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OCTOPUS, a polarly localised membrane-associated protein, regulates phloem differentiation entry in *Arabidopsis thaliana*

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SUMMARY

Vascular development is embedded into the developmental context of plant organ differentiation and can be divided into the consecutive phases of vascular patterning and differentiation of specific vascular cell types (phloem and xylem). To date, only very few genetic determinants of phloem development are known. Here, we identify OCTOPUS (OPS) as a potentiator of phloem differentiation. OPS is a polarly localised membrane-associated protein that is initially expressed in provascular cells, and upon vascular cell type specification becomes restricted to the phloem cell lineage. *OPS* mutants display a reduction of cotyledon vascular pattern complexity and discontinuous phloem differentiation, whereas *OPS* overexpressers show accelerated progress of cotyledon vascular patterning and phloem differentiation. We propose that OPS participates in vascular differentiation by interpreting longitudinal signals that lead to the transformation of vascular initials into differentiating protophloem cells.

KEY WORDS: Phloem, *Arabidopsis*, Cell differentiation

INTRODUCTION

The plant vasculature is a complex network that interconnects all plant organs. Within the vasculature, xylem and phloem cells are specialised for the transport of water and organic compounds, respectively. In the cotyledons of *Arabidopsis thaliana*, phloem and xylem are combined in vascular bundles. These are organised in a reticulate network with secondary veins branching from a central midvein and forming closed loops. In hypocotyls and roots, the vasculature shows a diarch pattern with two phloem poles and two xylem poles located in perpendicular planes (Busse and Evert, 1999).

The development of the vasculature starts during embryogenesis and repeats itself in every newly forming or growing organ. In a first step, which is referred to as vascular patterning, the position of the vasculature is laid down. During this process, provascular cells, uncommitted meristematic cells with the potential to develop into vascular cells, are specified within a homogenous population of undifferentiated cells (Esau, 1969). Subsequently, during the first steps of vascular differentiation, some of these provascular cells divide longitudinally and give rise to procambial cells, which divide to produce phloem and xylem precursor cells, thus functioning as meristematic vascular tissue (Esau, 1969; Scarpella et al., 2004). During the final stages of vascular differentiation, phloem and xylem precursor cells undergo distinct developmental programmes and terminally differentiate into mature phloem and xylem elements (Esau, 1969).

One factor involved in vascular patterning is auxin (Scarpella et al., 2010). Expression of the auxin efflux carrier PIN1 in leaves precedes and converges on sites of procambium formation, and the polar localisation of PIN1 suggests transport of auxin towards the developing vasculature (Scarpella et al., 2006). However, not all vascular patterning mutants show altered auxin transport or response, indicating that auxin-independent factors also play a role in this process (Candela et al., 1999; Carland et al., 1999; Carland et al., 2002). Notably, vascular development is embedded into a developmental context, as the formation of vascular strands usually occurs in growing tissues. In leaves, for example, the formation of vascular precursor cells arrests upon differentiation of the adjacent mesophyll cells, showing that vascular patterning can only occur within a tightly regulated developmental window (Scarpella et al., 2004).

The final differentiation of specific cell types from vascular precursor cells is not a synchronous process, but rather starts from distinct locations within the plant. For example, after germination, the differentiation of *Arabidopsis* protophloem precursor cells into mature protophloem sieve elements is initiated in two locations: the cotyledon midveins and the cotyledonary node. It then progresses from the midvein along the cotyledon veins and from the cotyledonary node towards hypocotyl and root until a continuous network of functional vascular cells is set up (Busse and Evert, 1999; Bauby et al., 2007). Later, metaphloem cells (i.e. metaphloem sieve elements and companion cells) differentiate progressively next to mature protophloem cells. These observations suggest the existence of inductive phloem differentiation signals that coordinate vascular differentiation by moving from differentiating phloem cells to trigger phloem differentiation in the next cells along the file. For xylem differentiation, such a signal has already been identified: the proteoglycan-like factor XYLOGEN can induce xylem cell differentiation in mesophyll cell cultures, and *Arabidopsis* plants that do not produce this factor display discontinuous xylem cell files (Motose et al., 2001b).

Although several mutants with defects in leaf vascular patterning have been isolated (Carland et al., 1999; Koizumi et al., 2000; Casson et al., 2002; Clay and Nelson, 2002; Steynen and Schultz, 2003; Alonso-Peral et al., 2006), only a very small number of mutants specifically impaired in phloem cell differentiation are known. As a consequence, our knowledge about the factors that control phloem development is very limited. *WOODEN LEG* (*WOL*) mutants are characterised by the complete absence of

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phloem cells in the root and lower hypocotyl of *Arabidopsis* seedlings (Scheres et al., 1995). WOL is required for the periclinal cell divisions in the root meristem that give rise to the phloem cell lineages. Consequently, the number of cells in the *wol* root stele is reduced, thus indirectly compromising the development of phloem tissue (Mähönen et al., 2000). The *altered phloem development* (*apl*) mutant shows a more specific defect in phloem differentiation: metaphloem cells are absent and protophloem cells develop characteristics of xylem cells (Mähönen et al., 2000; Truernit et al., 2008). These results suggest that APL promotes phloem identity and suppresses xylem identity in phloem cells (Bonke et al., 2003).

To identify new genetic determinants of phloem cell differentiation, we have previously isolated several genes that display expression during the early steps of phloem differentiation (Bauby et al., 2007). Here, we present a thorough analysis of the role of one of those genes, *OCTOPUS* (*OPS*). *OPS* belongs to a family of five *Arabidopsis* genes that share a domain of unknown function (DUF740) (Nagawa et al., 2006) and display no other known protein motifs. The 686 amino acid long OPS protein has a glycine-rich domain at its C terminus, but its structure and glycine content does not classify OPS as a glycine-rich protein (Sachetto-Martins et al., 2000). *OPS*-like genes are specific to higher plants and are present in all higher plants whose genome has been sequenced; however, at present we have no information about the expression patterns of these genes in species other than *Arabidopsis*.

Here, we show that *OPS* is expressed in provascular cells and, following cell type specific differentiation, *OPS* expression is restricted to the phloem cell lineage. *OPS* mutants display phloem developmental defects resulting in discontinuous phloem differentiation and reduced vascular pattern complexity. Overexpression of *OPS* leads to the opposite phenotype: increased vascular pattern complexity and premature phloem differentiation. These data clearly demonstrate a central role of *OPS* in phloem differentiation. Moreover, *OPS* acts as an integrator of vascular patterning and phloem differentiation, showing that these two processes are linked. Polar membrane localisation of OPS in provascular and phloem cells suggests its involvement in the inductive process that promotes protophloem differentiation.

MATERIALS AND METHODS

Plant material

If not stated otherwise, *Arabidopsis thaliana* ecotype Wassilevskaja was used as wild type. Other transgenic or mutant plant lines used were CycB1;1:uidA (Colon-Carmona et al., 1999), tmGFP9 (Stadler et al., 2005), ProATSUC2:GFP (Imlau et al., 1999) and ProPD1:GFPER (Bauby et al., 2007).

Isolation of *ops-1* and *ops-2*

ops-1 was isolated from the Versailles *Arabidopsis* promoter-trap collection (Bechtold et al., 1993). In this line, the promoter-trap construct was inserted as an inverted tandem repeat into the 5′ untranslated region of the intronless gene At3g09070, deleting a 16 bp fragment, including the first 12 bp of the coding region. *ops-2* (SALK_139316) was obtained from the SALK collection (Alonso et al., 2003). Insertion mutant information was from the SIGnAL website at http://signal.salk.edu. In this line, we identified the T-DNA insertion site after 614 bp of the At3g09070-coding region. Crosses between *ops-1* and *ops-2* gave rise to 100% of the F1 plants with short root phenotypes, confirming that the two lines were allelic.

RT-PCR was performed with primers binding after the T-DNA insertion sites: *OPS* primers (primer 1, TGACGCTTACTCAGGATCACTG; primer 2, TTCTTAGGTGAGTACCTTGAAC); *ACTIN* primers (primer 1, GGTGAGGATATTCAGCCACTTGTCTG; primer 2, TGTGAGATCCCGACCCGCAAGATC).

Growth conditions and plant transformation

Plants were germinated and grown in growth chambers (16 hours light, 8 hours dark, 200 μE m^{-2} s^{-1}, 20°C, 70% humidity) on media containing 0.5× Murashige and Skoog salt mixture (MS), 0.5 g/l 2-(N-morpholino) ethanesulfonic acid (MES) pH 5.7 and 0.7% agar. For plant transformation the *Agrobacterium tumefaciens* strain C58pMP90 was used. *Arabidopsis thaliana* was transformed by floral dip (Clough and Bent, 1998). At least 20 independent transformants were collected for each transformed construct.

Histochemical and histological analysis

GUS histochemical staining and mPS-PI staining were performed as described (Truernit et al., 2008). For live propidium iodide staining, propidium iodide (Molecular Probes, Eugene, USA) was used as a 10 μg/ml solution in water. Plants were stained for 5 minutes and imaged within 30 minutes. To study venation patterns, cotyledons were cleared in a chloral hydrate/glycerol solution.

For immunolocalisation 5- to 10 day-old *Arabidopsis* seedlings were fixed under vacuum in 4% paraformaldehyde, 0.5× MTSB (25 mM PIPES, 2.5 mM EGTA, 2.5 mM MgSO4, adjusted to pH 7 with KOH) and 0.1% triton for 1 hour. Samples were then washed with 0.5× MTSB, 0.1% triton for 10 minutes. For cell wall permeabilisation, samples were treated for 10 minutes with 80% methanol, washed with PBS and then digested (MES 25 mM pH 5.5, $CaCl_2$ 8 mM, mannitol 600 mM, pectolyase 0.02%, macerozyme 0.1%) for 30 minutes at 37°C. Samples were pre-incubated in 0.1% BSA/PBS for 20 minutes at room temperature and incubated with primary antibody (goat anti-Pin1 AP20, 1:200; Santa Cruz Biotechnology, sc-27163) for 16 hours at 4°C and for 1 hour at 37°C with the secondary antibody (Alexa 555 donkey anti-goat, 1:1000; Molecular Probes, A21432). After each antibody treatment, samples were washed for 10 minutes with glycine 50 mM/PBS. Samples were mounted in Citifluor/DAPI 20 μg/ml and observed with a confocal laser-scanning microscope.

Microscopy

For confocal microscopy, a Leica TCS-SP2-AOBS spectral confocal laser-scanning microscope (Leica Microsystems, Mannheim, Germany) was used. Excitation wavelengths were 405 nm for DAPI, 488 nm for GFP and propidium iodide, 514 nm for YFP and 543 nm for Alexa 555. For Fig. 1H, we used mPS-PI stained samples and the reflection mode of the confocal microscope to visualise GUS activity (Truernit et al., 2008). For light microscopy a Nikon Microphot-FXA microscope was used.

Plasmolysis experiment

Pro35S:OPSGFP and LTI6b (Cutler et al., 2000) plants were subjected to either water (control) or 0.8 M mannitol treatment for 10 minutes.

Root length and cell length measurements

For cell length measurements, images of mPS-PI stained samples were taken with the confocal microscope. Length measurements were performed on a Macintosh computer using the public domain NIH Image programme (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Constructs

Flanking sequences of T-DNA insertions were identified according to Liu et al. (Liu et al., 1995). For generating the promoter, complementation and overexpression constructs, the gateway system was used (Invitrogen). To generate *ProOPS:GUS* and *ProOPS:GFPER*, a 1880 bp promoter fragment was PCR amplified (5′ primer: ACAGTTTGTACAAAAAAGCAGGCTGCGGTGTAATCATTATTTCG and 3′ primer: ACCACTTTGTACAAGAAAGCTGGGTCGACGGGAAATGGTGGTTAAT) and cloned successively in pDONR207 and then in pBI-R1R2-GUS or pBI-R1R2-GFP (Bauby et al., 2007). For the overexpression construct *Pro35S:OPSGFP*, the *OPS*-coding region was PCR amplified (5′ primer, ACAAGTTTGTACAAAAAAGCAGGCTCCATGAATCCAGCTACTGACCC; 3′ primer, ACCACTTTGTACAAGAAAGCTGGGTGTCAATACAGCCTCATTACACT). The PCR products were cloned successively in pDONR207 and in PMDC83 (Curtis and Grossniklaus, 2003). For *ProOPS:OPS* and *ProOPS:OPSGFP*, promoter and gene were amplified

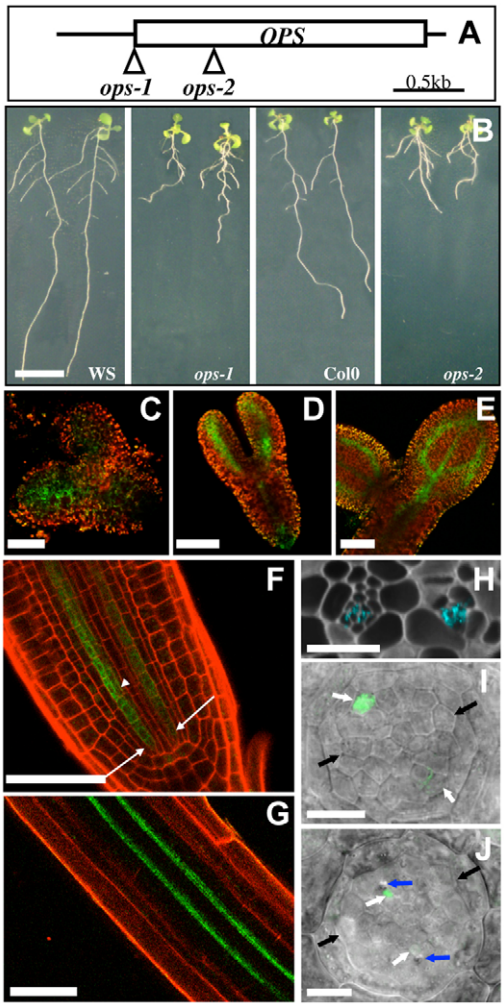

Fig. 1. Root phenotype of *OPS* loss-of-function mutants and expression pattern of *OPS*. (**A**) T-DNA insertion sites in *ops-1* and *ops-2*. (**B**) Phenotype of 10-day-old roots of *ops-1* with corresponding wild type WS, and of *ops-2* roots with corresponding wild type Col0. (**C-E**) Expression of *ProOPS:OPSGFPER* in embryos at (C) heart stage, (D) torpedo stage and (E) late torpedo stage. (**F**,**G**) Expression of *ProOPS:OPSGFPER* in roots. (F) Phloem cell files of a 5-day-old root meristem. Phloem initials are indicated with arrows; the asymmetric division of provascular cells is marked with an arrowhead. (G) Expression in the protophloem files in the differentiation zone. (**H**) Developmental progression of the expression of *ProOPS:GUS* in two cotyledon vascular bundles of a mature embryo. The vascular bundle on the right is not fully differentiated and expresses *ProOPS:GUS* throughout the provascular cells. The bundle on the left is more mature and shows expression only on the abaxial (i.e. phloem) side. (**I**,**J**) *ProOPS:OPSGFP* expression in root cross-sections: (I) in protophloem cells above the root meristem and (J) in metaphloem cells in the differentiated part of the root. The xylem pole is marked with black arrows; GFP expression is indicated by white arrows; differentiated protophloem cells are indicated with blue arrows. Scale bars: 50 μm in D-G; 1 cm in B; 10 μm in C,H-J.

(5′ primer, ACAGTTTGTACAAAAAAGCAGGCTGCGGTGTAATCATTATTTCG; 3′ primer, ACCACTTTGTACAAGAAAGCTGGGTGTCAATACAGCCTCATTACACT for *ProOPS:OPS*; ACCACTTTGTACAAGAAAGCTGGGTCATACAGCCTCATTACACTCC for *ProOPS:OPSGFP*). The PCR products were introduced into pDONR201 and then into pMDC99 or pMDC107 (Curtis and Grossniklaus, 2003). Homozygous single-insert lines were selected for all transgenic plants.

Accession Number

The Accession Number for *OCTOPUS* is At3g09070.

RESULTS

Identification of *octopus*

To identify genes that play a role during early phloem development, we screened the Versailles *Arabidopsis* promoter-trap collection (Bechtold et al., 1993) for *β-glucuronidase* (*GUS*) marker gene expression in differentiating phloem cells (Bauby et al., 2007). In this screen, we isolated line PD5, in which we found the promoter-trap construct inserted into the intron-less gene At3g09070 (Fig. 1A). PD5 had a short-root phenotype (Fig. 1B) that was linked to the T-DNA insertion and segregated as a monogenic recessive mutation. Both cell division in the root meristem and root cell elongation were impaired in PD5 roots (supplementary material Fig. S1A-C). Moreover, the roots at the root-hypocotyl junction grew out earlier than in wild type (not shown) and had almost the same length than the primary root (supplementary material Fig. S1A). Because of this characteristic root architecture with several short roots of nearly equal length, we named At3g09070 *OCTOPUS* (*OPS*) and PD5 *ops-1*. Introducing *OPS* under the control of its own promoter into *ops-1* rescued the root growth defect, thus demonstrating that loss of *OPS* function was indeed responsible for the mutant phenotype (supplementary material Fig. S2A). A T-DNA knockout line for *OPS*, *ops-2*, was also available (Alonso et al., 2003) (Fig. 1A,B). RT-PCR with RNA from *ops-1* and *ops-2* and primers binding after both T-DNA insertion sites showed a faint band in both mutant lines under saturating PCR conditions (supplementary material Fig. S1D). Therefore, we cannot fully exclude that residual *OPS* activity is present in the mutant lines. However, insertion of the T-DNAs in the beginning of the gene make it highly unlikely that a functional OPS protein is formed. Moreover, the recessive nature of the mutation excludes the possibility of a truncated OPS protein interfering with normal OPS function. *ops-1* and *ops-2* lines were allelic with respect to the root phenotype (see Materials and methods). We therefore decided to concentrate further phenotypic analyses on *ops-1*.

OCTOPUS is expressed in provascular cells and phloem initials

We have shown previously that the *OPS* promoter drives expression of reporter genes in protophloem cells and metaphloem initials of mature embryos (Bauby et al., 2007). Here, we looked at the developmental progress of *OPS* expression using plants expressing the gene for an endoplasmic reticulum localised GREEN FLUORESCENT PROTEIN (GFPER) or for GUS under control of the *OPS* promoter (Bauby et al., 2007). This promoter was also successfully used for complementation (see below) and thus could be reliably used for *OPS* expression analysis.

In all tissues analysed, the *OPS* promoter was active prior to phloem development in those cells that later gave rise to phloem cells. Upon specification and differentiation of protophloem and metaphloem cell types, promoter activity became restricted to those cells. This points towards an involvement of *OPS* in both the early events of vascular specification and in the differentiation of phloem cells.

In embryos, expression of *ProOPS:GFPER* was investigated from heart stage onwards. It was first seen in a relatively broad area delineating the position of the future vasculature (Fig. 1C). From torpedo stage onwards expression became restricted to the provascular cells of the embryo (Fig. 1D). From late torpedo stage

onwards marker gene expression in the cotyledon vasculature became abaxialized and thus restricted to the phloem precursor cells (Fig. 1E,H).

In seedling roots, vascular initials located adjacent to the quiescent centre (QC) produce new protophloem elements that differentiate at a distance from the meristem (Dolan et al., 1993; Mähönen et al., 2000; Bauby et al., 2007). Therefore, the root is a suitable organ to follow the stages of phloem development postembryonically. In the root, where xylem and phloem cell lineages are separated, we saw *GFP* expression only in the phloem pole, indicating that *OPS* is specific to the phloem developmental programme (Fig. 1F,G,I,J). Expression was already seen in the phloem vascular initials (Fig. 1F). These initials divide longitudinally at a distance from the QC and give rise to the protophloem cell lineage (Mähönen et al., 2000). After this asymmetric cell division, *OPS* promoter driven *GFP* expression was seen in both daughter cell files, but became restricted to the protophloem cell lineages two or three cells away from the asymmetric division (Fig. 1F,G,I). In the mature part of the root, where protophloem is differentiated, the *OPS* promoter was active in differentiating metaphloem cells (Fig. 1J).

Cotyledon vein complexity is reduced in *octopus*

As *OPS* was expressed in provascular cells, we checked if it plays a role in vascular patterning. Ten days after germination 69% of wild-type cotyledons displayed three or four completed vascular loops (n=202), while 73% of *ops-1* cotyledons had only two completed loops (n=167). In general, *ops-1* seedlings displayed a higher number of open loops than wild type, and we never found *ops-1* cotyledons with four completed vascular loops (Fig. 2A-C). mPS-PI staining (Truernit et al., 2008) confirmed that at the sites of non-closed loops it was not possible to detect any vascular cells (xylem, phloem or procambium) (supplementary material Fig. S3). This suggests that provascular cells failed to divide to give rise to procambial cells. Therefore, progression of vascular patterning was prematurely arrested or slowed down in *ops-1* mutants. Thus, OPS promotes the progression of vascular patterning.

Irregular early phloem differentiation in *octopus*

Because, upon vascular cell type-specific differentiation, *OPS* was expressed in the phloem cell lineage, we analysed whether OPS also specifically influenced phloem cell development. In cross-sections of *ops-1* seedlings, we did not see any alterations in phloem xylem ratios. This means that the radial differentiation of vascular tissue was not affected in *ops-1* (supplementary material Fig. S4). We next analysed the phloem cell files along their longitudinal axis. Protophloem cells specified during embryogenesis differentiate within the first three days after germination (Busse and Evert, 1999). Differentiation of the already specified protophloem cells in the basal part of the seedling is a gradual process starting from the upper part of the hypocotyl towards the root (Bauby et al., 2007). An integral part of the differentiation process is the thickening of protophloem cell walls, which is easily detectable in mPS-PI stained samples (Bauby et al., 2007). Interestingly, *ops-1* protophloem cell files differentiated discontinuously: in hypocotyls of 2-day-old seedlings, files of protophloem cells with thickened cell walls were interrupted by cells that did not display such cell wall thickening (Fig. 2F,G). To trace back the observed defect to the first specific steps of phloem development, we next looked at the phloem in mature *ops-1* embryos. In the mature embryo of wild-type plants, immature protophloem cells can be recognized according to their position and their characteristic elongated shape with bulging apical and basal ends (Busse and Evert, 1999; Bauby et al., 2007) (Fig. 2D). In *ops-1* embryos, cells that had failed to elongate and divide were found in an otherwise normally specified protophloem cell file (35/42 embryos) (Fig. 2E), thus demonstrating that already protophloem specification was impaired in *ops-1* embryos.

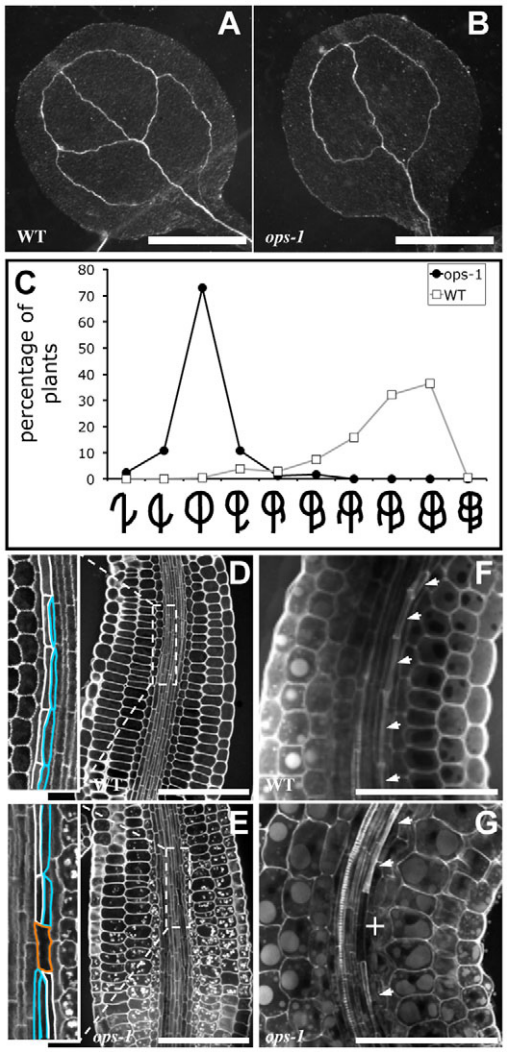

Fig. 2. Vascular phenotypes of *ops*. (**A**) Ten day-old wild-type (WS) cotyledon with four completed vascular loops. (**B**) Ten day-old *ops-1* cotyledon with two completed loops. (**C**) Percentage of plants that show the vascular patterns depicted on the *x*-axis (n=202 for WS, 167 for *ops-1*). WS vascular patterns are more complex. (**D**,**E**) Root-hypocotyl axis of mature embryos with detail of a phloem strand in the inset. Protophloem cells are outlined in white; their adjacent cells in blue. Supplementary material Movies 1 and 2 show the three-dimensional context of protophloem cells. (D) WS embryo showing a continuous specified protophloem cell file in the root-hypocotyl axis. (E) *ops-1* embryo shows unspecified cells (outlined in orange) within a specified protophloem cell file. Note also the higher number of starch granules. (**F**) WS seedling 2 days after germination. A continuous file of differentiated elongated protophloem cells with thicker cell walls can be seen in the hypocotyl (arrows). (**G**) *ops-1* seedling 2 days after germination. An undifferentiated cell (+) can be seen within the differentiated protophloem cell file of the hypocotyl (arrows). Scale bars: 100 μm in D-G; 1 mm in A,B.

Impaired phloem differentiation entry in *octopus* during root development

Approximately 2 days after germination, root meristems start dividing to produce new (i.e. postembryonic) cells (Bauby et al., 2007). To investigate whether irregularities in *ops-1* protophloem development occurred also in protophloem cells that were specified after embryogenesis, we looked at the protophloem cell files in roots after root meristem activity had started. Indeed, we also found irregular protophloem cell differentiation in the root tips of 5-day-old *ops-1* plants (Fig. 3A,B), showing that OPS is also important for protophloem differentiation after embryogenesis.

To confirm our observation with molecular markers, we crossed protophloem and metaphloem specific marker lines into *ops-1*. The *ProPD1:GFPER* marker line displays *GFP* expression in mature protophloem cells and in the metaphloem sieve tubes of wild-type roots (Bauby et al., 2007). In the *ops-1* background, this expression was interrupted by gaps at random positions (Fig. 3C-F). The same was observed for the metaphloem companion cell specific tmGFP9 marker line (Stadler et al., 2005) (Fig. 3G,H). Using the *ProPD1:GFPER* marker for quantification of the number of gaps in 10-day-old *ops-1* roots, we found on average 2.4 gaps per root protophloem file. Moreover, in about 40% of the *ops-1* roots, both protophloem cell files were interrupted at the same position. Approximately the same numbers were obtained when analysing the tmGFP9 marker in the *ops-1* background (Fig. 3I). Although a small percentage of wild-type roots also displayed interrupted *GFP* expression in one phloem cell file, the differences between *ops-1* and wild-type roots were extremely significant and we never found interruption of both files at the same time in wild-type roots.

We also analysed xylem cell files in 20 10-day-old roots that had been cleared and mounted in Hoyer's solution. *ops-1* proto- and metaxylem files displayed no differentiation gaps and were indistinguishable from wild type, demonstrating that the *ops-1* defect in roots was phloem specific (supplementary material Fig. S5).

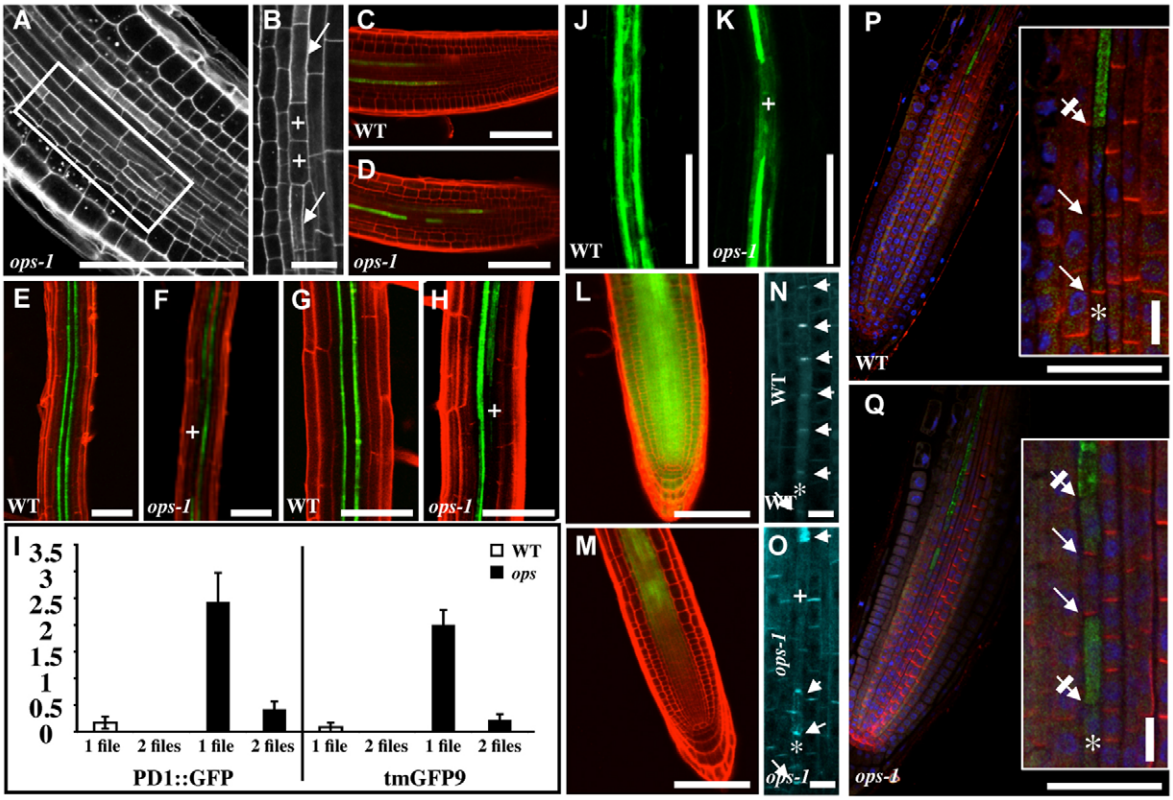

Fig. 3. Phloem discontinuity in *ops* phloem cells developing postembryonically. (**A**,**B**) Discontinuous phloem development in 5-day-old *ops-1* roots. Two cells in the protophloem cell file (+) do not develop protophloem characteristics. (**C-F**) *ProPD1:GFPER* mature protophloem marker gene expression in 5-day-old roots. (C) Two continuous *GFP*-expressing files in wild-type root tips. (D) Discontinuous expression in protophloem files of *ops-1* roots. (E) Continuity of *ProPD1:GFPER* marker gene expression in differentiation zone. (F) Discontinuity in corresponding *ops-1* roots. + indicates gap. (**G**,**H**) Metaphloem companion cell *tm9GFP* expression is continuous in wild type (G) and discontinuous in *ops-1* roots (H). + indicates gap. (**I**) Quantification of number of phloem file gaps per root (n=12 for each value) in 10-day-old roots using the *ProPD1:GFPER* marker and the *tmGFP9* marker. One file: gap in one phloem strand. Two files: gaps in both phloem strands at the same position in the root (P=0.007 for *ProPD1:GFPER* and P<0.001 for *tmGFP9*). Error bars are standard deviations. (**J-M**) Distribution of soluble GFP expressed under control of the *AtSUC2* promoter. (K) Leakage of GFP from a gap in the phloem files (+) of *ops-1* roots and control root without leakage (J). (L) Unloading of GFP in the root tip of a 5-day-old wild-type plant. (M) Less unloading occurs in *ops-1* roots. (J-M) Images were taken with exactly the same parameters. (**N**,**O**) Aniline Blue staining of developing sieve plates in the protophloem file (asterisk) of root tips. (N) Wild-type protophloem file. (O) In *ops-1* root tips, protophloem cells closer to the root meristem (bottom of picture) have already deposited callose, whereas cells in the gap (+) do not show this feature. (**P**,**Q**) Immunolocalisation of PIN1 (red), GFP fluorescence from *ProPD1:GFPER* construct (green) and nuclear stain DAPI (blue) in 5-day-old roots. The asterisk indicates the protophloem cell file in (P) wild type and (Q) *ops-1*. PIN1 is expressed in cells that did not develop into protophloem cells in *ops-1* (arrows), but not in the younger cell below that expresses the *PD1* marker (crossed arrows). The insets are magnifications of the region where *ProPD1:GFPER* starts to be active. Additional images can be seen in supplementary material Fig. S6. Scale bars: 100 μm in A,C-H,J-M,P,Q; 20 μm in B,N,O and in P,Q (insets).

To understand at which stage protophloem cell differentiation was arrested, we sought to identify earlier cytological hallmarks of the protophloem differentiation process. The transition from cell proliferation to elongation marks the initial stage of root cell differentiation. Once fully elongated, cells enter the maturation zone in which they differentiate into various cell types. The switch from cell division to elongation and differentiation occurs at slightly different points for each cell type (Ishikawa and Evans, 1995). For the root protophloem cell files, we previously showed that cell elongation and cell wall thickening occurred concomitantly (supplementary material Fig. S6A) (Truernit et al., 2008). Both processes did not take place in the gaps in the *ops-1* protophloem cell files, indicating an early slow-down of the differentiation process.

Additional events during the differentiation of protophloem cells are the deposition of callose in the transversal walls of adjoining sieve tube elements and disintegration of the nucleus (Esau, 1969). Both processes could be visualised in the elongating protophloem cells of 5-day-old *Arabidopsis* roots (supplementary material Fig. S6B-E). These events occurred close to the division zone of the root. By contrast, protoxylem elements differentiated much later in the zone where root hairs start to grow out from the epidermis (not shown), which suggests that root cell differentiation is regulated by a cell lineage autonomous process. Both, callose deposition and nucleus disintegration were not seen in the protophloem cell file gaps of *ops-1* roots (Fig. 3N,O; supplementary material Fig. S6H,I).

PIN-FORMED1 (*PIN1*) is expressed in immature root stele cells and its expression ceases as cells differentiate (Vieten et al., 2005; Dello Ioio et al., 2008). We therefore thought that expression of *PIN1* would be a good marker for non-differentiated cells. Indeed, in the root protophloem cell file, *PIN1* expression visualised with an anti-PIN1 antibody gradually decreased as protophloem cells differentiated (supplementary material Fig. S6D,E). In *ops-1* protophloem cell files, the cells that had not developed protophloem characteristics, and consequently did not express the *ProPD1:GFPER* protophloem marker, expressed *PIN1* (Fig. 3P,Q; supplementary material Fig. S6F-I), another indication that these cells did not undergo the protophloem developmental programme.

Taken together, cytological and molecular markers in *ops-1* roots confirmed that some cells in the phloem files remained largely undifferentiated and had not acquired phloem cell identity. The defect was phloem specific, as no discontinuity was found in the xylem cell files of *ops-1* roots.

OCTOPUS is impaired in phloem long-distance transport

The phloem being the major route for long-distance transport of photosynthates and signalling molecules, we expected to find evidence for shoot-to-root transport problems in *ops-1* due to the gaps in the phloem cell files. To assess this, we crossed the *ProAtSUC2:GFP* marker line into the *ops-1* background. Plants transgenic for *ProAtSUC2:GFP* produce soluble GFP under control of the metaphloem companion cell-specific *AtSUC2* promoter (Truernit and Sauer, 1995; Stadler and Sauer, 1996). Soluble GFP moves with the solute stream first from companion cells to sieve elements and then exits the phloem in sink tissues, thus reflecting solute transport (Imlau et al., 1999). Wild-type plants expressing the *ProAtSUC2:GFP* marker display brightly fluorescent root tips due to GFP unloading in this tissue (Fig. 3L). By contrast, root tips of *ops-1* plants expressing the marker were markedly less fluorescent (Fig. 3M), indicating that less solutes were transported into the root tips of *ops-1* mutants. A closer look at the gaps in the phloem files of these plants showed that in these areas GFP was leaking out of the phloem cells (Fig. 3J,K). We also noticed higher starch accumulation in mPS-PI-stained *ops-1* embryos. This may be another evidence for malfunctioning of solute allocation in *ops-1* (see, for example, Fig. 2E).

Overexpression of *OCTOPUS* leads to premature phloem differentiation and increased cotyledon vein complexity

To investigate whether OPS was sufficient to promote phloem differentiation, we generated plants expressing *OPSGFP* under control of the constitutive *35S* promoter (Odell et al., 1985) and analysed three independent transgenic lines. Although we did not find the formation of ectopic phloem in the *OPS* overexpressers, remarkably those plants showed a phloem phenotype opposite to the *ops-1* phenotype. In the hypocotyls of mature *Pro35S:OPSGFP* embryos, protophloem cells were prematurely elongated (Fig. 4E-

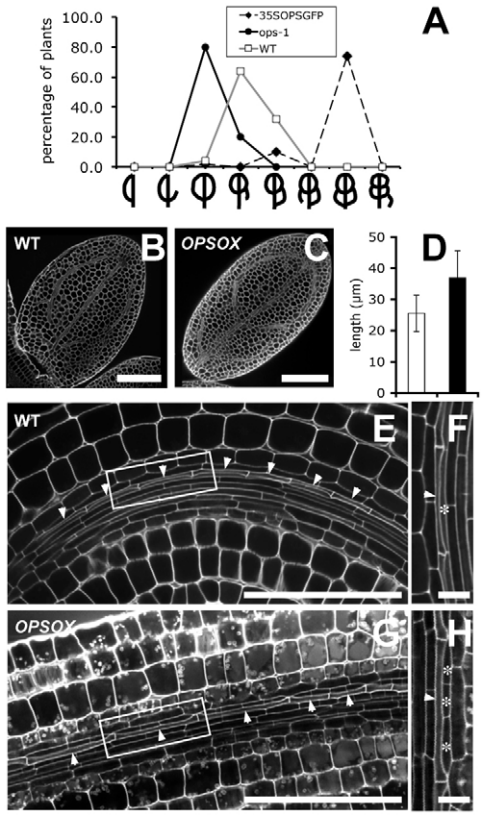

Fig. 4. *OPS* overexpression leads to premature vascular differentiation. (**A**) Percentage of mature embryo cotyledons that show the vascular patterns depicted on the *x*-axis. Overexpression of *OPS* leads to higher complexity (*n*=47 for wild type, 50 for overexpresser and *ops-1*). Data are from mature embryos and not from 10-day-old seedlings as in Fig. 2. (**B**,**C**) Cotyledons of mature wild-type (B) and *Pro35S:OPSGFP* (C) embryos. Average length of hypocotyl protophloem cells of wild type (white bar, *n*=72) and *Pro35S:OPSGFP* (black bar, *n*=60) in mature embryos. Error bars are standard deviations. (**E**,**G**) Protophloem cells in the hypocotyl of wild-type (E) and *OPS* overexpressing (G) embryos. (**F**,**H**) Magnifications of the rectangles shown in E,G. Protophloem cells are labelled with arrowheads; the adjacent cells with asterisks. Protophloem cells are longer and adjacent cells have already divided in *OPS* overexpressers. Scale bars: 100 µm in B,C,E,G; 10 µm in F,H.

H) and the cells located adjacent to the protophloem cells had already divided in all 10 embryos analysed. At this stage, these cells normally only start dividing (Bauby et al., 2007). In some embryos, the adjacent cells had even divided three times, which is usually not the case in wild type (Bauby et al., 2007) and which may indicate that the elongation of protophloem cells drives division of adjacent cells. We used one transgenic line for measurements of protophloem cell length (Fig. 4D). While in mature wild-type embryos the average length of protophloem cells was 25.5±6 μm (n=72), the average length of protophloem cells in the overexpresser was 36.8±9 μm (n=60) (P<0.0001).

To investigate whether vascular patterning was also advanced, we looked at the cotyledons of mature embryos. We used embryos instead of 10-day-old seedlings (unlike in Fig. 2) because in the mature embryo vascular patterning is not completed and therefore a premature complexity of vascular pattern is easier to visualise. Indeed, 74% (n=50) of the overexpressing lines already had four completed loops at the embryo stage, whereas 96% (n=47) of wild-type cotyledons had only 2.5 to 3 completed vascular loops. By contrast, vascular patterning in *ops-1* was already slightly delayed with 80% of cotyledons displaying only two completed vascular loops (n=50) (Fig. 4A-C). Together, these phenotypes provide more strong evidence for a role of OPS in driving phloem development and vascular patterning.

OCTOPUS is a membrane-associated protein with polar localisation

To learn more about OPS function, we generated a translational fusion of *OPS* with *GFP* and expressed it under the control of the *OPS* promoter (*ProOPS:OPSGFP*). In six independent *ops-1* lines, homozygous for the *ProOPS:OPSGFP* construct, root growth was partly or fully restored (supplementary material Fig. S2A). In addition, we did not find vascular patterning defects in the cotyledons (supplementary material Fig. S2B), nor irregular phloem development in embryos of those plants that showed full restoration of root growth (not shown). This shows that the OPSGFP fusion protein is functional. GFP fluorescence in *ProOPS:OPSGFP* plants was generally much weaker when compared with the ER-localised GFP fluorescence of the promoter fusion described earlier (Fig. 1), pointing towards a high turnover of the protein. Interestingly, OPSGFP showed polar plasma membrane associated localisation in provascular and phloem cells (Fig. 5A,B). Immunolocalisation with anti-PIN1 antibodies demonstrated that OPSGFP was located at the apical end of phloem cells, opposite to PIN1, which is known to be located at the basal end of root stele cells, including the protophloem cell file (Galweiler et al., 1998; Blilou et al., 2005) (Fig. 5C-E).

To confirm OPS membrane localisation, we performed a plasmolysis experiment with *Pro35S:OPSGFP* plant roots. We noted that OPSGFP was also polar localised in other cells of the root of these plants, suggesting that a general mechanism is responsible for addressing OPS to one side of the membrane. During plasmolysis, the OPSGFP fusion (Fig. 5F,G) behaved identically to the well-established plasma membrane GFP marker line LTI6b (Cutler et al., 2000) (supplementary material Fig. S7A,B), demonstrating that OPSGFP was indeed membrane associated.

DISCUSSION

In spite of the important function of the phloem, to date we know little about the genetic determinants of phloem cell differentiation. In this work we show that *OPS* plays a role in this process. In the absence of *OPS*, random cells within a file of root protophloem cells fail to acquire protophloem cell identity. Overexpression of *OPS*, however, leads to the opposite phenotype, i.e. accelerated phloem differentiation. These data show that *OPS* is required for proper differentiation of protophloem cell files and is sufficient to promote cell type-specific differentiation of cells that are programmed to develop into protophloem cells. Although loss-of-function of several genes has been shown to result in the formation of discontinuous vascular networks (Sieburth et al., 2006), *ops-1* represents a novel class of mutant, which is only affected in phloem continuity while xylem strands differentiate normally.

After a vascular pattern is set up, the cell-type specific differentiation of phloem and xylem strands is an inductive process that starts from distinct locations within the plant body (Esau, 1969; Bauby et al., 2007). Therefore, for proper vascular development, radial and longitudinal signals are required. Although radial signals determine the position of the vasculature within a plant organ, inductive cell type-specific differentiation suggests the existence of longitudinal signals that coordinate vascular development by moving from cell to cell along the developing vascular strands to ensure continuity of the network. For phloem development, the existence of such a signalling pathway has not been shown yet. However, the identification of XYLOGEN has demonstrated that such short-range signals exist for xylem development (Motose et al., 2001b). In planta, XYLOGEN is most likely secreted in a polar manner from developing xylem elements to induce xylem differentiation in the neighbouring cell. Interestingly, several parallels exist between our results and those obtained for the *XYLOGEN* genes. First, differentiation of both phloem and xylem

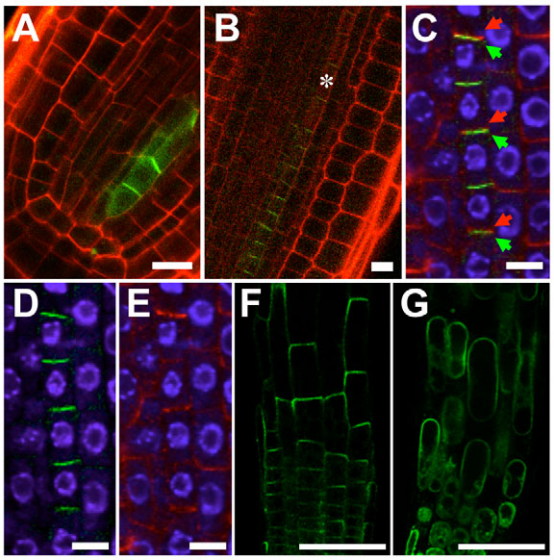

Fig. 5. Polar localisation of OPS. (**A**) Propidium iodide-stained root tip with localisation of *OPS* promoter driven OPSGFP to the apical end of the protophloem precursor cells. (**B**) Polar localisation of OPSGFP in a protophloem cell file (asterisk) of the root. (**C-E**) Immunolocalisation of PIN1 (red colour), fluorescence of OPSGFP driven by the *OPS* promoter in the protophloem cell file (green colour) and staining of nuclei with DAPI (blue) showing OPSGFP localised to the apical side (green arrows in C), whereas PIN1 is located at the basal side (red arrows in C) of the protophloem cells. (C) An overlay image of D,E. (**F**,**G**) Plasmolysis experiment showing that OPSGFP is associated with the plasma membrane. (F) Before plasmolysis; (G) after plasmolysis. Scale bars: 10 μm in A-E; 100 μm in F,G.

cells is an inductive process (Motose et al., 2001a; Bauby et al., 2007). Second, loss of function of the two redundant *XYLOGEN* genes in *Arabidopsis* leads to discontinuous xylem development. Last, XYLOGEN is polarly localised in xylem elements (Motose et al., 2004). Therefore, the identification of *OPS* may be the first step towards discovering a mechanism that ensures the coordinated differentiation of phloem strands, similar to what happens during xylem differentiation.

We found OPS to be associated with the plasma membrane at the apical end of provascular and protophloem cells. Membrane association of OPS was also independently found by others (Benschop et al., 2007). The mode of OPS plasma membrane association remains to be established. OPS has several putative palmitoylation sites determined by CSS palm2.0 (Ren et al., 2008) and therefore may be inserted into the apical phloem cell membrane by a palmitoyl anchor. Alternatively, or in addition, it may interact with another protein that is inserted into the membrane. A few plasma membrane integral or associated polar localised proteins have been described in plants. They play a role in the establishment of cell polarity and/or are involved in cell-cell communication by transporting or generating signalling molecules (Fu and Yang, 2001; Swarup et al., 2001; Motose et al., 2004; Vieten et al., 2005; Dong et al., 2009; Humphries et al., 2011; Wu et al., 2011). The OPS protein does not have any known functional domains that could give evidence of its role. Nevertheless, the *ops* phenotype, together with OPS localisation, make it likely that OPS is involved in the events that lead to inductive phloem differentiation. We propose that *OPS* loss of function reduces or delays the ability of a cell to respond properly to phloem differentiation signals within a given time window during which the already specified cells are sensitive to such signals (Fig. 6). Thus, although stochastically some cells in the vascular cell file would not reach a specific threshold that directs them towards phloem differentiation, the longitudinal differentiation signal would still be transported correctly across this cell towards the next cell in the file. The relatively mild phenotype of *ops* may have several reasons. (1) *ops-1* and *ops-2* may not be complete knockouts. (2) *OPS* belongs to a family of five genes whose expression patterns in seedlings have been described elsewhere (Nagawa et al., 2006). Although the other genes all seem to have somewhat broader or vascular unrelated expression patterns, there may still be redundancy within the gene family. This will be investigated. (3) OPS may be part of a backup system that guarantees integrity of the phloem network. The same holds true for XYLOGEN: although it has been demonstrated that XYLOGEN can induce xylem cell differentiation, loss of XYLOGEN does not result in a complete loss of xylem development but rather in a xylem phenotype that is similar to the *ops-1* phloem phenotype (Motose et al., 2004).

For our characterization of *ops-1* mutants, we have concentrated on two organs, roots and cotyledons. In cotyledons, protophloem and protoxylem cells originate from the same provascular cells, whereas in the active root meristem these cell types develop from separate initials surrounding the QC (Dolan et al., 1993). In developing cotyledons, *OPS* is expressed in provascular cells before xylem and phloem cell fates are morphologically distinguishable. However, in the root meristem where phloem and xylem cell lineages are spatially and ontogenically separated, *OPS* expression is restricted to the phloem cell lineage. This indicates that also during cotyledon provascular development OPS is likely to be specific to phloem differentiation and therefore must act downstream of early phloem specification signals. More evidence to support this hypothesis comes from *OPS* misexpression studies:

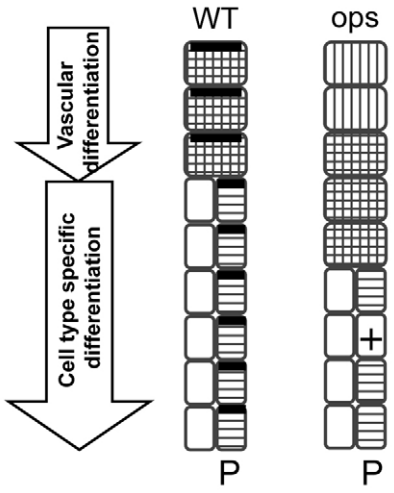

Fig. 6. Model of OPS action. OPS (black rectangles) interprets vascular differentiation signals that ultimately lead to phloem differentiation. In the absence of OPS, the first steps of vascular differentiation are generally slowed down. During phloem-specific differentiation, a crucial threshold for differentiation is not reached in some phloem cells (+). P, phloem cell lineage.

overexpression of *OPS* can accelerate protophloem development but it does not change the developmental fate of other cell types that have not been programmed to become protophloem cells.

The difference of phloem ontogeny in cotyledons and roots can explain the differences in vascular phenotypes we see in these organs. Although roots of *ops-1* and *OPS* overexpressers display phloem specific defects, in cotyledons xylem development also seems to be affected. As *OPS* is already expressed in cotyledon provascular cells, we cannot fully exclude the possibility that *OPS* plays a separate role in provascular development, similar to the role it plays during protophloem development. However, as in leaves, vein pattern formation terminates when mesophyll cells start to differentiate (Scarpella et al., 2004); a general slowdown of vascular development due to loss of *OPS* function (and thus a slowdown of protophloem differentiation) could result in the reduction of venation pattern complexity seen in *ops-1*, and thus *OPS* would indirectly affect provascular development. However, if protophloem development is accelerated due to overexpression of *OPS*, this would positively feed back to vascular patterning and result in an acceleration of both, xylem and phloem development. Our observations strongly suggest that vascular patterning and vascular cell type specific differentiation are linked and that *OPS* is one of the integrators of these processes (Fig. 6). A link between vascular patterning and vascular cell type specification is also supported by the observation that *XYLOGEN* mutants display reduced vascular complexity (Motose et al., 2004).

In roots, *OPS* was expressed earlier than other known protophloem markers, such as the *APL* gene (Bonke et al., 2003) or J0701 (Mähönen et al., 2000), which only start being expressed at a distance from the QC. Protophloem cells in *apl* plants initially develop normally and only show characteristics of xylem cells about two days after germination (Truernit, 2008). It is therefore likely that OPS acts upstream of APL in protophloem development. However, this needs to be demonstrated.

OPS was also found in an independent screen for vascular expressed genes (Nagawa et al., 2006). Nagawa and co-authors did not report any mutant phenotype for this gene, which may be

due to the relatively inconspicuous short-root phenotype of *ops* during the first days of plant growth. In all probability, *ops* root growth defects are the indirect result of impaired phloem function. Using phloem mobile GFP, we showed that indeed the phloem solute stream is reduced in *ops-1* and GFP is leaking out of the gaps in the phloem files. Solutes such as sucrose most probably will leak out in the same way and then will be taken up again by sucrose transporters expressed in the next functional phloem cell down the phloem strand. Phloem transport of signalling molecules may also be altered in *ops*. Impaired phloem transport into root tissue was shown to lead to a root system architecture similar to that of *ops* roots (Ingram et al., 2011). Alternatively, improper protophloem development may also have a more direct developmental effect on root growth. Protophloem cell files in the root meristem have been demonstrated to be the source of a yet unknown signal that promotes early root meristem growth (Scacchi et al., 2010).

Taken together, we propose a model in which OPS is involved in the process that leads to the differentiation of a continuous phloem network. Disruption of OPS function slows phloem differentiation, resulting in some cells in the root protophloem cell file that fail to develop into mature protophloem cells. As differentiation of the tissue surrounding the vasculature progresses in *ops* cotyledons, a crucial developmental window will be missed during which vasculature patterning can be completed (Fig. 6). *OPS* is the first phloem mutant described that displays more specific and subtle defects in phloem development and that is still viable and can produce seeds. It therefore represents a novel entry-point into the identification of the factors that control phloem development.

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Competing interests statement
The authors declare no competing financial interests.

Author contributions
E.T., H.B. and J.-C.P. designed experiments. E.T., H.B., J.B., K.B. and J.-C.P. performed experiments and analysed the data. E.T. and J.-C.P. wrote the manuscript.

Supplementary material
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