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The *IDA/IDA-LIKE* and *PIP/PIP-LIKE* gene families in *Arabidopsis*: phylogenetic relationship, expression patterns, and transcriptional effect of the PIPL3 peptide

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Abstract

Peptide ligands play crucial roles in the life cycle of plants by modulating the innate immunity against pathogens and regulating growth and developmental processes. One well-studied example is *INFLORESCENCE DEFICIENT IN ABSCISSION* (IDA), which controls floral organ abscission and lateral root emergence in *Arabidopsis thaliana*. IDA belongs to a family of five additional *IDA-LIKE* (IDL) members that have all been suggested to be involved in regulation of *Arabidopsis* development. Here we present three novel members of the *IDL* subfamily and show that two of them are strongly and rapidly induced by different biotic and abiotic stresses. Furthermore, we provide data that the recently identified *PAMP-INDUCED SECRETED PEPTIDE* (PIP) and *PIP-LIKE* (PIPL) peptides, which show similarity to the IDL and *C-TERMINALLY ENCODED PEPTIDE* (CEP) peptides, are not only involved in innate immune response in *Arabidopsis* but are also induced by abiotic stress. Expression patterns of the *IDA/IDL* and *PIP/PIPL* genes were analysed using *in silico* data, qRT-PCR and GUS promoter lines. Transcriptomic responses to PIPL3 peptide treatment suggested a role in regulation of biotic stress responses and cell wall modification.

Key words: *Arabidopsis*, evolution, gene expression, *INFLORESCENCE DEFICIENT IN ABSCISSION*, peptide ligand.

Introduction

Plants, like all other multicellular organisms, are dependent on cell-to-cell communication for growth and development, as well as for managing and surviving in a challenging and unpredictable environment. Plant cells are linked together by a cellulose wall, and signals between cells passes through plasmodesmata (Gallagher and Benfey, 2005; Kim, 2005) or through ligand-receptor interactions on the cell surface (Shiu and Bleecker, 2001a). Recent evidence indicates that there could be a connection between these two (Stahl and Simon, 2013). For many years, research focused on the classical phytohormones and their abilities to mediate physiological responses, but during the last decade, peptide...
ligands have emerged as important mediators of cell-to-cell communication in both development and defense (Butenko et al., 2009; Matsubayashi, 2014). Most peptide ligands are transcribed as prepropeptides and shuttled into the secretory pathway through their N-terminal signal peptide (SP). The SP is removed, followed by further structural modifications to yield the mature peptide ligand (Murphy et al., 2012). Peptide ligands can be divided into two main groups based on these modifications. Cysteine-rich peptides are characterized by an even number of cysteine residues that form intra-molecular disulfide bonds upon maturation (Matsubayashi, 2014). Small peptides may on the other hand be generated from the C-terminus of propeptides with a general absence of cysteine residues, and the active peptides may contain post-translational modifications of key amino acids, like tyrosine sulfation, proline hydroxylation and hydroxyproline arabinosylation (Matsubayashi, 2014; Tabata and Sawa, 2014). A large number of genes encoding putative RECEPTOR-LIKE KINASEs (RLKs) and peptide ligands have been identified in the Arabidopsis genome (Shiu and Bleeker, 2001b; Lease and Walker, 2006); still, only a few ligands have been characterized and linked to a receptor and a cellular response (Butenko et al., 2009, 2014).

A well-studied peptide of the second category is INFLORESCENCE DEFICIENT IN ABSICCISSION (IDA), known to regulate cell separation processes in A. thaliana (Aalen et al., 2013). The ida mutant fails to undergo floral organ abscission (Butenko et al., 2003), and overexpression of IDA leads to premature and ectopic abscission (Stenvik et al., 2006). Twenty amino acids in the C-terminal region, termed EPIP, were shown in genetic experiments to be sufficient to rescue the ida phenotype, thus suggesting that EPIP encompasses the active ligand motif of the peptide (Stenvik et al., 2008). IDA mediates its effect through the two LEUCINE-RICH REPEAT RLKs (LRR-RLKs) HAESA (HAE) and HAESA-LIKE 2 (HSL2), as the double knockout hae hsl2 is phenotypically similar to the ida mutant and overexpression of IDA is not able to rescue this phenotype (Cho et al., 2008; Stenvik et al., 2008). Moreover, a dodoca hydroxyprolinolated peptide within the EPIP domain can activate and bind HSL2, and also activate HAE, at substantially higher concentration (Butenko et al., 2014). So far, five genes encoding peptides with similarity to IDA, named IDA-LIKE 1 to 5 (IDL1 to 5), have been identified in the Arabidopsis genome (Butenko et al., 2003). It has previously been suggested that the IDL genes may share a common role in regulating cell separation events, as they are expressed at sites where cell separation occurs, such as during vascular development, stoma formation, root cap sloughing, lateral root emergence and seed shedding (Stenvik et al., 2008; Kumpf et al., 2013).

Bioinformatic tools have been used to identify ~1000 putative peptides in Arabidopsis, based on their general features and similarities to known peptides (Lease and Walker, 2010). Currently, only a small fraction of these have been assigned a function. In this paper we present three new members of the IDA-LIKE family named IDA-LIKE 6 (IDL6), IDA-LIKE 7 (IDL7) and IDA-LIKE 8 (IDL8), encoding putative proteins with a ligand motif similar to IDA. In addition, we have in parallel with Hou et al. (2014) identified a new family of 11 genes termed PAMP-INDUCED SECRETED PEPTIDES (PIPs) and PIP-LIKE (PIPLs) (Hou et al., 2014) encoding peptides with similarity to IDA/IDLs and the C-TERMINALLY ENCODED PEPTIDES (CEPs) (Ohyama et al., 2008; Delay et al., 2013; Imin et al., 2013; Roberts et al., 2013). These families can be recognized by the presence of one or both of two C-terminal, conserved core motifs: SGPS, a motif present in the functional peptide of IDA (Butenko et al., 2014), is found in IDA/IDLs and PIP/PIPLs, whereas the GxGH motif located at the extreme C-terminal is common for PIP/PIPLs and CEPs. Interestingly, while the IDA/IDL and CEP members characterized so far are involved in developmental processes, we show that the PIP/PIPL peptides are involved in stress responses.

Materials and methods
Identification of IDA/IDL and PIP/PIPL family genes and phylogenetic analyses
Full-length protein sequences and the conserved C-terminal domain of IDA and IDL1-5 were used in TBLASTN searches against expressed sequence tag (EST), genomic and non-redundant nucleotide databases at NCBI (Altschul et al., 1997). In order to further investigate the presence of IDA/IDL family members in other plant species, similar BLAST searches were performed on the Phytozone v9.1 genome (Goodstein et al., 2012) and OneKP EST (https://sites.google.com/a/ualberta.ca/onekp/ accessed 28 May 2015) databases. Protein alignments were made using the ClustalX programme (Larkin et al., 2007) and later manually refined with GeneDoc (Nicholas et al., 1997). Neighbour-joining (N-J) trees were produced from the protein alignments using the N-J method (Saitou and Nei, 1987) and Kimura’s correction for multiple substitutions as implemented in the ClustalX programme. In total 1000 bootstrap trials were run on the N-J tree. Maximum-likelihood (ML) analysis of the full-length IDA/IDL and PIP/PIPL protein alignments were performed using the RAxML programme (Stamatakis, 2006) with the PROTGAMMABLOSUM62 substitution model and running 1000 bootstrap replicas. BLOSUM62 substitution matrices were used in both ML and N-J analyses. Trees were visualized using TreeView 1.6.6 (Page, 2002) and refined in Adobe Illustrator CS6. SP sequences were identified through SignalP 4.0 (Petersen et al., 2011) (http://www.cbs.dtu.dk/services/SignalP/ accessed 28 May 2015). Protein sequence motif visualization was done using WebLogo (Crooks et al., 2004; Schneider and Stephens, 1990). Analysis of gene duplication events and identification of syntenic regions was done by screening the 40 nearest protein coding genes flanking each of the IDAI/IDL-PIP/PIPL gene loci for other closely related genes located next to the IDA/IDL and PIP/PIPL genes. Each region was analysed by BLASTP searches, and a custom-made Perl script was used to parse BLAST tables and identify high scoring proteins (included in the top 5 score list) that had corresponding genes mapping to IDA/IDL-PIP/PIPL genomic regions.

Plant material
Seeds of the Arabidopsis thaliana ecotype Col-0 (N1092) were obtained from the European Arabidopsis Stock Centre (NASC, Nottingham, UK).

The five pIDL-GUS constructs were made using Gateway technology. The promoters included 1555, 1864, 1908, 1980 and 2020bp upstream of the ATG start codon of IDL1 to IDL5, respectively (Stenvik et al. 2008).
Plant growth conditions and plant tissue collection for expression analysis during development

Seeds of Col-0 ecotype were surface sterilized and sown on half-strength MS plates supplemented by 2% (w/v) sucrose at a density of 44 seeds per Petri dish (14 cm diameter) and stratified for 3 d at 4°C before being transferred to a controlled in vitro growth room under a 16 h light (70 μmol m⁻² sec⁻¹): 8 h dark photoperiod at 22°C. At stage 1.10 (Boyes et al., 2001), plants were transferred to soil and grown further in a controlled growth chamber (VB1514, Vötsch Industrietechnik, Balingen, Germany) under the same light conditions at 22°C until the end of the experiment.

Tissue was harvested at different growth stages as defined by Boyes et al. (2001). For stages 1.0, 1.06 and 1.10, whole plantlets were harvested from in vitro cultivation medium. At the later stages roots, rosette leaves, cauline leaves, stem, inflorescences and silique were harvested separately. All material was immediately flash frozen in liquid nitrogen upon harvesting and stored at −80°C until further processing. Three biological replicates were harvested, where each replicate consisted of plant material pooled from eight Petri dishes (stage 1.0), four Petri dishes (stages 1.06 and 1.10) and five plants (stages 6.00 and 8.00), respectively.

Stress treatments

All treatments were conducted on 2-week-old wild-type seedlings corresponding to growth stage 1.06 (Boyes et al., 2001) unless otherwise stated. Seeds of Col-0 ecotype were surface sterilized and sown out on half-strength MS plates supplemented by 2% (w/v) sucrose at a density of 20 seeds per Petri dish (14 cm diameter). For chitin, cycloheximide (CHX) and anisomycin treatments, seedlings were sprayed with 10 μg/ml chitin, 10 μg/ml CHX or 15 μg/ml anisomycin in MilliQ (MQ) water added 0.02% Silwet L-77 (Lehle Seeds) and vacuum infiltrated at 20 inches Hg for 1 min before harvesting. For all stress experiments, gene expression was measured by microarray hybridization using an Agilent 44K Arabidopsis microarray and the Gene Expression Hybridization Kit (Agilent Technology, USA) as described by the supplier's instructions. cDNA samples were diluted 10-fold before use in qRT-PCR reactions.

Quantitative real-time PCR

Quantitative real-time PCR (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: pre-incubation was performed at 95°C for 5 min, followed by 50 amplification cycles, each consisting of 10 s denaturation at 95°C, 10 s annealing at 55°C and 10 s elongation at 72°C. cDNA synthesis was performed on 1 μg total RNA using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany), following the supplier's instructions. cDNA samples were diluted 10-fold before use in qRT-PCR reactions.

Gene ontology (GO) annotation analysis

Genome-wide expression analysis was performed using the Arabidopsis (V4) Gene Expression Microarray 4×44K (Agilent Technology, USA) as described by the supplier's manual: total RNA (<0.2 μg) was reverse transcribed, amplified and labelled using the Low Input Quick Amp Labeling Kit, two-colour protocol, (Agilent p/n 5190-2306) (Agilent Technologies, USA). Hybridization was performed with the Gene Expression Hybridization Kit (Agilent p/n 5188-5242). 825 ng cRNA from both mock-treated plants and PIPL3 peptide-treated plants were used. The cRNA mixture was fragmented and hybridized on Arabidopsis (V4) Gene Expression Microarray 4×44K arrays in a rotary oven at 65°C for ~15 h. cRNA from the mock- and PIPL3-treated plants were alternately labelled with Cy3 or Cy5, which makes it possible to assess dye bias effects during the statistical analysis. The slides were washed with Gene Expression Wash Buffer 1 (Agilent p/n 5188-5325). Gene Expression Wash Buffer 2 (Agilent p/n 5188-5326), acetonitrile (VWR International) and Stabilization and Drying Solution (Agilent Technologies) according to the manufacturer's instructions. The slides were scanned at 5 μm resolution on an Agilent DNA microarray scanner (Agilent Technologies). The image files were analysed with the Agilent Feature Extraction Software.

Prior to the statistical analysis spots from control spikes, landmarks and genes with low expression (absent) were filtered out. The data were analysed using the limma package (Smyth, 2005) and the R statistical data analysis programme package (R 2.10.1). No background subtraction was performed, and data were normalized using the Global Loess Normalization method. Benjamini and Hochberg's method to control the false discovery rate (FDR) was used to identify differentially regulated genes (Benjamini and Hochberg, 1995). Genes with dye bias effects were removed and genes with an adjusted P-value of less than 0.05 were regarded as significantly differentially expressed. The study is MIAME compliant. Raw data has been deposited in GEO (accession GSE66201).

GO analysis

Gene ontology (GO) annotation analysis was performed using the Cytoscape 3.1.0 (Smoot et al., 2011) plug-in Bingo 3.0.2 (Maere et al., 2005). Over-represented categories were identified using a hypergeometric test with a significance threshold of 0.05 after Benjamini and
Hochberg’s FDR correction (Benjamini and Yekutieli, 2001) using the whole annotated genome as the reference set.

**Results**

Identification of IDL and PIP/PIPL genes in Arabidopsis

Six members of the *IDA* gene family have been identified in *Arabidopsis* to date: *IDA* and IDL1 to IDL5. All members of the *IDA* gene family are intronless and encode small proteins (<110 amino acids) characterized by an N-terminal secretory SP, a variable region and a C-terminal, conserved region (Butenko et al., 2003). Through database searches, three new IDL genes were found (Table 1; Fig. 1A; Supplementary Dataset S1). In addition, we identified 11 genes with similarity to the IDLs. During preparation of this paper an article was published presenting these genes as a family encoding secreted PAMP-INDUCED PEPTIDES (PIPs) and PIP-LIKE (PIPL) peptides (Hou et al., 2014) (Table 1; Fig. 1A; Supplementary Dataset S1). For all members the N-terminal SP contains a stretch of aliphatic residues typical of secreted proteins (Fig. 1A, green box). This motif is followed by a conspicuously conserved arginine residue (Fig. 1A, red diamond). The C-terminal is characterized by the conserved core motif S(G,A,V)GPS (hereafter called the SGPS motif) conserved in both IDLs and PIP/PIPLs (Fig. 1A, blue box). The SGPS motif in IDL proteins is followed by four highly conserved residues ([R/K](R/K)HN] followed by up to 13 additional less conserved residues (Fig. 1A, Bi). The PIP/PIPL proteins lack the variable region C-terminal to the SGPS motif that is found for the IDL proteins (Fig. 1A, Bii, iii). Three of the PIP/PIPLs (PIP2, PIP3 and PIPL1) contain two SPGS motifs in a tandem orientation at the C-terminal (Fig. 1A, Bii), as identified by Hou et al. (2014).

Two studies of the CEP family of small peptides, consisting of 15 members, have recently been published (Delay et al., 2013; Roberts et al., 2013). Two of the CEP family members, CEP13 and CEP14, show similarity to the PIP/PIPLs. A sequence consensus logo of the SGPS motif and surrounding residues was made for the IDL and PIP/PIPL families as well as for the CEP family (Fig. 2A). The SGPS motif (position 4–7) is conserved in IDLs and PIP/PIPLs. A second conserved motif (GxGH, where x is any amino acid) was seen at the C-terminal end of all PIP/PIPLs. The SGPS motif was not found in the CEPs; however, the family was characterized by the C-terminal SP(G,I,V)GH sequence, which resembles the C-terminal end of the PIP/PIPLs. Thus, the putative ligand domain of the PIP/PIPLs shares features with both IDLs and CEPs.

**Table 1. The IDA/IDL and PIP/PIPL gene families in Arabidopsis**

| Gene name | Locus | Accession number | Signal peptide aa | Protein aa |
|-----------|-------|------------------|-------------------|------------|
| IDA       | At1g68765 | NM_105560.1       | 26                | 77         |
| IDL1      | At3g25666 | NM_113464.2       | 27                | 86         |
| IDL2      | At5g56467 | NM_00108327.2     | 36                | 95         |
| IDL3      | At5g09805 | NM_001085091.1    | 32                | 99         |
| IDL4      | At3g18715 | NM_001084711.1    | 36                | 93         |
| IDL5      | At1g76952 | AY642386.1        | 27                | 103        |
| IDL6      | At5g65300 | NM_120612.1       | 24                | 102        |
| IDL7      | At3g10930 | AK118348.1        | 21                | 97         |
| IDL8      | At5g02591 | AK221754.1        | 22                | 95         |
| PIP1      | At4g28460 | NM_118988.2       | 30                | 72         |
| PIP2      | At4g37290 | NM_118982.1       | 24                | 84         |
| PIP3      | At2g23270 | NM_127891.1       | 19                | 86         |
| PIP1L     | At1g49800 | NM_103864.2       | 27                | 108        |
| PIP2L     | At3g06090 | NM_111484.1       | 22                | 79         |
| PIP3L     | At4g37295 | NM_118982.1       | 22                | 86         |
| PIP4L     | At5g43066 | NM_00125892.1     | 21                | 74         |
| PIP5L     | At5g43068 | CB254609.1        | 21                | 79         |
| PIP6L     | At1g47178 | EF183199.1        | 22                | 88         |
| PIP7L     | At4g11402 | NC_003075.7b      | 23                | 69         |
| PIP8L     | At5g43064 | NC_003076.8b      | 21                | 72         |

a Preliminary AtID from TAIR

b Chromosomal coordinates of cds
Phylogenetic analysis of the IDA/IDL and PIP/PIPL families

In order to examine the relationship of the IDA/IDL and PIP/PIPL gene families in *Arabidopsis*, a phylogenetic analysis on the full-length protein sequences of all IDL and PIP/PIPL members was performed. Two methods were used: a distance-matrix method combined with the N-J algorithm as implemented in the ClustalX programme, and a ML method using a gamma model and the RAxML programme (Fig. 2B). The resulting tree topologies from the two analyses were highly similar (data not shown), although bootstrap confidence values were a bit lower for the ML analysis. The analysis shows that the IDA/IDL and PIP/PIPL families split into separate branches with a high level of bootstrap confidence, even though the highly variable central part of the proteins is included in the analysis. Due to the high sequence divergence of these genes, not all branches in the tree are supported with high confidence levels. However, the IDA/IDL cluster can be divided into two subgroups: one containing IDL2, IDL3, IDL4 and IDL5 and the other containing IDA, IDL1, IDL6 and IDL7. Identical ML and N-J tree topologies support this...
division. Of the IDL proteins, IDL8 can be regarded as an outlier, and does not cluster well with any of the other IDLs. The PIP/PIPL proteins can be broadly divided into two groups: those with a single SGPS motif (PIP1 and PIPL2–PIPL8) and those with two SGPS motifs (PIP2, PIP3 and PIPL1).

A phylogenetic analysis (using the ML method) was also performed on a full-length protein alignment of the IDA/IDL and PIP/PIPL families as well as the CEP family (Supplementary Fig. S1). CEP9 was not included in the analysis due to its aberrant length and number of peptide motifs (five). The IDA/IDL family was clearly separated from the PIP/PIPL and CEP families with a high bootstrap confidence value (100%). Furthermore, CEP13, CEP14 and CEP15 [defined as group II CEPs by Delay et al. (2013) and Roberts et al. (2013)] formed a clade with significant bootstrap values.

To further study the evolutionary relationship between the IDA/IDL and PIP/PIPL genes, the region surrounding the IDA/IDL-PIP/PIPL gene loci were analysed for ancient chromosomal or gene duplications. The results are summarized in Supplementary Table S2. The chromosomal localization of the genes are shown in Supplementary Fig. S2. PIPL5, PIPL4 and PIPL8 are organized in tandem repeats, as are PIPL2 and PIPL3. IDA and IDL1 are likely the result of a recent duplication event, as seven of the 40 genes flanking IDA and IDL1 are closely related to each other. This is the case for several other genes in the IDA/IDL family as well; IDL2 and IDL3 share 13–15 common neighbouring genes, as previously noted by Stenvik et al. (2008), while IDL6 and IDL7 shares six. PIPL6 have five neighbouring genes with corresponding homologues surrounding the PIPL5-PIPL4-PIPL8 loci and PIPL3 shares four to eight genes with PIPL3-PIPL2, depending on the region used for BLAST search.

The regions flanking IDL5 do not share any closely related genes located within the other IDA-PIPL regions, while IDL8 and PIPL6 only share a few. This lack of synteny suggests that these genes have evolved through mechanisms other than tandem gene duplication, such as movement via RNA intermediates like retrotransposable elements.

BLAST searches for IDA/IDL and PIP/PIPL family genes within the Viridiplantae were performed using full-length protein sequences as well as mature peptide sequences of Arabidopsis proteins. The searches showed that genes encoding both peptide families are present in seed plants, but absent in lycophytes (Selaginella), mosses (Physcomitrella) and green algae (Fig. 3). This distribution is similar to the one previously reported for CEPs (Delay et al., 2013; Roberts et al., 2013).

**Expression patterns of IDA/IDL and PIP/PIPL genes during development**

Since the expression pattern of a gene may provide an indication of its function, we conducted an in silico analysis of the transcription levels of the genes included on the Affymetrix ATH1 microarrays (IDA, IDL1, IDL6, IDL7, PIPL1-PIPL3) during plant development (Schmid et al., 2005), obtaining data from the eFP Browser (Winter et al., 2007). These genes are expressed at low levels both at post-germination stages and during embryogenesis (Fig. 4). However, IDL1, IDL6, PIP1 and PIPL3 are expressed in several embryonic tissues [data obtained from Casson et al. (2005)], with the highest expression found in root primordia during the torpedo stage. PIPL1 is also expressed in basal tissue during the globular stage. PIPL1 is the most strongly expressed gene during seed development. In contrast to the rest of the genes, PIPL1 is not expressed in the embryo, but shows high expression levels in the seed coat during early stages of seed development.

Although the publicly available gene expression databases provide valuable information about the IDA/IDL and PIP/PIPL genes, it is very incomplete. Ten of the genes (IDL2–5, IDL8, PIPL4–8) are not included on the Affymetrix ATH1 microarrays used to generate these data. To fully assess the expression pattern of all the genes, qRT-PCR was performed on RNA isolated from Col-0 ecotype tissue harvested at various growth stages during the plant...
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life cycle, as described by Boyes et al. (2001). Since none of the genes in the family contain introns, a control for genomic contamination was included in the analysis, using primers for CYP71A13 spanning the third intron in this gene.

The expression levels of the IDA/IDL and PIP/PIPL genes under normal growth conditions were in general very low (Fig. 5); for some of the genes (IDL1, IDL5 and PIPL1) hardly any transcripts were detected. The highest transcript levels were found at the later stages of Arabidopsis development (i.e. IDL6, IDL7 and IDL8), indicating a possible role for these genes during seed development or senescence. Other genes, like IDL3 and PIPL3, were weakly expressed during all stages.
GUS expression analyses of IDL genes

To further investigate the expression pattern of the different IDL genes during early Arabidopsis development, plants expressing promoter:GUS reporter constructs for IDA and IDL genes (pIDA/pIDL-GUS) were investigated from germination up until 14 d after germination (Supplementary Table S3). pIDA:GUS expression was observed in the cortex and epidermal cells overlaying the lateral root primordia in accordance with the recently reported function in cell separation allowing lateral root emergence (Fig. 6A; Kumpf et al., 2013). pIDL1:GUS had a specific pattern of expression in the columella root cap cells of the primary root (Fig. 6A), where cells undergo cell separation during root cap sloughing, which allows the primary root to penetrate the soil (del Campillo et al., 2004). After germination, pIDL2:GUS, pIDL4:GUS and pIDL5:GUS were expressed in the vascular tissue of the primary and lateral roots (Fig. 6A). Consistent with the expression in leaves identified by qRT-PCR (Fig. 5), the pIDL:GUS constructs were all expressed in the vascular tissue of the expanding cotyledons and/or primary leaves (Fig. 6B, C), and IDL4 expression was also observed in the guard cells (Fig. 6D) (Stenvik et al., 2008). The IDL promoter:GUS constructs were all expressed in the shoot apical meristem region, here represented by IDL4 and IDL5 in
A subset of IDA/IDL and PIP/PIPL genes are induced by biotic and abiotic stress

In general, the members of the IDA/IDL and PIP/PIPL families were found to be only weakly expressed under normal growth and development, so the response of IDA/IDL and PIP/PIPL genes to different abiotic and biotic stresses were therefore investigated (Fig. 7). Figure 7A summarizes the in silico analysis of abiotic stresses (Kilian et al., 2007). Cold stress induces expression of IDL7, PIP1 and PIP3 in roots, whereas UV induces eight out of 11 of the IDA/IDL/PIP/PIPL genes present on the Affymetrix ATH1 microarrays. The highest expression is observed in roots during salt stress. IDA, IDL1, IDL7, PIP1 and PIP3 are especially highly induced upon such stress, with expression levels up-regulated 500–1000 times compared to the control. PIPL1 is not expressed during any of the specified treatments. Biotic stress and treatments with elicitors (Fig. 7B; Supplementary Fig. S3) induces fewer genes than abiotic stress. Treatments with both virulent and avirulent strains of the biotrophic pathogen Pseudomonas syringae induce IDL6 and IDL7 expression. It should be noticed that expression of IDL6 is induced 1 h after treatments with the pathogen-derived elicitors flg22, HrpZ and NPP1. PIP1 is up-regulated upon treatment with NPP1 and HrpZ (Supplementary Fig. S3). The necrotrophic pathogens Botrytis cinerea and Phytophthora infestans both induce expression of PIP2 and PIP3. This is in accordance with data recently published by Hou et al. (2014), showing that PIP1, PIP2 and PIP3 are involved in amplification of the immune response. IDL6 and IDL7 are in general induced at earlier time points than the rest of the genes (Fig. 7; Supplementary Fig. S3). Transcripts of IDL6 and IDL7 are detected as early as 15 min post UV exposure, while PIPL2 and PIP3 are detected 30 min (PIPL2) and 3 h (PIP3) after exposure (Fig. 7A). Data obtained from Genevestigator (Hruz et al., 2008) confirmed these results (Supplementary Dataset S2).
**IDA/IDL/PIP/PIPL** responses to hormone treatments were also studied using *in silico* data (Supplementary Fig. S4). Treatments with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), zeatin, abscisic acid (ABA), gibberellin A3 (GA-3) or brassinolide (BL) does not lead to any change in expression. **IDL7** is weakly induced by methyl jasmonate, whereas **PIPL3** and to a lesser extent **PIP2** are induced by indole-3-acetic acid (IAA).

In order to complement the *in silico* data, *Arabidopsis* Col-0 seedlings were subjected to various biotic and abiotic treatments and a qRT-PCR analysis was performed to obtain information on all **IDA/IDL** and **PIP/PIPL** members (Fig. 8). Treatments with the elicitor chitin induced several of the genes, with the highest log2 ratio observed for **IDL6** and **PIPL6**. The aphid *Brevicoryne brassicae*, which is a specialist on Brassicaceae species, was used as biotic treatment, and induced the expression of a large subset of the genes. Ten genes were up-regulated with log2>1; among these were **IDA1**, **PIP2**, **PIP3** and **PIPL1**. Salt stress induced eight of the genes log2>1, confirming the *in silico* data.

**CHX** is a known inhibitor of protein synthesis (Schneider-Poetsch et al., 2010). Interestingly, all the **IDL** genes present on the ATH1 array, as well as **PIP1**, **PIP2** and **PIP3**, are strongly induced upon CHX treatment (Fig. 7C). Treatment of seedlings with CHX led to an increase in the expression level of most of the **IDA/IDL/PIP/PIPL** genes 2-fold or more (Fig. 8). A subset of 12 genes showed an astounding response upon CHX treatment, with >1000-fold increase in expression levels. Generally, the genes found to be most inducible, either by abiotic or biotic stress, were strongly induced upon CHX treatment as well. These results were confirmed using another known protein synthesis inhibitor, anisomycin (Grollman, 1967). Similar to CHX, treatments with anisomycin highly induced the expression of ten of the **IDA/IDL/PIP/PIPL** genes (Fig. 8).

**Transcriptomic responses to PIPL3 peptide treatment**

In order to investigate possible roles of **IDA/IDL** and **PIP/PIPL** peptides, the transcriptomic response of *Arabidopsis* seedlings to treatment with **PIPL3** peptide was analysed. **PIPL3** was chosen, as no functional data was available for this peptide; furthermore, **PIPL3** was expressed in leaf tissue during seedling stages.

Treatment with 100nM **PIPL3** peptide (containing a hydroxyproline in position 15) for 3 h led to a widespread response compared with mock peptide-treated seedlings: 1599 genes were significantly (*P*<0.05) induced, whereas 1133 genes were significantly repressed. Genes showing low expression ratios (>|log2 1.0, <|log2 -0.7) were removed, and the filtered dataset (291 induced and 129 repressed genes, respectively, **Supplementary Dataset S3**) was subjected to a Gene Ontology (GO) enrichment analysis (Ashburner et al., 2000). The ten GO biological process categories most over-represented in the up-regulated gene set indicated that the peptide treatment induced processes related to biotic stress and responses to chemical substances (possibly derived from other organisms) (Fig. 9A). The GO categories most enriched among the down-regulated genes were related to cell wall modification and loosening (Fig. 9B).

A more detailed analysis of the significantly regulated genes revealed that a majority of the genes encoding enzymes of the camalexin biosynthetic pathway were strongly induced by **PIPL3** peptide treatment (Supplementary Fig. S5; **Supplementary Dataset S3**). Camalexin, an indole
Phytoalexin, is synthesized from tryptophan via indole-3-acetaldoxime (IAOx) (Glawischnig et al., 2004; Ahuja et al., 2012). IAOx is a branching point between the biosynthetic pathways of camalexin and two other groups of compounds: indole glucosinolates and IAA. Whereas the first biosynthetic components of the indole glucosinolate pathway were not transcriptionally responsive, the last steps, from indol-3-ylmethyl glucosinolate (I3M) to 4-methoxy-indol-3-ylmethyl glucosinolate (4MO-I3M), were induced (Supplementary Fig. S5). Indole glucosinolate and camalexin biosynthetic genes have been reported to be positively regulated by HIG1, WRKY33 and ANAC042, respectively (Gigolashvili et al., 2007; Qiu et al., 2008; Saga et al., 2012). The expression of all three transcription factors was induced by PIPL3 treatment (Supplementary Dataset S3). In contrast, no genes encoding enzymes of the IAA biosynthetic pathway showed any response to PIPL3 treatment.

**Discussion**

For the last decade, peptide ligands have been found to act as important regulatory factors in plants as well as in animal systems. A well-studied peptide is IDA, found to regulate floral organ abscission in Arabidopsis (Butenko et al., 2003). In this study, we searched the Arabidopsis genome for genes encoding peptide ligands related to IDA, using both in vivo and in silico expression data to investigate these genes. In addition to three novel IDL genes, this search identified the recently described PIP/PIPL gene family. Of these genes, IDL8 has not previously been annotated.

Our phylogenetic analyses indicate that the CEPs (Delay et al., 2013; Roberts et al., 2013) constitute a related peptide family, as they share the C-terminal GxGH motif with the PIP/PIPLs (Fig. 2A; Supplementary Fig. S1). CEP13, CEP14 and CEP15 might be considered to be a subclade of the CEP family, as suggested by Delay et al. (2013) and Roberts et al. (2013), or as a separate family. IDA/IDLs, PIP/PIPLs and CEPs all first appear in seed plants; the founder of these peptide families is therefore difficult to predict. A phylogenetic tree including IDA/IDL, PIP/PIPL and CEP members presented by Hou et al. (2014) differs topologically somewhat from ours (Supplementary Fig. S1). The tree produced by Hou et al. (2014) also includes members of the CLE and PEP families. Furthermore, it is based on an alignment of the C-terminal peptide motif, in contrast to the tree in Supplementary Fig. S1, which was generated from a full-length protein alignment. Thus, a direct comparison of these trees is difficult.

![Fig. 9. Transcriptional responses to PIPL3 peptide treatment. GO enrichment analysis of significantly regulated (P<0.05) genes in 2-week-old seedlings 3h after treatment with 100nM PIPL3 peptide. (A) Up-regulated genes; (B) Down-regulated genes. Control seedlings were treated with 100nM mock peptide. The dataset was filtered for expression ratio ratios (>log2 1.0, <log2 −0.7). The 10 most significantly enriched terms are listed from top to bottom. The bars show the frequency ratios of each GO term in the PIPL3-responsive gene set versus the genome.](image)
Families of post-translationally modified peptides are characterized by multiple paralogous genes encoding small, cysteine-poor peptides with high sequence diversity outside of the C-terminal domain, which contains the mature peptide (Matsubayashi, 2014). All IDA/IDL and PIP/PIPL family genes fulfil these criteria; they encode putative propeptides with an N-terminal SP followed by a variable sequence and a C-terminal, conserved motif related to the EPII motif of IDA (Butenko et al., 2003; Stenvik et al., 2008). The highly conserved SGPS core motif contains a proline that is an attractive candidate for post-translational modification. Several characterized plant peptides, such as systemin (Pearce et al., 1991), TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF) (Ito et al., 2006) and CEPI (Ohyama et al., 2008) have been shown to contain hydroxyproline. Furthermore, hydroxyprolines of PLANT PEPTIDE CONTAINING SULFATED TYROSINE 1 (PSY1) (Amano et al., 2007), CLAVATA 3 (CLV3) and CLV3/E mbryo SURROUNDING REGION-RELATED 2 (CLE2) (Ohyama et al., 2009) are modified by the addition of an O-linked L-arabinose chain. These post-translational modifications increase the activity and/or specificity of the peptides (Shinohara and Matsubayashi, 2010, 2013).

We have previously noted that IDA/IDL peptides and several CLE peptides share a similar core with (hydroxy)prolines and small amino acids (Ala, Gly, Ser) (Stenvik et al., 2008). These amino acids are also found in mature peptides of the PIP/PIPL and CEP families, and one may speculate that they are involved in receptor binding. Biochemical evidence has recently been provided for the binding of PIPL to RLK7 (Hou et al., 2014) and CEP peptides to CEPRI and CEPRII (Tabata et al., 2014). Intriguingly, these three receptors are highly similar LRR-RLKs belonging to a subgroup of the LRR-RLK subclass XI that also includes HAE and HSL2 (Yamaguchi et al., 2006; Butenko et al., 2009). Sequence alignment (Supplementary Fig. S6) indicates that the LRR ectodomains of these three receptors are more similar to HAE and HSL2 than for instance to BAM1, which is documented to bind the CLE9 peptide (Shinohara et al., 2012). Other members of the HAE/HSL2 branch of LRR-RLKs should be promising candidate receptors for peptides belonging to the IDA/IDL, PIP/PIPL and CEP families.

Interestingly, PIPL2, PIPL3 and PIPL1 apparently possess two SGPS motifs (Fig. 1; Hou et al., 2014). The double peptide motif might be processed into two independent peptides that may bind different receptor complexes, thus activating different pathways. As a result, the response induced by the peptides may be wider and/or stronger. Alternatively, the double peptide acts as one functional unit that may interact with two different binding sites, or even to different partners in a receptor dimer. Multiple peptide motifs have also been identified in members of the CEP family (Delay et al., 2013; Roberts et al., 2013). A member of the CLE family, CLE18, encodes a precursor protein that contains two functional peptide motifs. The 13 aa CLE18 peptide located in the CLE18 variable region inhibits tracheary element differentiation and suppresses root growth (Ito et al., 2006), whereas the newly discovered C-terminal 12 aa CLE-LIKE peptide motif, promotes root growth (Meng et al., 2012), suggesting that one gene can encode two peptides with different roles. Crosstalk between different pathways is an important way to fine-tune the response to a given signal, and is normally mediated by common components in signalling pathways (Knight and Knight, 2001; Fujita et al., 2006). The double peptide motifs of PIPL2, PIPL3 and PIPL1 might provide plants with an extra dimension in crosstalk; one gene encodes two possible peptides that may modulate two different pathways.

Many of the IDA/IDL/PIP/PIPL genes have tissue-specific expression (Figs 4–6), suggesting that these genes may play roles during plant growth and development (Stenvik et al., 2008). We were hardly able to detect expression of IDA, IDLI, IDL2 and IDL5 in different organs during development (Fig. 5), but as shown in Fig. 6, expression of IDA and IDL1–IDL5 is restricted to very specific tissues or cell types, indicating a strict developmental regulation of transcription. This is further confirmed by the abiotic and biotic stress assays (Fig. 8), where IDL2–IDL5 appear to be non-responsive. IDA is strongly regulated by IAA in roots (Kumpf et al., 2013), but our analysis of in silico data suggests that this is not the case for green tissue (Supplementary Fig. S5; Supplementary Dataset S2). PIPL1 shows the highest expression levels of the 20 genes in our in silico analysis (Fig. 4), but as for the IDL genes, the expression is too specific to be detected in our qRT-PCR analysis (Fig. 5). Transcriptome studies of Arabidopsis seed development indicate that PIPL1 expression is restricted to seed coat tissue during early stages of seed development (Le et al., 2010; Belmonte et al., 2013).

Stress-induced genes (Figs 7, 8) include PIPL1, PIPL2, PIPL3, PIPL5 and PIPL6, while IDA, IDL1, IDL6 and IDL7 are up-regulated both during development and stress. The stress-induced genes can be separated into two categories: those induced by abiotic stress (like IDA, IDL1, PIPL1) and those induced by biotic stress (like IDL6). IDL7, PIPL2 and PIPL3 are induced by both abiotic and biotic stress. PIPL1 and PIPL2 have been implicated in immune responses and pathogen resistance (Hou et al., 2014). Our results suggest that IDA/IDL and PIP/PIPL peptides also may be involved in regulation of responses to abiotic stresses such as salt stress. The peptides could act in positive or negative feedback loops for temporal and/or spatial fine-tuning of stress signalling pathways.

A subset of the IDA/IDL and PIP/PIPL genes were strongly induced by treatment with the translational inhibitors CHX and anisomycin (Figs 7, 8). Such superinduction has previously been reported in plants, for cold-induced genes (Berberich and Kusano, 1997; Zarka et al., 2003) and immediate-early response genes (Horvath et al., 1998; Uquillas et al., 2004), amongst others. The mechanism behind CHX superinduction could be related to the presence of a labile transcriptional repressor or increased mRNA stability.

Treating seedlings with PIPL3 peptide led to the induction of genes involved in defence responses, including the camalexin and indole glucosinolate biosynthesis pathways (Supplementary Fig. S5). Neither in silico data (Fig. 7; Supplementary Fig. S3) nor our own experiments (Fig. 8) indicate that PIPL1 expression is activated by biotic or...
abiotic treatments; instead, expression appears to be auxin-induced (Supplementary Fig. S4; Supplementary Dataset S2). However, PIPI and PIP2, the other family members for which functional data exist, have been strongly implicated as positive regulators of defence responses (Hou et al., 2014). Both camalexin and indole glucosinolates are important parts of the chemical defence system of crucifers (Sønderby et al., 2010; Ahuja et al., 2012). Whereas glucosinolates are stored after synthesis and are detected in most tissues (Brown et al., 2003), camalexin is produced upon biotic or abiotic stress (Glawischning, 2007). Why are these pathways induced by a peptide that shows developmentally regulated expression? It is tempting to speculate that PIPL3 is involved in regulation of the trade-off between growth and defence during development. PIPL3 may activate a limited set of defence-related pathways to a basal expression level in tissue surrounding meristematic regions. The down-regulation of cell wall loosening genes by PIPL3 indicate that the target cells could be differentiated cells that have reached their final size and shape.

In this study, we have characterized the IDA/IDL and PIP/PIPL families of peptide ligands in Arabidopsis. The family is characterized by one or both of two C-terminal conserved peptide motifs, SGPS and GxG. Three PIP/PIPL members contain two tandem peptide motifs. Members of the IDA/IDL and PIP/PIPL gene families are expressed during development or induced by stress, suggesting distinct biological roles. Transcriptome analysis of PIPL3 peptide-treated seedlings indicates that although PIPL3 expression appears to be developmentally regulated, it activates defence-related processes.

Supplementary data

Supplementary data are available at JXB online.

Supplementary Figure S1. Phylogenetic relationship between IDA/IDL, PIP/PIPL and CEP peptides in Arabidopsis.

Supplementary Figure S2. Chromosomal localization of Arabidopsis IDA/IDL and PIP/PIPL genes.

Supplementary Figure S3. Elicitor-induced expression of IDA/IDL and PIP/PIPL genes based on in silico data.

Supplementary Figure S4. Transcriptional responses of IDA/IDL and PIP/PIPL genes to hormone treatments.

Supplementary Figure S5. Transcriptional activation of the camalexin and indole glucosinolate biosynthetic pathways by PIPL3 peptide treatment.

Supplementary Figure S6. IDA, PIPI and CEP1 bind related proteins.

Supplementary Table S1. List of PCR primers used in this study.

Supplementary Table S2. Gene duplication events of the IDA/IDL and PIP/PIPL gene families.

Supplementary Table S3. Expression of IDA and the IDL1 to IDL5 genes fourteen days after germination.

Supplementary Dataset S1. Protein sequences of IDA/IDL and PIP/PIPL in Arabidopsis.

Supplementary Dataset S2. Transcriptional responses of IDA/IDL/PIP/PIPL genes to perturbations, based on in silico data from Genevestigator.

Supplementary Dataset S3. Transcriptome responses to PIPL3 peptide treatment.

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