been reported for CLAVATA3/ESR-RELATED 18 (CLE18). The CLE18 peptide was first reported as an inhibitor of tracheary element differentiation and root growth (Ito et al., 2006), whereas another active peptide form of CLE18 has been described to promote root growth (Meng et al., 2012). Another study showed that synthetic peptides for the C-terminal SGPS motifs of PIP2 and PIP3 were found to inhibit lateral root development, while no effect was observed for the N-terminal SGPS motif peptides (Ghorbani et al., 2015). Thus, the C-terminal SGPS motif might be the major contributor to the biological activity; alternatively, the N-terminal SGPS motif might be important in other processes or tissues. Structural flexibility of prepropeptides to produce different final active forms provides an extra layer of complexity in studies of signal transduction pathways initiated by peptide hormones.

Plant immunity consists of a complex network of different hormonal and protein signalling cascades with synergistic or
antagonistic cross-talk depending on the nature of the invader and their lifestyle (Jonak et al., 2002; Spoel et al., 2003; Pieterse et al., 2012; Berens et al., 2017). Activation of the plant immune system is a highly energy-demanding process and can affect plant growth adversely. Due to the limited available resource pool, a tight regulatory mechanism should control growth–defence trade-offs in plants to avoid unnecessary activation of the immune system (Huot et al., 2014). Our results show that ectopic application of the conserved C-terminal domains of PIP1, PIP2, and PIP3 peptides leads to differential regulation
of many genes involved in defence-related phytohormone (SA, JA and ET) biosynthesis and signalling pathways, as well as redox homeostasis (Table 1; Figs 1, 2A).


**Fig. 9.** PIP1 and PIP3 expression is repressed by the WRKY18/40 and WRKY33 complex. Wild-type and knockout plants were grown on ½MS agar plates for 2 weeks. Rosette leaf tissue was harvested for RNA isolation and qRT-PCR analysis. Bars and error bars represent mean and SD, respectively (n=3). Expression level was assessed relative to wild-type plants grown under similar conditions. Statistical differences (Student’s t-test: *P*<0.05) between wild-type and mutants are indicated.

flg22 and *B. cinerea* treatment of WT plants strongly induced the expression of PIP1, PIP2, and PIP3 (Fig. 3). In addition, seedling growth inhibitory assays in the presence of flg22 revealed that plants overexpressing PIP3 are hypersensitive to flg22 (Fig. 5A), suggestive of a role for PIP3 in the regulation of plant response to pathogens. Recognition of PAMPs or pathogen-derived effectors by their cognitive receptors initiates a biphasic oxidative burst composed of fast and slow steps that are correlated to PTI and ETI, respectively (Grant and Loake, 2000). During the early stage of PAMP perception, a rapid oxidative burst is triggered by plasma membrane localized Respiratory Burst Oxidase Homologs (RBOHs) that leads to accumulation of H$_2$O$_2$ in the apoplastic space. Elevated ROS levels in the apoplast are toxic to pathogens, and mediate fast, long-distance, cell-to-cell propagation of a ROS signalling wave (Miller et al., 2009). Vie et al. (2017) showed that IDL6 and IDL7 act as negative modulators of stress-induced ROS signalling in Arabidopsis. Our data showed a significant decrease of ROS production in response to flg22 in PIP3:OX plants (Fig. 5B). Whether this effect is caused by activation of ROS scavenging mechanisms or repression of ROS-producing RBOHs remains to be shown. However, co-treatment of WT plants with flg22 and PIP3 did not change the ROS accumulation significantly (Supplementary Fig. S3).

PIP1 is perceived by RLK7, which activates the MAP kinases MKP3 and MKP6 through a MAP kinase cascade (Hou et al., 2014). RBOH activity is regulated at the post-translational level by phosphorylation (Baxter et al., 2014, Kadota et al., 2014). The MAP kinase kinase MKKK7 was found to interact with FLS2 and attenuate both ROS bursts and MKP6 activation downstream of FLS2 (Mithoe et al., 2016). In view of our results, MKKK7 would be an attractive downstream candidate for PIP3 and its receptor. Another interesting target that can correlate ROS accumulation levels to plant response to pathogens is Redox Responsive Transcription Factor 1 (RRTF1). The expression of RRTF1 is significantly induced by exogenous application of PIP1–3 peptides (Table 1; Fig. 2). It has been shown that RRTF1 inactivation restricts and RRTF1 overexpression induces ROS accumulation in response to stresses. Furthermore, plants overexpressing RRTF1 were highly susceptible to infection by the necrotrophic fungus *Alternaria brassicaceae* (Matsuo et al., 2015). Regulation of RRTF1 expression by *A. brassicaceae* infection, high light, and H$_2$O$_2$ was also shown to require WRKY18/40/60. In line with this hypothesis, our promoter analysis showed that WRKY18/40 heterodimer and WRKY33 act as negative regulators of PIP1, PIP2, and PIP3 expression (Fig. 9). In addition, WRKY factors can bind to the promoter of PIPs during biotic stresses (Supplementary Figs S6, S7). Altogether, our data suggest that PIP3 might be involved in attenuation of ROS production and subsequent systemic signalling, possibly as part of a negative feedback controlling system to avoid runaway responses.

The altered susceptible phenotype of PIP3:OX plants when challenged by pathogens with different lifestyles (Figs 5B, 6) supports the hypothesis that PIP3 is involved in the modulation of plant immunity through regulation of SA and JA signalling pathways (Figs 7, 8). Plants have evolved different mechanisms to fight off pathogens based on their invasion strategies. In order to discriminate biotrophs from necrotophs, plants use phytohormones to activate and regulate appropriate responses. SA is a major regulator when plants are challenged by biotrophs (Tsuda et al., 2008; Vlot et al., 2009), while responses to necrotophs and herbivorous insects mainly are mediated by JA and ET (Farmer et al., 2003; Kachroo and Kachroo, 2009; Berens et al., 2017). SA and JA signalling pathways are generally antagonistic to each other. When plants encounter multiple pathogens with different lifestyles simultaneously, internal cross-talk between SA and JA signalling pathways optimizes the proper response. It has been shown that under this situation, synergistic and compensatory relationships between signalling pathways drive the final response by the host plant (Tsuda et al., 2009). Simultaneous activation of JA and SA biosynthesis results in suppression of JA signalling by the SA pathway. SA triggers cell death, which acts in favour of necrotophs (Glazebrook, 2005; Spoel and Dong, 2008; Caarls et al., 2015). The observed susceptible phenotype of PIP3:OX plants infected by *B. cinerea* can be explained in line with this concept. However, the temporal regulation of SA and JA biosynthesis and signalling genes by overexpression of PIP3 is not able to explain the observed susceptible phenotype of PIP3:OX plants challenged by the biotroph pathogen *Pst* DC 3000. A recent study has shown that when Arabidopsis plants are challenged by *Pst* DC 3000 avirulent strain carrying avrRpt2, both SA and JA pathways are induced simultaneously but in different zones relative to the infection site (Betsuyaku et al., 2018). This study showed that 13 h after infection, the SA active zone surrounds the infection site where the hypersensitive reaction takes place, while JA signalling is exclusively activated in cell layers outside of the SA active zone. According to our
gene expression analysis after PIP3 and flg22 treatments (Figs 2, 3), we hypothesize that upon perception of pathogens, PIP3 might play a positive role in spatiotemporal regulation of SA and JA signalling pathways to mount a proper defence response based on pathogen invasion strategy. Our data indicate that this spatiotemporal regulation is compromised in PIP3:OX plants. The actual mechanistic role of PIP3 in this process remains to be tested experimentally. Hou et al. (2014) reported that PIP1 signals through RLK7, causing an amplification of the immune response against pathogens. There are differences in the experimental set-ups that may explain the contradictory observed responses to the synthetic peptides. Hou et al. used shorter PIP1 and PIP2 synthetic peptide (13 and 15 aa, respectively) that were hydroxylated at the last proline (P-OH) within the SGPS motif. It has been shown that post-translational modifications can change peptide activities. Furthermore, Hou et al. applied synthetic peptides at micromolar concentrations in all of their assays, whereas we used unmodified longer synthetic peptides at 100 nM concentration. Studies on synthetic CLE40 peptide and its effect on root growth have revealed that depending on the nature of modifications and concentrations of applied peptide in the growth medium, CLE40 can repress or induce root growth of soybean plants (Corcilius et al., 2017). However, our data suggest that RLK7 is less likely to be a major receptor for PIP3, as rlk7 plants responded similarly to WT plants when treated with PIP3 peptide (Fig. 2B).

In conclusion, our results show that signalling triggered by the small peptides PIP1, PIP2, and PIP3 can reprogram the expression of genes encoding proteins involved in regulation of plant immunity. Furthermore, ectopic expression or exogenous application of PIP3 peptide changes the expression of genes involved in immune response towards attenuation of immunity. Based on our data, we propose a model in which expression of PIP3 is cooperatively repressed by WRKY18/40 and WRKY33 under normal conditions (Fig. 10A). Perception of PAMPs by corresponding PRRs induces the expression of PreproPIP3. Preproproteins are then transported to the apoplast and proteolytically processed to produce mature peptides. Perception of mature PIP3 peptide by its corresponding cell surface receptors initiates new signalling pathways that result in the suppression of PAMP-induced genes. Induction of WRKY18, WRKY40, and WRKY33 expression by PAMPs as well as PIP3 results in a negative feedback loop that restricts PIP3 expression temporally and/or spatially. When PIP3 overexpression plants are challenged by B. cinerea, both SA and JA biosynthesis and signalling pathways are transcriptionally induced (Fig. 10B). Simultaneous activation of SA and JA pathways upon pathogen attack prioritize SA over JA. One outcome of this interaction is hypersensitive reaction and cell death, facilitating growth and colonization of the host plant by necrotrophic pathogens (Fig. 10). An alternative explanation is the spatiotemporal regulation of SA and JA activities by PIP3 as discussed above. The potential role of these peptides in growth and development should be unveiled by analysis of PIP1 and PIP2 knockout/silencing plants, alone or in combination with pip3. In addition, identification of the postulated PIP3 receptor(s) will shed light on the downstream events and components involved in signalling, as well as their possible cross-talks with phytohormones.

Fig. 10. Proposed model for PIP3-modulated signalling of plant immunity. (A) PIP3 transcript level is induced by perception of PAMPs and/or pathogens through PRRs. Mature PIP3 is sensed by its corresponding receptors located at the plasma membrane. Exogenous application of PIP3 peptide leads to induction of JAZ genes as well as WRKY18 and WRKY40. WRKY18, WRKY40, and WRKY33 may act in a negative feedback loop to terminate PIP3 expression. (B) PIP3 overexpression plants challenged by B. cinerea simultaneously activate the expression of JA and SA biosynthesis and signalling genes. The outcome of the interaction between SA and JA is hypersensitive reaction, cell death, and susceptibility to B. cinerea.
Supplementary data

Supplementary data are available at JXB online.
Dataset S1. PIP1–3 and PEP1–3 co-expression tables.
Dataset S2. Differentially regulated genes upon PIP1 and PIP2 peptide treatments.
Fig. S1. Co-expression analysis of PIP1, PIP2, and PIP3.
Fig. S2. Expression and phenotype analysis of PIP3 overexpression lines.
Fig. S3. Temporal production of ROS in response to flg22 and PIP3 peptides.
Fig. S4. Susceptibility of 5-week-old pip3 and PIP3:OX plants to P. syringae.
Fig. S5. Disease symptoms of 5-week-old plants 5 d after inoculation with 5×10^5 ml−1 of B. cinerea spores.
Fig. S6. Binding of WRKY18, WRKY40, and WRKY33 to PIP1, PIP2, and PIP3 promoter regions in response to flg22 treatment.
Fig. S7. Binding of WRKY33 to PIP1 and PIP3 promoter regions in response to B. cinerea infection.
Table S1. List of primers used in this study.
Table S2. Expression pattern of PIP1, PIP2 and PIP3 in response to the different elicitors in wild-type (Col-0) and ftl2 backgrounds obtained from public databases.

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