Protophloem Differentiation in Early Arabidopsis thaliana Development

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During Arabidopsis embryogenesis, procambial cells undergo coordinated, asymmetric cell divisions, giving rise to vascular precursor cells (protophloem and protoxylem precursors). After germination, these cells terminally differentiate into specialized conducting cells, referred to as protophloem and protoxylem cells. Few readily identifiable markers of the onset of specification and differentiation are available, hampering the molecular genetic analysis of protophloem development. Confocal microscopy was used to investigate the patterning and differentiation of phloem cells during early plant development. Longitudinal divisions of phloem initials allowed the identification of protophloem precursor cells and adjacent metaphloem initials along the length of the plant. During germination, protophloem differentiation was observed at two independent locations, in the cotyledons and the hypocotyl. In both locations, differentiation was concomitant with cell elongation. We identified five gene-trap lines (PD1–PD5) with marker gene expression in immature protophloem elements. The spatio-temporal marker expression pattern of the lines divides them into two groups. The early specification markers PD4 and PD5 were expressed in developing organs before procambium formation and then became restricted to phloem initial cells. The protophloem precursor markers PD1–PD3 were expressed in differentiating protophloem cells at different stages of their development. All markers were expressed transiently and iteratively during the differentiation of protophloem in newly formed organs. Flanking genes were identified for four out of five gene-trap insertion lines. The possible function of these genes with respect to phloem differentiation is discussed.

Keywords: Phloem — Differentiation — Development — Vascular tissue — Arabidopsis.

Abbreviations: AB, aniline blue; DAG, days after germination; GFP, green fluorescent protein; GPI, glycosylphosphatidylinositol; GUS, β-glucuronidase; HAG, hours after germination; PD, phloem differentiation; PIP, 4,5-kinase, phosphatidylinositol phosphate-4,5-kinase; PP, protophloem precursor.

Introduction

The development of a flexible and efficient vascular system in higher plants was probably one of the most significant improvements leading to the conquest of land by terrestrial plants. The vascular system connects plant organs separated by distances of up to several meters and provides for the transport and allocation of water, nutrients and signaling molecules. Vascular strands are composed of specialized elongated cells: the xylem, phloem and meristematic vascular cells. These cell types form a continuous network from roots to shoots (Esau 1969).

Clonal fate and positional information control plant organization. During Arabidopsis embryogenesis, procambial cells are characteristically arranged in continuous strands. They acquire their elongated shape through coordinated cell divisions, oriented parallel to the axis of the emerging strand (Berleth et al. 2000). At the end of embryogenesis, asymmetric divisions occur at particular locations in the procambium, generating several files of cells that become precursors of phloem and xylem cells (Scheres et al. 1995). In the mature embryo, the specialized conducting cells are specified but not yet fully differentiated. In Arabidopsis ecotype Ler, terminal differentiation of the first vascular elements occurs within 3 d of germination (Busse and Evert 1999).

The mechanisms by which certain procambial cells are selected for phloem differentiation remain unclear. However, the stability of the patterning process in most species suggests that positional cues and growth conditions are of critical importance. The molecular details of the specification of phloem and xylem cells are particularly intriguing, as the underlying mechanism must reconcile the stringent requirement to keep all parts of the vascular system interconnected with the constraints imposed by the developmental pattern of the various organs.

Vascular tissue patterning models have been developed from examining natural variability and mutant phenotypes (Carlsbecker and Helariutta 2005). These mutants have
provided evidence for the involvement of sterols (Clouse et al. 1996, Carland et al. 2002, Cano-Delgado et al. 2004), small peptides (Casson et al. 2002), cytokinin (Mahonen et al. 2000, Inoue et al. 2001) and auxin (Hardtke and Berleth 1998, Deyholos et al. 2000, Hamann et al. 2002) in vascular patterning. However, very few vascular pattern mutants have so far been identified, possibly due to the difficulties in visualizing vascular tissue.

Mutant descriptions and screens, and molecular genetic analyses of cell patterning processes are dependent upon the proper identification of cell types and differentiation stages. The genetic dissection of cell identity acquisition in internal tissues is possible if distinct cell states can be distinguished by identifying cell type characteristics and/or genetic markers of cell fate. If gene expression profiles associated with specific differentiation stages are identified, it should be easy to mark individual cell states along vascular differentiation pathways. However, most gene expression profiles analyzed to date are associated with the terminal differentiation stage of various vascular cell types, and very few mark the early stages of phloem formation. Two mutants specifically involved in phloem formation have been identified. The wol mutant generates too few procambial cells and is therefore affected in the specification of phloem tissue in the axis (root/hypocotyl) (Mahonen et al. 2000). In the apl mutant, protophloem determination seems to be correct but sieve elements are modified. Instead, apl mutants develop a hybrid cell type with a xylem-like structure (Bonke et al. 2003).

To gain more insight into the pattern and the process of phloem development, we have identified characteristic cellular features of protophloem precursors that are suitable for rapid and high-resolution confocal microscopic analysis. We have assigned cytological and molecular markers to specific stages of protophloem development and followed their progression in the early stages of Arabidopsis development. Protophloem cell files are organized in a longitudinal and radial pattern that shows organ-specific features. Screening and identification of gene-trap lines with marker expression in immature protophloem elements have validated our results and provide new data for the analysis of spatial and temporal specificities of phloem formation.

Results

In order to characterize the organization and the process of phloem development, we monitored early phloem patterning and differentiation in Arabidopsis thaliana (ecotype Ws). Studies of the vascular tissue in plants are limited by difficulties obtaining access to internal plant tissues by classical microscopic techniques. The use of an aniline blue (AB) staining technique (Bougourd et al. 2000) in combination with confocal laser scanning microscopy enabled us to study the differentiation state of phloem cells in mature embryos and young seedlings of A. thaliana. Confocal microscopy allowed us to collect stacks of optical longitudinal sections (Z-stacks). With this, phloem strands could be followed directly along the longitudinal axis of the plant without the need for transversal sectioning.

Radial and longitudinal pattern of protophloem elements in the mature embryo

The mature embryo of A. thaliana is composed of two cotyledons and a root/hypocotyl axis. The cotyledons show a bilateral symmetry in contrast to the radial symmetry of the axis. Thus, we defined two planes of organization in the mature embryo: a frontal plane that splits the cotyledons into two, and a perpendicular sagittal plane (Fig. 1A). In the axis of 20 embryos tested, xylem and phloem were composed of two poles organized in bilateral symmetry. The radial organization of the poles could be inferred from the position of the cotyledons. The future xylem poles were located in the frontal plane and the phloem poles were located on both sides of the xylem plate in the sagittal plane.

To study the differentiation state of phloem cells in the mature embryo, high-resolution confocal imaging (Bougourd et al. 2000) was utilized. Embryos were stained with AB and cleared with chloral hydrate. Detailed observation of one phloem pole in the median part of the hypocotyl indicated that this pole consisted of at least two different cell types (Fig. 1B, C; Supplementary Movie SM 1). One cell type was elongated and bone-shaped, with a thinner middle section and bulging apical and basal ends. Based on cell shape, spatial relationship to other procambium and pericycle cells, and the known locations of protophloem cells at later stages of development (Esau 1969, Busse and Evert 1999, Baum et al. 2002), we identified cells of this type as immature protophloem cells or protophloem precursors (PPs). The nucleus of these PPs was brightly fluorescent in AB-stained samples, indicating the undifferentiated state of these cells (Fig. 1C). A second cell type was observed in close proximity to the PPs, molding itself to the curves of these cells (Fig. 1C). We identified these cells as metaphloem initials. These cells continued to divide and gave rise to functional metaphloem cells.

By studying stacks of longitudinal sections, we were able to trace both cell types back along the root/hypocotyl axis. An initial cell located above the quiescent center undergoes a first longitudinal division, giving rise to the phloem initial and to a more internal cell. This phloem initial then undergoes a second longitudinal division, 3–5 cells above the quiescent center (Fig. 1D). A third longitudinal division in the vicinity of the collar then generates the PP and the metaphloem initial.
Fig. 1  Organization of the vasculature in the mature Arabidopsis thaliana embryo (Ws). (A) Scheme of the symmetry planes (frontal and sagittal) of a mature embryo of A. thaliana. (B–G) Longitudinal section of AB-stained mature embryos of wild-type A. thaliana, showing phloem organization. (B) Hypocotyl. (C) Magnification of an immature protophloem cell (bone-shaped, white arrow) and its adjacent metaphloem initial (white arrowhead). (D) Root and collar. The black arrowheads show the consecutive longitudinal cell divisions that give rise to protophloem and metaphloem precursor cell files. (E) Upper hypocotyl and cotyledonary node showing the branching of immature phloem cells at the transition zone. (F) Abaxial face of the cotyledon, showing a central immature protophloem cell (white arrowhead) and two flanking derivative cells (black arrowheads). (G) Cell wall thickening of protophloem precursors is observed in developing seedlings (black arrows). QC, quiescent center; P, pericycle; C, cortex; EN, endodermis; EP, epidermis. Scale bars = 10 μm.
These longitudinal divisions are not strictly oriented in the same plane. Therefore, cells adjacent to the PPs may be either internal or external to the precursors (Fig. 3B, E). Taken together, PPs initiate at the root/hypocotyl junction and are not yet specified in the embryonic root.

The organization of the phloem and xylem changes significantly in the cotyledonary node. The two strands of vascular tissue join to form a collateral vascular bundle in the cotyledons (Esau 1969). We visualized this structure by following the file of PPs in the cotyledonary node in stacks of longitudinal optical sections. Each protophloem strand was seen to split into two strands, with one strand supplying one cotyledon and the other strand supplying the other cotyledon (Fig. 1E). Thus the two initial PPs strands in the axis produce four strands, with two strands leading into each cotyledon. Both strands of phloem were visible in each cotyledon until the branching of the distal loop of secondary veins. Beyond this branching point, only one strand of protophloem cells was generally observed in the midvein (Fig. 1G). This strand was located on the abaxial side of the cotyledon and was accompanied by two adjacent cells. The three cells were tightly linked, and were probably generated by two longitudinal divisions during late embryogenesis (Fig. 1F). The specification of PPs was completed in the midvein and in the distal loops forming the secondary veins. Usually, PPs in the proximal loops were not yet completely specified.

Size distribution of PPs reflects the organ-dependent differentiation stage

One of the characteristics of differentiating protophloem cells is their ability to elongate and to give rise to a functional conducting unit. Thus, we hypothesized that cell length would be a relevant parameter of the differentiation state. Therefore, we determined the average length of PPs/phloem initials in each organ of five mature embryos (Fig. 2A). PP size repartition shows that the most elongated cells are located in the cotyledons, with longer cells in the midvein than in the distal loop. Shorter cells were present in the hypocotyl and even shorter phloem initials in the root. Along the root/hypocotyl axis, PP cell sizes increased from the collar to the upper middle part of the hypocotyl (Fig. 2B). Cells were shorter at the cotyledonary node. The increase of phloem cell size coincided with the acquisition of the characteristic bone shape. No correlation was observed between size and inverted position of protophloem cells. These results suggest that cell length is a relevant parameter for the study of protophloem differentiation.

Initiation and progression of vascular tissue differentiation during early seedling development

As we showed that PPs are not in the same differentiation state in the different organs of the mature embryo, we determined the kinetics of their differentiation in 20 synchronized seedlings at 24, 48 and 72 h after germination (HAG). In particular, we focused on three consecutive features of differentiation that we could identify in AB staining: (i) cell elongation; (ii) cell wall thickening; and (iii) loss of the nucleus coinciding with a bright fluorescence of the cell content. In parallel, we followed the progression of protoxylem differentiation (secondary cell wall thickening of xylem cells). At 24 HAG, protophloem precursors in the cotyledons displayed cell wall thickening all along the midvein and the distal loop. No nucleus was visible in these cells. In the middle/upper part of the hypocotyl, PPs were highly fluorescent (Fig. 3A, B). The fluorescent cells also exhibited thickened cell walls (Fig. 3A, C). In the lower part of the hypocotyl, PPs were still nucleated, less elongated and did not exhibit fluorescence. No major modifications were detected in the embryonic root. At the same time, metaphloem initials were already elongated in the cotyledons. In the hypocotyl, whilst protophloem cells elongated, metaphloem initials divided transversally, rapidly doubling in number along the axis (Fig. 3A). Annular cell wall thickening of tracheary elements started at the proximal midvein at the adaxial side of the cotyledon (Fig. 3D). No secondary cell wall thickening of protoxylem was detected in the hypocotyl. These observations were consistent with previous descriptions of differentiating protoxylem (Dharmawardhana et al. 1992; Pyo et al. 2004) (see also Supplementary Movie SM2).

At 48 HAG, protophloem cell contents in the hypocotyl and the cotyledons were no longer fluorescent (Fig. 3E). In the root, meristem activity increased and procambial cells and phloem initials divided basipetally (data not shown). Phloem initials gave rise to PPs that started to differentiate. Thus, protophloem differentiation now progressed basipetally from the root meristem. In parallel, secondary cell wall thickening of protoxylem was detected in the middle/upper part of the hypocotyl.

At 72 HAG, differentiated protophloem cells appeared completely dark in most parts of the seedling (Fig. 3F). In the root, protophloem still differentiated basipetally, as demonstrated by progressive cell wall thickening and cell content darkening. Xylem cells were differentiating distally from the phloem cells (Fig. 3G).

Taken together, these results suggest that protovascular elements are specified and begin to differentiate earlier in the cotyledons than in the axis. Protophloem and protoxylem differentiation was first observed in the midvein and then extended to the distal loops and the cotyledonary node (Fig. 6A1, A2). A second independent locus of differentiation was observed in the upper part of the hypocotyl. From there, protophloem differentiation progressed towards the root (Fig. 6A2–A4). Later, a third
starting point of differentiation was observed coming from the root meristem (Fig. 6A5).

Identification of phloem markers by gene-trap screening

In order to identify reporter gene expression profiles suitable for the study of phloem development and its genetic determinism, we screened the Versailles collection of Arabidopsis gene-trap mutants for plant lines expressing the uidA reporter gene in immature vascular tissues (Bechtold et al. 1993). Lines in which the gene-trap construct is located inside a transcriptional unit can generate chimeric proteins consisting of a partial protein product of the transcriptional unit fused to the β-glucuronidase (GUS) protein. Of 10,000 independent transformants, 100 transgenic lines displayed reporter gene expression in the vascular tissues during vegetative development 7d after germination (DAG).
Phloem-specific expression was confirmed by analyzing the GUS staining pattern in cross-sections of inflorescence stems (Supplementary Fig. S1). We then screened these lines for GUS activity between 0 and 72 HAG. We isolated five marker lines that showed GUS expression in phloem tissue during early differentiation (annotated PD1–PD5, for phloem differentiation).

Fig. 3  Confocal sections of AB-stained seedlings of wild-type Arabidopsis thaliana (Ws). (A) Hypocotyl of a 24-h-old seedling. Bone-shaped protophloem precursors can be clearly observed. Black arrowheads show the corresponding transversal division of metaphloem initials. (B) Hypocotyl of a 24-h-old seedling. The white arrow shows the classic orientation of a protophloem precursor and the white arrowhead shows a protophloem precursor in inverted orientation. (C) Longitudinal section through the hypocotyl of a 36-h-old seedling. White arrowheads point towards cell wall thickening of protophloem cells. (D) Adaxial section through the cotyledon midvein of a 36-h-old seedling showing the cell wall thickening of the first differentiating protoxylem element. (E) Hypocotyl of a 48-h-old seedling. White arrows indicate protophloem cells with thickened cell wall and dark cell content. The black arrowhead shows adjacent cells in inverted orientation. (F, G) Two different Z-stacks through a root of a 72-h-old seedling, showing that protophloem cells are differentiated earlier than protoxylem cells. (F) A row of differentiating protophloem cells shows dark staining (black arrowhead). (G) Section showing differentiating xylem cells with highly fluorescent secondary cell wall thickenings (white arrowhead). Scale bars = 10 µm.
Phloem specificity and patterning of PD markers during early development

We determined the expression of each PD marker during early phloem formation in 100 embryos/seedlings. All PD marker lines, with the exception of PD1, displayed GUS expression in the mature embryo. In PD1–PD4, the GUS-expressing cells were identified as PPs on the basis of their characteristic bone shape (Fig. 4B, E, H, I, L). In PD5, reporter gene expression was detected in both PPs and metaphloem initials (Fig. 4F–H).

While PD5 marker expression was seen throughout the protophloem of the mature embryo (Fig. 4F), PD2 and PD3 marker expression was restricted to the midveins of the cotyledon, the cotyledonal node and the upper part of the hypocotyl (Fig. 4A, C). PD4 marker expression was also restricted to the upper/middle part of the embryo. It was detected in the hydathodes and in the distal protophloem cells of the cotyledons (Fig. 4D). Its expression was also present independently in two internal zones at both sides of the meristem and was also present in the columella. Scale bars = 100 μm (A, C, D, F, J, K), 10 μm (B, H), 20 μm (E, G, I).
Each construct was used to transform \textit{Arabidopsis} \textit{thaliana} (ecotype Col-0) in the region close to the cotyledon midvein and/or in the vascular strand of the upper part of the hypocotyl (Fig. 4J). It was not possible to discriminate statistically whether the first expression event was in the hypocotyl or in the cotyledon (data not shown).

Between 36 and 48 HAG, PD1, PD2, PD3 and PD4 marker expression progressed basipetally from the hypocotyl to the hypocotyl–root junction as shown for PD1 (Fig. 4J, K), whereas PD5 showed continuous marker expression in the axis. Further progression of PD marker expression was observed from the hypocotyl to the root. As the seedlings developed, \textit{GUS} expression in mature protophloem cells gradually disappeared in all marker lines. At 4 DAG, PD markers showed similar expression patterns in the primary root. \textit{GUS} expression initiated above the root division zone in two symmetrical strands that could be identified as protophloem strands. \textit{GUS} activity gradually disappeared towards the mature root (Fig. 4M). PD2 and PD3 markers were also expressed in the differentiating root cap (Fig. 4N) and root epidermis (data not shown).

Our data show that all PD markers are expressed in the protophloem, but their spatial and temporal expression patterns make them different and reflect the complexity of phloem formation. Most of the PD marker expression patterns progressed from the hypocotyl to the root, supporting our previous observation in AB-stained seedlings. Furthermore, PD1 and PD3 marker expression patterns indicated the presence of two independent foci of protophloem differentiation during early seedling development: one coming from the midvein of the cotyledons and another one from the upper part of the hypocotyl.

\textit{Progression of PD marker expression during shoot development}

In order to follow the chronology of PD marker expression during the formation of a new organ, we took advantage of the accurate description of vascular patterning during \textit{Arabidopsis} leaf development (Scarpella \textit{et al.} 2004). We followed the initiation and the progression of PD marker expression during leaf primordia development from 3 to 7 DAG. At 3 DAG, no vascular precursors are present in the primordia, whereas at 7 DAG, differentiating protoxylem and protophloem cells are present in the midvein and in the first distal loop.

We identified and cloned the promoters driving \textit{GUS} expression in the PD1, PD3, PD4 and PD5 lines upstream of the \textit{GFPER} marker gene (see Materials and Methods). Each construct was used to transform \textit{Arabidopsis} (ecotype Ws) by floral dipping. Independent transformants for each construct were obtained and homozygous plants containing the construct \textit{pPDx::GFP} were selected for further investigation (PDx for PD1, PD3, PD4 and PD5). For technical reasons, we were not able to identify the PD2 locus. \textit{pPDx::GFP} expression in the transformants reflected marker expression in the \textit{PDx} lines (not shown).

\textit{pPD1::GFP} expression and \textit{pPD3::GFP} expression were detected in the proximal protophloem of leaf primordia at 4 DAG (Fig. 5A, E). It proceeded to the distal protophloem (Fig. 5B, F) at 5 DAG and into the distal loops at 6 DAG (Fig. 5C, H). In addition, \textit{pPD3::GFP} expression was found in the epidermis of leaf primordia (Fig. 5D, G).

At 3 DAG, \textit{pPD4::GFP} expression was detected in the tip of leaf primordia (Fig. 5I). It was also seen in the future midvein and started to invade the distal loops (Fig. 5I). At 3.5 DAG, it invaded the proximal loops (Fig. 5J) with a high \textit{GFP} expression in the hydathode of the leaf tip. At 4 DAG, a strong \textit{GFP} expression appeared along the leaf margins and in the secondary distal loops (Fig. 5K). At 7 DAG, \textit{GFP} expression was strong in leaf lobes and defined the pre-pattern of the vascular tissue in the leaf. Interestingly, \textit{GFP} expression in the differentiated midvein was restricted to the protophloem (Fig. 5L, M), and in the margin of the leaf it was restricted to large isodiametric cells (Fig. 5N).

\textit{pPD5::GFP} expression was detected in the future midvein axis in large isodiametric cells at 2.5 DAG (Fig. 5O). The progression of this expression pattern during development was thus very similar to the \textit{pPD4::GFP} expression pattern. At 7 DAG, \textit{GFP} expression was strong in the distal part of the leaf margin. In lower order veins, expression was seen in large isodiametric cells that were not yet specified as procambial cells (Fig. 5P, Q). In the midvein of the same leaf, \textit{pPD5::GFP} expression was also restricted to the protophloem (Fig. 5R, S).

These results indicate that PD1 and PD3 markers are expressed sensu stricto during protophloem differentiation. In contrast, PD4 and PD5 reporter gene expression was detected in the location of midveins and higher order veins before procambium differentiation, and thus defined the pre-patterning of the future veins. At later stages of differentiation, the expression of these markers was restricted to the phloem.

\textit{Genetic and molecular analyses of the gene-trap lines}

For four out of five gene-trap lines, the \textit{GUS} reporter gene and resistance to kanamycin were co-segregating as a typical single Mendelian trait. No differences were found in the reporter gene expression analyses in segregating populations of hemizygous plants, suggesting that patterns were stable even in mutant backgrounds. Genomic regions flanking the gene-trap insertions were identified. Detailed loci analyses showed that in most cases the \textit{GUS} gene
was inserted close to the 5' end of a gene in the correct orientation with respect to the transcriptional unit (Supplementary Fig. S2). Two genes were annotated as coding for putative membrane-bound proteins: first, a small glycosylphosphatidylinositol (GPI)-anchored protein (PD1) that could act as a potential signaling protein; and secondly, a putative phosphatidylinositol phosphate (PIP)-4,5-kinase (PD3) involved in intracellular signaling. Two genes (PD4 and PD5) were annotated as proteins of unknown function that are specific to multicellular plants. The PD5 protein is a glycine-rich protein and the PD5 line displays a short-root phenotype. The PD4 protein has already been identified as corresponding to the \textit{BREVIS RADIUS} locus in the Uk-1 ecotype and the protein was described as a transcriptional activator (Mouchel et al. 2004). In Uk-1, the primary root length is reduced and the root system is more branched. This phenotype was not observed in PD4 lines, which suggests that PD4 is either still functional in this line or that the Ws ecotype could compensate for the phenotype. Lines PD1–PD3 had a wild-type phenotype. These data are summarized in Supplementary Fig. S3.

**Discussion**

In this study, we provide a framework for the analysis of vascular development in \textit{A. thaliana}. Utilizing a combination of confocal microscopy and new genetic markers, we were able to visualize phloem precursor cells at a high resolution. This has allowed studies of the behavior of these cells during development without the need for physical sectioning of plant tissues (Bougourd et al. 2000). Furthermore, the method makes it possible to analyze the structure and patterning of vascular tissue early in plant development and should provide a good tool for further characterization of vascular mutants.

We have used the term ‘specification’ to define the stage at which a cell adopts a predictable phenotype. For example, some procambial cells are destined to become phloem precursor cells. The differentiation of a proto-phloem cell starts with the acquisition of its final cell shape. This is achieved by modification of the cell wall, which ultimately leads to the formation of sieve plates. Functional phloem tissue is formed by a continuous, rapid and transient process, and this process may also be influenced...
by pre-existing mature phloem structures. These characteristics may hamper studies of phloem differentiation. These problems were overcome by studying phloem specification and differentiation at the very beginning of phloem development. This approach can be used to study vascular patterning in detail and to identify the key steps of protophloem differentiation.

**Pattern of phloem development**

We have described the expression patterns of five new molecular markers specific to several steps of phloem differentiation. Based on our analyses, we were able to determine the chronological order of expression of these genes (Fig. 6B, and Supplementary Fig. S3). GUS expression in the PD4 and PD5 lines marks early provascular patterns and is later restricted to the future phloem strand. PD1, PD2 and PD3 markers are specific to protophloem cells throughout plant development (Fig. 6B).

Only a few early phloem markers have been described so far: the *APL* gene is expressed from the torpedo stage onwards at the site of PP specification (Bonke et al. 2003). It was also found to be expressed in metaphloem precursors and companion cells. The *AtHB8* gene was reported to be expressed in the pre-procambial stage (Scarpella et al. 2004) as early as the PD4 and PD5 markers, but its expression was restricted to the procambium (Baima et al. 1995). Conversely, the GT5211 reporter line shows expression in the procambium and is progressively restricted to xylem initials or precursors (Scarpella et al. 2004). Furthermore, expression of the auxin efflux facilitator gene *PIN1* coincides with the probable sites at which pre-procambial cells are being specified. In addition, *DR5rev::GFP* expression, that reflects sites of auxin accumulation, indicates the sites of vein formation (Scarpella et al. 2006). PD4 and PD5 markers display expression domains similar to *DR5rev::GFP* and *PIN* expression patterns, respectively. Thus, it is tempting to speculate that expression of the respective PD4 and PD5 genes might be regulated by auxin concentrations, or that the genes play a role in polar auxin transport and its effects on phloem formation.

To explain the mechanism that controls the radial pattern of the primary vascular tissues in the root, Aloni et al. (2006) proposed that the primary xylem and phloem strands are induced by alternating streams of high vs. low IAA concentrations. Consistent with this hypothesis, there is increasing evidence that the auxin concentration could act as the driving force for the specification of vascular poles: (i) the DR5 reporter gene is expressed in root protoxylem cells; (ii) high auxin concentrations induce xylem differentiation (Aloni et al. 2003); and (iii) low levels of auxin are needed for phloem differentiation (Aloni 2001). We show in this study that xylem and phloem initials are specified at two opposite locations in the mature embryo of *A. thaliana*.

Xylem is localized in the frontal plane and phloem in the sagittal plane (Fig. 1A). Where does this symmetry come from? We suggest that the acquisition of bilateral symmetry at the heart stage of embryo development and consequently the strong accumulation of auxin in the frontal plane determines the position of xylem and phloem poles. Consistent with this hypothesis, in leaves, xylem can differentiate in the absence of phloem at the freely ending veinlets (Horner et al. 1994) and hydathodes (Aloni et al. 2003), sites of presumably high auxin concentrations.

**Protophloem specification and differentiation occur gradually from the cotyledon to the root**

Our data show that during embryogenesis phloem differentiation in the cotyledons occurs earlier than in the rest of the plant: (i) bone-shaped protophloem precursors are longer in cotyledons than in the other organs; (ii) expression of some PD markers is initially restricted to the upper part of the plant; and (iii) metaphloem precursors are already specified in cotyledons but not in the hypocotyl. This suggests that protophloem development in cotyledons is already underway before germination.
The existence of a gradual differentiation process was further confirmed during germination. The observed pattern of transient fluorescence in protophloem cells, the cell wall thickening and the progression of reporter gene expression for most PD markers are consistent with a time gradient of differentiation from the cotyledons down to the root.

According to the PD1 marker expression pattern, protophloem is initiated at distinct loci after germination and then progresses almost simultaneously along the cotyledons, hypocotyl and root. In the root, new protophloem elements are added acropetally from the meristem onto the previously differentiated elements in the same cell file, and consequently a continuous, uninterrupted protophloem is established in the root (Fig. 6A1–A5). The progression of expression of the PD1 marker is reminiscent of the previously described ZCP4 marker of xylem differentiation (Pyo et al. 2004), suggesting that initiation and progression of phloem and xylem differentiation are tightly linked. Consistent with our results is the previous description of polar differentiation of the first protophloem and protoxylem elements in Arabidopsis (Busse and Evert 1999). However, this previous anatomical description suggests that protophloem is differentiating almost simultaneously in cotyledon and hypocotyl. In the present study, we demonstrate that protophloem is initiated discontinuously and then differentiation progresses simultaneously. This new finding might be due to the use of a suitable molecular marker (PD1) and the ability to scan the whole phloem using confocal microscopy, with which we were able to easily follow the early stages of phloem differentiation in detail.

Identification of potential genes involved in specification and differentiation of protophloem

To date, few genes have been identified as being involved in phloem specification and/or differentiation (APL and WOL). In our study, we identified four new genes that are expressed during phloem formation. Molecular analysis of the marker genes revealed that they are plant specific and belong to multigene families.

The PD1 marker gene expression is localized in PPs, indicating a proclivity to protophloem differentiation. The PD1 gene encodes a small putative GPI-anchored protein of 7 kDa that belongs to a family of plant-specific proteins. Analogous small GPI-anchored proteins in mammals, such as CD24, are located on the cell surface and are signal-transducing molecules. They communicate external signals to the cell and function to control cell proliferation or differentiation (Kay et al. 1991).

PD3 encodes a putative PIP-4,5-kinase annotated as AtPIPK7 that belongs to a family of 10 genes (Mueller-Roeber and Pical 2002). This class of proteins has been shown to be involved in numerous signal transduction pathways in eukaryotes, and one of them has been localized to the procambium and may play a role in cell proliferation (Elge et al. 2001). In plants, this family of proteins has an additional N-terminal domain known as the membrane occupation and recognition nexus (MORN) motif. MORN repeats are thought to play a role in the targeting of proteins to the plasma membrane of junctophilins, a class of proteins that is present in the junctional complexes between the plasma membrane and the endoplasmic reticulum (Takeshima et al. 2000).

PD4/BRX belongs to a family of five proteins. It was recently shown that all members of this family contain a BRX domain, which is likely to be involved in protein–protein interactions (Briggs et al. 2006). BRX is nuclear localized and might act as a transcriptional activator (Briggs et al. 2006).

PD5 is an unknown protein that belongs to a family of five higher plant-specific genes. Preliminary observations suggest that primary root development is altered in the PD5 line (unpublished results). Such a phenotype is encountered in other phloem mutants, such as apl and wol. It may be that a modification of phloem transport interferes with nutrients and/or auxin transport.

Materials and Methods

Plant material and growth

Arabidopsis thaliana ecotype Wassilevskaja (Ws) was used as the wild type. The markers were obtained from insertion lines of the Versailles collection (Ws ecotype). The original names of the lines in the Versailles collection were CUM12 (PD1), ERA4 (PD2), EHD280 (PD3), EHH20 (PD4) and DYG25 (PD5). Seeds were surface-sterilized and incubated in the cold for 2 d. They were then sown on Arabidopsis medium (Estelle and Sommerville 1987) in Petri dishes and allowed to germinate and grow in a growth chamber (200 μE m−2 s−1, 16 h day/8 h night, 20°C, 70% humidity).

Cytoenzymological localization of GUS activity

GUS activity was detected as follows. Whole seedlings were permeabilized in ice-cold (−20°C) 90% (v/v) acetone for 1 h at −20°C (Hemerly et al. 1993) and washed twice, for 5 min each, in 100 mM sodium phosphate buffer pH 7.2. They were then infiltrated with 100 mM sodium phosphate buffer pH 7.2, 10 mM sodium EDTA, 0.1% Triton X-100 and 1 mg ml−1 5-bromo-4-chloro-3-indolyl-β-d-glucuronic acid (X-gluc, Duchefa), to which 2.5 mM potassium ferrocyanide and potassium ferricyanide were added, and incubated in the dark at 37°C. PD5 seedlings were incubated for 2 h. Other PD marker plants were incubated overnight. Samples were rinsed three times in water, treated with 70% ethanol for 24 h and cleared in chloral hydrate for 24 h. They were then fixed in Hoyer solution (chloral hydrate, arabic gum, glycerol and H2O) on a glass slide. Samples were viewed under differential interference contrast (DIC) optics, with a Zeiss Axiosplan II microscope (Zeiss, France). Images were acquired with a Jenoptik ProGress C10plus digital camera (Clara Vision, France).
Histological analysis
Floral stalks for resin embedding were fixed in 2% (v/v) glutaraldehyde and 1% (v/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. After incubation at 4°C for 24 h, samples were washed in phosphate buffer, dehydrated using a series of graded ethanol solutions and embedded in Technovit 7100 resin (Heraeus Kulzer, Wehrheim, Germany), according to the manufacturer’s instructions. Sections (5 μm) were made on a rotary Jung RM 2055 microtome (Leica Microsystems, Heidelberg, Germany) equipped with metallic blades (Heraeus Kulzer).

Aniline blue staining and confocal microscopy
Mature embryos and 24-, 48- and 72-h-old seedlings were stained with AB as previously described (Bougourd et al. 2000) and mounted in Hoyer solution.

AB staining was observed by confocal microscopy, using an inverted Leica TCS-SP2-AOBS spectral confocal laser scanning microscope (Leica Microsystems, Mannheim, Germany). Samples were excited with a 488 nm argon laser with emission at 516–721 nm for AB detection.

AB staining was combined with GUS staining in some cases. GUS staining of seedlings was performed first. The seedlings were then rinsed in water and AB staining was performed. GUS staining resulted in the formation of crystals that could be observed with the reflection mode of the confocal microscope. Samples were excited with a 488 nm argon laser and the reflection of the signal was detected at wavelengths between 485 and 491 nm.

GFP detection
Confocal microscopy was performed on an inverted Leica TCS-SP2-AOBS spectral confocal laser scanning microscope (Leica Microsystems, Mannheim, Germany). Samples were excited with a 488 nm argon laser with emission at 502–547 nm for green fluorescent protein (GFP) detection.

Plasmid construction and plant transformation
For all promoter GFP constructs, a region upstream of the ATG start codon (promoter and 5′-untranslated region) was cloned in the entry vector pDONR207 with the gateway BP enzyme clonase mix (Invitrogen). AttB1 and attB2 sites were added, respectively, to the 5′ and 3′ ends of PD promoter sequences via PCR for Ws genomic DNA using Taq DNA polymerase (Invitrogen) according to the manufacturer’s instructions. These regions were subsequently cloned in the pb101-R1R2-GFP binary vector using the gateway LR enzyme clonase mix (Invitrogen), according to the manufacturer’s instructions. The R1R2 cdB recombination cassette was inserted upstream from the GFP gene in the pb101-GFP binary vector, to obtain pb101-R1R2-GFP. This vector was then propagated in Escherichia coli DB3.1.

For PD1, a region of 1,329 bp (primers 5′-AAAAAAGCAGCTTGATCACACAGACTGC-3′ and 5′-AAGAGACG TGGTTCTCTTTGCTTTCTGTT-3′) was used. For PD3, a 1,926 bp region (primers 5′-AAAAACAGCGCTTTCAACCTTACACACAA-3′ and 5′-AAGAGAACGTTGGCTCTGGGATTG-CTTGGTGT-3′) was used. For PD4, a region of 3,859 bp (primers 5′-AAAGATCCGTTCTCTTTGCTTTCTTCTGTTG-3′ and 5′-ACCACT TGGATTCAAAAGAGCGGGTCTGTTGTTGATGTTCAT-3′) was used. For PD5, a 1,880 bp region (primers 5′-AAAAAAGCGGCGCTCGTTCCAATACATTCCCTG-3′ and 5′-AAGAAGCTGCTCGCGACGAAAATGTTGATTTTA-3′) was used.

These constructs were used to transform E. coli DH10B (Invitrogen) and were then used with Agrobacterium tumefaciens C58RPM90, for the transformation of wild-type Arabidopsis plants (Ws) by floral dipping.

Supplementary material
Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

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