RESEARCH PAPER

Overexpression of the *Arabidopsis thaliana* signalling peptide TAXIMIN1 affects lateral organ development

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Received 26 February 2015; Revised 19 May 2015; Accepted 21 May 2015

Editor: Ruediger Simon

Abstract

Lateral organ boundary formation is highly regulated by transcription factors and hormones such as auxins and brassinosteroids. However, in contrast to many other developmental processes in plants, no role for signalling peptides in the regulation of this process has been reported yet. The first characterization of the secreted cysteine-rich TAXIMIN (TAX) signalling peptides in *Arabidopsis* is presented here. TAX1 overexpression resulted in minor alterations in the primary shoot and root metabolome, abnormal fruit morphology, and fusion of the base of cauline leaves to stems forming a decurrent leaf attachment. The phenotypes at the paraclade junction match TAX1 promoter activity in this region and are similar to loss of LATERAL ORGAN FUSION (LOF) transcription factor function. Nevertheless, TAX1 expression was unchanged in *lof1lof2* paraclade junctions and, conversely, LOF gene expression was unchanged in TAX1 overexpressing plants, suggesting TAX1 may act independently. This study identifies TAX1 as the first plant signalling peptide influencing lateral organ separation and implicates the existence of a peptide signal cascade regulating this process in *Arabidopsis*.

Key words: Boundary genes, cysteine-rich peptide, fruit development, lateral organ fusion, paraclade junction.

Introduction

Development of multicellular organisms requires tight spatiotemporal control of cell differentiation. In plants, this is established by gradients of morphogens, such as hormones or noncoding RNAs, and polypeptides, such as secreted peptides or mobile transcription factors (Vernoux et al., 2010; Van Norman et al., 2011; Skopelitis et al., 2012; Long et al., 2015). Two distinct classes of secreted peptides can be distinguished in plants: small post-translationally modified peptides such as the CLAVATA3/ENDOSPERM SURROUNDING REGION (CLE) family, and cysteine-rich peptides...
fusion, while gain-of-function mutants show an increased size of the boundary domain (Laufs et al., 2004). Loss-of-function mutants of other genes specifically expressed in boundaries (boundary genes) are also often characterized by organ fusion defects (Žádníková and Simon, 2014). These include the transcription factors LATERAL ORGAN FUSION1 (LOF1) and LOF2 (Lee et al., 2009), JAGGED LATERAL ORGANS (Borghì et al., 2007), and BLADE ON PETIOLE1 and 2 (Ha et al., 2007; Khan et al., 2012).

In participation with these transcriptional regulators, polar auxin transport carriers also play a role in boundary establishment (Žádníková and Simon, 2014). Auxin gradients originating from the meristem and organ primordia intersect at the boundary, forming a local auxin minimum. ABCB19 is an ATP-binding cassette transporter required for normal basipetal auxin transport from the meristem auxin maximum (Noh et al., 2001). Loss of ABCB19 function increases auxin levels in both meristems and boundary regions, disturbs the auxin minimum, and results in the fusion of cauline leaves to the primary stem and in pedicel–stem fusions, ultimately accompanied by reduced expression of boundary genes (Zhao et al., 2013).

So far, no signalling peptides have been described to play a role in organ separation. Overexpression of the signalling peptide TAX1 in Arabidopsis is here reported to result in fusion of the cauline leaves to stems with only minor effects on the primary metabolome of leaf and root tissue. Accordingly, TAX1 promoter activity is higher at the base of the cauline leaf and axillary stem and in the apical meristem. Interestingly, the developmental defects caused by TAX1 overexpression are expanded in the Landsberg erecta (Ler) background. Finally, although the TAX1 overexpression phenotype at the paracline junction phenocopies that of lof1lof2 mutants, these data suggest that TAX and LOF signalling pathways converge independently.

Materials and methods

Plant materials and growth conditions

Plants in this study were either the Columbia (Col-0) or Ler ecotype. For in vitro growth, seeds were gas-sterilized, stratified, and germinated on full-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). Plants were cultivated in a growth room at 22°C with a 16-h light/8-h dark photoperiod (110 µmol m⁻² s⁻¹). For analysis of adult plants, 10-day-old in vitro-germinated seedlings were transferred to soil in a growth chamber at 20–22°C and a photoperiod of 16-h light/8-h darkness.

T-DNA insertion lines for TAX1 (SALK_016616) and TAX2 (SALK_113004C) were obtained from Nottingham Arabidopsis Stock Center (NASC; Alonso et al., 2003). Seedlings were PCR-genotyped using a T-DNA- and gene-specific primer (Supplementary Table S1). Amplicons were sequenced to confirm the location of the T-DNA.

DNA constructs

The open reading frames of Arabidopsis TAX1 (At1g31090), TAX2 (At2g20562), and TAX1ASP (lacking the N-terminal signal) were amplified between attB sites from Col-0 cDNA using Phusion polymerase (New England Biolabs) and Gateway recombined in the Entry vector pDONR207 (Invitrogen) and then in the pFAST-G02 Entry vector pDONR207 (Invitrogen) and then in the pFAST-G02 vector (Invitrogen). The open reading frames of At2g20562 from the Fragaria x ananassa (strawberry) genome were cloned into the pDONR207 (Invitrogen) Entry vector using the Gateway recombination system to generate a functional Arabidopsis TAX2 homolog. Constructs were then introduced into Arabidopsis via Agrobacteriummediated transformation as previously described (Agrobacterium-mediated transformation as previously described (Ha et al., 2007; Khan et al., 2012).
destination vector (Shimada et al., 2010) for overexpression. The *TbTAX* open reading frame was amplified from *T. baccata* cDNA and fused to 6xHis by PCR and cloned into the Entry clone pDONR221 (Invitrogen) and then in the pK7WG2D (Karimi et al., 2005) destination vector. Promoter sequences of *TAX1* (1575 bp upstream of the ATG) and *TAX2* (2000 bp upstream of the ATG) were PCR amplified between *attB* sites from Col-0 gDNA, Gateway recombined in pDONRP4P1R and then in pmK7S*NFm14GW as the destination vector for promoter activity analysis (Karimi et al., 2007). For subcellular localization, the *Venus* sequence was fused to that of *TAX1* and *TAX1ΔSP* by PCR amplification and cloned as an entry clone in pDONR207 and recombined to destination vector pFAST-R02 (Karimi et al., 2005; Shimada et al., 2010).

**Plant transformation**

All constructs were transformed into *Agrobacterium tumefaciens* strain C58C1 (pMP90) for subsequent transformation of Col-0 plants by floral dip (Clough and Bent, 1998). The 35S::TAX1 construct was used to transform both Col-0 and Ler plants. Transformatants were selected on MS media based on *OLE1::GFP* expression in seeds. Homozygous plant lines with one T-DNA locus were selected and used in all assays. All primers used for cloning are listed in Supplementary Table S1 at JXB online.

**Gene expression analysis**

RNA was isolated from plant material using the Plant RNeasy Kit (Qiagen, Germany) following the manufacturer’s instructions with the addition of a DNase treatment step. cDNA was synthesized from 1 µg RNA using the iScript reverse transcriptase kit (Bio-Rad). Quantitative real-time PCR (qRT-PCR) was performed on a LightCycler 480 (Roche Applied Science, USA) using Fast Start SYBR Green I fluorescent dye (Roche). At least three biological repeats and three technical repeats were used for each analysis. Expression data were normalized through two reference genes, *UBC* (At5g25760) and *PP2A* (At1g13320). For RT-PCR, cDNA was amplified with the Go-Taq PCR mix (Promega) using different amplification cycles, and loaded on an agarose gel containing SYBR Safe (Life Technologies). cDNA was synthesized through a DNase treatment step. cDNA was synthesized following the manufacturer’s instructions with the Plant RNeasy Kit (Qiagen, Germany) following the manufacturer’s instructions with the addition of a DNase treatment step. cDNA was synthesized from 1 µg RNA using the iScript reverse transcriptase kit (Bio-Rad). Quantitative real-time PCR (qRT-PCR) was performed on a LightCycler 480 (Roche Applied Science, USA) using Fast Start SYBR Green I fluorescent dye (Roche). At least three biological repeats and three technical repeats were used for each analysis. Expression data were normalized through two reference genes, *UBC* (At5g25760) and *PP2A* (At1g13320). For RT-PCR, cDNA was amplified with the Go-Taq PCR mix (Promega) using different amplification cycles, and loaded on an agarose gel containing SYBR Safe (Life Technologies). The cycle number showing the highest contrast without saturation was used. *ACTIN* (At3g18780) was used as the reference gene for RT-PCR experiments. Primers for expression analysis are listed in Supplementary Table S1 at JXB online. Paraclade junctions (1 cm of nodal tissue that includes the primary stem and part of the axillary stem and cauline leaf) were collected from eight plants to form one replicate and immediately frozen in liquid nitrogen.

**GUS expression**

Plant material was harvested in 90% (v/v) acetone and kept at 4°C for up to 1 week to remove chlorophyll. For the GUS staining, plants were first rinsed in NT buffer (100 mM Tris, pH 7.0 and 50 mM NaCl) and incubated in ferricyanide solution (1.94 mM potassium ferricyanide (K₃[Fe(CN)]₆) prepared in NT buffer) for 30 min at 37°C. Next, plants were transferred to a staining solution (2.47 mM X-Gluc prepared with ferricyanide solution) and kept at 37°C for at least 8 hours. Plants were kept in 70% (v/v) ethanol prior to visualization under a Bino Leica stereomicroscope (Leica MZ16) equipped with a digital camera.

**Microscopy**

Imaging of living SAMs of 5-week-old plants was performed using a LSM700 laser-scanning confocal microscope (Zeiss, Jena, Germany). TAX-Venus fusions were imaged in 5- or 10-day-old seedlings with an Olympus FV10 ASW confocal microscope. Images in Fig. 1 and Supplementary Fig. S1 at JXB online are from independent transformed lines. Before imaging, seedlings were briefly incubated in propidium iodide (3 mg/L, Sigma) and subsequently washed and mounted in water. For scanning electron microscopy of gynoecia, flowers at stage 13 were collected from wild-type and *TAX1* overexpressing plants cultivated in the greenhouse. Sepals, petals, and stamen were removed to reveal the carpel, which was directly mounted on the steel stubs. Images were collected using a Hitachi TM-1000 table-top scanning electron microscope (Hitachi High-Technologies Corporation).

**Metabolite profiling**

Metabolite profiling was performed exactly as described by Joshi et al. (2006), using the modifications for root tissue described in Joshi et al. (2006). Metabolite identities were verified via comparison to spectral libraries of authentic standards housed in the Golm Metabolome Database (Kopka et al., 2005). Metabolite information is provided following recent recommendation standards (Supplementary Table S2; Fernie et al., 2011).

**Results**

**The TAX genes encode putative signalling peptides**

Recently, a novel putative signalling peptide termed TAXIMIN (TAX) was identified in the medicinal tree *T. bacata*. This peptide co-regulates with taxol biosynthesis genes and overexpression of *TbTAX* in *N. tabacum* hairy roots enhances production of alkaloids (Onrubia et al., 2014). Analysis using the PLAZA comparative genomics workbench (Proost et al., 2014) indicated that this peptide is highly conserved across the plant kingdom. Homologues with a remarkable sequence identity can already be found in the lower land plants *Selaginella moellendorffii* and *Physcomitrella patens* (Supplementary Fig. S1 at JXB online). In this study, the model species *Arabidopsis thaliana* was used to further characterize the function of the TAX signalling peptides, and two homologous sequences were discovered at the loci *At2g31090* and *At2g20562*, which were renamed *TAX1* and *TAX2*, respectively (Fig. 1A).

Both *Arabidopsis TAX* genes consist of two exons flanking one intron (Fig. 1B). *TbTAX* encodes a peptide of 73 amino acids (7.82 kDa), whereas *TAX1* and *TAX2* encode 75 (8.15 kDa) and 73 (7.84 kDa) amino-acid peptides, respectively (Fig. 1A). The *TAX2* peptide has the highest sequence similarity to TbTAX with 51 amino acids identical to its gymnosperm homologue (Fig. 1A).

TAXIMIN peptides have an *in silico*-predicted N-terminal secretion peptide (Fig. 1A, Onrubia et al., 2014), generating equally sized mature peptides of 46 amino acids located at the C-terminus (Fig. 1A). The hydrophobicity of this mature peptide is striking, with up to 29 amino acid residues being hydrophobic. This hydrophobicity and equal mature peptide length is conserved in all TAXIMIN family members (Onrubia et al., 2014). Currently, it is not known if and/or how the TAX peptides are post-translationally modified, but chemical synthesis of the *TbTAX* peptide was only possible when the prolines were hydroxylated (Onrubia et al., 2014). The TAX peptides are cysteine-rich with six conserved cysteines and three conserved prolines (Fig. 1A), suggesting that this peptide belongs to the cysteine-rich family of peptides.
To validate the functionality of the predicted N-terminal signal peptide, fusions of TAX1 to the Venus fluorescent protein were constructed. TAX1-Venus with and without signal peptide were expressed with a 35S Cauliflower mosaic virus promoter in Arabidopsis seedlings and the localization of the fusion protein was determined by confocal microscopy in root cells. Similar to earlier observations of TbTAX subcellular localization (Onrubia et al., 2014), TAX1-Venus was targeted to the plant cell membrane (Fig. 1C and Supplementary Fig. S1B at JXB online) and this was dependent on the presence of the N-terminal signal peptide (Fig. 1D and Supplementary Fig. S1C at JXB online).

It can be concluded that, like TbTAX, TAX1 is a putative signal peptide that is likely secreted through the canonical secretion pathway.

**TAX1 overexpression causes developmental phenotypes**

First, full-length TAX1, TAX2, and TbTAX were constitutively expressed under control of the 35S promoter in Arabidopsis ecotype Col-0. For each construct, several independent lines were selected for the presence of a single T-DNA locus and showing clear overexpression in seedlings.

The highest expressing TAX1 overexpression lines OE-2 and OE-3 showed reduced growth of the seedlings on basal MS plates (Fig. 2A, Supplementary Fig. S2A at JXB online). After transfer to the greenhouse, these lines were delayed in development (Supplementary Fig. S2B, C at JXB online). Importantly though, TAX1 overexpressing lines OE-2 and OE-3 showed developmental defects at paraclade junctions and altered fruit morphology (Figs 2 and 3).

Fruits of wild-type Arabidopsis Col-0 were narrow, cylindrical, and elongated (Fig. 2B). The siliques of the highest TAX1 overexpressing lines OE-2 and OE-3 were shorter and wider at the tip, due to an outgrowth of both the valves (Fig. 2B) and the replum (Fig. 2C). The ovules inside the sique had a normal organization at the base of the fruit, but they were disordered at the wider tip (Fig. 2D). This was also associated with preferential opening of mature siliques at the site of seed crowding (Supplementary Fig. S2D at JXB online). The number of carpels in the 35S::TAX1 siliques was unaffected. The valve outgrowths were visible at early stages of gynoecium development (Fig. 2E) and could be followed over the course of fruit development (Supplementary Fig. S3 at JXB online).

The 35S::TAX1 transgene was also overexpressed in the Ler background. Four lines were generated expressing TAX1 at different levels (Fig. 2F) and similar fruit phenotypes were
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observed in these lines compared to the Col-0 background. Again, the severity correlated with TAX1 expression levels (Fig. 2F, G). The silique phenotype observed in lines OE-2 and OE-3 in Col-0 and in OE-1L, OE-3L, and OE-4L in Ler had complete penetrance: all siliques in all plants over several generations displayed this phenotype.

**TAX1 constitutive expression results in lateral organ fusion**

Besides the changes in fruit morphology, different types of lateral organ fusion were present in TAX1 overexpressing lines at the paraclide junctions between the primary stem, axillary shoot, and cauline leaf (Fig. 3A). These phenotypes had reduced penetrance, and occurred in most plants of line OE-3 (Fig. 3A) and sporadically in OE-2 and Ler (Supplementary Fig. S4 at JXB online). Of 23 OE-3 plants, 17 showed pedicel–stem fusions with outgrowths subtending some fruits, possibly corresponding to bract-like structures (Fig. 3B). At the first formed node on the main stem, 12 plants showed a protrusion of the main stem with the cauline leaf fused to a down- or side-wards deflected axillary shoot (Fig. 3C). At the second node, the cauline leaf had a broader leaf base and also deflected downwards (Fig. 3D). Finally, the most frequently occurring defect was a fusion of the cauline leaf to a stem together with the leaf extending down the insertion point along the stem, forming a decurrent leaf attachment. For the primary stem this was observed in 4 out of 23 plants (Fig. 3E). Interestingly, when no third node was visible, fusion occurred in 18 cases between a secondary shoot originating from the rosette, a tertiary axillary shoot, and the subtending cauline leaf (Fig. 3F). All these defects were associated with downward bending of the stems early during outgrowth of the axillary stem (Supplementary Fig. S4 at JXB online).

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**Fig. 2.** TAX1 overexpression in Col-0 and Ler backgrounds results in an alteration of fruit morphology. (A) Relative expression of TAX1 in 19-day-old seedlings compared to the Col-0 wild type for three independent 35S::TAX1 lines. Expression values were normalized to those of the wild type (Col-0), set to 1. Values represent the average of three biological replicates ±SE. (B) Medial and lateral view of mature siliques of TAX1 overexpression (OE) lines. (C) Lateral view with seeds removed. Arrow indicates protrusion of the replum at the tip of the silique in TAX1 overexpressing lines. (D) Lateral silique view with one valve removed. (E) Scanning electron microscopy images of early stage gynoecia of TAX1 OE lines. Arrow indicates protrusion. The scale bar is 400 μm. (F) Relative expression of TAX1 in 19-day-old seedlings compared to the Ler wild type for four independent 35S::TAX1 lines. Expression values were normalized to those of the wild type (Ler). Values represent the average of four biological replicates ±SE. (G) Medial and lateral view of mature siliques of TAX1 OE lines in the same order as in (F). Scale bars in B-D and G are 5 mm.
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Fig. S2B at JXB online). The axillary stem bent upwards again later during development, probably owing to phototropism (Supplementary Fig. S2C at JXB online). No fusion of pedicels to stems was observed. In the Ler background, additional phenotypes besides those seen in Col-0 were occasionally observed, such as bending of the axillary stem at tertiary branch points (Supplementary Fig. S4E at JXB online) and twisting of the primary stem (Supplementary Fig. S4F at JXB online).

**Lateral organ phenotypes are specific for TAX1 overexpression**

First, fruits and lateral organs of Arabidopsis lines overexpressing TAX2 or TbTAX were examined (Supplementary Fig. S5A–E at JXB online). Although high transgene expression was detected, the lines displayed a wild-type phenotype. These results suggest that specifically TAX1 is capable of triggering organ fusion defects. Arabidopsis lines overexpressing TAX2 did not display any visible phenotype in root or leaf growth.

Next, whether the signal peptide of TAX1 is essential for its activity was examined. A construct lacking the N-terminal signal, 35S::TAX1ΔSP, was constitutively expressed, but failed to show any of the phenotypes observed for the full-length peptide (Supplementary Fig. S5F, G at JXB online). This result confirms that TAX1 represents a signalling peptide that is most likely secreted through the canonical pathway and that high peptide levels inside the cell do not result in observable phenotypes. Notably, wild-type phenotypes were observed in the TAX1-Venus lines (data not shown), suggesting that the fusion to Venus might interfere with TAX1 processing and/or function.

For both TAX1 and TAX2, a T-DNA insertion line was isolated, tax1 and tax2 respectively, with the T-DNAs located in the sole intron (Supplementary Fig. S6A at JXB online). A cross was made between tax1 and tax2 to exclude functional redundancy between the two genes. Expression analysis by qRT-PCR in paraclade junctions (Supplementary Fig. S6B, C at JXB online) and RT-PCR in seedlings (Supplementary Fig. S6D at JXB online) confirmed the absence or at least severely reduced expression of both TAX genes in tax1tax2.
Notwithstanding, single nor double mutant plants did not display any mutant phenotype in lateral organs or fruits (Supplementary Fig. S6E, F at JXB online). Likewise, no visible mutant phenotypes in root or leaf growth in tax1 and tax2 mutant lines could be observed.

**TAX1 and TAX2 have distinct expression patterns**

The tissue-specificity of the promoter activities of **TAX1** and **TAX2** was then investigated. Promoter fragments of the 1575 bp upstream of the start codon for **TAX1** and 2000 bp upstream of the start codon for **TAX2** were cloned and used to drive a nuclear-localized GUS–GFP fusion (Karimi et al., 2007).

In 10-day-old in vitro-germinated p**TAX1**:GUS::GFP seedlings, the **GUS** expression was detected mainly in the SAM region (Fig. 4A, B, I). Using GFP, **TAX1** promoter activity was detected in the entire inflorescence meristem of 5-week-old plants, but was stronger in the organ primordia (Fig. 4C-E). **TAX1** expression was mostly specific to the L1 layer in the centre of the meristem but was also detected in the L2 layer in organ primordia (Fig. 4C-E).

In mature plants cultivated for 28 days in the greenhouse, **TAX1** expression was observed in the anthers and in the nectaries in the floral tissue but no expression was visible in the gynoecium (Fig. 4F, G). The paraclade junctions between the primary stem and axillary stems showed **GUS** expression at the base of the cauline leaf and the emerging axillary shoot (Fig. 4H). The latter **TAX1** expression pattern supports a role of **TAX1** in the lateral organ fusion phenotype observed following **TAX1** overexpression. Accordingly, no expression was observed in the pedicel–stem junctions, corresponding to the absence of fusion phenotypes there (Supplementary Fig. S7C at JXB online).

In contrast, in 10-day-old p**TAX2**:GUS::GFP seedlings, **GUS** was highly expressed in the vasculature in the cotyledons, first true leaves, and hypocotyl (Fig. 4J). In the floral tissue of mature plants, **GUS** was visible in the vasculature of the sepals, petals, and style (Fig. 4K). Also in paraclade junctions, **GUS** was detected mainly in the vasculature of the cauline leaf (Fig. 4L). For both **TAX1** and **TAX2**, **GUS** was observed in main and lateral roots in seedlings, mainly in vasculature, but was absent from the root tip (Supplementary...
Expression in root hair cells was only observed for TAX2 (Supplementary Fig. S7B, G at JXB online).

Overall, the difference in TAX1 and TAX2 expression patterns suggests that they play distinct roles in plant development, which is in agreement with the different effects caused by their overexpression.

Expression of known boundary genes does not change in TAX1 overexpression lines

The fusion of cauline leaves to the stem has been previously reported in LOF loss-of-function lines (Lee et al., 2009) or to be associated with reduced LOF expression in abcb19 (Zhao et al., 2013). The expression of LOF1 and LOF2 in junctions of the TAX1 overexpressing lines was therefore determined. The boundary gene CUC3 was also included, because it has been reported to be downstream of the LOF transcription factors and because loss of CUC3 function causes fusion defects (Hibara et al., 2006).

First, overexpression of TAX1 in the junctions was confirmed by qRT-PCR (Fig. 5A). Line OE-3 showed very high expression of TAX1 at this site, corresponding to the severity of the fusion phenotypes observed in these lines associated with downward bending of the axillary stem (Fig. 3). However, expression of none of the tested boundary genes was significantly altered at this site (Fig. 5B-D). To confirm this finding, a pLOF2::GUS reporter line (Lee et al., 2009) was crossed into the OE-3 background. Both the expression intensity and pattern of pLOF2-driven expression remained unaltered under TAX1 overexpression (Fig. 5E, F), confirming the qRT-PCR results. Likewise, also earlier during development, LOF expression was unchanged in seedlings overexpressing TAX1 (Fig. 5G, H). Finally, when expression of LOF1 and LOF2 was tested in paraclade junctions of the tax1tax2 double mutant, no changes could be observed (Fig. 5I, J). Conversely, to assess whether TAX1 or TAX2 could act downstream of the LOF transcription factors, the paraclade junctions of lof1lof2 mutant plants that show similar fusion defects (Lee et al., 2009) were harvested, but, again,
no significant effect on TAXI and TAX2 expression could be detected (Fig. 5K, L).

These expression data suggest that the similar phenotypes observed for TAXI overexpression lines and lof1lof2 are independent and probably result from converging signalling pathways.

TAX1 overexpression has only minor effects on the leaf and root metabolome

Having documented developmental phenotypes for the TAXI overexpressors, and given the link of the T. baccata homologue TAXIMIN with plant metabolism (Onrubia et al., 2014), an established GC-MS protocol was used to assess whether TAXI overexpressing seedlings contained changes in the levels of primary metabolites in their leaves (Supplementary Table S3) and roots (Supplementary Table S4).

The results of these analyses are presented in the heat-map of Fig. 6. Four, eight, and 22 of 61 measured metabolites were significantly different in the leaves of weak (OE-1), intermediate (OE-2), and strong (OE-3) TAXI overexpressing lines, respectively, and only serine and serine being altered (enhanced in both instances) in all three lines. That said, sucrose and glucose 6-phosphate were increased in both line OE-2 and line OE-3, whilst threonine was significantly decreased in both lines. Line OE-3 was additionally characterized by increased levels of histidine, putrescine, glucose, 4-hydroxyproline, ribulose 5-phosphate, asparagine, pyroglutamate, glutamine, glycercate, β-alanine, proline, malate, glutamate, and arginine. In contrast, this line displayed decreased levels of dehydroascorbate, threonate, and threitol. In roots, the changes were even less marked, with two, six, and 10 of 61 metabolites significantly different in the weak, intermediate, and strong overexpressing lines, respectively, and only serine being altered (again enhanced) in all three lines. That said, glycocolate, succinate, succinolate, and β-alanine were increased in both line OE-2 and line OE-3, whilst hydroxyproline, pyroglutamate, glutamine, proline, asparagine, and fumarate were increased and histidine decreased only in line OE-3.

Discussion

More than 1000 signalling peptides, most of them still uncharacterized, are encoded in the Arabidopsis genome (Czyzewicz et al., 2013). A first characterization of a family of two Arabidopsis peptides, TAX1 and TAX2, the homologues of the TbTAX (Onrubia et al., 2014), has been presented here. TbTAX was shown to localize to the plasma membrane through the secretory system and ectopically activate specialized metabolite pathways in yew and tobacco cells (Onrubia et al., 2014).

Regulatory neofunctionalization of the Arabidopsis TAX genes

Because of the relative sequence similarity between Arabidopsis TAX1 and TAX2, possible functional redundancy was investigated. During evolution, a parologue may have lost all functionality or gained a new function. Other paralogues share the ancestral function or remain redundant in Arabidopsis (De Smet and Van De Peer, 2012). The promoters of the Arabidopsis TAX genes have very distinct expression patterns, with TAX2 being expressed mainly in the vasculature and TAX1 rather at specific sites, such as anthers and nectar in flowers, the paraclade junction on the primary stem, and the L1 layers of the SAM, implying at least regulatory
neofunctionalization of the two Arabidopsis TAX genes. Furthermore, overexpression of TAX1, but not of TAX2 or TbTAX, led to severe developmental phenotypes, suggesting functional specialization of the Arabidopsis TAX peptides, with roles in diverging target pathways. The pronounced effects caused by TAX1 overexpression on the Arabidopsis developmental programmes that are reported here raise the possibility that the effect of TbTAX on metabolism might be indirect and caused by preceding developmental rewiring. Accordingly, the biosynthesis of paclitaxel in Taxus spp. is tissue-dependent (Vidensek et al., 1990) and overexpression of TbTAX in tobacco hairy roots leads to morphological changes (Onrubia et al., 2014).

TAX1 overexpression mimics loss of LOF function

Several developmental phenotypes are apparent in TAX1 overexpressing lines at paraclade junctions: (i) cauliflower leaves are partially fused to stems; (ii) the cauline leaf base is widened and can form a decurrent strand extending down along the primary stem; and (iii) axillary stems bend downwards before growing upwards again, probably due to phototropism. The severity of these phenotypes was associated with the TAX1 expression levels in the paraclade junctions. Similar phenotypes have been described for the lof1-1 and lof1-1lof2-1 mutants (Lee et al., 2009). These lines are defective in the closely related MYB-domain transcription factors LOF1 and LOF2, which are expressed in Arabidopsis organ boundaries. In lof1-1, the cauline leaves are also fused to the axillary branch at the base, which bends down and completely lacks accessory shoots (Lee et al., 2009). Additional phenotypes that are also presented in TAX1 overexpressing lines, such as the decurrent leaf attachment, were only observed in the lof1-1lof2-1 double mutant.

Nonetheless, no reduction in LOF1 and LOF2 expression levels in TAX1 OE paraclade junctions or seedlings was observed. Therefore, down-regulation of LOF1 and LOF2 is not causal for the phenotype. Conversely, TAX1 expression was not de-regulated in lof1-1lof2-1 paraclade junctions, suggesting that high TAX1 expression was not causal for fusion defects in this line. Another boundary gene that has been linked to fusion of cauline leaves to the primary stem is CUC3 (Hibara et al., 2006). CUC3 expression was also down-regulated in lof1-1 and lof1-1lof2-1 paraclade junctions (Lee et al., 2009) and LOF1 expression was down-regulated in the cuc3-105 mutant (Gendron et al., 2012). The cuc3-105 allele also enhanced paraclade fusions in the lof1-1 background (Lee et al., 2009). However, CUC3 expression did not change in paraclade junctions of the TAX1 overexpression lines. Fusion phenotypes are often observed in loss-of-function mutants for genes expressed in the boundary (Zádníková and Simon, 2014). However, promoter activity in the SAM shows that TAX1 is not a boundary gene itself. The fact that the most well-known boundary genes affecting fusion of cauline leaves to neighbouring organs do not show a change in expression in TAX1 overexpressing lines suggests that TAX1 might work in a converging, yet unknown, signalling pathway.

TAX1 overexpression also affects fruit development

TAX1 overexpression also resulted in shorter siliques with protrusions at the tip of both valve tissue and the replum resulting in seed crowding. Basal parts of the fruit were however normal. In contrast to lateral organ separation, there is a known role for plant peptides in Arabidopsis fruit development. For instance, the CLE peptide family was discovered due to the club-shaped fruit of the clv3 mutant, resulting from an enlarged flower meristem and extra floral whorls (Clark et al., 1996). Overexpression of members of the DEVIL (DV1)/ROTUNDIFOLIA4 family resulted in an alteration of silique morphology with different members causing different phenotypes, including protrusions at the tip in DV1I overexpressors (Wen et al., 2004). Although the exact cellular and molecular bases of these phenotypes are currently not well understood, DV1I expression was associated with down-regulation of the valve identity regulator AGAMOUS-LIKE8 (AGL8)/FRUITFUL (Wen et al., 2004). Several organ-meristem boundary genes also influence fruit development. Gain-of-function lines of CUC1 and CUC2 prevent congenital fusion of carpels (Nikovics et al., 2006; Sieber et al., 2007; Larue et al., 2009) and the LOF1 gain-of-function line constricted fruit 1 displays small misshapen fruits with increased replum size and enhanced expression of the valve margin identity markers SHATTERPROOF1 (SHP1/AGL1) and SHP2/AGL5 (Gomez et al., 2011). The effect of TAX1 overexpression on fruit development could therefore be linked to its influence on lateral organ separation.

TAX1 overexpression mildly affects the primary metabolome

Given the link between TbTAX and plant metabolism (Onrubia et al., 2014), the primary metabolome of TAX1 overexpressing seedlings was profiled. The changes observed in the primary metabolites were comparatively mild in the overexpressors with only two of the changes conserved across the genotype in leaves, namely phosphate and serine, and only one (again serine) in the roots. However, the extent of metabolic change was consistent with the degree of overexpression and the severity of the developmental phenotypes in the lines.

When assessed at a pathway level, the leaf data clearly suggest an elevated rate of photosynthesis on a per gram fresh weight basis, with increases in pentose- and hexose-phosphates as well as in sucrose. In addition, the intimately connected pathway of photorespiration appears to be up-regulated as indicated by the above-mentioned increases in serine and also in glycerate. The enhanced levels of phosphate would also be anticipated to facilitate the operation of photosynthesis, which can be phosphate-limited in vivo, suggesting that the increase in sucrose was not due to an inhibition of sucrose export driven by the lower sink strength, but rather indicative of an increased rate of sucrose synthesis. Of note, but only in the strongest line, was a general increase in the levels of the amino acids intimately associated with the TCA cycle. Such changes have previously been observed following increases in leaf sucrose (see for example Purdy et al., 2013).
and have been noted to invoke changes in the levels of some phytohormones, such as gibberellic acid (Araújo et al., 2012). However, these metabolic changes were only seen in line OE-3 and not in line OE-2, which has a very similar, albeit less severe, developmental phenotype, and as such it is difficult to envisage them being causal of these phenotypes. Similar arguments preclude a strong case for a role of putrescine in the determination of the phenotype, despite considerable evidence being presented that this metabolite can exhibit bioactivity (Handa and Mattoo, 2010).

The root data presented fewer metabolic differences; however, two were highly notable. First, consistent with recent reports on the functionality of the enzymatic reactions of photorespiration in roots (Nunes-Nesi et al., 2014), considerable changes were seen not only in serine but also in glycolate within this tissue. However, given that the exact function of these reactions in root tissue is currently not established, the significance of this observation remains unclear. Second, a much clearer up-regulation of the TCA cycle intermediates and closely associated metabolites was observed, albeit only significantly in the strongest overexpressing line. Previous work on tomato lines exhibiting reduced expression of any of the TCA cycle enzymes revealed that this resulted in decreased root growth, most likely as a compound result of decreases in cell wall biosynthesis and an alteration in the balance of phytohormone levels (van der Merwe et al., 2009).

Peptide signalling in lateral organ separation

Additional phenotypes were caused by TAXI overexpression in the Ler background compared to Col-0, such as bending at a tertiary branch point and twisting of the stem. It has been reported that the Ler ecotype influences lateral organ phenotypes. For example, the Ler background possibly increases organ fusion between the cauline leaf and the axillary stem in lob mutants defective in the transcription factor LATERAL ORGAN BOUNDARIES (LOB) (Bell et al., 2012). LOB negatively regulates brassinolide (BR) biosynthesis in organ boundaries by activating the expression of the BAS1 gene encoding a BR-inactivating enzyme. Consequently, loss of LOB leads to hyperaccumulation of BR in the boundary (Bell et al., 2012). Similarly, hyperactivation of BR signalling in the bzl1-D mutant or BR treatment also leads to fusion of the cauline leaf to the axillary stem and is associated with bending of the primary stem (Gendron et al., 2012). The bzl1-D mutation constitutively activates the BZR1 transcription factor, capable of targeting the promoters of a plethora of genes, including CUC3 (Gendron et al., 2012; Guo et al., 2013). Accordingly, not only CUC3 expression, but also LOF1 expression was reduced in bzl1-D paraclade junctions by BR treatment (Gendron et al., 2012). Further work will be required to determine the relationship between TAXI overexpressing phenotypes and hormone signalling.

The lack of any obvious phenotype for a taxl loss-of-function mutant raises the possibility of functional redundancy or that overexpression of TAXI leads to ectopic receptor activation (Rowe and Bergmann, 2010; Torii, 2012). Notwithstanding, the presented work implicates the existence of a peptide signal cascade regulating lateral organ separation in Arabidopsis.

Supplementary data

Supplementary data are available at JXB online.

Supplementary Fig. S1. Distribution of the TAXIMIN peptide family in the plant kingdom.

Supplementary Fig. S2. Phenotypes of TAX1 overexpressing seedlings and flowering plants.

Supplementary Fig. S3. Fruit developmental series of Col-0 and TAXI overexpression lines.

Supplementary Fig. S4. Paraclade junction phenotypes in line OE-2 and Ler background with reduced penetrance.

Supplementary Fig. S5. Effects of constitutive overexpression of TAX2, TbTAX-His, or TAX1dSP in Arabidopsis thaliana.

Supplementary Fig. S6. Generation and characterization of tax loss-of-function lines.

Supplementary Fig. S7. TAXI and TAX2 expression.

Supplementary Table S1. Primers used in this study.

Supplementary Table S2. Reporting metabolite data presented in this study.

Supplementary Table S3. Primary metabolite profiling of Col-0 and TAXI overexpressing lines in leaf.

Supplementary Table S4. Primary metabolite profiling of Col-0 and TAXI overexpressing lines in root.

Acknowledgements

We thank Patricia Springer for providing seeds of lof1-lof2-1 and pLOF2-GUS; Paul Wirtmans, Amparo Cuéllar Pérez, Astrid Nagels Durand, and Robin Vanden Bossche for excellent technical assistance; Karel Spruyt for help with photography; and Aniek Bleys for help in preparing the manuscript. This work has been supported by funding from the Research Foundation-Flanders through the project G005312 and the Special Research funds from Ghent University and the National Research Foundation (NRF) from South-Africa for a North-South "Sandwich"-type postdoctoral scholarship to JC. LP is a postdoctoral fellow of the Research Foundation-Flanders.

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