Journal of Plant Biology, June 2007, 50(3) : 266-273 **REVIEW**

Cell-to-Cell Transport of Macromolecules during Early Plant Development

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Plant cells exchange developmental signals, distribute nutrients and ribonucleoprotein complexes through dynamic intercellular channels termed plasmodesmata (PD). Multidisciplinary investigations over the last decade have provided evidence that plasmodesmatal regulation is critical to various basic plant functions, such as development, host–pathogen interactions, and systemic RNA-silencing. This review highlights the cell-to-cell transport of micro- and macromolecules via PD during embryo and seedling growth.

Keywords: Arabidopsis, cell-to-cell transport, embryo, plasmodesmata, seedling

INTERCELLULAR COMMUNICATON IN PLANTS

Embryogenesis sets up basic body axes that enable the development of multicellular organisms (Wolpert, 2002). In vertebrates, this axis formation involves maternal factors, environmental influences, and cell-to-cell interactions. During animal embryogenesis, two major types of communication play important roles in cell-fate decisions. These are 1) extracellular signals recognized by corresponding receptors on the cell surface (Johnston and Nusslein-Volhard, 1992), and 2) the formation of gradients in signaling molecules and their linked threshold responses. Plants share these mechanisms, but because their cells are encased in rigid walls, they also have a unique third mechanism for exchanging developmental cues and distributing nutrients via dynamic intercellular channels called plasmodesmata (PD) (Heinlein and Epel, 2004; Lucas and Lee, 2004; Oparka, 2004; Zambryski, 2004). The review presented here describes the intercellular transport of macromolecules via PD during embryo and seedling development.

CELL-TO-CELL TRANSPORT VIA PLASMODESMATA

Generic simple plasmodesmata have two major components, membranes and spaces (Roberts, 2005) (Fig. 1). The plasma membrane (PM) of two neighboring cells forms the outer boundary of the PD channel. An appressed endoplasmic reticulum (ER), termed the desmotubule (D), runs through the axial core of the PD and forms the inner boundary. The space between PM and D is the cytoplasmic sleeve (CS). This primary passageway for molecular transport is continuous with the cytoplasm between adjacent cells. The CS is not empty but is, instead, filled with proteinaceous molecules that likely regulate transport via PD. For example, actin and myosin along the length of PD (Baluska et al., 2001), and centrin nanofilaments at the neck region (Blackman et al., 1999), may provide contractile elements to control PD apertures.

The functional measure of PD is its size exclusion limit (SEL), i.e., the maximum size for macromolecules that can freely diffuse. PD SEL is regulated temporally, spatially, and physiologically throughout a life cycle. PD selectively allow the movement of proteins, e.g., transcription factors, and RNAs, such as mRNAs and silencing RNAs, all critical in cell-fate determination (Ding et al., 2003; Kim, 2005; Kurata et al., 2005). PD in various tissue types may be regulated differently, possibly by the involvement of developmentally controlled factors.

The determination of which cells and tissues are in communication via PD is an area of active investigation. Such experiments are revealing where and when developmental signaling may occur. Because the PD passageway is continuous, when adjacent cells and tissues exhibit cell-to-cell transport of micro- or macromolecular tracers, they are said to form "symplastic domains" of shared cytoplasm. Cells within such domains have a common PD aperture (SEL) compared with cells in the surrounding regions. Symplastic

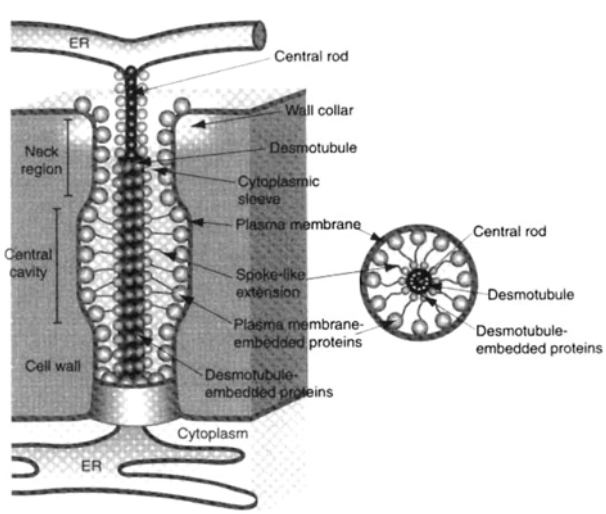

Figure 1. Diagram of simple plasmodesmata: longitudinal view at left; transverse view at right. Reprinted with permission from Roberts (2005).

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domains are thought to form during the differentiation of tissues/organs, and studies have now located communication domains for this developmental/morphogenetic signaling.

Characterizing the formation of distinct symplastic domains requires observations over a certain developmental period. Embryogenesis offers a superb model system because embryos develop from a single cell, and divisions result in symplastic coupling via PD. Jurgens and Mayer (1994) have demonstrated that early-heart-stage embryos comprise about 250 cells, which are interconnected by open PD and constitute a single symplast of continuous cytoplasm bounded by the plasma membranes (McLean et al., 1997). As embryonic development proceeds, groups of cells become symplastically isolated from neighboring cells to form symplastic sub-domains. Such sub-domains are characterized by PD with distinct SELs.

MACROMOLECULAR TRANSPORT DURING SEEDLING DEVELOPMENT

Phloem-loading, together with novel fluorescent probes, has made it possible for researchers to track the cell-to-cell movement of symplastic probes, both locally and long-distance, in whole seedlings just after germination (Oparka et al., 1994). This approach is especially suited to the model plant *Arabidopsis*, where seedlings are small (~1 cm for the shoot and ~3 cm for the root of a 1-week-old seedling) and the whole plantlet can be viewed easily under a fluorescence microscope. Earlier examinations have used small (~0.5 kDa) tracers, such as carboxyfluorescein (CF) diacetate and 8-hyroxypyrene-1,3,6-trisulfonic acid (HPTS). There, the ester (uncharged) form of CF diacetate freely moves across the plasma membrane. A cytosolic esterase then converts this probe to the anionic membrane-impermeable form, trapping CF in the cytoplasm. CF diacetate that is applied to the cut leaves of *Arabidopsis* seedlings translocates via the phloem and unloads into the growing root tip (Oparka et al., 1994), thus indicating symplastic coupling between young root cells. However, HPTS is a more reliable tracer because it is highly anionic and localizes entirely to the cytoplasmic compartment for intercellular transport via PD. HPTS-loading has shown that the epidermis of the root becomes symplastically isolated from the inner cells as development proceeds (Duckett et al., 1994). While such phloem-loading offers an excellent non-invasive means for monitoring PD-functioning, especially over long distances, this method can measure the transport of only small probes.

An elegant series of experiments has revealed macromolecular movement in *Arabidopsis* seedling roots, and the precision whereby PD can control such action. In particular, the SHORTROOT (SHR) transcription factor (TF), which moves cell-to-cell in developing roots, is required for the normal differentiation of cortex/endodermal initial cells that control the formation of the endodermis. Surprisingly, transcription of *SHR* (as shown by *in situ* mRNA localization and a transcriptional fusion of GFP to the promoter of *SHR*) is absent from the cortex/endodermal initial cells or daughter cells. Instead, *SHR* mRNA is present in the internally adjacent cells of the stele (Fig. 2, inset). However, SHR protein (found through the immunolocalization and a translational fusion of GFP to the coding sequence of SHR) localizes to the stele and the cells of the adjacent layer in the endodermis, which includes the cortex/endodermal initial cells and the quiescent center. These results imply that SHR-GFP traffics from the stele to a single adjacent layer of cells, where it functions to promote asymmetric divisions and endodermal cell fate.

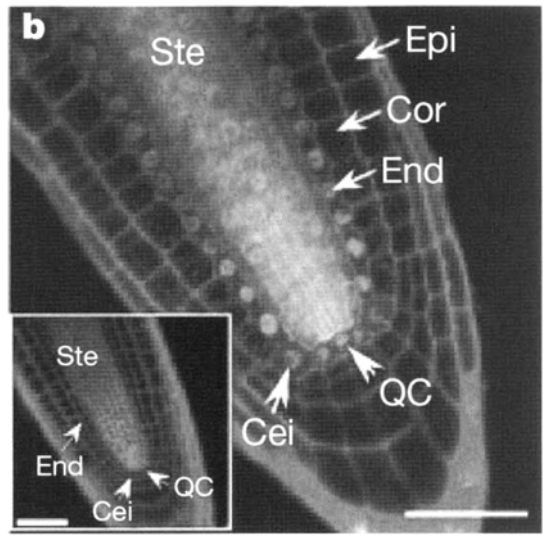

Figure 2. Intercellular movement of SHORTROOT (SHR) protein in *Arabidopsis* root. SHR proteins as GFP fusions (shown as grey regions) localize both in stele (ste) and endodermis (end), while *SHR* transcript is detected only in stele (inset). Modified with permission from Nakajima et al. (2001).

Additional studies using genetics and *in situ* gene expression have shown that other plant TFs can move cell-to-cell via PD. The classic example is the maize KNOTTED1 (KN1) protein, which moves one cell layer in the shoot apex. KN1 is a homeodomain-containing protein that regulates the development of leaf and shoot meristems. Along with its mRNA, KN1 travels from cell to cell in the shoot apical meristems and leaves (Kim et al., 2003). Two other examples are LEAFY (Wu et al., 2003), which controls floral meristem identity, and CAPRICE (Wada et al., 2002), which is central to the formation of root hair cells. The movement of TFs via PD has been widely reviewed by Hake (2001), Wu et al. (2002), Cilia and Jackson (2004), and Kim (2005).

To elucidate the role of PD during post-embryonic development, researchers have analyzed the transport of GFP tracers in young seedling leaves (Kim et al., 2005b; Stadler et al., 2005b) and roots (Stadler et al., 2005b). The role of symplastic communication in the morphogenesis of postembryonic tissues has also been examined in the gametophytes, leaves, roots, stems, flowers, and shoot apical meristems of land plants and algae (Erwee and Goodwin, 1985; Tilney et al., 1990; van der Schoot and van Bel, 1990; Duckett et al., 1994; van der Schoot et al., 1995; Kwiatkowska, 1999; van der Schoot and Rinne, 1999). When considering all of these previous studies, one must note that symplastic isolation occurs in different manners and to varying degrees -- permanent vs. transient, complete

vs. reduced PD aperture -- and that symplastic domains differentiate into tissues with distinct structures and functions.

MACROMOLECULAR TRANSPORT DURING EMBRYO DEVELOPMENT: A TRANSIENT ASSAY TO IDENTIFY PD GENES

Although PD-functioning and ultrastructure have been extensively analyzed, until recently few studies have addressed the genes that control these plasmodesmata. Despite having a powerful tool such as genetics for isolating potential genes that regulate PD, such PD research is quite limited (Russin et al., 1996; Provencher et al., 2001; Kim et al., 2002). One obstacle is that, given that PD are essential for plant growth, most mutants are unlikely to grow to adult plants. Nonetheless, even though they do not attain adulthood, their PD mutation should be manifested early in embryogenesis. These lethal mutants can then be propagated as heterozygous plants to display their homozygous defective phenotype, segregating in the seedpod or fruit. For example, one pea pod-like *Arabidopsis* fruit (i.e., the silique) contains 40 to 60 seeds, in which the embryos are enclosed (Fig. 3A). Thus, 10 to 15 homozygous mutant embryos will segregate within a single silique.

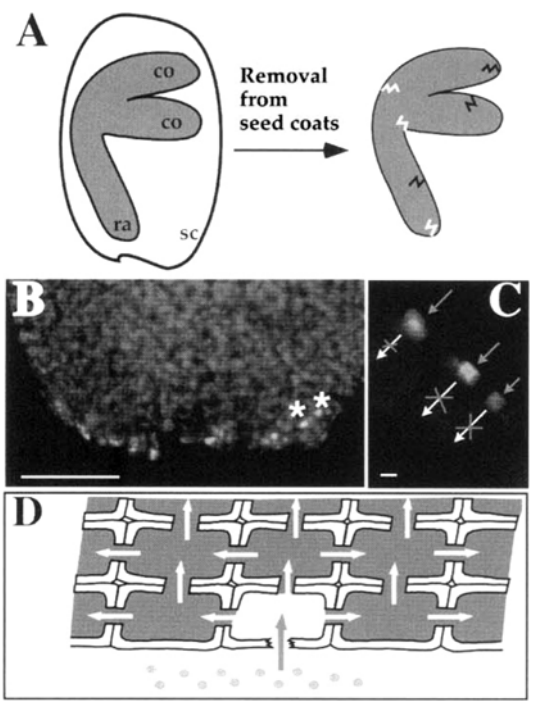

Figure 3. Uptake of symplastic probes in cells of *Arabidopsis* mid-torpedo embryos.
(**A**) When embryos are released from seed coats, physical damage occurs in subset of cells. Consequently, small regions of cell walls and plasma membranes are broken at sub-lethal level to provide initial entrance site for uptake of symplastic tracers, such as HPTS and F-dextran, which do not cross plasma membranes. Jagged lines indicate most common site of damage. co, cotyledon; ra, radicle; sc, seed coat. (**B**) Small number of cells at base of detached cotyledons from mid-torpedo embryos are cytoplasmically loaded with 10-kDa F-dextran (asterisks), yet further movement to neighboring cells does not occur. Scale bar, 50 μm. (**C**) Typical example of loaded cells in region containing abrasion at edge of protodermal layer, marked as jagged lines in (**A**). Individual cells in protodermal layer take up 10-kDa F-dextrans (arrows), and show cytoplasmic localization of probe. However, subsequent movement of probe is inhibited (arrows with X). Scale bar, 5 μm. (**D**) Diagram showing partially broken cell wall and plasma membrane (jagged edge), which may provide initial entrance site for uptake of symplastic tracers, F-dextran or HPTS (circles). Further symplastic transport is then determined by PD SEL and size of introduced symplastic tracers.

The next research hurdle is to develop a strategy for testing PD functions during embryogenesis. First, the embryos must be released from their seed coats. This is accomplished by removing the seeds from the silique, collecting them on a glass slide, and applying slight pressure with a cover glass. This extrusion process induces sub-lethal tears in the plasma membrane and in the wall of the outermost cell layer of the embryo. Such breaks provide initial entries for symplastic tracers of various sizes (Fig. 3D). While probes larger than the PD SEL at the break site are trapped in the initial cells (Fig. 3B, C) and cannot move, any smaller tracers can travel cell-to-cell via the PD (Fig. 3D).

In this transient assay, HPTS- (0.5 kDa) or fluorescently (F)-labeled 10-kDa dextrans can be exogenously introduced into the developing embryos. HPTS moves through all cells and throughout all stages of embryonic development (early-heart to mid-torpedo), demonstrating that the embryo is single-symplast. However, the use of higher-molecular-weight tracers reveals that the PD aperture is also down-regulated over time. For example, 10-kDa F-dextrans can be transported cell-to-cell in 50% of the heart, 20% of the early-torpedo, and 0% of the mid-torpedo embryos. Thus, while symplastic connectivity remains (as measured by small tracers such as HPTS), PD SELs are altered during the development period.

Over 5000 lines of *Arabidopsis* with an embryo-defective phenotype have been screened by the above assay to detect mutants that continue to traffic 10-kDa F-dextran at the mid-torpedo stage. Kim et al. (2002) have found 15 lines, called *increased size exclusion limit of plasmodesmata* (*ise*). Two of these, *ise1* and *ise2*, are currently under investigation to identify their defective genes and characterize their role in PD-functioning and/or structure.

MACROMOLECULAR TRANSPORT DURING EMBRYO DEVELOPMENT: ANALYSIS OF SYMPLASTIC DOMAIN FORMATION

Besides serving as genetic tools, embryos are innately interesting subjects for examining intercellular-transport patterning. Embryogenesis is a critical stage that establishes basic body axes to direct the development of different tissues and organs. *Arabidopsis* embryos have a regular pattern of divisions that allows one to trace the origin of seedling structures back to specific, early groups of cells (Mansfield and Briarty, 1991; Jurgens and Mayer, 1994). Seedlings show an apical-basal pattern along the main axis that is composed of structures such as the shoot apical meristem (SAM), cotyledons, hypocotyl, and root (Fig. 4I). Clonal analyses and histological techniques can predict the contribution of each embryonic cell to this body plan (Scheres et al.,

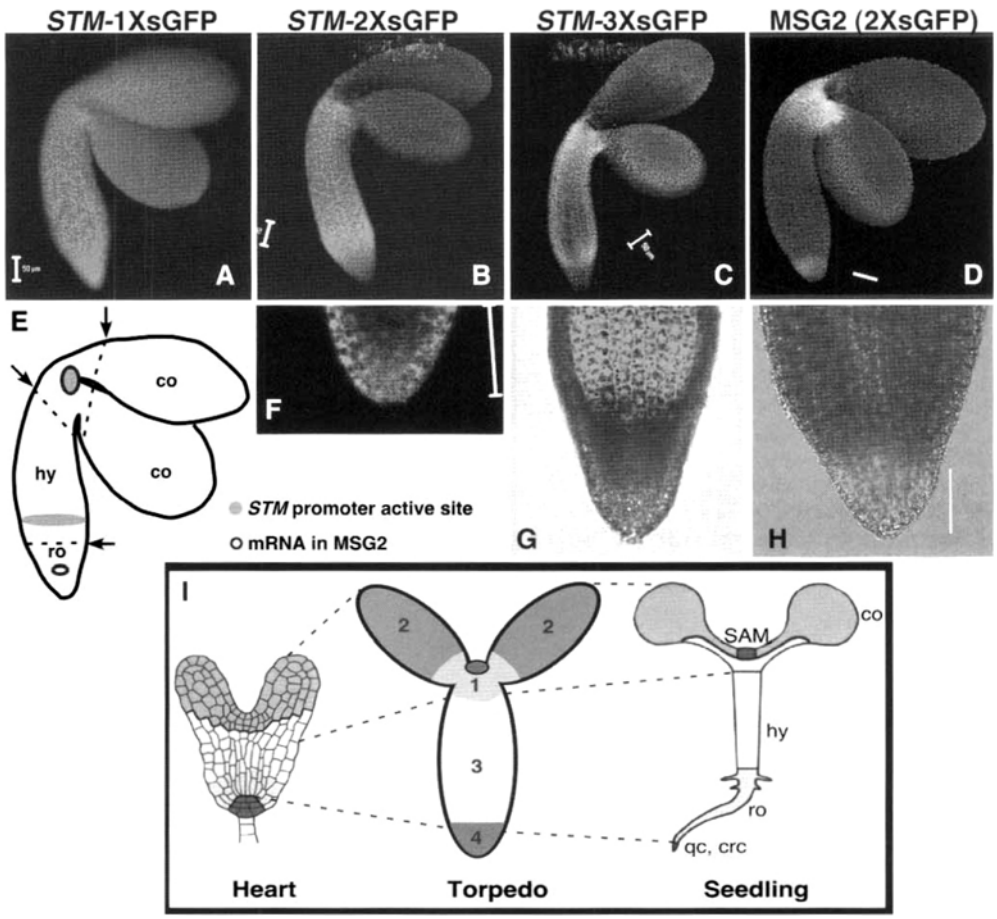

Figure 4. sGFP movement in *Arabidopsis* mid-torpedo embryos. 1XsGFP expressed by *STM* promoter in SAM and at base of hypocotyl (hy) (**E**) freely moves throughout entire embryo (**A**). 2XsGFP fails to move into cotyledons (co) (**B**) but moves to root tip (**F**). 3XsGFP fails to move to root (ro) as well as cotyledons (**C**, **G**). These results indicate formation of at least two symplastic sub-domains, e.g., cotyledon and root. 2XsGFP expressed in SAM and RAM in MSG2 line (**E**) stays within sub-domains of shoot apex and root, respectively (**D**, **H**). These results, together with those in (**B**), confirm boundary between shoot apex and hypocotyl sub-domains. Root sub-domains from embryos in (**C**) and (**D**) are shown under larger magnification for each whole mid-torpedo image, and include quiescent center (qc), part of RAM, and central root caps (crc). (**E**) Origin of MSG2-mediated expression is indicated by empty circles at SAM and RAM; origin of *STM*-mediated expression is indicated by shaded circles at SAM (same as MSG2) and lower part of hypocotyl. (**I**) Four symplastic sub-domains in torpedo embryos, shoot apex (1) including SAM (dark-green circle), cotyledons (2), hypocotyl (3), and root (4) are extrapolated to organs in heart embryos and seedlings. Same shading in heart embryo and seedling represent regions of development with common clonal origins. Sub-domains of torpedo embryo, determined by their cell-to-cell transport via PD, also correspond, by their positions, to apical-basal body pattern of heart embryo (and seedling); these regions are diagrammed with various shadings to indicate they were defined by different assays. Scale bars, 50 μm.

1994). In this review, Figure 4I presents a comparison between a heart embryo and a seedling. Generally, positional information determines the overall body pattern, and lineage-dependent cell fate specifies local patterning (Poethig et al., 1986; Scheres et al., 1994; Saulsberry et al., 2002). Auxin-signaling, as well as differential gene expression, then facilitates specific morphogenesis (Berleth and Chatfield, 2002; Laux et al., 2004).

Sub-domains corresponding to axial body pattern

Two different promoters can be used to drive GFP expression in specific meristematic regions of *Arabidopsis* embryos. For example, the *SHOOT MERISTEMLESS* (*STM*) promoter is utilized for expressing 1X, 2X, and 3XsGFP (single 27-kDa, double 54-kDa, and triple 81-kDa forms) in the shoot apical meristem (SAM) and a subset of cells in the hypocotyl (Kim et al., 2005b). In addition, a cell-type-specific enhancer of Line J2341 induces the expression of 2XsGFP in the SAM and root apical meristem (RAM) of Line MSG2 (Fig. 4E) (Kim et al., 2005b). The subsequent movement of these various-sized tracers from their site of synthesis has been monitored at three stages of embryogenesis. The 2XsGFP (54 kDa) moves throughout the entire early-heart embryo, demonstrating that PD apertures (interconnecting cells to form a single symplast) in those early embryos are quite dilated. Furthermore, different regions of the embryo have distinct PD SELs that define symplastic sub-domains by the mid-torpedo stage. These sub-domains correspond to major regions in the apical-basal body axis, the shoot apex, cotyledons, hypocotyl, and root (Fig. 4 and legend). They can be extrapolated to regions of the early embryo (and seedling) as

determined by gene-expression profiles and clonal analyses (Fig. 4I).

Boundaries between symplastic sub-domains of cell-to-cell transport

The above data imply that boundaries exist between the four symplastic sub-domains in which the embryo controls intercellular transport (Kim et al., 2005a). Each boundary has a distinct PD SEL. For example, the boundary between the shoot apex and the cotyledons has a SEL ranging from 27 to 54 kDa. That is, 1XsGFP, but not 2XsGFP, moves from the SAM to the cotyledons (Fig. 4A, B, E). The boundary between the hypocotyl and the root has a SEL of 54 to 81 kDa, with 2XsGFP, but not 3XsGFP, moving from the hypocotyl to the root (Fig. 4F, G). The hypocotyl and shoot apex sub-domains are indicated by the movement of 2XsGFP from its site of synthesis at the SAM and surrounding cells in MSG2, and by its failure to move to the hypocotyl (Fig. 4D, E). Movement of 2X and 3XsGFP in the hypocotyl sub-domain results from upward transport from its site of synthesis (under the *STM* promoter) near the hypocotyl-root junction (Fig. 4B, C, E). The existence of root and cotyledon sub-domains has been further investigated in transgenic plants expressing 1X or 2XsGFP fused to the P30 movement protein (MP) of *tobacco mosaic virus* (TMV), under the control of the same *STM* promoter (Kim et al., 2005a). TMV P30 localizes to PD in both virus-infected cells (Tomenius et al., 1987) and in uninfected transgenic plants expressing P30 (Atkins et al., 1991). It acts as a molecular chaperone to bind the single-stranded viral RNA genome and targets this ribonucleoprotein complex to PD, where it triggers an increase in PD SEL (i.e., "gating") to facilitate movement of the TMV genome into adjacent uninfected cells (Ghoshroy et al., 1997). In embryos (as in the adult plants), GFP-P30 targets to PD, and moves more extensively than similarly sized GFP tracers, thereby confirming the functionality of P30. However, 1XGFP-P30 (57 kDa) and 2XGFP-P30 (84 kDa) behave as the similarly sized 2XsGFP (54 kDa) and 3XsGFP (81 kDa) in their inability to be transported into the cotyledons and root, respectively (Kim et al., 2005a). These data reinforce the existence of boundaries between symplastic sub-domains in embryos.

Further refinement of local symplastic sub-domains

Additional symplastic sub-domains have been observed that correspond to the protodermis and stele. For example, when 1XsGFP is expressed in the outermost protodermal layer of the hypocotyl, under the control of the *Arabidopsis GLABRA2 (AtGL2)* promoter, it moves uniformly inward to internal ground tissues and to neighboring protodermal cells in cotyledons at the heart stage (Fig. 2F; Stadler et al., 2005a). However, in the early-torpedo stage, centripetal movement of 1XsGFP from the protodermis is reduced, such that the GFP signal intensity is much weaker in ground tissues, while movement among cells in the protodermis continues (Fig. 5A). Similarly, 1XsGFP, expressed in the root tip by the *Arabidopsis SUCROSE TRANSPORTER3 (AtSUC3)* promoter, freely moves to the hypocotyl at earlier stages (Stadler et al., 2005a), but becomes restricted to the stele in the mid-torpedo stage (Fig. 5B).

We note that the extent of this symplastic movement is significantly influenced by the location of the initial site of sGFP synthesis. 1XsGFP freely moves to every cell in the embryo following expression in the SAM (Kim et al., 2005a,

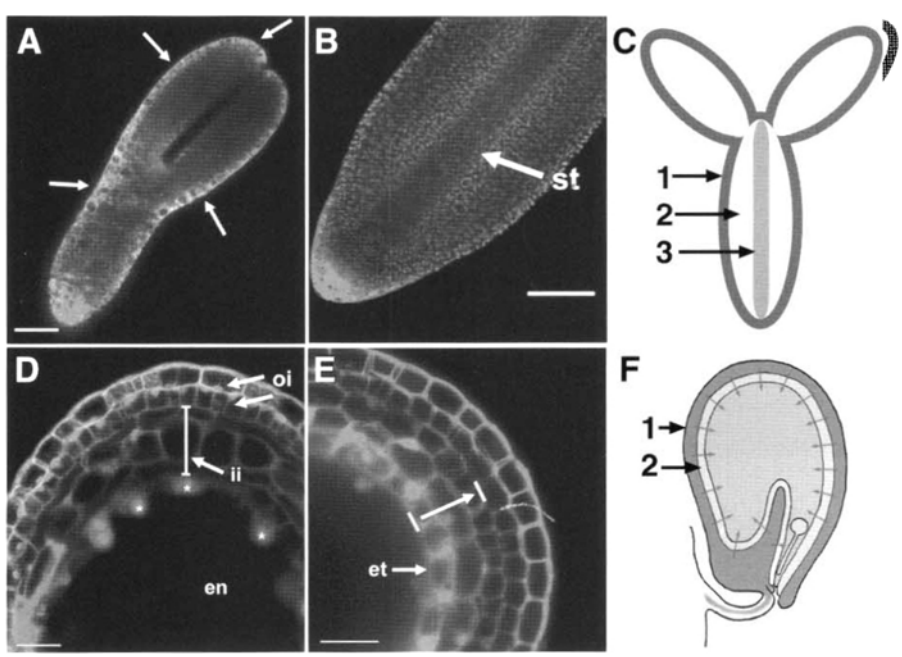

Figure 5. Additional sub-domains in embryos and seed coats. Protodermis (**A**, arrows, and **C1**) and stele (st) (**B**, **C3**) form sub-domains where movement of 1XsGFP, expressed by *AtGL2* and *AtSUC3* promoter, respectively, is allowed within domains but is reduced (**A**) or blocked (**B**) to cells beyond each domain. Outer integuments (oi) (**D**, **F1**) and inner integuments (ii) (**E**, **F2**) form separate symplastic domains where 1XsGFP movement is blocked across boundary between those domains. C2, ground tissues; en, endosperm; et, endothelium. Scale bars, 40 μm (**A**), 50 μm (**B**), 25 μm (**D**), and 20 μm (**E**). Modified with permission from Stadler et al. (2005a).

b), but its movement is limited to within the stele upon expression from a subset of cells in the root tip (Stadler et al., 2005a) (Fig. 4A, 5B). Clearly, PD in and around the SAM are more active than those in the root tip because meristems are likely the source of morphological signals to enable patterning during embryogenesis. Future studies are needed to address how the SAM (and RAM) contribute to the formation of symplastic sub-domains to determine the apical-basal body pattern, and to determine how symplastic sub-domains corresponding to various tissue types are controlled locally.

Symplastic domains in developing seed coats

The *Arabidopsis* seed coat consists of five cell layers: the innermost endothelial layer, followed by two cell layers each of inner and outer integuments. Two symplastic domains, corresponding to those outer and inner integuments, have been identified in developing seed coats (Stadler et al., 2005a). GFP expressed in the outer integument cannot move to the inner integument layers (Fig. 5D). Similarly, GFP expressed in the innermost endothelial layer moves to the inner integument layers, but cannot move to the outer integument layers (Fig. 5E). Even small tracers, such as HPTS (0.5 kDa), are not transported across the boundary between the outer and inner integuments. Therefore, those outer integuments may provide a symplastic route for nutrient transport from maternal tissues to the developing seed, but transfer between outer and inner integument, and from inner integument to embryo, may be apoplastic (Stadler et al., 2005a).

MORE APPROACHES TO IDENTIFYING PD COMPONENTS

Although it is now established that plasmodesmata have dynamic and critical roles in various aspects of plant life, no components specific to PD are known. In addition to the genetics approach mentioned above, several other methods are being explored in an effort to uncover those structural or functional components. One biochemical technique, affinity purification, has been used to reveal that a *Nicotiana tabacum* NON-CELL AUTONOMOUS PATHWAY PROTEIN 1 (NtNCAPP1), from PD-enriched cell wall extracts, serves as an interacting partner with a PD-trafficking protein (CmPP16) (Lee et al., 2003). NtNCAPP1 locates to the cell periphery and contains an ER trans-membrane domain for which deletion blocks the movement of specific PD-trafficking proteins. This suggests that protein movement via PD is both selective and regulated. A plasmodesmal-associated protein kinase (PAPK) specifically interacts with plant viral proteins, such as TMV P30, and localizes to PD. Because P30 is known to manipulate PD, PAPK may act to regulate PD-functioning (Lee et al., 2005).

A collection of random plant cDNA-GFP fusions and their localization in cells has been used to generate a library composed of GFP tags to specific plant organelles, including PD (Cutler et al., 2000). Another high-throughput screening, in which plant cDNA-GFP fusions are expressed by a viral expression system, has enabled researchers to identify 12 proteins specifically localized to PD (Escobar et al., 2003). A punctate pattern in the cell wall is diagnostic for PD labeling and localization. Half of those 12 encoded proteins share no similarity with known proteins, and they may represent novel components of PD.

Proteomics technology is another approach for identifying PD-specific proteins from purified PD or cell-wall fractions enriched for PD (Faulkner et al., 2005a). One protein found by several research groups is a Class 1 reversibly glycosylated polypeptide (RGP). Although these RGPs normally associate with the Golgi, one targets to PD (Sagi et al., 2005). The giant-celled green alga *Chara corallina* provides an advantageous system for applying proteomics (Faulkner et al., 2005b) because its cells are arranged in a single linear file and PD are localized to the cross walls between adjacent cells. Peptides isolated from PD-enriched cell-wall fractions include previously known PD-associated proteins, thereby validating these experiments. Likewise, novel proteins provide new candidates for PD components.

CONCLUDING REMARKS

The critical role of plasmodesmata in plant development is supported by accumulating data of the cell-to-cell movement of transcription factors critical to cell-fate determination. Recent data also suggest that RNAs, mRNAs, and gene-silencing RNAs (Baulcombe, 2002; Voinnet, 2002; Lucas and Lee, 2004) traffic via the vascular system and its connected PD. Other than the identities of PD cargo, little else is known about any potential regulatory molecules that signal PD to allow the selective movement of macromolecules. Further questions to be addressed concern the exact mechanics of transport via PD. To do so, we must synergistically apply diverse approaches that include cellular, genetics, and genomics tools.

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