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Plasmodesmata during development: re-examination of the importance of primary, secondary, and branched plasmodesmata structure versus function

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REVIEW ARTICLE

# Plasmodesmata during development: re-examination of the importance of primary, secondary, and branched plasmodesmata structure versus function

**Tessa M. Burch-Smith · Solomon Stonebloom · Min Xu · Patricia C. Zambryski**

**Abstract** Plasmodesmata (PD) structure and function vary temporally and spatially during all stages of plant development. PD that originate during, or post, cell division are designated as primary or secondary according to classical terminology. PD structure may be simple, twinned, or branched. Studies of PD during leaf, root, and embryo development have lead to the generalization that cells in less mature tissues contain predominantly simple PD. New quantitative analyses reveal that twinned and branched PD also occur in immature tissues. New data also highlight the versatility of viral movement proteins as tags for labeling PD in immature tissues as well as PD in mature tissues. A summary of the formation and function of primary, secondary, and branched PD during leaf, trichome, embryo, apical meristem, vascular cambium, and root development underscores the remarkable and indispensible plant-specific intercellular communication system that is mediated by PD.

**Keywords** Plasmodesmata · TMV-MP · Plant development · Primary and secondary plasmodesmata · Intercellular movement

## Introduction

Coordinated growth, development, response to the environment, and defense in multi-cellular organisms require the exchange of information between cells. In plants, cell-to-cell communication must occur despite the presence of cellulosic walls surrounding each individual cell. This difficulty is circumvented by the use of cell wall spanning, membrane-lined, cytoplasmic channels known as plasmodesmata (PD). PD connect nearly all the cells of a plant, allowing the transport of nutrients, signaling molecules, and developmentally important macromolecules such as transcription factors and RNA (reviewed in (Cilia and Jackson 2004; Lucas and Lee 2004; Kim and Zambryski 2005)).

One of the most critical and unique functions of PD during plant development is the formation of symplastic domains. During embryonic development, all cells share common cytoplasm due to PD interconnections and thus form one single symplastic unit allowing transport of small fluorescent tracers and green fluorescent protein (GFP) (McLean et al. 1997; Kim et al. 2005a). As growth and further differentiation occurs, individual cells or groups of cells become isolated from their immediately neighboring cells by the loss of functional PD. Such subsets of cells form distinct symplastic domains where cells within the domain communicate with each other via PD, while cells at the borders of the domains restrict intercellular transport between domains.

That PD have essential roles in plant growth and development is underscored by the lethal phenotypes of mutants exhibiting altered PD or plasmodesmal transport (Kim et al. 2002a; Kobayashi et al. 2007; Benitez-Alfonso et al. 2009, Stonebloom et al. 2009). In this review, we discuss the formation, structure, and distribution of PD during development. We focus on cell-to-cell movement and not long distance movement via the vascular system. There is a vast PD literature and wherever possible, we try to give only the most recent relevant references. For the reader interested in additional references and details, we

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suggest a book entitled "Plasmodesmata" (Oparka 2005). Other reviews are mentioned in specific subsections. We sincerely apologize if we have missed a relevant publication due to our unawares. As we pay special attention to PD nomenclature, we begin here.

## PD nomenclature, structure, and function during development
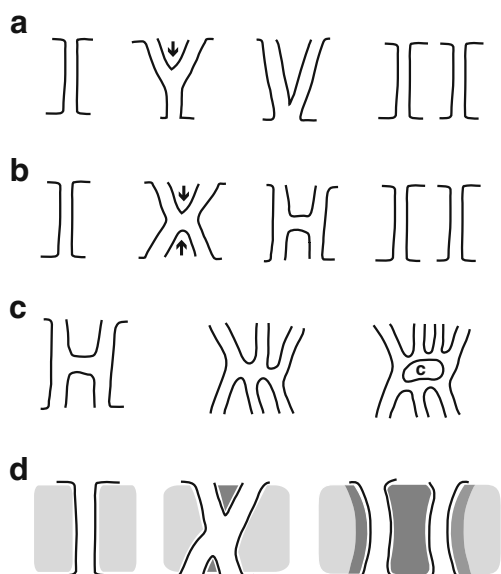
Primary and secondary PD have been described and defined in a comprehensive review by Ehlers and Kollmann (2001); primary PD originate during cell division, and secondary PD refer to all PD that originate post cytokinesis. Besides differing origins, PD have numerous structures, from simple, to branched, to highly branched with central cavities (Fig. 1). The least complex branched PD are Y-, V-, X-, or H-shaped. Simple PD are most often found alone, but they also occur in pairs, called twinned PD, PD not more than 100-nm apart (Faulkner et al. 2008). Y-, V-, X-,



Fig. 1 PD structures and how they form. **a**, **b** Present different intermediate PD structures that may form during the creation of twinned PD by fission of a simple PD. **a** Initiation of PD doubling at one site that results in Y- and V-shaped intermediates. **b** Initiation of PD doubling at two sites that results in X- and H-shaped intermediates. **c** How an H-shaped PD may become further branched and ultimately contain a central cavity (**c**). **d** A mechanistic model for how twinned PD or branched PD arise that derives from the laying down of new cell wall material (*dark grey shading*) as the cell wall (*light grey shading*) expands laterally. New cell wall material may initiate the branching of PD and then ultimately separate the two newly formed PD channels. While the cell wall is not drawn in panels **a**, **b**, and **c**, cell wall expansion likely also drives the formation of the Y-, V-, X-, and H-shaped PD, twinned PD, and multiple-branched PD. Note the PD channels diagrammed in all panels represent the cell membrane bound channel without the central ER-derived desmotubule. The model drawn in D is a simplification of a similar model in Ehlers and van Bel (2010)

or H-shaped PD may represent intermediates in fission of simple PD to form twinned PD. Figure 1a diagrams twinned PD formation from a single initiation point that then results in PD doubling via Y- to V-shaped intermediates. Figure 1b presents a scenario that involves two opposite initiation sites forming an X-shaped intermediate that then becomes H-shaped prior to resolving into twinned PD. Figure 1c diagrams that complex branched PD with multiple channels and central cavities that may arise from H-shaped branched PD.

Here, we suggest that H-shaped PD represent intermediates in the formation of twinned PD (Fig. 1b) or they may represent modifications of simple PD that occur as cells and tissues mature to form complex multiply branched PD (Fig. 1c). Previously, numerous authors propose that H-shaped branched PD arise by fusion of twinned PD, possibly by an X-shaped intermediate (Ding and Lucas 1996; Ehlers and Kollmann 2001; Faulkner et al. 2008); such PD fusion implies H-shaped PD arise post-twin PD formation. However, here we put forward the hypothesis that PD fusion is an unlikely event as such fusions would imply that the cell wall regions between PD contract. Cell wall contraction may occur under certain circumstances such as during senescence, or environmental stress. However, during the bulk of plant development as plant cells grow, their cell walls expand, and PD are likely to double during cell wall expansion (Ehlers and van Bel 2010) (Fig. 1d) (discussed further below). Quantitative time course studies may resolve these conflicting views.

The frequency of simple to branched PD strongly correlates with plant development. In young, immature tissues like embryos (Burch-Smith and Zambryski 2010), sink leaves (Oparka et al. 1999), shoot (Ormenese et al. 2000), and root meristems (Duckett et al. 1994; Zhu et al. 1998a) PD are mostly morphologically simple, consisting of a single linear channel connecting adjacent cells. In mature tissues, PD are mostly branched, adopting more complex structures of multiple channels connected by central cavities (Ding et al. 1992). One of the best illustrations of PD structural alterations during development from immature to mature cells, is a quantitative study of simple versus branched PD during the sink to source transition in tobacco leaves (Oparka et al. 1999). These authors found that sink leaf cells contain over 90% simple PD. In contrast, source leaf cells contain predominantly highly branched PD with central cavities, and only approximately 20% simple PD.

The molecular details of how primary and secondary PD are formed are unknown. Models to date derive from descriptive ultrastructural studies that give only a static snapshot of PD structure and development following harsh chemical fixation of tissues. Nevertheless, we briefly summarize what is known. Primary PD always arise during

cell division, when strands of endoplasmic reticulum (ER) become entrapped in the cell plate during Golgi-mediated deposition of new cell wall material; Golgi membranes then surround the ER strands to form the outer limits of the PD membrane (reviewed in Ehlers and Kollmann 2001).

An even bigger question is how secondary PD form? We now know that there are two types of secondary PD. Secondary PD that arise from simple PD to form twinned PD, and secondary PD that arise entirely de novo in cell walls without pre-existing PD. We use the terms "twinned secondary PD" and "de novo secondary PD" to distinguish these two types of secondary PD. Figure 1d presents a simple model for how twinned secondary PD arise. In this model (from Ehlers and van Bel 2010), PD form as the cell wall loosens and expands horizontally and new cell wall material becomes inserted in between PD membranes. Cell wall expansion may also provoke the insertion of new PD channels as occurs during complex branched PD formation (Fig. 1c). Another model proposes new strands of ER become inserted nearby an existing PD localized ER strand, followed by insertion of new cell wall material (Faulkner et al. 2008). Ehlers and van Bel (2010) distinguish the role of the ER in these two models in PD fission, as "passive" and "active". We favor the passive model (cell wall deposition, not ER drives PD formation) presented in Fig. 1d as we never observe ER insertion into the cell wall during examination of thousands of PD that are forming in cells from developing immature tissues in embryos and sink leaves. It remains possible that ER insertion and/or entrapment may occur in other cells and tissues. Genetic, molecular, and advanced imaging approaches are clearly needed to resolve how twinned PD arise and subsequently develop.

How de novo secondary PD form is even more poorly understood. The critical question is how does the formation of a plasmodesma initiate? Does PD formation initiate in one cell and then progress through the cell wall to the adjacent cytoplasm? A more intricate and unlikely scenario would involve signaling of PD initiation at identical locations in adjacent cells resulting in PD fusion/formation in the central wall region. A plausible scenario involves localized thinning of the cell wall bringing the plasma membranes between two adjacent cells into proximity to allow PD formation to initiate in a focused region with subsequent vertical extension as new cell wall material is laid down. This intriguing possibility is supported by abundant data from graft unions, and fusions between cells growing in tissue culture (reviewed in Ehlers and Kollmann 2001).

However, few studies monitor de novo secondary PD formation during wild-type development. The most obvious cells to study de novo secondary PD formation are those undergoing cell wall elongation post cell division. Two such systems have been investigated in astonishing detail, the elongation zone of the developing root (Zhu et al. 1998a) and the development of secondary xylem and phloem (Ehlers and van Bel 2010). Both studies quantify PD in different cell types along apical, basal, and side cell walls, and are described in more detail in later sections of this review. Apical and basal root cell walls predominantly contain primary PD resulting from cell division in the horizontal plane, whereas elongating sidewalls contain de novo secondary PD. Other systems to study de novo secondary PD formation are those which characterize PD between cells that are unambiguously not clonally related, such as between the epidermal cell layer and the underlying mesophyll cells of the leaf (Burch-Smith and Zambryski 2010). Like primary PD, secondary PD can have various morphologies, from simple to branched (Ehlers and van Bel 2010; Burch-Smith and Zambryski 2010).

How then can primary PD be distinguished from secondary PD? In immature cell walls that are clearly formed as a result of cytokinesis, such as the mesophyll cells of a newly arising sink leaf or the transverse cell walls of a file of elongating root cells in the differentiation zone, PD can be classified most likely as primary. Twinned, Y-, or H-shaped PD in rapidly dividing cells from embryos or sink tissues may represent different stages in the modification of primary PD to produce secondary twin or branched PD (Fig. 1). In contrast, in mature cell walls it is impossible to unequivocally assign the origin of PD as primary or secondary unless the lineage of the cell wall and the developmental history of the PD is known.

Finally, PD function is described by the types of molecules are transported by PD. We introduced the terms targeted and nontargeted PD movement (Crawford and Zambryski 2000). Proteins that interact with PD and induce their own efficient movement are designated "targeted" PD proteins. Targeted proteins, such as tobacco mosaic virus movement protein (TMV-MP) have a punctate appearance in cell walls and such puncta are coincident with PD. Non-targeted proteins, exemplified by exogenous proteins such as GFP, move cell to cell by diffusion and do not target/localize to PD. Targeted movement is thought to be an active process compared with passive non-targeted movement. Whether a protein can move via PD or not is dictated by its cellular address (Crawford and Zambryski 2000); thus, proteins with ER retention or organelle targeting signal sequences do not move cell to cell as they are sequestered into different cellular compartments. Likewise, proteins that are anchored onto cellular structures such as the cytoskeleton do not move cell to cell via PD. Thus, only "soluble" nonsequestered cytoplasmic proteins can move cell to cell. Interestingly, the nuclear pore allows proteins to move into the cytoplasm and vice versa, so that nuclear proteins also can move cell to cell via PD (Crawford and Zambryski 2000). Notably, numerous transcription factors

have been demonstrated to move to adjacent cells via PD (see below), and when expressed ectopically they move extensively. These latter data have led to the suggestion that transcription factors move cell to cell via diffusion, unless retained by their respective binding sites in active chromatin (Wu et al. 2003).

Two new results provoke renewed attention to PD nomenclature. Firstly, we (Kobayashi et al. 2007), and others (Benitez-Alfonso et al. 2009), previously assumed that embryos only contain simple unbranched PD; both reports mention the presence of branched PD specifically as a feature of the mutant phenotype. However, quantitative and statistically significant measurements of PD structures during embryogenesis now reveal that wild-type embryos contain 5–9% twinned and branched PD between early and late torpedo stages of development (Burch-Smith and Zambryski 2010). Thus, the 9% branched PD reported by Benitez-Alfonso et al. (2009) is not significantly different from the frequency observed in wild-type embryos. While Kobayashi et al. (2007) noted 15% branched PD in their mutant, they incorrectly reported no branched PD occur in wild-type tissues because insufficient numbers of PD were analyzed in wild-type tissues. Thus, to accurately determine the frequencies of different PD structures in particular cell types it is essential to analyze a statistically significant number (at least 500) of PD in both experimental and wild-type tissues.

Secondly, we have new results on the properties of the movement protein (MP) of tobacco mosaic virus (TMV). TMV-MP is an exceptionally valuable tool for PD research as TMV-MP labels PD, and TMV-MP can also manipulate PD aperture to increase intercellular movement (reviewed in (Epel 2009)). While TMV-MP previously has been used to identify PD as "secondary" (Ding et al. 1992), we present data (Fig. 2 below) showing that TMV-MP also extensively labels primary PD.

Developmental regulators that traffic through PD

Over the last two decades the importance of PD has been augmented by abundant and clear evidence that endogenous plant macromolecules use PD to move from cell to cell to influence development. Table 1 highlights two critical developmental regulators of cell identity, transcription factors, and small interfering RNAs (siRNAs), that traffic via PD. Most noncell-autonomous transcription factors are members of the KNOTTED1 homeobox (KNOX) or MADS domain families of proteins (reviewed in Jackson 2005). That multiple members of a given protein family traffic intercellularly from one tissue layer to another underscores such movement as essential for function. Movement of transcription factors is tightly regulated as most move only one to a few cells beyond their initial

expression site (Nakajima et al. 2001; Kim et al. 2002b; Kim et al. 2003). The position of a plant cell is the key factor governing its developmental fate (van den Berg et al. 1995, 1997). Therefore the movement of transcription factors is a mechanism for conveying positional information to neighboring cells to reinforce other signals directing a differentiation process (Mezitt and Lucas 1996; Sessions et al. 2000). While numerous transcription factors move cell to cell via PD, none have been observed at sufficient microscopic resolution to reveal whether or not they accumulate to form PD puncta. However, KN1 dilates PD (Lucas et al. 1995), and this result implies a specific active interaction/targeting to PD.

Different types of siRNAs move cell to cell and act to silence endogenous and exogenous homologous sequences. Remnants from transposon inverted repeat sequences (IR) form a large fraction of eukaryotic genes, and such IRs are targets of endogenous gene silencing; we now design RNAinterference (RNAi) experiments that mimic this endogenous gene silencing strategy (Dunoyer et al. 2010b). Gene silencing is also critical to silence exogenous RNAs such as plant viruses (Mlotshwa et al. 2008). siRNAs likely move through PD as double-stranded RNA molecules and to date no proteins are known to associate with the siRNA complex (Dunoyer et al. 2010a). MicroRNAs (miRNAs) are regulators of developmentally important transcription factors and most act cell autonomously (reviewed in Voinnet 2009). However, new evidence suggests two miRNAs, miR165/6, move across cell files to regulate root development (Carlsbecker et al. 2010) discussed further below. Finally, some trans-acting silencing (tasi) RNAs move from the upper leaf surface to the lower leaf surface to signal auxin-mediated lower leaf development (Chitwood et al. 2009). Local PD-mediated and systemic movement of small (~17–27 nucleotides) RNAs is a rapidly advancing new area of research in plant developmental biology.

PD aperture is regulated during development

PD were first described as channels allowing the passive movement of water and small solutes between cells (reviewed in Roberts and Oparka 2003). The advent of modern cell biological techniques combined with probes derived from plant viruses and membrane-impermeant fluorescent molecules of various sizes revealed the dynamic nature of PD. The upper limit of the size of molecules transported by PD is called the size exclusion limit (SEL), and SEL reflects PD aperture. Changes in PD aperture/SEL occur during all stages of plant growth and development, most often studied in tobacco leaves of different ages (Oparka et al. 1999 and references therein). However, it is important to note that within mature or immature tobacco

**Table 1** Developmental regulators of cell identity, transcription factors, and small interfering RNAs

| Molecule | Description | Function | Tissue | References |
|---|---|---|---|---|
| Transcription factors | | | | |
| KN1 | KNOX family | Cell division and cell fate | Maize SAM | Lucas et al. 1995 |
| GLO/DEF | MADS domain family and B type | Petal and stamen identity | *Antirrhinum* inflorescence meristem | Perbal et al. 1996 |
| LFY | Unique and plant specific | Floral meristem identity | *Arabidopsis* inflorescence | Sessions et al. 2000 |
| STM | KNOX family | SAM initiation and maintenance | *Arabidopsis* SAM | Kim et al. 2003 |
| KNAT1/BP | KNOX family | SAM initiation and maintenance; inflorescence cell fate | *Arabidopsis* meristem | Kim et al. 2003 |
| SHR | GRAS family | Cell division and endodermis specification | *Arabidopsis* root | Helariutta et al. 2000; Nakajima et al. 2001 |
| CPC | MYB family | Root hair development | *Arabidopsis* root | Wada et al. 2002 |
| AG | MADS domain family and C type | Cell division and cell fate; flower development | *Arabidopsis* inflorescence | Urbanus et al. 2010 |
| RNAs | | | | |
| Endogenous siRNAs | 21–24 nt RNA from transcripts with stem-loop structures | Silence endogenous IR sequences | All tissues | Tretter et al. 2008; Dunoyer et al. 2010a, b; Molnar et al. 2010 |
| tasiRNA | 20–24 nt RNA, form by miRNA activity, and siRNA machinery | Leaf development | Leaf | Tretter et al. 2008; Chitwood et al. 2009 |
| miRNAs | 20–24 nt RNA encoded by *MIR* genes | Cell fate and root development | Root | Carlsbecker et al. 2010 |

*KN1* KNOTTED1, *KNOX* KNOTTED1 homeobox, *SAM* shoot apical meristem, *GLO* GLOBOSA, *DEF* DEFICIENS, *LFY* LEAFY, *STM* SHOOTMERISTEMLESS, *KNAT1* KNOTTED1-like homeobox protein 1, *BP* BREVIPEDICELLUS, *SHR* SHORTROOT, *CPC* CAPRICE, *AG* AGAMOUS, *siRNAs* small interfering RNAs, *tasi* transacting siRNAs, *miRNAs* microRNAs

leaves individual cells may have PD with different states of aperture/SEL such as closed, open, or dilated, and the frequency of the different states of PD aperture varies in young versus older tissues (Crawford and Zambryski 2001). Remarkably, viral MPs can overcome reduced aperture even in mature leaves; 2X-GFP (54 kDa) normally moves out of only 2% of the cells expressing it in mature source leaves, but TMV-MP fused to GFP (58 kDa) moves out of 52% of such cells (Crawford and Zambryski 2001). These data suggest that PD aperture is dynamic and not fixed.

As already mentioned, one important function of the dynamic regulation of PD aperture/SEL is the establishment of symplasmic fields, cytoplasmically coupled cells that execute a common developmental program. Symplasmic fields occur in the shoot apex (Rinne and van der Schoot 1998; Gisel et al. 1999; Gisel et al. 2002), root epidermis (Duckett et al. 1994), different regions of developing embryos (Kim et al. 2005a), seeds (Stadler et al. 2005), and the tissues surrounding the male and female gametophytes (Imlau et al. 1999). PD within a field will likely have similar SEL but reduced PD aperture at the borders likely modulates the passage of morphogens, allowing the establishment of morphogenetic gradients required for tissue development. Sometimes symplastic isolation is

fundamental to the development of a particular cell type including root hairs (Duckett et al. 1994), stomatal guard cells (Palevitz and Hepler 1985), cotton fiber cells (Ruan et al. 2001), pollen mother cells that become isolated from the surrounding tapetum during their development (Regan and Moffatt 1990), and the female megaspore that becomes isolated from the surrounding cells of the ovule (Werner et al. 2010).

## The viral connection: confusing primary and secondary PD

TMV-MP localizes to the central cavities of complex branched PD in the source leaves of transgenic tobacco plants constitutively expressing MP but is absent from simple PD in the sink leaves of the same plants (Ding et al. 1992). These data have led to the idea that TMV-MP only localizes to complex branched PD with central cavities, called "secondary" PD by the authors, in mature tissues (Ding et al. 1992). Many reports have subsequently asserted that viral MPs preferentially localize to "secondary" plasmodesmata in mature tissues (e.g., Epel 1994; Hofius et al. 2001). Based on these earlier reports, TMV-MP was used to distinguish branched ("secondary") PD from primary PD (Roberts et al. 2001).
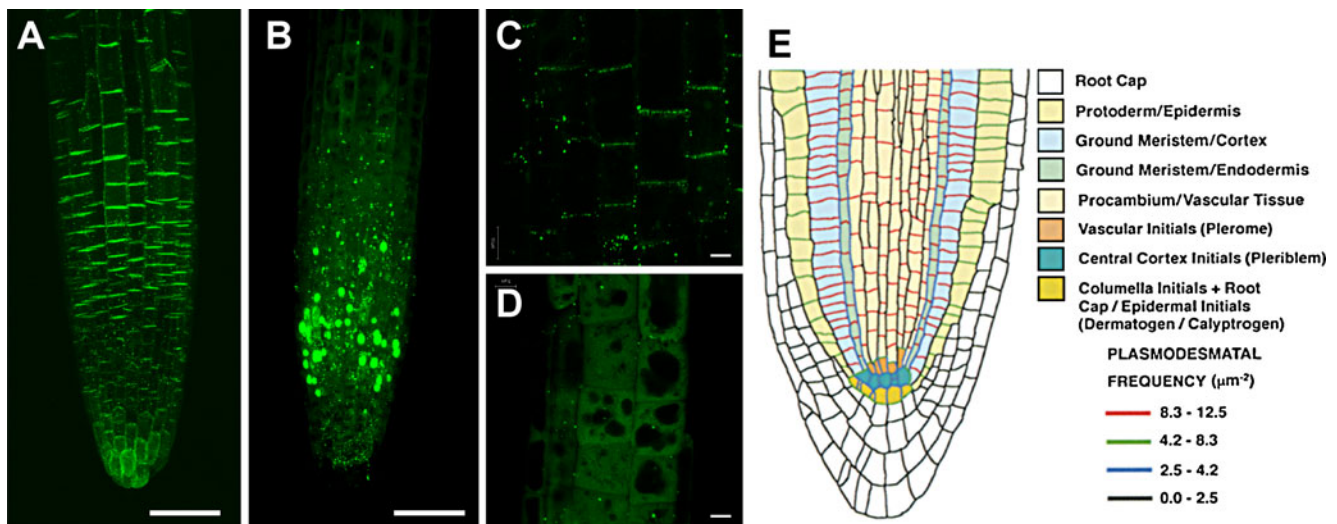
**Fig. 2** Localization of viral movement proteins in *Arabidopsis* seedling root tips. TMV-MP-GFP constitutively expressed from the CaMV minimal 35S promoter in a 5-day-old transgenic *Arabidopsis* seedling root tips localizes predominantly to plasmodesmata in transverse cell walls (**a**) containing high densities of primary PD while PLRV MP17-GFP expressed from the same promoter fails to localize to plasmodesmata in root tips (**b**). Images are projections of confocal stacks; *scale bar* represents 50 μm. Higher magnification confocal images of TMV-MP-GFP (**c**) and PLRV MP17-GFP expression (**d**) in root tip epidermal cells reveal that TMV-MP-GFP is localized largely to punctae in transverse cell walls while PLRV MP17-GFP is retained in the cytoplasm and forms cytoplasmic aggregates. Scale bar represents 5 μm. **e** A graphic representation of the distribution and frequency of predominantly primary plasmodesmata in 1-week-old *Arabidopsis* root tips demonstrates that transverse cell walls in all cell layers of the root tip contain a higher density of plasmodesmata than longitudinal cell walls. **e** Adapted from Fig. 4 of Zhu et al. 1998b and used with kind permission from Springer: Protoplasma

However, it is critical to point out that TMV-MP is also capable of labeling simple PD (without branches or cavities) in immature cells. For example, TMV-MP fused to GFP (MP-GFP) significantly labels PD in cells from tobacco sink leaves (see Fig. 4 in Crawford and Zambryski 2001). Furthermore, TMV-MP-GFP extensively labels PD in embryos (Kim et al. 2005a), even though very few PD in embryonic tissues are secondary (Zhu et al. 1998b; Burch-Smith and Zambryski 2010). Significantly, here we show that TMV-MP-GFP also labels the PD in transverse walls of cells in the differentiation zone just above the meristem of 1-week-old *Arabidopsis* seedling roots (Fig. 2a, c). These PD have been established as primary PD (Zhu et al. 1998a) and their distribution frequencies in the different cell files of seedling roots are illustrated in Fig. 2e. In support and in contrast the movement protein of, Potato leaf roll virus (PLRV) MP17, another MP that accumulates at complex PD in transgenic plants (Hofius et al. 2001), does not localize to PD in the same region of the developing root (Fig. 2b, d). Instead, PLRV MP17-GFP appears throughout the cytoplasm or in aggregates in the developing root (Fig. 2b, d).

While the literature claims that both TMV-MP and PLRV MP17 specifically label secondary PD, it is clear that TMV-MP can also label primary PD in sink leaves, embryos, and young seedling roots. Thus, P30 and P17 have different and distinct PD-labeling specificities. Results that depend on the use of these probes to distinguish between PD of different origins should therefore be interpreted cautiously.

**PD in different developing cells and tissues have differing transport capacities**

PD of different origins and structures have different transport capabilities in different tissues. Arguably, strictly controlled differences in PD function have important roles in development to allow specific distribution of developmentally important molecules.

*Leaves*

Cell-to-cell movement has been studied most in tobacco leaves. In young leaves or in regions of maturing leaves that are still net photosynthetic sinks, greater than 90% of PD are simple (Oparka et al. 1999). As these cells become net exporters of photosynthate, simple PD become complex and sometimes elaborately branched PD (Ding et al. 1992; Itaya et al. 1998). While the origins of these latter PD were not determined, the authors refer to them as secondary. At least some of these complex PD may derive from modified (i.e., branched) primary PD. Nonetheless, it is clear that modified PD have different transport abilities from their simple progenitors.

Large soluble molecules like GFP move through PD by diffusion without specifically interacting with the PD,

termed non-targeted movement (Crawford and Zambryski 2000). Cell-to-cell spread of GFP fusions revealed that the aperture/SEL of mesophyll PD of tobacco sink leaves is 47 to 54 kDa (Oparka et al. 1999; Crawford and Zambryski 2001). However, source tissues showed a marked decrease in the ability to transport molecules of this size (Oparka et al. 1999; Crawford and Zambryski 2001). These results suggest that sink PD have a high basal aperture/SEL allowing diffusion-driven non-targeted transport of relatively large molecules, while source tissues have more restricted cell-to-cell movement.

In contrast to non-targeted movement of soluble GFP, TMV-MP-GFP targets to PD forming punctate patterns in the cell wall and remains associated with PD as it spreads to neighboring cells (Crawford and Zambryski 2000). When fluorescent membrane-impermeable dyes were microinjected into the cytoplasm of mature tobacco mesophyll cells, dyes of only approximately 500 Da move freely from cell to cell (Wolf et al. 1989). However, in transgenic tobacco plants constitutively expressing TMV-MP, PD aperture/SEL increases to allow molecules of approximately 10 kDa to move cell to cell following microinjection (Wolf et al. 1989). Thus in addition to mediating its own intercellular movement TMV-MP can "gate" PD, causing large increases in their apparent aperture/SEL and such "gating" stimulates movement of molecules (such as large fluorescent dextrans) in *trans*. PD gating was limited to fully expanded or mature leaves in TMV-MP transgenic tobacco when studied by microinjection assays (Deom et al. 1990). However, quantitative studies using biolistic bombardment to introduce TMV-MP for transient expression revealed that MP-GFP dramatically gates PD in source leaves (52% of cells exhibited targeted movement of 58-kDa MP-GFP versus only 2% exhibited non-targeted movement of similarly sized 54-kDa 2X-GFP), and also causes significant gating in sink leaves (76% of cells exhibited targeted movement of MP-GFP versus 30% exhibited non-targeted movement of 2X-GFP) (Crawford and Zambryski 2001). Thus, TMV-MP causes a striking manipulation of aperture/SEL in complex PD of mature source leaf cells, but can also manipulate PD that already exhibit high aperture/SEL in immature sink leaf cells. That Deom et al. (1990) did not observe gating in immature tissues as in Crawford and Zambryski (2001) is likely due to several factors. Deom et al. (1990) used transgenic plants with long-term over-expression of TMV-MP that may lead to inactivation of TMV-MP by host factors, and fluorescent dextrans were added to cells by microinjection that may result in pressure induced PD closure (Oparka and Prior 1992). In contrast, Crawford and Zambryski (2001) studied TMV-MP activity following transient expression of DNA constructs introduced by biolistic bombardment at very low pressure. Note, early studies using biolistic bombardment

at much higher pressure resulted in no diffusive non-targeted cell-to-cell movement of GFP (Itaya et al. 1997).

Independent dye coupling analyses in mature leaves, where the intercellular movement of an injected dye is monitored, reveal PD transport between specific cells: mesophyll to mesophyll, mesophyll to bundle-sheath cells, bundle-sheath cells to each other, but not bundle-sheath to phloem-parenchyma cells (Ding et al. 1992). This latter group of PD is truly secondary (Ehlers and Kollmann 2001), and is critical for unidirectional sugar transport (Volk et al. 1996). Thus secondary PD may have functions that are different from modified primary PD, despite their apparently identical structure.

### Trichomes

Tobacco trichomes contain a linear array of five to eight cells that arise by cell division; thus, the PD connecting the cells are mainly primary, or secondary if they are formed post cell division. Trichome cells are connected by a gradient of simple (at the less mature tip cell) to complex, branched PD (in the more mature basal cell) (Waigmann et al. 1997). The basal aperture/SEL of PD between trichome cells is relatively large at 7 kDa (determined by microinjection) and is not increased by TMV-MP (Waigmann and Zambryski 1995). Thus TMV-MP cannot gate PD in all cell types.

A comprehensive study of PD at the tobacco leaf trichome-epidermal cell interface illuminates PD development during trichome maturation (Faulkner et al. 2008). The first PD formed at this interface are randomly distributed primary PD. As the surface area of the trichome-epidermal cell wall interface increases, there is an increase in the number of secondary PD due to the insertion of new PD directly adjacent to existing channels to produce twinned PD. These twinned PD likely represent one primary PD and one secondary PD and Faulkner et al. (2008) suggest they may fuse to give rise to branched PD with central cavities. Interestingly, there is unidirectional transport across this wall interface. The small (~45 Da) fluorescent dye Lucifer yellow CH failed to enter epidermal cells when microinjected into the basal trichome cell, although it moved apically into other trichome cells (Christensen et al. 2009). However, if the dye was injected into the supporting epidermal cell, it entered the trichome cells. TMV-MP had no effect on this symplastic barrier, suggesting that the PD at this boundary are resistant to the gating effect of MP consistent with previous data (Waigmann and Zambryski 1995). Directionality of PD transport develops as leaves mature and PD undergo modification since PD in young single-celled trichomes, containing simple PD, allow transport into epidermal cells (Faulkner et al. 2008; Christensen et al. 2009). Another factor that may drive directional transport in mature trichomes is bulk solute transfer to tip cells for excretion.

## Embryos

The embryo has traditionally been viewed as a collection of immature tissues that establish the basic body plan of the adult plant. However, these developing tissues exhibit intricate regulation of their PD aperture/SEL (Kim et al. 2002a; Kim et al. 2005a, b; Kim and Zambryski 2005; Stadler et al. 2005) that is critical for normal *Arabidopsis* embryogenesis. For example, at the mid-torpedo stage PD aperture/SEL is significantly decreased (Kim et al. 2002a). Furthermore, the embryo contains distinct symplastic domains where single, double, and triple-sized GFP display differential movement into different regions of the embryo at different stages of embryogenesis (Kim et al. 2005a). Remarkably, these domains of PD-mediated transport correspond to the areas that will give rise to the basic tissues of the seedling established by clonal analyses, i.e., cotyledons, shoot apical meristem, hypocotyl, and root. Thus, the size of PD aperture and consequent transport capacity likely facilitates morphogenesis.

The identification of embryo-lethal mutants with altered patterns of movement of fluorescent tracers compared with wild type reinforces the importance of regulation of PD aperture/SEL in embryo development (Kim et al. 2002a). Two mutations, *increased size exclusion limit* (*ise*)*1* and *2*, allow transport of fluorescent tracers beyond the time of the mid-torpedo transition when wild-type PD aperture/SEL is decreased. *ISE1* encodes a mitochondrial DEAD-box RNA helicase (Stonebloom et al. 2009), and *ISE2* encodes a DEVH-type RNA helicase (Kobayashi et al. 2007). Benitez-Alfonso et al. (2009) mutagenized seedlings expressing GFP in phloem specific cells that lead to GFP movement cell to cell into surrounding tissues. They screened for mutants, called *GFP-arrested trafficking1* (*gat1*) that exhibit reduced PD function by failing to unload GFP from the phloem.

Ultrastructural analyses examined the relationship between PD form and function in wild type versus *ise1* and *ise2* embryos (Burch-Smith and Zambryski 2010). Interestingly twinned and branched (Y- and H-shaped) PD occur in wild-type embryos of all stages representing 9%, 10%, and 8% of PD in hypocotyls of early, mid, and late torpedo embryos (Burch-Smith and Zambryski 2010). Thus, in contrast to the widely held view that branched PD are synonymous with mature tissues, branched PD also occur in very young developing tissues. Notably *ise1* and *ise2* mutations result in a significant increase in the occurrence of twinned and branched PD in embryos (Burch-Smith and Zambryski 2010); *ise1* embryos have 18%, 15%, and 26% twinned and branched PD in hypocotyls of early, mid, and late torpedo stages, and *ise2* hypocotyls have 25%, 28%, and 13% twinned and branched PD at the same stages. Interestingly there is a temporal pattern in the frequency of

twinned and branched PD during embryogenesis; the hypocotyl contains more twinned and branched PD than the cotyledon in early and mid-torpedo embryos, and this pattern is reversed in late torpedo embryos. This pattern is conserved in the *ise2* mutant compared with wild type, but it is altered in the *ise1* mutant. Thus, PD structure/function may regulate plant development or vice versa.

What is the origin of these twinned and branched PD in the embryo? RNA silencing experiments in sink leaves of *Nicotiana benthamiana* revealed that ISE1 and ISE2 act in pathways to control the formation of de novo secondary plasmodesmata; we were able to unambiguously assign PD as de novo secondary PD since we scored PD in the cell walls connecting the epidermal layer to the underlying mesophyll cells. Epidermal and mesophyll cells are not clonally related and divide independently of each other ((Marcotrigiano 2001) and references therein). Thus, we propose that the increased proportions of twinned and branched PD in *ise1* and *ise2* mutant embryos are due to the upregulation of secondary PD formation. Secondary PD formed in *ISE1*- and *ISE2*-silenced leaves have significantly increased transport abilities compared with nonsilenced controls, as measured by TMV-MP-2XGFP movement in the horizontal and vertical planes representing PD-mediated movement between epidermal cells and from the epidermal layer to the underlying mesophyll cells. These data in silenced leaves are consistent with the increased transport of fluorescent tracers in *ise1* and *ise2* mutant embryos (Burch-Smith and Zambryski 2010).

## PD in the shoot apex

PD connecting the L1 cells to underlying L2 cells in the shoot apical meristem (SAM) of dicotyledonous plants are unambiguously secondary as cells in the L1 layer almost exclusively divide anticlinally (Marcotrigiano 2001). Secondary PD connecting L1 to L2, such as those connecting the epidermis to the mesophyll layer of leaves can be simple or branched (Burch-Smith and Zambryski 2010). Secondary L1–L2 PD in the vegetative and inflorescence apical meristems facilitate movement of developmentally important transcription factors belonging to the KNOX (Jackson et al. 1994; Lucas et al. 1995) and the MADS families (Perbal et al. 1996) among others (Table 1). KNOTTED1 (KN1) was the first transcription factor shown to move intercellularly: *KN1* RNA is expressed exclusively in L2 cells of maize meristems but KN1 protein is detected in both L1 and L2 (Lucas et al. 1995). Similarly, *Antirrhinum* DEFICIENS moves from L2 and L3 to the L1 (Perbal et al. 1996). When a GFP-KN1 fusion was expressed specifically in *Arabidopsis* leaf mesophyll cells (L2/L3) it was detected in the epidermis (L1), but it could not move in the opposite direction from epidermis to mesophyll (Kim et al. 2003). In contrast, when

GFP-KN1 was expressed in the L1 of the SAM it moved to the L2/L3 layers. Thus, leaf L1–L2 secondary PD exhibit directionality of PD transport compared with L1–L2 secondary PD in the SAM (Kim et al. 2003). Free GFP and a GFP-viral MP fusion could move in both directions in the leaf, suggesting there is a specific mechanism regulating movement of the GFP-KN1 fusion and that PD are not generally closed in one direction.

PD and intercellular trafficking in the SAM are also regulated in response to environmental cues like seasonal day length changes that influence development. The frequency of PD throughout the *Sinapis alba* SAM undergoing the floral transition increases dramatically (Ormenese et al. 2000). While some PD may be primary due to cell division, Ormenese et al. (2000) suggest that the vast majority of these newly formed PD are secondary. Such secondary PD formation may be prompted by the transient increase in a floral stimulus (Ormenese et al. 2000), most likely cytokinin (Ormenese et al. 2006).

Detailed time course experiments indicate that PD transport is temporally regulated at the *Arabidopsis* SAM during development (Gisel et al. 1999; Gisel et al. 2002). There is little unloading of fluorescent tracers from the vascular system into young vegetative apices (plants with less than 12 visible leaves) but there is a dramatic increase of unloading into apices in older plants with greater than 12 visible leaves. (Loading is a term used to denote movement of nutrients, signal molecules and tracers into the vascular system from the surrounding cells of source leaves; the corollary is unloading which occurs when molecules move from the vascular system into the surrounding cells of sink tissues. For the experiments described tracer was loaded into the vascular system of leaf petioles, and then observed for unloading in the SAM sink.) Remarkably, unloading into the apex ceases for several days during the transition from vegetative to floral development in *Arabidopsis* (Fig. 3), and then resumes again once floral morphogenesis is underway (Gisel et al. 1999; Gisel et al. 2002). As the inflorescence meristem undergoes profound changes in architecture and gene expression to produce floral meristems (that will produce multiple organ types) versus the single type of organ (leaves) produced by the vegetative meristem, it may be advantageous to sequester the SAM during the establishment of reproductive development. Secondary PD formed during this time (Ormenese et al. 2000) may then facilitate highly regulated transport of micro- and macromolecular signaling molecules.

## PD in vascular development

The vascular cambium is a lateral meristem that gives rise to the secondary vascular tissues. An outstandingly detailed study utilized newly arising secondary xylem and phloem in
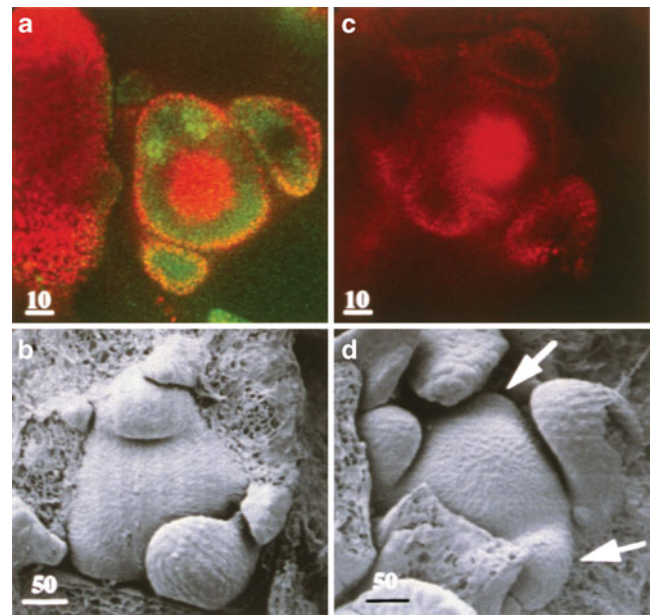


**Fig. 3** Movement of symplastic tracers during the transition to floral development in *Arabidopsis*. *Arabidopsis* grown under short-day (SD) conditions were loaded through their leaf petioles with 8-hydroxypyrene 1,3,6,-trisulfonic acid (HPTS), molecular mass 520 Da. Following 39 and 42 SDs, seedlings have 15 (**a**, **b**) and 19 leaves (**c**, **d**). **a** At 39 SD, plants are still vegetative and unload HPTS into their apices following transport via the vascular system from leaf petioles. The image is a confocal optical section and documents that HPTS does not unload in the center of the meristem (Gisel et al. 1999); however, HPTS does unload throughout leaf primordia seen around the circumference of the meristem. **c** At 42 SD, plants have initiated reproductive development and no longer unload HPTS into their apices. Vegetative versus reproductive apices are distinguished by the types of primordia they produce; leaf primordia are pointy versus floral primordia have a rounded shape (*arrows* in (**d**)). **b** Scanning electron micrograph (SEM) of a 39-SD-old apex reveals leaf primordia around the SAM. **d** SEM of a 42-SD apex reveals leaf primordia as well as newly arising floral primordia. Data were obtained as described in Gisel et al. 1999

the tomato stem to follow the production and function of PD during vascular development (Ehlers and van Bel 2010). PD development follows a strict chronological pattern that coincides with cell development, as also noted during embryo development (Burch-Smith and Zambryski 2010). Notably, most of the PD deployed in the cambial zone arise by modification of existing PD and extensive insertion of secondary PD (Ehlers and van Bel 2010). The authors describe four phases of PD development in tangential walls beginning with PD connecting the xylem and phloem initials and ending with PD connecting mature vascular cells. The radial patterning of cells during vascular development is dependent on increased branching of PD and insertion of secondary PD during cell expansion, and is concomitant with decreased intercellular transport (measured by injection of Lucifer Yellow and its movement in radial sections). In contrast, in transverse cell walls connecting cells of the same developmental stage, secondary PD are inserted in all

developmental phases, not always coincident with cell expansion. Despite the large numbers of PD connecting longitudinal cell files, transport in cambial tissue is preferentially in the radial direction via PD between different cell types representing distinct developmental stages. This elaborate pattern of PD formation and modification likely serves to establish symplastic domains that are important for the differentiation of the secondary vasculature. There also are seasonal changes in PD trafficking in the cambium of poplar trees (Fuchs et al. 2010). Storage proteins that accumulate during the winter months are mobilized from vacuoles and storage bodies and are transported to growing buds during a 3-week period in the spring coincident with reactivation of PD transport in cambial cells.

### PD in roots

Cell division patterns, cell expansion and PD distribution in the roots of *Arabidopsis* seedlings have been extensively characterized (Baum and Rost 1996; Rost et al. 1996; Zhu et al. 1998a; Zhu et al. 1998b; Benfey and Scheres 2000). The distribution of PD in developing *Arabidopsis* seedling roots as observed by Zhu et al. (1998a, b) is summarized in Fig. 2e. Since the cells of a developing root file are the product of successive transverse divisions, the PD in the transverse cell walls are predominantly primary, while those in the longitudinal walls connecting neighboring files of tissue are mainly secondary. There are more PD in the transverse walls than in longitudinal walls in young roots (Fig. 2e). This pattern is maintained through root development as cell expansion occurs, although the relative number of PD per $\mu m^2$ in transverse walls decreases (Zhu et al. 1998b).

The cells of a particular vertical file or lineage share similar gene expression patterns (Rost and Bryant 1996; Brady et al. 2007) and are connected by numerous PD. After unloading from the phloem carboxyfluorescein moves preferentially through the transverse cell walls within a cell file of the root, consistent with the abundance of primary PD in those walls (Zhu et al. 1998a). Enhanced transport via PD may serve to reinforce both developmental programs within groups of cells and positional signaling from older to younger cells (van den Berg et al. 1995, 1997; Zhu et al. 1998a, b).

A striking example of the importance of PD-mediated intercellular traffic during development is how radial patterning is established in the root. The SHORTROOT (SHR) transcription factor moves from cell files in the central stele to the single layer of adjacent endodermal cells (Nakajima et al. 2001) (Table 1). Interestingly, SHR transport occurs via the secondary PD that connect the vertical walls in adjacent cell files, just as secondary PD are used for transcription factor movement in the apical meristem (see above). Note again that traffic through the secondary PD seems to be more tightly regulated than through primary PD, since there is no

dye coupling between cell files (Zhu et al. 1998a). SHR acts in the nucleus of the endodermis with its partner, the SCARECROW (SCR) transcription factor, to induce the expression of other transcription factors to promote root cell differentiation. Moreover, SHR and SCR induce the expression of specific miRNAs 165 and 166. miRNA165/166 then move cell to cell in the reverse direction to regulate the activity of the PHABULOSA (PHB) transcription factor by binding to PHB mRNA to promote root xylem development in a dose dependent manner (Carlsbecker et al. 2010). Lateral movement of miRNA 165/166 likely occurs via secondary PD.

### How are PD apertures/SELs and transport regulated?

One of the outstanding questions about PD function is how aperture/SEL is regulated. The emerging picture suggests numerous cellular processes coordinate intercellular and intracellular signals that affect PD function/aperture.

### Callose

In the dormant birch SAM, callose is deposited around PD neck regions leading to the isolation of individual cells and the breakdown of symplasmic fields (Rinne and van der Schoot 1998; Rinne et al. 2001). However, in the active birch SAM, callose is not associated with PD at the boundaries of active symplasmic fields (Rinne and van der Schoot 1998; Rinne et al. 2001). Also, callose deposition was not observed when PD transport was decreased in cells with clear differences in PD aperture/SEL that connect the differing cell types in the cambium and developing secondary vasculature of the tomato stems (Ehlers and van Bel 2010). Thus, while callose may be involved in long-term occlusion of PD during dormancy, it is not likely to be the main mechanism used for the transient regulation of PD during normal development. The importance of callose as a regulator of PD aperture is evidenced by the identification of a PD localized b-1,3-glucanase that regulates callose degradation (Levy et al. 2007). Reversibly glycosylated proteins (RGPs) can also localize to PD (Sagi et al. 2005), and they may regulate callose accumulation and PD function. In fact, RGP over-expression leads to decreased cell-to-cell movement (Zavaliev et al. 2010) and silencing of RGP leads to enhanced PD-mediated transport (T.B.-S.,unpublished). The deposition of callose following wounding and during pathogen attack is an active area of investigation (see (Chen and Kim 2009) for review). Herein, Benitez-Alfonso, Jackson, and Maule review callose deposition at PD in response to changes in redox state.

### Cargo modification

The best-studied PD interacting proteins are the viral MPs and several are phosphorylated in planta ((Lee and Lucas

2001) and references therein). TMV-MP is phosphorylated at specific residues at its C terminus resulting in loss of its ability to move cell to cell and gate PD (Waigmann et al. 2000). TMV-MP is phosphorylated by a PD-resident kinase that is also active on a subset of other viral MPs and plant transcription factors including LEAFY (Lee et al. 2005). These results suggest an endogenous pathway uses phosphorylation to regulate cargo movement through PD.

### Energy and reactive oxygen species

Inhibitor studies suggest that ATP-dependent mechanisms (apart from the previously mentioned phosphorylation) are important for regulating PD function. The general metabolic inhibitor sodium azide increases PD aperture/SEL while drastically reducing cellular ATP levels in a variety of tissues including wheat roots (Cleland et al. 1994), tobacco leaves (Christensen et al. 2009), and *Setcreasea* stamen hairs (Tucker 1993). ATP-dependent proteins such as actin may regulate PD transport in specific cell types. Application of the actin inhibitor latrunculin B caused an increase in PD aperture/SEL between leaf mesophyll cells (Ding et al. 1996) but not at the trichome-epidermis boundary (Christensen et al. 2009).

Recent evidence further implicates the general energy status of the cell, i.e., ATP production by mitochondria, in regulating PD transport. Embryo defective *ise1* mutants increase PD transport and exhibit delayed development (Kim et al. 2002a). *ISE1* encodes a nuclear encoded mitochondria localized RNA helicase that is likely essential for mitochondrial mRNA synthesis, and *ise1* mutants are defective in the formation of mitochondrial electron transport gradients necessary for ATP production (Stonebloom et al. 2009). In addition, reactive oxygen species (ROS) likely act as critical signaling molecules between cellular energy status and PD development and/or function as *ise1* mutants produce increased levels of ROS (Stonebloom et al. 2009), and another *Arabidopsis* PD mutant, *gat1*, also produces increased levels of ROS (Benitez-Alfonso et al. 2009). *GAT1* encodes a plastid thioredoxin and *gat1* mutants increase callose production (Benitez-Alfonso et al. 2009). However, *ise1* mutants have increased intercellular transport while *gat1* mutants have decreased intercellular trafficking (Benitez-Alfonso et al. 2009; Stonebloom et al. 2009). Thus, the site of ROS production, mitochondria versus chloroplasts, likely differentially regulates cellular processes and associated specific targets that in turn affect PD function. In addition, the relative amount of ROS may differentially affect PD function as the levels of ROS produced in *gat1* are higher than those produced in *ise1*.

Interestingly, thioredoxins, which are general regulators of the oxidative state of cells, can themselves move between cells (Ishiwatari et al. 1998; Meng et al. 2010). However, various thioredoxins differ in function and PD are implicated in the movement of only some thioredoxins in the vasculature (Ishiwatari et al. 1998). Further supporting the integral role of ROS in co-ordinating PD function, oxidative stress induced by anaerobic conditions increases PD aperture/SEL in wheat roots (Cleland et al. 1994).

### Perspectives

Exciting advances in understanding PD form and function during development have been made recently. We look forward to progress in answering numerous outstanding questions. Now independent reports document the occurrence of twinned and branched PD in several immature tissues, leaves, roots, embryos, and vascular cambium. Does PD function differ in simple versus twinned or branched PD in such young tissues? Does PD function differ in primary versus secondary or twinned and branched PD in immature versus mature tissues? As PD-specific components are identified (Faulkner and Maule 2010) it will be interesting to determine their localization patterns during primary and secondary PD formation and PD modification throughout development. How do energy and ROS levels (reviewed by Benitez-Alfonso et al. 2010) signal PD formation and function during development? How widespread is miRNA movement? How do small RNAs interact with and move via PD? What factors control the movement of cell nonautonomous transcription factors that regulate development? How do secondary PD form? What are the roles of membranes and/or cell wall synthesis and degradation in secondary PD formation? What signals secondary PD to form? What signals PD to form branches or cavities? All these studies will benefit from the development of advanced imaging techniques as described by Oparka et al. (1992, 1999; Fitzgibbon et al. 2010). Finally, new data show that TMV-MP also labels PD in immature tissues, thus increasing its value as a versatile probe to study PD formation and function during all stages of developmental programming.

As we continue to make strides in elucidating and characterizing the critical roles of PD in plant development, it is important to adopt standard nomenclature for PD. We strongly encourage the use of the terms utilized by Ehlers and Kollmann (2001) to describe PD based on their origin and structure. The terms "primary" and "secondary" should be reserved for use when the origin of PD is unambiguously known. Simple and twinned PD are easiest to distinguish. Branched PD can be specified as Y- or H-shaped, or complex branched PD with central cavities. The latter degree of branching or cavity formation can be further specified, as they are clearly less complex in immature versus mature tissues. Moreover, as there are 2 types of secondary PD, it may be useful to adopt the terms "twinned

secondary PD" and "de novo secondary PD". Clarity in PD nomenclature will ensure consistency and allow coherent and incisive discussions of existing and forthcoming data.

**Conflicts of interest**   The authors declare that they have no conflict of interest.

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