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The IDA-LIKE peptides IDL6 and IDL7 are negative modulators of stress responses in Arabidopsis thaliana

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Abstract

Small signalling peptides have emerged as important cell to cell messengers in plant development and stress responses. However, only a few of the predicted peptides have been functionally characterized. Here, we present functional characterization of two members of the IDA-LIKE (IDL) peptide family in Arabidopsis thaliana, IDL6 and IDL7. Localization studies suggest that the peptides require a signal peptide and C-terminal processing to be correctly transported out of the cell. Both IDL6 and IDL7 appear to be unstable transcripts under post-transcriptional regulation. Treatment of plants with synthetic IDL6 and IDL7 peptides resulted in down-regulation of a broad range of stress-responsive genes, including early stress-responsive transcripts, dominated by a large group of ZINC FINGER PROTEIN (ZFP) genes, WRKY genes, and genes encoding calcium-dependent proteins. IDL7 expression was rapidly induced by hydrogen peroxide, and idl7 and idl6 idl7 double mutants displayed reduced cell death upon exposure to extracellular reactive oxygen species (ROS). Co-treatment of the bacterial elicitor flg22 with IDL7 peptide attenuated the rapid ROS burst induced by treatment with flg22 alone. Taken together, our results suggest that IDL7, and possibly IDL6, act as negative modulators of stress-induced ROS signalling in Arabidopsis.

Key words: Abiotic stress, Arabidopsis, IDA-LIKE, peptide ligand, ROS, transcriptome, ZAT12.

Introduction

Plants cannot escape their environment, and are thus exposed to herbivore grazing, pathogen attacks, and other environmental perturbations such as drought, temperature changes, and high salinity. Plants have evolved sophisticated mechanisms in order to meet these challenges. At the cellular level, environmental cues are perceived either directly or indirectly and transduced through a complex signalling network, resulting in an appropriate response by changing the chemical environment of the cells. This transduction typically involves alternations of secondary messengers and signalling molecules such as calcium and phytohormones, but also reactive oxygen species (ROS).

Most types of environmental stresses such as wounding, drought, salinity, heat, cold, and pathogen attack lead to
early and rapid accumulation of ROS (Miller et al., 2008; Torres, 2010; Sierla et al., 2013; Gilroy et al., 2014), known as the oxidative burst. Depending on the type of stress and the host involved, the oxidative burst relies on different types of enzymes, including cell wall peroxidases (Bindschedler et al., 2006) and plasma membrane NADPH oxidases (Torres et al., 2002; Miller et al., 2009; Torres, 2010). NADPH oxidases, often referred to as respiratory burst oxidase homologues (Rbohs), are transmembrane proteins responsible for the production of extracellular superoxide (O₂⁻) upon pathogen attack (Torres et al., 2002) or abiotic stresses (Kwak et al., 2003; Miller et al., 2009). Both abiotic and biotic stresses trigger a systemic autoregulating wave of ROS, mediated by the NADPH oxidase RBOHD, that travels rapidly in the apoplasm from the affected tissue to the entire plant and activates a systemic response to the stress (Miller et al., 2009; Dubiella et al., 2013; Gilroy et al., 2014).

External stress triggers and activates intercellular signalling, essential for cell to cell communication. Since the discovery of systemin in tomato (Pearce et al., 1991), small post-translationally modified peptides have been recognized as important mediators of cell to cell communication (Butenko et al., 2009; Matsubayashi, 2014). They are characterized as small proteins with an N-terminal signal peptide directing the protein to the secretory pathway, a variable region, and a conserved C-terminal part containing the active peptide that after translation undergoes a series of modifications (Matsubayashi, 2014). The translated propeptide is proteolytically processed into the shorter active peptide, which may be further modified (Murphy et al., 2012) before the mature peptide can bind its receptor(s). One well-studied peptide is CLAVATA3 (CLV3) (Clark et al., 1995; Fletcher et al., 1999). Mature CLV3 is modified with two hydroxyprolines; one of these is further modified with three arabinose molecules, enhancing the binding affinity of the peptide for its receptor (Ohyama et al., 2009).

Although several peptides have been linked to growth and development, there are a few studies in Arabidopsis linking signalling peptides to plant stress responses. In Arabidopsis, the first defence-related peptide identified was the plant elicitor peptide PROPE1 (Hulfaker et al., 2006). AtPep1 binds the two leucine repeat-rich receptor-like kinases (LRR-RLKs) PEPR1 and PEPR2 (Yamaguchi et al., 2006, 2010), and acts as a damage-associated molecular pattern (DAMP) promoting the expression of pathogen defence genes such as PLANT DEFENSIN 1.2 (PDF1.2) and PATHOGEN-RELATED 1 (PR1), thus amplifying the defence response upon pathogen attack (Yamaguchi et al., 2006, 2010). The growth-promoting phytosulfokine (PSK) peptide has also been linked to plant defence. It has been shown that PSK through its receptor PSK RECEPTOR 1 (PSKR1) attenuates pattern-triggered immunity in Arabidopsis, suggesting that PSK/PSKR1 is part of a mechanism that controls the allocation of resources between growth and immunity (Igarashi et al., 2012). Knockout plants of the C-TERMINALLY ENCODED PEPTIDE family member CEP3 show increased root and shoot growth compared with the wild type under several abiotic stress conditions, suggesting a role for CEP3 as a negative regulator of growth in response to changing environmental conditions (Delay et al., 2013). Overexpression of oxidative stress-induced peptide OSIP108 resulted in increased tolerance to the ROS inducer paraquat (De Coninck et al., 2013), and proteolytic processing of apoplastic GRIM REAPER by METACASPASE-9 produced an 11 amino acid ligand for its receptor PRK5 that is active in RbohD-dependent cell death (Wraczaczek et al., 2015). The PAMP-induced secreted peptide 1 (PIP1) and 2 (PIP2) have been shown to be induced by a variety of pathogens and elicitors, and PIP1 was found to amplify the immune response in a PEP1-like fashion through the LRR-RLK RLK7 (Hou et al., 2014). Finally, the cysteine-rich peptide AtCAPE1 confers salt sensitivity, in line with a negative regulatory role in salt stress tolerance (Chien et al., 2015).

We have recently performed a genome-wide screen for genes encoding small signalling peptides with similarity to INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) in Arabidopsis thaliana (Vie et al., 2015). IDA belongs to a family of nine genes, where IDA-LIKE 1 (IDL1) to IDL5 have been postulated to play roles during plant development (Butenko et al., 2003; Stenvik et al., 2008). Two newly discovered members of this subfamily, IDL6 and IDL7, are closely related, and expression analysis revealed that these two genes are induced by various stress treatments (Vie et al., 2015). In this study, we aimed to characterize the putative ligands IDL6 and IDL7 and their roles in modulating stress responses.

**Materials and methods**

**Plant material**

Seeds of the Arabidopsis thaliana ecotype Col-0 (N1092), idl6-1 (SALK_074245), idl6-2 (SALK_126026) (Alonso et al., 2003), and idl7 (WDL293-296; Woody et al., 2007) mutants were obtained from the European Arabidopsis Stock Centre (NASC, Nottingham, UK), and T-DNA insertions were confirmed by PCR using gene- and T-DNA-specific primers (see Supplementary Table S1 at JXB online). Verified homozygous lines were back-crossed to Col-0 wild type to ensure single knockout lines. Double knockout lines were obtained by crossing idl7 (pollen) to idl6-1 (mother plant) and idl6-2 (pollen) to idl7 (mother plant). Double homozygous lines were verified by PCR using gene- and T-DNA-specific primers (Supplementary Table S1).

IDL6 and IDL7 promoter:β-glucuronidase (GUS) fusions were generated using Gateway technology (Invitrogen). The intergenic regions upstream of IDL6 (869 bp) and IDL7 (1306 bp) were amplified from genomic DNA from the Col-0 ecotype using the primers pIDL6attB1 and pIDL6attB2 for the IDL6 promoter region, and pIDL7attB1 and pIDL7attB2 for the IDL7 promoter region (Supplementary Table S1). The fragments were cloned upstream of the GUS gene in the destination vector pMDC163 (Curtis and Grossniklaus, 2003) via the pDONR/ZEo vector (Invitrogen). Complementation lines were generated by amplifying the region surrounding IDL6 (860 bp upstream and 142 bp downstream coding sequence) and IDL7 (1306 bp upstream and 336 bp downstream coding sequence) from genomic DNA from the Col-0 ecotype using the primers pIDL6attB1 and IDL6comp attB2 for IDL6 and pIDL7attB1 and IDL7comp attB2 for IDL7 (Supplementary Table S1). The fragments were cloned into the destination vector pMDC99 (Curtis and Grossniklaus, 2003). The constructs were introduced into Agrobacterium tumefaciens strain
C58C1 pGV2260 and transformed into Arabidopsis Col-0 ecotype using the ‘floral dip’ method (Clough and Bent, 1998). Positive transformants were selected on half-strength solid Murashige and Skoog (MS) medium containing the T-DNA-specific selection marker hygromycin (20 µg ml⁻¹).

Subcellular localization

Full-length (IDL₇FL) or mutated [IDL₇ΔSP, IDL7 lacking the predicted signal peptide (SP); IDL₇ΔSPΔC, IDL7 lacking both the SP and the four last C-terminal amino acids; IDL₇ΔC, full-length IDL7 lacking the four last C-terminal amino acids] coding sequences of IDL7 were amplified from Col-0 ecotype genomic DNA using the primers SPIIDL7attB1, IDL7DSattB1, IDL7UattB2, and IDL7EPEIattB2 ( Supplementary Table S1), and cloned into the destination vector pEG103 (Earley et al., 2006) by Gateway technology (Invitrogen). The vectors were introduced into A. tumefaciens strain C58C1 pGV2260, and transformed cultures [optical density (OD₆₀₀)=0.05, 10 mM MES, 10 mM MgCl₂, and 100 µM acetosyringone] were used for infections of leaves of 3- to 4-week-old Nicotiana benthamiana plants grown on soil under a 16 h photoperiod (70 µmol m⁻² s⁻¹) at 22 °C as described (Sparkes et al., 2006).

Infiltrated leaves were investigated 2–4 d post-infection using a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) with a ×50 water immersion objective. Green fluorescent protein (GFP) was excited using an argon laser at 488 nm, and emission was detected at 495–550 nm.

Histochemical GUS assays

Histochemical GUS assay was performed as described by Butenko et al. (2003).

RNA decay analysis

RNA decay assays were carried out as described (Zhang et al., 2010). Four-day-old seedlings were pre-treated in incubation buffer (1 mM PIPES, pH 6.25, 1 mM sodium citrate, 1 mM KCI, 15 mM sucrose), and then supplied with cordycepin (Sigma-Aldrich, St Louis, MO, USA) to a final concentration of 1 mM. Samples were collected before (0 min) and 30 min and 60 min after cordycepin addition. Tissues for each time point were flash-frozen in liquid nitrogen and stored at −80 °C.

Peptide treatments

Synthetic peptides (Biomatik, Canada) used in this study are described in Table 1. Seeds of the Col-0 ecotype were surface-sterilized and sown out on half-strength MS plates at a density of 20 seeds per Petri dish (14 cm diameter), and stratified for 3 d at 4 °C. Plates were grown under a 16 h photoperiod (70 µmol m⁻² s⁻¹) at 22 °C for 2 weeks. Seedlings were sprayed with an aqueous peptide solution (100 nM) supplemented with 0.02% Silwet L-77 (Lehle Seeds, Round Rock, TX, USA) and vacuum infiltrated at 20 inches Hg for 1 min. Whole rosettes were collected 1, 2, and 3 h after treatment, snap-frozen in liquid nitrogen, and stored at −80 °C.

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<th>Position</th>
<th>Sequence</th>
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<td>MOCKID7</td>
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Expression analyses

RNA isolation, cDNA synthesis, real-time quantitative reverse transcription–PCR (qRT–PCR), microarray experiments, statistical analysis, and Gene Ontology (GO) analysis were performed as described in Vie et al. (2015). In brief, RNA was extracted from 100 mg of frozen plant tissue each from four biological replicas using the Spectrum Total RNA kit (Sigma-Aldrich). cDNA synthesis was performed on 1 µg of total RNA using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany), following the supplier’s instructions. qRT–PCR was performed on a LightCycler 480 using the LightCycler 480 SYBR Green I Master kit (Roche Applied Science, Mannheim, Germany), with PCR parameters as recommended by the supplier. PCR efficiencies and Ct values were calculated by linear regression using the LinRegPCR software (Ramsak et al., 2013; Ruijter et al., 2009), and mean PCR efficiency was calculated for each pair of primers. Ct values and PCR efficiencies were then imported into the REST 2008 software (Pfaffl et al., 2002) to calculate the statistical significance of differences in expression levels upon various treatments. Primers used are listed in Supplementary Table S1. Genome-wide expression analysis was performed using the Arabidopsis (V4) Gene Expression Microarray 4 × 44K (Agilent Technology, USA) as described in the supplier’s manual. The data were analysed using the limma package (Smyth, 2004) and the R statistical data analysis program package (R 2.10.1). The Benjamini and Hochberg method to control the false discovery rate was used to identify differentially regulated genes (Benjamini and Hochberg, 1995). Genes with an adjusted P-value <0.05 were regarded as significantly differentially expressed. The microarray study is MIAME compliant. Raw data have been deposited in GEO (accession GSE77467).

Luciferase assay

Three-week-old transgenic plants containing the ZAT12::luc reporter system (Miller et al., 2009) were grown under a 16 h photoperiod regime and sprayed with 1 mM luciferin the evening prior to the experiment. The next day, plants were uniformly sprayed with luciferin supplemented with each of the three peptides (100 nM). At 65 min after treatment, the plants were injured by pricking one leaf. In total three plants per peptide were treated in each live imaging experiment. Luminescence imaging and measuring was conducted using the NightOWL in vivo imaging system (Berthold Technologies, Germany).

flg22 and H₂O₂ treatment

Seeds of the Col-0 ecotype were surface-sterilized and sown out on half-strength MS plates at a density of 20 seeds per Petri dish (14 cm diameter), and stratified for 3 d at 4 °C. Plates were then grown under a 16 h photoperiod (70 µmol m⁻² s⁻¹) at 22 °C for 2 weeks. Seedlings were sprayed with either flg22 (100 nM) or H₂O₂ (20 mM) in deionized (DI) water containing 0.02% Silwet L-77 and vacuum infiltrated at 20 inches Hg for 1 min. As control, plants were treated with DI water supplemented with 0.02% Silwet-L77 and vacuum infiltrated at 20 inches Hg for 1 min. Whole rosettes were harvested from four biological replicas at 5, 10, 15, 30, and 60 min after treatment, snap-frozen in liquid nitrogen, and stored at −80 °C.

Seedling pathogen assay

The protocol was modified from Lee et al. (2011): surface-sterilized seeds were sown in 24-well plates containing 2 ml of liquid half-strength MS, with a density of three seedlings per well, and stratified for 3 d at 4 °C. Plants were grown in a growth chamber (VB1514, Vötsch Industrietechnik, Balingen, Germany) under a 16 h photoperiod (70 µmol m⁻² s⁻¹) at 22 °C. Pseudomonas syringae pv. tomato DC3000 and P. syringae pv. tomato AvrRPM1 cultures were grown overnight in liquid King’s B medium supplemented with the appropriate antibiotics (50 µg ml⁻¹ rifampicin for the DC3000 strain,
50 µg ml⁻¹ rifampicin, and 50 µg ml⁻¹ tetracycline for the AvrRPM1 strain) in a shaker at 28 °C. The cultures were washed twice with 10 mM MgCl₂ and diluted to OD₆₀₀=0.002 (~2.5 × 10⁶ CFU ml⁻¹). Seven-day-old seedlings were transferred to new 24-well plates containing fresh half-strength MS without sucrose, and 100 µl of diluted bacterial culture was added. For measurements of bacterial growth, co-cultivated seedlings were washed with 70% ethanol for 10 s and rinsed in water. Three seedlings were put into eppendorf tubes containing 100 µl of 10 mM MgCl₂ and ground with a pestle. A 10 µl aliquot of serial dilutions was plated out on LA medium containing the appropriate antibiotics and incubated for 2 d at 28 °C. All pathogen assays were repeated at least three times with similar results.

Stress assays

Seeds of wild-type Col-0 and loss-of-function mutants of IDL6 and IDL7 were surface-sterilized, placed in rows on square half-strength MS plates (control) and half-strength MS with added NaCl (100 mM), mannitol (300 mM), or flg22 (100 nM), and stratified for 3 d at 4 °C. Plates were then placed vertically in a growth chamber (VB1514, Vötsch Industrietechnik) under a 16 h photopheriod for 100 µM, mannitol (300 µM), or flg22 (100 nM), and stratified for 3 d at 4 °C. Plates were then placed vertically in a growth chamber (VB1514, Vötsch Industrietechnik) under a 16 h photopheriod for 4 °C. Root lengths were scored 1 week after seed plating for NaCl and mannitol treatments, and at 10 d for flg22 treatments. The X/XO assay was performed as described (Overmyer et al., 2003). Briefly, rosette leaves from 4-week-old plants grown at 22 °C, with a 12 h light/12 h dark photoperiod were detached and rinsed in DI water. Leaf disks were made using a puncher, incubated for 2 h in DI water, and transferred to a 96-well plate containing DI water. The plate with leaf disks was incubated in darkness overnight. DI water was then replaced with seed plating for NaCl and mannitol treatments, and at 10 d for flg22 treatments. The X/XO assay was performed as described (Overmyer et al., 2000). All stress assays were repeated at least three times with similar results.

Results

**IDL7 is processed C-terminally prior to export from the cell**

One of the hallmarks of the IDA family proteins is the presence of an N-terminal SP for the secretory pathway. IDA has previously been shown to be localized to the apoplast (Butenko et al., 2003). To investigate whether IDL7 is secreted out of the cell, we created constructs containing translational fusions between GFP and full-length IDL7 cDNA (IDL7FL, corresponding to amino acids 1-97) or IDL7 without the predicted N-terminal SP (IDL7ASp, corresponding to amino acids 22-97), as shown in Supplementary Fig. S1. The protein fusions were transiently expressed in N. benthamiana leaves, and the subcellular localization was examined by confocal microscopy. All constructs produced a strong fluorescence signal; the GFP control was located in the cytosol and nucleus (Fig. 1A), IDL7ASp::GFP was, as expected, located in the cytosol, and could also be found in the nucleus (Fig. 1C), resembling the GFP control. This localization was confirmed by plasmolysis with 1 M NaCl (GFP control, Fig. 1B; and IDL7ASp::GFP, Fig. 1D). IDL7FL::GFP localization appeared to be intracellular (Fig. 1E), with the formation of fluorescent aggregates, in addition to a weak extracellular localization. Plasmolysis verified the intracellular localization (Fig. 1F).

It has been suggested that small signalling peptides might be processed C-terminally as well as N-terminally (Matsubayashi, 2014). The fluorescent aggregates seen in IDL7FL::GFP-transformed cells might thus result from proteolytic cleavage of the IDL7 C-terminal end fused with GFP. We therefore generated a new IDL7–GFP fusion without the four C-terminal amino acids of IDL7, corresponding to the C-terminal end of the IDA EPIP (extended PIP) peptide (Stenvik et al., 2008; Supplementary Fig. S1). In cells expressing IDL7C::GFP, however, the fluorescent signal was detected at the surface of the cell (Fig. 1G). In plasmolysed cells expressing IDL7C::GFP, fluorescence was observed in the extracellular space, between the detached plasma membrane and the cell wall (Fig. 1H). Altogether, these results show that IDL7 is indeed exported from the cell, and that IDL7 is processed C-terminally as well as N-terminally during maturation.

**Rapid turnover of IDL6 and IDL7 mRNA**

IDL6pro::GUS and IDL7pro::GUS lines were generated to investigate the expression pattern of these genes. However, the expression patterns indicated by GUS staining did not match the expression pattern found by qRT–PCR (Vie et al., 2015); instead, GUS appeared to be constitutively expressed at high levels in most tissues (Supplementary Fig. S2). This, together with the elevated expression level of IDL6 and IDL7 observed upon cycloheximide (CHX) treatment (Vie et al., 2015), led us to perform a comparison of the expression levels of IDL6/IDL7 and GUS in four independent lines each of IDL6pro::GUS and IDL7pro::GUS. GUS expression in IDL6pro::GUS and IDL7pro::GUS lines was 1000-fold and 200-fold higher than the IDL6 and IDL7 expression levels, respectively (Fig. 2A). In comparison, the GUS expression after CHX treatment in IDL6pro::GUS lines was only up-regulated 13-fold compared with IDL6, and 5-fold up-regulated in the IDL7pro::GUS lines compared with IDL7 (Fig. 2A; Supplementary Fig. S2). One consequence of the inhibitory effect of CHX on translation is that mRNA molecules become trapped on polysomes, thereby preventing mRNA degradation (Cochran et al., 1983; Edwards and Mahadevan, 1992). Therefore, we measured mRNA decay on IDL6 and IDL7 at early growth stages using the transcription inhibitor cordycepin (Johnson et al., 2000). mRNA from both IDL6 and IDL7 showed a rapid decay shortly after cordycepin treatment (Fig. 2B), confirming the GUS assay results. Taken together, the results suggest that IDL6 and IDL7 are under strong regulation, at both transcriptional and post-transcriptional level.

**IDL7 peptide down-regulates many stress-responsive genes**

The bioactive part of the IDA peptide has been identified to be within the C-terminal part, between amino acids 50 and 69. This 20 amino acid peptide, named IDA EPIP, is
sufficient to rescue the floral abscission-deficient phenotype of the ida mutant in vitro (Stenvik et al., 2008). The corresponding EPIP motifs from IDL6 and IDL7, while somewhat longer (24 amino acids), are similar to IDA in 12 positions (Fig. 3A). Peptides corresponding to the EPIP motifs of IDL6 and IDL7 were synthesized and tested for bioactivity in a pilot experiment by spraying wild-type plants with a 10 µM peptide solution. No phenotypes were observed on the plants after treatment; however, a microarray analysis of plants 3 h after treatment suggested that both peptides induced significant transcriptome changes (results not shown). Among the most down-regulated genes were the stress-related transcription factor genes WRKY33, WRKY40, ZAT10, and ZAT12. These genes were selected for a closer analysis of the timing of the response to IDL7 peptide. The peptide concentration was reduced to 100 nM, which is comparable with other studies (Yamaguchi et al., 2006; Delay et al., 2013; Chien et al., 2015; Wrzaczek et al., 2015). qRT–PCR showed that the strongest transcriptional response was observed 2 h after application of peptides (Fig. 3B). This time point was chosen for global transcription profiling.
Global transcriptome profiles were obtained from Arabidopsis seedlings following 2 h treatments with 100 nM IDL6-EPIP or IDL7-EPIP. Treatment with the IDL7 EPIP peptide resulted in significant changes in the transcriptome compared with mock peptide-treated plants; in total, 939 genes were found to be significantly regulated (log2>0.5 and log2< –0.5; \( P <0.05 \); Supplementary Dataset S1). Table 2 presents the most highly regulated genes. Interestingly, a majority of the genes (73%) were down-regulated. Treatment with the IDL6-EPIP peptide resulted in a much weaker response, with only 42 significantly regulated genes (log2>0.5 and log2< –0.5; \( P <0.05 \); Supplementary Dataset S2). When using less stringent statistical criteria for IDL6-regulated genes (log 2>0.5 and log2< –0.5; \( P <0.1 \)), the number of significantly regulated genes was still lower (493 genes; 270 up-regulated, 298 down-regulated) compared with IDL7-treated plants. More than half of the genes regulated by IDL6-EPIP treatment (using the less stringent statistical criteria) were also regulated by IDL7-EPIP treatment (Supplementary Fig. S3). This suggests that despite the strong homology of IDL6 and IDL7 peptide, the effect of IDL7-EPIP on the transcriptome is much stronger at the given developmental stage and environmental conditions than IDL6-EPIP.

To elucidate a possible function of the IDL7-EPIP peptide, the dataset was analysed for over-representation of Gene Ontology (GO) terms (Ashburner et al., 2000) on genes with log2< –0.5; \( P <0.05 \); Supplementary Dataset S1). Table 2 presents the most highly regulated genes. Interestingly, a majority of the genes (73%) were down-regulated. Treatment with the IDL6-EPIP peptide resulted in a much weaker response, with only 42 significantly regulated genes (log2>0.5 and log2< –0.5; \( P <0.05 \); Supplementary Dataset S2). When using less stringent statistical criteria for IDL6-regulated genes (log 2>0.5 and log2< –0.5; \( P <0.1 \)), the number of significantly regulated genes was still lower (493 genes; 270 up-regulated, 298 down-regulated) compared with IDL7-treated plants. More than half of the genes regulated by IDL6-EPIP treatment (using the less stringent statistical criteria) were also regulated by IDL7-EPIP treatment (Supplementary Fig. S3). This suggests that despite the strong homology of IDL6 and IDL7 peptide, the effect of IDL7-EPIP on the transcriptome is much stronger at the given developmental stage and environmental conditions than IDL6-EPIP.

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The most prominent gene families in the dataset included WRKY transcription factors, ZINC FINGER PROTEINS (ZFP), MYB transcription factors, and ethylene-responsive transcription factors (Table 2; Supplementary Dataset S1). We found 11 significantly down-regulated WRKY genes after treatment with the IDL7 peptide; all of them are associated with stress responses. Nine out of 20 genes encoding ZFPs belonging to the C2H2 subfamily C1-2i, all associated with
Table 2. The most up- and down-regulated genes 2 h after treatment with IDL7-EPIP peptide (P<0.05)

Genes with expression ratios of log2>1.0 or log2<–1.5 are listed

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<th>Description</th>
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<tr>
<td>ALAAT2 (ALANINE AMINOTRANSFERASE 2)</td>
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<td>Unknown protein</td>
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<td>ST (steroid sulphotransferase)</td>
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</tr>
<tr>
<td>DIC2 (Mitochondrial Dicarboxylate Carrier Protein 2)</td>
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<td>Unknown protein</td>
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<td>At1g72330</td>
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<td>At2g66650</td>
<td>–2.456</td>
<td>0.121</td>
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*a*Significantly regulated 2 h after treatment with IDL6 peptide (P<0.1).

*b*Identified as PIF-regulated PIF4 target gene (Oh *et al.*, 2012).
abiotic stress responses, were down-regulated by IDL7. Many other genes associated with stress adaptation or defence were down-regulated, including a large group encoding Ca^{2+}-binding proteins, genes encoding heat shock proteins, cytochrome P450-encoding genes, and genes encoding receptor proteins (Table 2; Supplementary Dataset S1).

Genes up-regulated by IDL7-EPIP treatment included GLUTAMINE-DEPENDENT ASPARAGINE SYNTHASE 1 (ASN1), SMALL AUXIN UP RNA (SAUR) and INDOLEACETIC ACID-INDUCED PROTEIN 29 (IAA29) (Table 2). Since SAURs have been shown to be regulated by the basic helix–loop–helix (bHLH) transcription factor PHYTOCHROME INTERACTING FACTOR 4 (PIF4; Franklin et al., 2011), we compared the genes in Table 2 with a list of PIF4 target genes identified by ChIP sequencing (Oh et al., 2012). Six of 13 genes up-regulated by log2 > 1.0 by IDL7 treatment were PIF4 targets. In contrast, only one of 39 genes down-regulated by log2 < −1.5 was a PIF4 target. PIF4 itself was moderately, but significantly induced by IDL7-EPIP treatment (Supplementary Dataset S1).

To gain a further overview of the genes regulated by IDL7-EPIP, a network analysis of the most down-regulated genes in the gene set (log2 < −1) was performed using the STRING database (Szklarczyk et al., 2015). It is a general assumption that genes involved in the same processes are co-regulated or interact in one way or another, also known as the ‘guilt-by-association’ principle (Saito et al., 2008). As shown in Supplementary Fig. S4, a tight cluster of highly connected genes was found within our array, indicating that IDL7 is regulating this network. The cluster includes the ZFP genes ZAT6, ZAT12, ZAT10/STZ, and SZF1, the WRKY transcription factor genes WRKY33, WRKY40, and WRKY53, and CML38, ERF5, and ERF6.

**IDL7 suppresses the expression of ZAT12 upon wounding**

One of the most down-regulated genes in our array was the ZFP gene ZAT12. ZAT12 is highly responsive to different environmental conditions, and is rapidly induced after wounding and accumulation of ROS (Davletova et al., 2005a; Miller et al., 2009). Furthermore, in a study using the luciferase reporter gene, ZAT12 expression was shown to be systemically induced at a rate up to 8.4 cm min^{-1} upon wounding (Miller et al., 2009). We wanted to investigate the effect of treatment with IDL6-EPIP and IDL7-EPIP peptide prior to wounding on the expression of ZAT12 using the ZAT12::luciferase reporter plants used in the study by Miller and co-workers (Miller et al., 2009). Three plants per peptide were treated with luciferin and peptides (IDL6-EPIP, IDL7-EPIP, and MOCKIDL7), and 1 h later one rosette leaf per plant was wounded. Interestingly, as shown in Fig. 5, IDL7-EPIP was able to suppress spreading of the luciferase signal, verifying the negative effect of IDL7-EPIP on ZAT12 expression. IDL6-EPIP was also able to suppress ZAT12 expression, but the response was weaker. This trend was observed in three independent experiments.

**IDL6 and IDL7 are stress-responsive genes**

*In silico* data indicate that IDL6 and IDL7 are moderately expressed during development, and that both genes are rapidly induced by stresses including exposure to *P. syringae* and the pathogen-associated elicitor flg22, salt, UV, wounding, and ROS (Vie et al., 2015). To analyse the stress-induced expression of IDL6 and IDL7, a time course experiment with Arabidopsis seedlings treated with flg22 or H2O2 was performed. Expression analyses using qRT–PCR analysis showed that the expression of both IDL6 and IDL7 was rapidly induced 10–15 min after flg22 treatment, and reached a peak after 30 min (Fig. 6A). The transcriptional response of IDL7 to H2O2 treatment was even faster, reaching maximal levels after 10 min, whereas induction of IDL6 expression was both weaker and slower (Fig. 6B).

T-DNA insertion lines of IDL6 and IDL7 were used for functional analysis. Loss of gene expression was verified using qRT–PCR. Two available T-DNA insertion lines were obtained for IDL6. Both idl6 [SALK_074245 (idl6-1) and SALK_126026M (idl6-2)] lines contain a T-DNA insertion 1 bp upstream of the start codon, and low IDL6 transcript levels were detected. However, IDL6 expression in these mutant lines was not inducible with flg22 (Supplementary Fig. S5A), suggesting that idl6-1 and idl6-2 are knockdown mutants. Only one T-DNA insertion line was available for
IDL6 and IDL7 modulate ROS signalling in Arabidopsis

IDL7. idl7 (WDL293-296) has the T-DNA inserted in the exon (after 188 bp), and is a null line with no detectable IDL7 mRNA with or without induction of flg22 (Supplementary Fig. S5B). Neither idl6 nor idl7 plants displayed any observable phenotypical differences compared with wild-type control plants (Col-0) under normal growth conditions. Therefore, crosses between idl6 and idl7 plants were performed. However, neither idl6-1 idl7 nor idl6-2 idl7 double mutants displayed any obvious phenotypes during normal growth conditions, and no additive effect of the double mutation was found (results not shown). To investigate the potential change in gene expression in the mutant lines compared with Col-0, 2-week-old seedlings were treated with flg22 (100 nM) and samples harvested after 3 h, 1 h after the peak response to IDL7-EPIP. Surprisingly, the responses of ZAT10 and WRKY40 were slightly, but not significantly, reduced in all the mutant lines compared with Col-0 wild type (Supplementary Fig. S6).

IDL7-EPIP peptides. Plants pre-treated with IDL6-EPIP, IDL7-EPIP, or MOCKID7 (100 nM) by spraying were simultaneously wounded in one leaf (marked with arrows), and changes in luminescence were immediately measured using the NightOwl in vivo imaging system. (A) Representative pictures are shown. Injured leaves are marked with arrows. (B) Quantification of the wound-induced signals in rosette leaves over the time course before and after wounding. Error bars indicate the SD between three biological replicates. (This figure is available in color at JXB online.)

Fig. 5. Suppression of the rapid wound-induced signal in ZAT12::luc plants by IDL6-EPIP and IDL7-EPIP peptides. Plants pre-treated with IDL6-EPIP, IDL7-EPIP, or MOCKID7 (100 nM) by spraying were simultaneously wounded in one leaf (marked with arrows), and changes in luminescence were immediately measured using the NightOwl in vivo imaging system. (A) Representative pictures are shown. Injured leaves are marked with arrows. (B) Quantification of the wound-induced signals in rosette leaves over the time course before and after wounding. Error bars indicate the SD between three biological replicates. (This figure is available in color at JXB online.)

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Fig. 6. Time series analysis of IDL6 and IDL7 expression by qRT-PCR after flg22 treatment (A) and H2O2 treatment (B) compared with untreated tissue (n=4). Statistical differences (REST analysis: *P-value <0.05; **P-value <0.01) between the time point samples and control are indicated. Error bars indicate SDs.
pathogen-derived elicitor flg22 (Supplementary Fig. S7A). Similarly, no significant differences in growth of *P. syringae* were observed between wild-type plants and the mutant lines, neither with the virulent nor with the avirulent strain (Supplementary Fig. S7B, C). As shown in Supplementary Fig. S8A, both *idl6* and *idl7* lines showed significantly (*P<0.01*) increased tolerance to salinity stress, producing longer roots than the wild type under the same growth conditions. However, no additive effect was observed for the double loss-of-function mutant *idl6-2 idl7*. Complementation lines of *idl6* and *idl7* loss-of-function lines were used to verify the observed phenotype (Supplementary Fig. S8B). No significant differences in root growth were observed between *idl6* and *idl7* lines and the wild type grown in the presence of mannitol (Supplementary Fig. S8C). Hypocotyl elongation in the dark was also investigated, with no differences found between the wild type and the mutant lines (Supplementary Fig. S8D). Germination rates of *idl6* and *idl7* mutant lines on medium containing NaCl, mannitol, or abscisic acid (ABA) did not differ from the wild type (results not shown).

**IDL7 acts as a negative modulator of stress-induced ROS signalling**

Since *IDL6* and especially *IDL7* expression was rapidly and strongly induced by H$_2$O$_2$, we investigated the response of *idl6* and *idl7* mutants to oxidative stress, using the xanthine/xanthine oxidase (X/XO) system. X/XO is an O$_2^·$-generating system that mimics and induces production of extracellular superoxide by NADPH oxidases, similar to O$_2$. X/XO treatment results in cell death, which can be measured by electrolyte leakage (Overmyer *et al.*, 2005). X/XO treatments of *idl6-1, idl6-2, idl7*, and the idl6-2 idl7 double mutant showed that *idl7*, but not *idl6*, was more tolerant to ROS, as indicated by less electrolyte leakage from *idl7* compared with the wild type and *idl6* over time (Fig. 7A).

In order to investigate further the role of the IDL7 peptide in ROS responses, we employed a luminol-based assay to measure ROS production in Arabidopsis leaf disks. In addition to *IDL7-EPIP*, we included two shorter synthetic peptides and IDA, IDL6 and IDL7 contain an N-terminal SP (Vie *et al.*, 2015). However, in addition to localization in the apoplast, a full-length fusion of IDL7::GFP was accumulated in a vesicular compartment (Fig. 1E, F). An IDL7sp::GFP fusion displayed cytosolic localization resembling GFP (Fig. 1A–D). Deletion of the four C-terminal amino acids of IDL7 resulted in a GFP fusion protein with apoplastic localization resembling GFP (Fig. 1A–D). Deletion of the four C-terminal amino acids of IDL7 resulted in a GFP fusion protein with apoplastic localization (Fig. 1G), which was confirmed through plasmolysis experiments (Fig. 1H). These results suggest that IDL7 is processed at both the N- and the C-terminus before or during the transport out of the cell. The EPIP/SGPS motif of all IDL subfamily members contains a C-terminal asparagine followed by between 4 and 13 poorly conserved residues (Vie *et al.*, 2015). Removal of Asn69 abolishes IDA activity in a ROS burst assay (Butenko *et al.*, 2014). Furthermore, IDA in complex with the ectodomain of its receptor HAESA (HAE) suggests that Asn69 constitutes the C-terminal residue of the mature IDA peptide *in planta* (Santiago *et al.*, 2016). Thus, it seems likely that C-terminal processing is a general and important feature of the maturation process of these peptides. Some of the C-terminal residues may confer specificity to the mature peptides. The N-terminus of the mature IDL6 and IDL7 peptides remains unresolved.

Our analyses indicate that the *IDL6* and *IDL7* promoters show high basal activity (Fig. 2); a similar observation was recently made by *Wang et al.* (2017) using an *IDL6* promoter::GUS construct. This is compensated by a high turnover rate for the transcripts. Thus, expression of a stable reporter gene transcript under control of the *IDL6* or *IDL7* promoter will lead to artificially high reporter levels (Supplementary Fig. S2). Alternatively, the binding site for a strong transcriptional repressor or another important regulatory unit, e.g. the 3′-untranslated region, could be missing in the promoter regions used in the *IDL6pro::GUS* and *IDL7pro::GUS* plants. A peptide ligand involved in stress signalling should be rapidly induced in order to ensure a quick response to biotic or abiotic challenges to the plant. However, the signal should also be quickly attenuated to ensure specificity and avoid runaway responses. Both *IDL6* and *IDL7* are transiently expressed upon H$_2$O$_2$ or flg22 treatment (Fig. 6; *Wang et al.*, 2017), in line with a role in early responses to these stresses.

**Discussion**

In this study we describe two new putative peptide ligands belonging to the extended family of IDA and IDL peptides (Vie *et al.*, 2015) and show evidence suggesting that they act as negative modulators of ROS responses.

Small transcriptionally modified peptides are characterized by an N-terminal SP that directs the peptide out of the cell. Analyses of subcellular localization of IDA and CLE peptides indicate that they are localized to the extracellular space (Butenko *et al.*, 2003; Sharma *et al.*, 2003). Similar to the CLE peptides and IDA, IDL6 and IDL7 contain an N-terminal SP (Vie *et al.*, 2015). However, in addition to localization in the apoplast, a full-length fusion of IDL7::GFP was accumulated in a vesicular compartment (Fig. 1E, F). An IDL7sp::GFP fusion displayed cytosolic localization resembling GFP (Fig. 1A–D). Deletion of the four C-terminal amino acids of IDL7 resulted in a GFP fusion protein with apoplastic localization (Fig. 1G), which was confirmed through plasmolysis experiments (Fig. 1H). These results suggest that IDL7 is processed at both the N- and the C-terminus before or during the transport out of the cell. The EPIP/SGPS motif of all IDL subfamily members contains a C-terminal asparagine followed by between 4 and 13 poorly conserved residues (Vie *et al.*, 2015). Removal of Asn69 abolishes IDA activity in a ROS burst assay (Butenko *et al.*, 2014). Furthermore, IDA in complex with the ectodomain of its receptor HAESA (HAE) suggests that Asn69 constitutes the C-terminal residue of the mature IDA peptide *in planta* (Santiago *et al.*, 2016). Thus, it seems likely that C-terminal processing is a general and important feature of the maturation process of these peptides. Some of the C-terminal residues may confer specificity to the mature peptides. The N-terminus of the mature IDL6 and IDL7 peptides remains unresolved.

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Transcriptome analyses of seedlings treated with either IDL6-EPIP or IDL7-EPIP indicated that these peptides act as negative regulators of genes associated with early responses to stress in Arabidopsis seedlings (Figs 3, 4; Table 2; Supplementary Dataset S1, S2). Treatments with the synthetic IDL7-EPIP resulted in a striking transcriptome response, in which 75% of the significantly regulated genes were down-regulated. Several studies have proposed a ‘universal stress response transcriptome’ cluster in plants, which includes IDL7, ZAT10, ZAT12, WRKY11, WRKY18,
WRKY33, WRKY40, and CML38 (Kilian et al., 2007; Ma and Bohnert, 2007; Walley et al., 2007, Hahn et al., 2013). In addition, network analysis of the genes most down-regulated by IDL7-EPIP (Supplementary Fig. S4) illustrates that these genes are highly co-expressed and probably involved in the same biological processes. Expression of ZFP and WRKY genes is highly responsive to a great variety of stresses; these transcription factor families have emerged as key regulators and integrators of ROS signalling in plants (Miller et al., 2008). ZAT12 has been described as a hyper-responsive gene to ROS accumulation, and is expressed both locally and systemically within minutes after increased ROS production (Miller et al., 2009). ROS-induced expression of ZAT12 was strongly reduced in response to treatment with IDL7-EPIP. Both the transcriptional changes triggered by IDL7-EPIP and the increased tolerance to ROS and salinity stress in idl6 and idl7 lines (Fig. 7A; Supplementary Fig. S7) suggest that IDL6 and IDL7 are negative modulators of stress responses in Arabidopsis.

Pathogen attack or environmental perturbations such as increased salinity induce a large network of different pathways containing both positive and negative regulatory components, where negative regulators may be important factors preventing excessive and unrestricted activation of defence mechanisms. Activation of the plant defence system has been shown to have a major impact on plant growth and fitness. In line with this, overexpression of ZAT7 leads to severe growth defects (Ciftci-Yilmaz et al., 2007). Mutants for the MAP kinase MPK4 display severe growth retardation, possibly through overaccumulation of the defence-related phytohormone salicylic acid (SA) and H2O2 (Petersen et al., 2000; Ichimura et al., 2006; Suarez-Rodriguez et al., 2007), and overexpression of DREB2A, a positive regulator of stress tolerance, showed reduced growth and dwarfism similar to mpk4 mutants (Sakuma et al., 2006).

Expression analyses of IDL6 and IDL7 show that these genes are responsive to a wide variety of biotic and abiotic stresses (Fig. 6; Vie et al., 2015). However, both the single and double loss-of-function mutants displayed minor phenotypes in only a subset of the stress assays performed during this study. There are several possible explanations for the lack of phenotypes. One possibility is that IDL6 and IDL7 are part of a complex signalling network with redundant pathways, and that altered expression of IDL6 and IDL7 is not enough to disrupt this network noticeably. The gene expression of ZAT10 and WRKY40 was not different in the mutant lines compared with Col-0 after flg22 (Supplementary Fig. S6), possibly reflecting the lack of phenotypes observed in our stress assays. It is possible that IDL6 and IDL7 have multiple roles in tolerance to different stresses, and that our functional characterizations have not included all of the specific stresses linked to IDL6 and IDL7. In a recent study, IDL6-overexpressing plants were found to exhibit reduced resistance to P. syringae pv. tomato DC3000, whereas IDL6 dsRNA-silenced plants showed increased resistance (Wang et al., 2017). There are major differences between the two studies, with regard both to the growth stage of the plants (1 versus 5 weeks) and to the nature of the knockdown mutants (T-DNA versus dsRNA), which could explain the different outcomes.

The fact that treatments with X/XO and NaCl (Fig. 7; Supplementary Fig. S7) led to a phenotype in idl7 lines is supported by the fact that a large fraction of the genes down-regulated by IDL7-EPIP treatment are associated with these stresses. WRKYS and ZFPs have been linked to oxidative and salinity stress (Rizhsky et al., 2004; Davletova et al., 2005a, b; Ciftci-Yilmaz et al., 2007; Jiang and Deyholos, 2009; Chen et al., 2010). Overexpression of ZAT7 and ZAT12 has been reported to increase tolerance to salin and oxidative stress (Rizhsky et al., 2004; Davletova et al., 2005b; Ciftci-Yilmaz et al., 2007). The increased tolerance to salinity stress in the idl6 and idl7 loss-of-function lines are in line with these data.

In spite of its similarity to IDL7, IDL6-EPIP displayed a far weaker effect on transcriptional changes compared with IDL7-EPIP, indicating that IDL6 has lower biological activity than IDL7. Alternatively, IDL6 and IDL7 might have different roles in stress regulation, mediated through differences in expression patterns, receptor availability, and/or peptide modifications, or that the peptide is active at different growth stages from those investigated in this study. It is worth noting that IDL6-EPIP differs in four amino acids in comparison with IDL7-EPIP, where IDL7-EPIP contains one charged amino acid (arginine versus valine) more than IDL6-EPIP in front of the SGPS motif (Fig. 3A). It is also likely that many of the transcriptional targets of the two peptides are expressed at their basal, non-induced levels during normal growth conditions, explaining the few regulated genes for IDL6 and the moderate response for IDL7.

Several lines of evidence point toward a role for IDL7, and possibly IDL6, as a modulator of ROS signalling. The transcriptional response of IDL7 to H2O2 treatment is rapid (Fig. 6B). The temporal difference in IDL7 induction by H2O2 and flg22 could correspond to the time needed for flg22 to induce a ROS burst (Gómez-Gómez et al., 1999; Fig. 7B). Application of exogenous IDL7-EPIP (and to a minor extent IDL6-EPIP) peptide blocked the rapid induction of ZAT12 expression after wounding (Fig. 5), which has been shown to be mediated by ROS (Miller et al., 2009). idl7 and idl6-2 idl7 double mutants displayed decreased sensitivity to ROS produced by X/XO (Fig. 7A). Importantly, the IDL7 peptides attenuated the flg22-induced ROS burst and partially rescued the enhanced ROS production in the idl7 background (Fig. 7B, C). The fact that the synthetic IDL7 peptide fails to rescue the idl7 ROS phenotype completely suggests that it is not identical to the mature IDL7 peptide with regard to length and/or post-translational modification. However, counterintuitively, both the shorter IDL7-PiP and the hydroxyprolinated IDL7-PIPo resulted in a similar response to the longer IDL7-EPIP peptide (Fig. 7B). At this point, we cannot draw any conclusions regarding the length of the biological peptide. It is possible that the bioactive IDL7 peptide is longer than IDA, but still contains modifications, or that the sensitivity of our assays is not high enough to detect the possible stronger effects of the more active peptide.

The receptor(s) for the IDL7 peptide are unknown; however, IDL7 perception is likely to involve LRR-RLKs that
phosphorylate downstream targets. Binding of IDA to its receptors HAE or HAESA-LIKE2 (HSL2) activates a mitogen-activated protein (MAP) kinase cascade that results in the phosphorylation of transcription factors (Cho et al., 2008; Patharkar and Walker, 2015). Furthermore, the effect of IDL6 on resistance to *P. syringae* and pectin degradation was found to be dependent on HAE and HSL2 (Wang et al., 2017). A pathway possibly involving HAE/HSL2 could act downstream of IDL7 to down-regulate genes of the ‘universal stress response transcriptome’, through either activation of a transcriptional repressor, or inactivation of a transcriptional activator. However, this scenario does not explain the attenuating effect of the IDL7 peptides on ROS production when added together with flg22. Alternatively, downstream targets of IDL7 signalling could be involved in regulation of ROS levels. An attractive group of target candidates would be NADPH oxidases, especially RBOHD. In addition to calcium binding, RBOH activity is regulated by phosphorylation by several classes of protein kinases (Baxter et al., 2014; Kimura et al., 2017). IDL7 signalling could regulate RBOH phosphorylation status, either by inactivating one or more of these kinases, or by activating a phosphatase with specificity toward RBOHs. Instead of inhibiting ROS production, IDL7 signalling could potentially activate ROS scavenging enzymes.

What could be the role of IDL6/IDL7-regulated ROS attenuation? IDL7 (and possibly IDL6) may act in a negative feedback loop terminating the fast phase of the biphasic ROS burst. Mild salt treatment induces a biphasic RBOHD-dependent ROS burst similar to the one induced by flg22 (Xie et al., 2011), and the *rbodh rbohf* double mutant displays increased salt sensitivity (Ma et al., 2012; Ben Rejeb et al., 2015). The strong negative effect of IDL6/IDL7 treatment on wound-induced ZAT12 expression also suggests that these peptides might be involved in attenuation of the autopropagating ‘ROS wave’ leading to systemic acquired acclimation (Baxter et al., 2014; Gilroy et al., 2014). Such attenuation could be important for specificity of the signal, as well as avoiding runaway responses.

**Supplementary data**

Supplementary data are available at JXB online.

Fig. S1. Overview of the constructs used for localization studies.

Fig. S2. Distribution of *GUS* mRNA directed by the *IDL6* and *IDL7* promoters in 10-day-old seedlings.

Fig. S3. Venn diagram of IDL6- and IDL7-responsive genes.

Fig. S4. Network analysis of genes down-regulated by IDL7-EPIP treatment.

Fig. S5. Verification of T-DNA insertion lines.

Fig. S6. Expression of IDL7-responsive genes in *idl6* and *idl7* mutant backgrounds.

Fig. S7. Growth arrest phenotype and susceptibility of *idl6* and *idl7* mutants to the phytopathogen *Pseudomonas syringae*.

Fig. S8. Growth arrest phenotype of *idl6* and *idl7* mutants to abiotic stress.

Fig. S9. Modulation of flg22-induced ROS burst by IDL7-EPIP and MOCK

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