High-Resolution Whole-Mount Imaging of Three-Dimensional Tissue Organization and Gene Expression Enables the Study of Phloem Development and Structure in Arabidopsis

Elisabeth Truernit,* Hélène Bauby,† Bertrand Dubreucq, Olivier Grandjean, John Runions,[2] Julien Barthélémy,[*] and Jean-Christophe Palauqui*[†,3]

[*] Laboratoire de Biologie Cellulaire, Institut Jean-Pierre Bourgin, Unité de Recherche 501, Institut National de la Recherche Agronomique, 78026 Versailles cedex, France
[†] Laboratoire de Biologie des Semences, Institut Jean-Pierre Bourgin, Unité Mixte de Recherche 204, Institut National de la Recherche Agronomique/AgroParistech, 78026 Versailles cedex, France
[3] Laboratoire Commun de Cytologie, Institut Jean-Pierre Bourgin, Unité de Recherche 254, Institut National de la Recherche Agronomique, 78026 Versailles cedex, France
[‡] Department of Plant Sciences, University of Cambridge, Cambridge CB2 3EA, United Kingdom

Currently, examination of the cellular structure of plant organs and the gene expression therein largely relies on the production of tissue sections. Here, we present a staining technique that can be used to image entire plant organs using confocal laser scanning microscopy. This technique produces high-resolution images that allow three-dimensional reconstruction of the cellular organization of plant organs. Importantly, three-dimensional domains of gene expression can be analyzed with single-cell precision. We used this technique for a detailed examination of phloem cells in the wild type and mutants. We were also able to recognize phloem sieve elements and their differentiation state in any tissue type and visualize the structure of sieve plates. We show that in the altered phloem development mutant, a hybrid cell type with phloem and xylem characteristics develops from initially normally differentiated protophloem cells. The simplicity of sieve element data collection allows for the statistical analysis of structural parameters of sieve plates, essential for the calculation of phloem conductivity. Taken together, this technique significantly improves the speed and accuracy of the investigation of plant growth and development.

INTRODUCTION

During plant development, cells interpret positional cues and differentiate accordingly. As a result, multicellular plants are composed of various cell types with specific functions and characteristic gene expression profiles. To understand the mechanisms that underlie plant growth and development, it is essential to visualize gene expression and the organization of plant organs on a cellular level.

For the study of plant tissue organization, microscopy observation of plant tissue sections has been used for centuries. However, the histological sectioning of plant tissue is labor intensive, and it is difficult to obtain a three-dimensional (3D) picture of cellular arrangements and gene expression patterns from tissue sections. Optical sectioning of plant tissue using confocal laser scanning microscopy (CLSM) overcomes these problems. It does not require physical tissue sectioning, and it allows the collection of a series of z axis images and the subsequent 3D reconstruction of the sample using specialized computer software.

To visualize living plant tissue using CLSM, fluorescent dyes are routinely used. One of the limitations of confocal microscopy using living plant material is the difficulty of observing cellular details in deeper tissue layers ($>50$ to $100 \mu m$; Haseloff, 2003). Most plant cells, especially in the aerial parts of the plant, produce a variety of substances that protect them from excess light radiation and that therefore prevent laser light and fluorescent emission from getting through the sample (Moreno et al., 2006). In addition, cellular contents and cell walls cause spherical aberration and light scattering (Haseloff, 2003). As a consequence, confocal imaging of living plant tissue only works well for thin and semitransparent organs, such as Arabidopsis thaliana roots (Helariutta et al., 2000; Birnbaum et al., 2003; Kurup et al., 2005; Laplaze et al., 2005; Stadler et al., 2005), and for the observation of external tissue layers, such as the epidermis and subepidermal cells (Grandjean et al., 2004; Tian et al., 2004).

To allow visualization of internal tissue layers of more complex organs, several advances have been made in the last years. Optical projection tomography (OPT) allows optical sectioning and 3D reconstruction of plant organs of up to $15 \text{ mm}$ thickness (Sharpe et al., 2002; Sharpe, 2003; Lee et al., 2006). Usually,
tissue samples need to be fixed and cleared, but, in case of semitransparent structures, OPT can also be used for imaging of live tissues (Lee et al., 2006). However, the resolution of OPT is limited to 5 \times 5 \times 5 \ \mu m; therefore, smaller cells or intracellular details are not resolvable. The combination of confocal microscopy of fixed plant material with clearing agents of high refractive indices is a way to achieve imaging of deeper tissue layers at higher resolution (0.3 \times 0.3 \times 0.5 \ \mu m). Aniline blue can be used as a stain of cell contents of Arabidopsis embryos (Bougourd et al., 2000) and very young (\approx 2 \text{d old}) seedlings (Bauby et al., 2007). Aniline blue-stained samples can be cleared with chloral hydrate to allow high-resolution confocal microscopy up to a depth of \geq 200 \ \mu m (Bougourd et al., 2000). More recently, we have used a pseudo-Schiff propidium iodide staining technique for the staining of cell walls of fixed plant material. The technique is based on the covalent labeling of cell wall material with fluorophors and the subsequent clearing of the tissue with chloral hydrate. Fixed plant tissue is treated with periodic acid, which leads to the formation of aldehyde groups in the carbohydrates of cell walls. These aldehyde groups can then react covalently with fluorescent pseudo-Schiff reagents, such as propidium iodide, resulting in samples with highly fluorescent cell walls that are well suited for confocal microscopy (Haseloff, 2003; Moreno et al., 2006; Truernit et al., 2006). This method works well for the staining of Arabidopsis embryos and roots but was not suitable for the staining of other plant organs or developmental stages.

To study gene expression within plant tissue, reporter genes such as green fluorescent protein (Haseloff et al., 1997) or \beta-glucuronidase (GUS) are used in plants. Green fluorescent protein expression studies can only be made using live tissue samples with the constraints described above. GUS expression studies can be combined with the fixing and clearing of plant material, but tissue sectioning is often necessary for a detailed cellular analysis. The acquisition of vascular tissue was a prerequisite for the development of land plants. During phloem development, protophloem sieve element cells develop prior to metaphloem sieve elements, which are the main solute conducting cells in the vasculature of differentiated plant organs (reviewed in Bauby et al., 2007). Both protophloem and metaphloem cells are long and narrow cells stretched in parallel to the longitudinal axis of plant organs. Perforated cell walls at their apical and basal ends, the sieve plates, allow for solute conductivity (Esau, 1969). As the vasculature is the most internal tissue of plant organs, it is especially difficult to access with classical microscopy techniques. Moreover, due to the characteristic shape of phloem cells, it is hard to obtain physical sections through the whole length of phloem cells. With the aim to study early phloem development, we have systematically developed and adapted the pseudo-Schiff propidium iodide (PS-PI) staining technique for the visualization of internal tissue structures of all Arabidopsis organs at all stages of development. We show that the modified PS-PI (mPS-PI) staining technique is a highly valuable tool for the study of the cellular organization of plant tissue. The method is fast and simple, and the images obtained are of high resolution and of a quality suitable for 3D reconstruction of cellular arrangements within a plant organ. Moreover, we show that the mPS-PI staining method can be combined with GUS marker gene expression analysis. This makes it possible to obtain a 3D view of gene expression patterns at the cellular level. Protophloem cells and their differentiation state can be easily identified in mPS-PI–stained tissue samples; moreover, even the visualization of sieve plates was achieved. We demonstrate the usefulness of the mPS-PI technique for the characterization of phloem development and for phloem mutant analysis.

RESULTS

The mPS-PI Staining Technique Allows the Study of Arabidopsis Cellular Architecture at All Stages of Plant Development

The PS-PI staining technique described previously (Haseloff, 2003; Moreno et al., 2006; Truernit et al., 2006) was suitable for the staining of Arabidopsis embryos and roots. Aboveground organs of seedlings, however, were not satisfactorily stained, suggesting poor stain penetration. The differences in staining efficiency were also visible with the eye: Embryos and roots appeared pink after the staining procedure, but the aboveground organs looked white (see Supplemental Figure 1 online). We therefore systematically tested and improved the PS-PI staining technique for the staining of all organ types at all stages of plant development. A treatment with ethanol was necessary to increase staining efficiency for most aboveground organs. The length of this treatment had to be adapted to the plant organ. For good staining of ovules and developing embryos in intact ovules, an alternative method that incorporated sodium hydroxide, sodium dodecyl sulfate, and sodium hypochlorite treatment was more effective (see Methods).

Figure 1 and corresponding movies (see Supplemental Movies 1 to 4 online) show optical sections of several Arabidopsis tissue types stained with the mPS-PI staining method. CLSM shows that the staining method labels cell walls and starch in plastids (examples of plastid staining can be seen in Figure 1A in petioles and in Figure 1H in columella cells). All tissue types are well stained, and different cell types are easily distinguishable, independent of their size and their vacuolation state. The cellular structure of leaf primordia, developing leaves, roots, and lateral root primordia was clearly visible (Figures 1A, 1B, 1H, and 1I). Small cells in meristematic tissues, such as the floral meristem (Figure 1C), could also be resolved. Ovules could be stained and imaged without the need to dissect them out of the silique (Figures 1E and 1F). Even developing embryos inside intact seed coats were stained (Figure 1G). When scanning toward deeper tissue layers, the signal intensity decreased only slightly (see Supplemental Movies 1 to 4 online). Therefore, in all cases, the working distance of the objective, and not the quality of the staining, limited the depth of image collection.

The method gave reproducible results, and stained and mounted tissue could be stored for several months in the dark without significant loss of stain intensity. Furthermore, very little photo bleaching occurred while laser scanning a sample. Taken together, the mPS-PI staining method is highly suitable for the
analysis of the cellular structures of all Arabidopsis organ types during all stages of development.

The mPS-PI Staining Method Can Be Combined with Gene Expression Studies

From a collection of promoter-trap lines (Bechtold et al., 1993), we recently isolated some GUS gene expressing marker lines (PD1 to PDS) that display GUS activity in protophloem cells at different stages of their development (Bauby et al., 2007). To investigate if we could analyze gene expression in mPS-PI–stained samples, we used line PD2, which shows GUS expression in immature protophloem cells (Bauby et al., 2007). GUS activity staining of this line was combined with mPS-PI staining. To monitor GUS expression in the mPS-PI–stained sample, the reflection mode of the confocal microscope was used. This

Figure 1. mPS-PI–Stained Arabidopsis Organs at Different Developmental Stages.

(A) Leaf primordium.
(B) Mature leaf with vascular strand.
(C) Flower bud.
(D) Anther with pollen grains.
(E) Silique with developing ovules.
(F) Developing ovule in silique.
(G) Developing embryos inside their seed coats.
(H) Primary root.
(I) Lateral root primordium.

All images are optical sections taken with a confocal microscope. Bars = 20 μm.
makes it possible to visualize the crystals that result from GUS activity assays using 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid as a substrate (Pautot et al., 2001).

Figure 2A shows the cotyledon of a mature embryo of marker line PD2. The long and narrow GUS-stained immature protophloem cells can be recognized easily within the mPS-PI–stained sample (Figure 2C). Moreover, by taking stacks of confocal images at steps of 0.1 to 0.2 μm, optical sections along the z axis of the sample can be reconstructed. The small GUS expressing protophloem cells can also be identified in these digital z-sections (Figure 2B). Also, with this technique, it is possible to follow cells or tissue types through stacks of z-sections, eliminating the need for exact sections through the tissue under investigation.

3D Reconstruction of mPS-PI–Stained Samples

Having a 3D view of plant tissue and gene expression is essential to understand cell–cell interactions. The suitability of the mPS-PI staining technique for the use with 3D reconstruction software was therefore important to test. Stacks of images from Arabidopsis embryos were taken along their z axis at steps of 0.2 μm, and the OsiriX software was used to reconstruct a 3D image of the embryonic organs (Rosset et al., 2004). Figures 2D, 2E, and Supplemental Movies 5 and 6 online show that we were able to obtain high-quality 3D images from mPS-PI–stained samples. GUS marker gene activity was also easily visible in these 3D reconstructions (shown for the PD2 GUS marker in Figure 2E). Virtual sections through the samples revealed internal tissue organization and gene expression patterns in cellular detail.

Characteristics of Protophloem Cells in mPS-PI–Stained Samples

After germination, protophloem precursor cells differentiate, and this process is associated with a thickening of the protophloem cell walls (Esau, 1969; Busse and Evert, 1999; Bauby et al., 2007). To investigate if we could recognize protophloem cells and their differentiation state in mPS-PI–stained samples, we analyzed roots of promoter-trap line PD1, which shows GUS expression in protophloem cells from the onset of their differentiation (Bauby et al., 2007). In the root, two protophloem cell files start to differentiate basipetally; 2 d after germination. Figure 3A shows one of these protophloem cell files. PD1 marker gene expression marks the start of the differentiation process (arrow) at a distance from the meristem. Basipetal differentiation is accompanied by cell wall thickening that is clearly visible in the mPS-PI–stained samples. To confirm our observation, we measured the signal intensity coming from the transverse cell walls of the developing

Figure 2. GUS Marker Gene (PD2) Expression and 3D Reconstruction.

PD2 marker gene expressing marks specified protophloem cells. Shown are CLSM images showing propidium iodide fluorescence (white) and GUS reflection (blue). Bars = 50 μm.

(A) Overview of a cotyledon of PD2 marker line. Digital z-sections through the cotyledon are shown on top and on the right.

(B) Magnification of digital z-section showing GUS expression in vascular bundle.
A protophloem cell file and compared it to a neighboring cell file. Signal intensity increased significantly specifically in the protophloem cell file as protophloem cells differentiated (Figures 3C and 3D). Protophloem cell elongation and cell wall thickening were linked processes (Figure 3E).

We also looked at other organ types, such as developing leaf primordia (Figure 3F), mature leaves (Figure 3G), or floral stems (Figure 3H). In all cases, the protophloem cells could be identified. Therefore, the mPS-PI staining technique allowed us to recognize protophloem cells and their differentiation state in all tissue types, even without the use of marker genes. Moreover, cellular features, such as cell length or fluorescence intensity of the mPS-PI–stained cell walls, could easily be quantified.

Visualization of Sieve Plates Suitable for High-Throughput Analysis

Sieve plates are part of sieve elements. They are mostly found in transverse and occasionally also in longitudinal orientation. Sieve plates are perforated by sieve pores, which are essential for the fluid conducting function of the phloem. In younger sieve elements, a thin lining of callose is associated with the sieve pores, while in older sieve elements, callose can be found as more or less massive deposit that can also cover the whole area of the sieve plate (Esau, 1969).

The study of sieve plates is extremely difficult and time consuming. The rare occurrence of this structure within a plant tissue necessitates extensive tissue sectioning to obtain sections that contain visible sieve plates. Moreover, the small size of the sieve pores (<0.5 μM) (Esau, 1969; Thompson and Holbrook, 2003), which is at the resolution limit of light microscopy, mostly requires the use of electron microscopy. Transverse sieve plates were seen in longitudinal optical CLSM sections through mPS-PI–stained phloem cells (see Supplemental Figure 2 online). Due to the lower z axis resolution when scanning through tissue samples, however, we were unable to obtain satisfactory images of transverse sieve plate surfaces when using 3D reconstruction. To visualize sieve plates in a more convenient way, transverse...
Arabidopsis (Wassilewskija [Ws]) floral stem sections of 60 to 100 μm were cut and stained. The use of relatively thick sections increased the probability of finding several sieve plates in one tissue sample. As CLSM was used, there was no need to cut exactly at the level of a sieve plate. Figure 4 shows that we were able to image transverse and longitudinal sieve plates at a very good resolution and sieve pores were clearly visible. We found that sieve plates in 2-d-old stems (4 to 5 cm long) were significantly (P = 0.0001) smaller than sieve plates in stems 1 week after bolting: On average, the first sieve plates had a surface of 20 μm² (n = 10) (Figure 4B), while sieve plates at later stages (n = 11, stems 10 to 15 cm long) had an average surface of 31 μm² (Figure 4F; see Supplemental Figure 3 online). Sieve pore area also increased significantly (P = 0.002) from 0.17 to 0.21 μm² (see Supplemental Figure 3 online). At the same time, sieve pore density remained relatively constant (see Supplemental Figure 3 online). In 1-week-old stems, we occasionally found sieve plates that were clogged with a structure that most likely represented callose (Figure 4H). To confirm this, we performed a double labeling with aniline blue, which is known to stain callose (Stadler et al., 1995). Callose deposits could be found on those sieve plates (see Supplemental Figure 4 online).

Early Phloem Development in the woodenleg and altered phloem development Mutants

To date, only a few mutants with defects in phloem development have been described. The woodenleg (wol) mutant lacks phloem tissue in roots and the lower part of the hypocotyl (Scheres et al., 1995). It has been demonstrated that WOL is required for the asymmetric division of cells in the stele tissue and that the lack of phloem in wol mutants is the indirect consequence of the reduced number of cells in the vascular cylinder (Scheres et al., 1995; Mähönen et al., 2000). So far, the mutant phenotype of wol has mainly been studied in transverse sections. Therefore, it is not clear how the transition of phloem cells to nonphloem cells occurs in wol mutants.

We used the mPS-PI staining technique to look more carefully at this transition in stacks of longitudinal optical sections of wol mutants. While in the wild type two continuous files of differentiated protophloem cells could be seen throughout the root hypocotyl axis (Figures 5C and 5D), only a few differentiated protophloem cells in the upper part of the hypocotyl were seen in wol (Figures 5A and 5B; see Supplemental Movie 7 online). These cells were continuous with a file of cells that was not differentiated as protophloem. The end of protophloem cell differentiation was abrupt. We did not find cells that showed partial protophloem cell differentiation. We also could not detect any protophloem cell specification in the lower part of the root hypocotyl axis when using our PD marker lines (data not shown). In most cases, protophloem cell differentiation in the two protophloem cell files did not stop at the same time, consistent with our observation that the number of cells in the vascular cylinder of wol mutants decreased gradually.

The altered phloem development (apl) mutant shows defects that are more specific to phloem cell specification. In this mutant, cells with characteristics of tracheary elements of xylem were found in the position normally occupied by phloem sieve
elements (Bonke et al., 2003). Metaphloem and protophloem cell markers were shown to be absent in the apl mutant background. Expression of APL in protoxylem cells prevented those cells from developing xylem characteristics. These results suggested that APL promotes phloem differentiation and is required for the inhibition of xylem differentiation in phloem poles.

To find out how cells at the position of protophloem cells developed in the apl background, we analyzed a segregating apl population (apl homozygous plants are seedling lethal) from the mature embryo stage to 4 d after germination using the mPS-PI technique ($n = 50$ for each stage). Until 2 d after germination, all plants from the segregating apl/APL population looked like wild-type plants. Moreover, all plants (50 of 50) showed continuous cell files of elongated protophloem cells with brightly fluorescent cell walls (Figures 5E and 5F). This suggests that neither the number of cells nor the position of phloem cell files or the first steps of protophloem differentiation were compromised in this mutant. This is in agreement with our observation that early protophloem PD markers were expressed in the apl background (data not shown). Three days after germination, plants homozygous for the apl mutation could be identified due to their shorter primary root (12 of 50). All of those plants now showed defects in protophloem differentiation: Cells with the appearance of protophloem cells developed spiralled cell wall thickenings reminiscent of the cell wall modifications found in xylem tracheary elements (Figure 5J). In the root, this secondary modification appeared at the same time in xylem and phloem cell files, suggesting that both cell types underwent the same developmental program at this stage. Taking into account the initially normal development of the cells in protophloem cell position in apl mutants, we were curious to know if those cells showed other features of phloem cells. Surprisingly, protophloem cells of 7-d-old apl mutants displayed sieve plates (Figures 5K and 5L), suggesting that although this mutant seems to have nonfunctional phloem, it still shows some phloem-specific structures.

**DISCUSSION**

We present a plant tissue staining technique that can be used in combination with CLSM to visualize the cellular structure of plant organs at high resolution. Initially, this technique was used for the staining of embryonic tissue (Haseloff, 2003). To use it for other developmental stages and tissue types, some crucial modifications to the staining procedure had to be introduced. A treatment of plant tissue with hot ethanol is conventionally used to isolate cuticle components. In our case, this treatment significantly increased stain penetration into aboveground organs, suggesting that the cuticle was interfering with this process. Treatment with sodium hydroxide and sodium dodecyl sulfate had the same effect for the staining of ovules. Subsequent sodium hypochlorite treatment most likely bleached tannins that accumulate in the seed integuments (Lepiniec et al., 2006).

With the mPS-PI staining technique, internal tissue layers of all organ types at all developmental stages from embryogenesis to seed set were well stained. Cellular organization, even of the long and narrow vascular cells in the center of the plant, could be discerned with great detail in optical sections through image stacks taken along the z axis of the samples. Moreover, the
staining method can be combined with GUS staining to analyze gene expression on the cellular level. The 3D reconstruction of image stacks is also possible, giving a detailed 3D view of tissue structure. Here, we have concentrated our analysis on Arabidopsis organs and tissue types, but the technique can be used equally for the staining of other plant species, such as tomato (Solanum lycopersicum) or Brachypodium (see Supplemental Movie 8 online).

Using the mPS-PI staining technique together with CLSM has many obvious advantages when compared with conventional histological tissue sectioning. It delivers a view of cellular arrangements and gene expression in three dimensions without the need for labor-intensive tissue sectioning. Whole organs can be scanned and optical sections can be made through the sample at any desired position. The production of a stack of images suitable for 3D reconstruction through the cotyledon of a mature embryo, for example, did not take us more than 5 min, and this time is likely to decrease with the improvement of confocal microscopy. Analysis of 3D gene expression and tissue organization will be useful for the study of asymmetric organs, of bilateral symmetry, or of gene expression patterns with axial preferences.

So far, phloem development has been mainly studied using histological sections in combination with light or electron microscopy (Esau, 1969; Busse and Evert, 1999; Helariutta et al., 2000; Bonke et al., 2003). Most likely because of the difficulty of producing longitudinal sections through the long and narrow phloem cells, many studies of phloem development have been limited to the use of transverse sections (Scheres et al., 1995; Helariutta et al., 2000; Bonke et al., 2003). Confocal microscopy eliminates the need for the production of physical sections through the long and narrow phloem cells. Moreover, it allows following the continuity of vascular strands through a series of z-stack images. Thus, even if the specimen is not completely flat, vascular cells can still be followed along their longitudinal axis. Differentiating and mature protophloem cells in different plant organs can be unequivocally recognized on the basis of their characteristic shape and their thickened cell walls.

Moreover, also for the analysis of transverse sections, our method shows clear advantages. Confocal microscopy eliminates the need to produce sections at exactly the point where the tissue should be analyzed, and this is especially useful for the study of sieve plate structures. To study sieve plates with CLSM, recent advances have been made using plants transgenic for a membrane-anchored protein fused to a yellow fluorescent protein expressed under the control of a companion cell–specific promoter (Thompson and Wolniak, 2008). The method presented here will be a valuable alternative because it is not limited to the use of transgenic plants. It will allow for a detailed and rapid study of sieve plate structure throughout plant development, in conditions of physiological constraints, and in different genetic backgrounds. Due to the relatively easy and fast sample collection, statistical analysis of sieve plate and sieve pore parameters, such as number, shape, or size, will now be possible. Knowing these parameters will be important for the calculation of sieve tube conductivity. For example, we show that sieve plates in young stems are significantly smaller and have smaller sieve pores than those of older stems. This means that the conductivity of the sieve tubes of older stems will be higher than those of stems shortly after bolting (Thompson and Wolniak, 2008).

We also used the mPS-PI technique to analyze mutant phloem phenotypes. In longitudinal optical sections through the vasculature of wol mutants (Scheres et al., 1995; Mähonen et al., 2000), we show that the developed protophloem cell file in the upper part of the hypocotyl of wol mutants is continuous with cells that do not show any protophloem cell specification or differentiation. During plant development, radial and longitudinal signals need to be integrated to obtain a continuous vascular network. While radial signals most likely determine the position of the vasculature in the center of the plant, longitudinal signals ensure the continuity of the vascular network. In agreement with this, the differentiation of already specified protophloem cells in young seedlings is a gradual process that starts from distinct locations in the plant (Bauby et al., 2007). In the root hypocotyl axis of wol mutants, radial signals seem to be dominant over longitudinal signals. Although differentiation of the two protophloem cell files in the upper part of the hypocotyl is normal, it cannot continue toward the lower part of the hypocotyl, since protophloem cells do not seem to be specified in this part of the seedling.

The apl mutant has previously been described as being defective in proto- and metaphloem development (Bonke et al., 2003). We confirm this observation; however, we show that the early steps of protophloem differentiation seem to occur normally in this mutant. Until 2 d after germination, cells in the position of protophloem cells display the characteristic shape and cell wall thickening of protophloem cells. Only after that time do they start to develop xylem characteristics, while at the same time sieve plates can be found in this cell type. Consequently, these cells can be described as hybrid cells with both phloem and xylem characteristics. Therefore, APL may be required for later steps of sieve element differentiation but may not be necessary for the first steps of this process. While the protophloem cell marker J0701 was not expressed in apl (Bonke et al., 2003), we could identify early PD marker gene expression in apl plants. One explanation for this may be the timing of the onset of expression of those markers, with J0701 being expressed later than some of our early PD marker genes. The other explanation may be that not all genes that are normally expressed during protophloem differentiation are expressed in the hybrid cell type found in apl plants.

Our data show that the use of the mPS-PI staining technique will open exciting new avenues for the study of phloem development. Currently, only a few mutants impaired in phloem development have been identified. This might be due to the difficulty of imaging phloem cells and also due to the paucity of criteria that can be applied to the search for new phloem mutants. More subtle deviations from normal phloem cellular structure might not be obvious using conventional imaging techniques. Moreover, to date, it is difficult to assess vascular continuity, as it is difficult to obtain longitudinal histological sections through a row of narrow phloem cells. The technique described here can be used for a detailed high-throughput screen for mutants impaired in vascular development.

While we focus our attention on vascular development, the technique presented here will facilitate and improve the detailed study of plant anatomy, and it will increase the number of
questions we will be able to ask about almost any aspect of plant development. Because of its speed, the screening of a large number of samples will be made possible. Therefore, it will also be easier to perform statistical analysis of cellular architecture and gene expression patterns of plants grown under different conditions or of plants with different genetic backgrounds. Because of the possibility of visualizing 3D tissue structures, the relative position of cells in a tissue context and their volumes or sizes can be studied and evaluated statistically. Taken together, the method will significantly improve the speed and accuracy with which plant developmental processes can be studied.

METHODOLOGY

Plant Material and Growth Conditions

If not stated otherwise, Arabidopsis thaliana ecotype Ws was used as the wild type. The phloem mutants wol and api (Scheres et al., 1995) were a gift from Yka Helariutta. The phloem marker lines used were described previously (Bauby et al., 2007).

Plants were grown on Murashige and Skoog medium supplemented with 1% sucrose in growth chambers (20°C, 70% humidity, 16 h light/8 h dark).

GUS Staining

Tissue was immersed in GUS staining solution (100 mM sodium phosphate buffer, pH 7.2, 10 mM sodium EDTA, 0.1% Triton X-100, and 1 mg/mL 5-bromo-4-chloro-3-indolyl-D-glucuronic acid [Duchefa], to which potassium ferrocyanide and potassium ferricyanide to a final concentration of 2.5 mM were freshly added). The staining solution was infiltrated into the tissue by subjecting samples to a vacuum for 2 min. The staining solution was infiltrated with 1% sucrose in growth chambers (20°C, 70% humidity, 16 h light/8 h dark).

mPS-PI Staining

Whole seedlings or plant organs were fixed in fixative (50% methanol and 10% acetic acid) at 4°C for at least 12 h. Tissue could also be stored in the fixative for up to 1 month. The tissue was then transferred to 80% ethanol and incubated at 80°C for 1 to 5 min, depending on tissue type (for example leaves, 1 min; floral stalks, 5 min). Tissue was transferred back to fixative and incubated for another hour. Next, tissue was rinsed with water and incubated in 1% periodic acid at room temperature for 40 min. The tissue was rinsed again with water and incubated in Schiff reagent with propidium iodide (100 mM sodium metabisulphite and 0.15 N HCl; propidium iodide to a final concentration of 100 µg/mL was freshly added) for 1 to 2 h or until plants were visibly stained. The samples were then transferred onto microscope slides and covered with a chloral hydrate solution (4 g chloral hydrate, 1 mL glycerol, and 2 mL water). Slides were kept overnight at room temperature in a closed environment to prevent drying out. Next, excess chloral hydrate was removed and several drops of Hoyler’s solution (30 g gum arabic, 200 g chloral hydrate, 20 g glycerol, and 50 mL water) were placed over the tissue, and a cover slip was placed on top. Slides were left undisturbed for a minimum of 3 d to allow the mounting solution to set. For the staining of roots and emerged lateral roots, the ethanol step was omitted. For staining of ovules and seeds, siliques were transferred into 70% ethanol and incubated at 80°C for 1 to 5 min, and seeds were then blotted dry. For staining of embryos, embryos were fixed as described above and then subjected to an overnight treatment of 1% SDS and 0.2 N NaOH at room temperature. Siliques were rinsed in water, incubated in 25% bleach solution (2.5% active Cl 2 ) for 1 to 5 min, rinsed again, and then transferred to 1% periodic acid. The samples were then further processed as described above.

Imaging of Sieve Plates and Aniline Blue Staining

Sieve and hypocotyl cross sections (60 to 100 µm thick) were obtained with a vibratome (Leica VT1000S). Samples were then subjected to PS-PI staining as described above. Callose staining was performed as described by Stadler et al. (1995).

Confocal Microscopy

A Leica TCS-SP2-AOBS spectral confocal laser scanning microscope (Leica Microsystems) was used. The excitation wavelength for PS-PI-stained samples was 488 nm, and emission was collected at 520 to 720 nm. GUS staining was imaged with the AOBS reflection mode of the confocal microscope. The excitation wavelength was 488 nm, and the reflection signal was collected between 485 and 491 nm. Callose fluorescence was collected between 480 to 515 nm using a 405-nm laser.

Data Processing

Data were processed for some two-dimensional orthogonal sections, 3D rendering, and movie exports using the open source software Osiris (Rosset et al., 2004; http://homepage.mac.com/rossetantoine/osirix/) on a quadxeon 2.66-GHz, 2-GB RAM Apple Mac pro workstation. RGB stacks of confocal images were imported as DICom files into Osiris prior to surface rendering.

For the production of optical sections, for signal quantification, and for cell length measurements, we used the Leica Confocal Software version 2.61.

Supplemental Data

The following materials can be found in the online version of this article.

Supplemental Figure 1. Treatment with Hot Ethanol Increases Stain Penetration into Aboveground Organs.

Supplemental Figure 2. Sieve Plate in Longitudinal Optical Section.

Supplemental Figure 3. Parameters of Sieve Plates in 2-d-Old and 1-Week-Old Stems.

Supplemental Figure 4. Sieve Plate with Callose.

Supplemental Movie 1. Z-Scan through Arabidopsis Leaf from Abaxial to Adaxial Epidermis.

Supplemental Movie 2. Z-Scan through Arabidopsis Inflorescence Meristem.

Supplemental Movie 3. Z-Scan through Arabidopsis Siliques with Developing Ovules.

Supplemental Movie 4. Z-Scan through Arabidopsis Ovule with Developing Embryo at Late Globular Stage.

Supplemental Movie 5. 3D Reconstruction of an Arabidopsis Embryonic Root Using OsirIX Software.

Supplemental Movie 6. 3D Reconstruction of Cotyledon of PD2 Marker Line with GUS Expression in Immature Protophloem Cells Using OsirIX Software.

Supplemental Movie 7. Z-Scan through Root Hypocotyl Axis of the wol Mutant Showing the Abrupt End of Protophloem Differentiation.

Supplemental Movie 8. Z-Scan through Brachypodium Shoot Meristem with Leaf Primordia.
ACKNOWLEDGMENT

We thank “La Région Ile de France” for the cofinancing of the confocal microscope and Ykä Helariutta for wol and apl seeds. We also thank Gregory Mouille for helpful discussions and Céline Savattero for help in the lab as a summer student. We thank Patrick Laufs for critical reading of the manuscript. E.T. was funded by a Marie Curie Intra-European Fellowship.

Received October 3, 2007; revised May 6, 2008; accepted May 14, 2008; published June 3, 2008.

REFERENCES


**High-Resolution Whole-Mount Imaging of Three-Dimensional Tissue Organization and Gene Expression Enables the Study of Phloem Development and Structure in *Arabidopsis***

Elisabeth Truernit, Hélène Bauby, Bertrand Dubreucq, Olivier Grandjean, John Runions, Julien Barthélémy and Jean-Christophe Palauqui

*Plant Cell* 2008;20;1494-1503; originally published online June 3, 2008;
DOI 10.1105/tpc.107.056069

This information is current as of June 29, 2017

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