Chapter 2
Embryogenesis As a Model System to Dissect the Genetic and Developmental Regulation of Cell-to-Cell Transport Via Plasmodesmata

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1 Introduction

1.1 Embryogenesis

Arabidopsis offers a remarkably accessible model system to study plant embryogenesis largely because its seedpod, called a silique, carries 2 rows of 20 embryos at exactly the same stage of development along its length. This arrangement is a general feature of the Brassicaceae family. Also notably, each flowering *Arabidopsis* plant has up to 50 siliques along its inflorescence axis, and each silique will carry embryos from a different stage of development, from older (at the base) to younger (at the tip). This extremely ordered array of embryos allowed detailed studies to map the regular pattern of cell divisions in specific groups of cells in the early embryo that develop into specific cells and tissues of more mature embryos and seedlings (Jurgens and Mayer 1994). The mature embryo shows an apical-basal organization along the main axis that is composed of structures such as shoot apical meristem (SAM), cotyledons, hypocotyl and root, and clonal analyses predict the contribution of each embryonic cell to this body plan (Laux et al. 2004) (Fig. 2.1). Generally, positional information determines the overall body pattern, and lineage-dependent cell fate specifies local patterning (Mansfield and Briarty 1991; Scheres et al. 1994; Poethig et al. 1986; Laux et al. 2004). Auxin signaling in conjunction with differential gene expression then facilitate specific morphogenesis (reviewed in Saulsberry et al. 2002; Capron et al. 2009).

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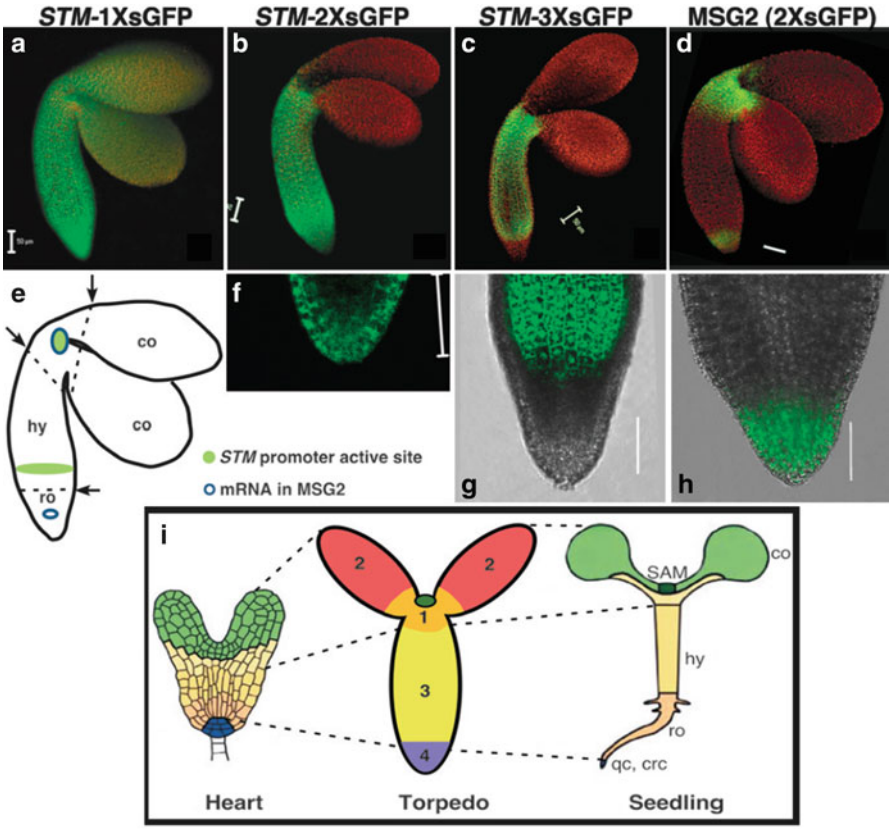

Fig. 2.1 *sGFP movement in Arabidopsis midtorpedo embryos*.This figure is reprinted in its entirety from Kim and Zambryski 2005, with permission (from Elsevier, and includes data previously published in Kim et al. 2005a, b). 1XsGFP expressed by the *STM* promoter in the SAM and the base of hypocotyls (hy) (see *green* areas in (**e**)) freely moves throughout the whole embryo (**a**). 2XsGFP fails to move into cotyledons (co) (**b**) but moves to the root tip (**f**). 3XsGFP fails to move to the root (ro) as well as cotyledons (**c**, **g**). These results indicate the formation of at least two symplastic sub-domains, the cotyledon and root. 2XsGFP expressed in the SAM and RAM in MSG2 line (see *dark blue* circles for where expression occurs in (**e**)) stays within sub-domains of the shoot apex and the root, respectively (**d**, **h**). These results, together with (**b**), reveal the boundary between the shoot apex and hypocotyl sub-domains. Root sub-domains from embryos in (**c**) and (**d**) are shown in larger magnification views in (**g**) and (**h**) under each whole mid torpedo image, and include quiescent center (qc), part of the RAM, and central root caps (crc). (**i**) Four symplastic sub-domains, shoot apex including SAM (*1*), cotyledons (*2*), hypocotyl (*3*), and root (*4*) are extrapolated to the body parts in heart embryos and seedlings shown to the *left* and *right*. Same colors in heart embryo and seedling represent regions of development with common clonal origins. Sub-domains of the torpedo embryo, as determined by their cell-to-cell transport via PD, also correspond to the apical-basal body pattern of the heart embryo (and seedling) by their positions; these regions are diagrammed with different colors to indicate they were defined by a different assay. Scale bars, 50 μm

Plasmodesmata (PD) directly connect the cytoplasm of adjacent cells, and convey positional information during axial patterning in embryogenesis by determining whether or not specific cells are symplastically coupled. The cytoplasm between adjacent plant cells is continuous due to PD connections; such adjacent cells share cytoplasm and thus are symplastically coupled. Embryos develop from a single cell, and cell division results in symplastic coupling between cells. Oriented patterns of cell division then result in morphogenesis. Early heart stage embryos, comprised of about 250 cells, are interconnected by open PD and constitute a single symplast of continuous cytoplasm bounded by the plasma membranes of connected cells (McLean et al. 1997). However, as embryonic development proceeds to the later torpedo stages, groups of cells become symplastically isolated from neighboring cells to form symplastic sub-domains (described below).

Arabidopsis embryos provide a very tractable system to study the genetic and developmental regulation of PD. Importantly, maternal transport of macromolecules ceases by the early heart stage. Thus, one can monitor cell-to-cell transport in isolated embryos independent of maternal effects by observing fluorescent tracers that are either added transiently or stably expressed in transgenic lines. Also, embryos are easily released from developing seed coats by mild pressure.

We review the establishment of symplastic domains in *Arabidopsis* embryos, genetic identification of mutations that alter PD function during embryogenesis, PD ultrastructure during embryogenesis, and discuss novel findings that have emerged from studies of PD transport during embryogenesis.

2 Symplastic Domain Formation During Arabidopsis Embryogenesis

We first studied cell-to-cell transport during embryogenesis by transiently and exogenously adding fluorescent (F) tracers. How are F-tracers introduced into embryos? We developed a simple strategy (Kim et al. 2002) that relies on the fact that certain cells of the embryo are more likely to break during extrusion of embryos from their seed coats. First, developing seeds are removed from the silique by running a needle horizontally along its length. Then the seeds are placed in plant liquid media under a cover slip on a slide. Gentle pressure to the cover slip then releases the embryos from their seed coats. This pressure often causes breaks at the junction between cotyledons and the meristem as the cotyledons are bent at a severe angle in the small space of the seed coat. Similarly the tip of the embryonic root often sustains small breaks in its terminal cells during extrusion. Following extrusion embryos are incubated with F-tracers and then washed extensively to remove unincorporated tracer. The broken cells provide entry points for the tracer, and then depending on the SEL of their connecting PD, the tracer either moves into the tissue or not.

The small F-tracer 8-hyroxypyrene-1,3,6-trisulfonic acid (HPTS, 0.5 kDa) moves through all cells of embryos during all stages of embryonic development examined from early heart to mid and late torpedo. However, the use of higher molecular weight tracers, such as 10-kDa F-dextran, reveals that PD aperture is down regulated, for such larger macromolecules, as development proceeds. 10-kDa F-dextrans are transported cell-to-cell in 50% of heart, 20% of early torpedo, and 0% of mid torpedo embryos (Kim et al. 2002). This developmental transition was used as a screen to select for mutants with altered cell-to-cell transport via PD (discussed in Sect. 3 below).

We then used transgenic approaches to express fluorescent proteins such as green fluorescent protein (GFP). In fact, GFP is a boon for PD research. GFP is an exogenous protein with no particular cellular address so that its expression results in nonspecific cytoplasmic and nuclear localization, and depending on the aperture of PD, it can move between cells by diffusion (Crawford and Zambryski 2000). Since GFP is not sequestered in cellular compartments, its movement between cells gives an indication of the PD-mediated connectivity between groups of cells. Observations of changes in PD aperture during embryonic development especially illustrate the usefulness of GFP. Note, PD aperture is also described as PD size exclusion limit (SEL), reflecting the molecular weight of the largest molecules that can be transported via PD connecting particular sets of cells. We use both terms in this review, but prefer the term "aperture" as it describes PD themselves versus the size of molecules they transport.

We used two different promoters to drive GFP expression in meristematic regions. The *SHOOT MERISTEMLESS (STM)* promoter was used to express 1X, 2X, and 3XGFP in the shoot apical meristem (SAM) and a subset of cells in the hypocotyl just above the root meristem (Kim et al. 2005b). In addition, the cell-type-specific enhancer of the Haseloff enhancer trap line J2341 (Cho and Zambryski 2011) induced expression of 2XGFP in the SAM and the root apical meristem (RAM) in the MSG2 line (Fig. 2.1e). The subsequent movement of these various sized tracers from their site of synthesis was monitored at three stages of embryogenesis to reveal several major findings (Kim et al. 2005a, b). Remarkably, 2×GFP (54 kDa) moves throughout the entire early heart embryo demonstrating that PD apertures in early embryos are quite dilated (Kim et al. 2005b). Importantly, these data reinforce the observations made with exogenously applied HPTS and F-dextran tracers, again revealing that the entire embryo is a single symplast, where all cells can freely transport GFP cell-to-cell via PD. Significantly, different regions of the embryo have distinct PD apertures/SELs defining specific symplastic sub-domains by the mid torpedo stage.

Figure 2.1 a–d illustrate the movement of 1X to 3XGFP at the late-torpedo stage. Mid-torpedo cotyledons do not allow transport of probes larger than 1XGFP (27 kDa). Mid-torpedo roots do not allow transport of probes larger than 2XGFP (54 kDa). Finally, the movement of 3XGFP (81 kDa) is more restricted to the regions immediately surrounding its site of synthesis at the SAM and at the base of the hypocotyl. Strikingly, these sub-domains of cytoplasmic continuity correspond to

the basic layout of the organs of the adult body along the apical-basal body axis: the shoot apex, cotyledons, hypocotyls and root (Fig. 2.1i and legend). These sub-domains can be extrapolated to morphogenetic regions of the early embryo (and seedling) defined by gene expression profiles and clonal analyses (Fig. 2.1i) (Poethig et al. 1986; Mansfield and Briarty 1991; Scheres et al. 1994; Laux et al. 2004).

An independent study of GFP transport following expression mediated by the cell-type-specific enhancer of the Haseloff reporter line J2341 (Kim et al. 2005a) provides salient additional evidence for a boundary with distinct PD aperture between the root tip and the hypocotyl. Figure 2.1f, g shows that 2XGFP and 3XGFP expressed from the STM promoter can, or cannot, move down into the root tip, respectively. Figure 2.1h shows that 2XGFP expressed at the root tip under the control of the J2341 enhancer cannot move up into the hypocotyl. Thus, PD at the root-hypocotyl boundary allow directional movement of 2XGFP downward, but not upward.

In summary, there are boundaries between each of four symplastic sub-domains, SAM, cotyledons, hypocotyl, and root, that control embryo-specific intercellular transport (Fig. 2.1i). Each boundary likely has a distinct PD aperture/SEL. The boundary between the shoot apex and the cotyledons has a SEL between 27 and 54 kDa, as 1XGFP but not 2XGFP moves from the SAM to the cotyledons (Fig. 2.1a, b, and e). The boundary between the hypocotyl and the root has a SEL between 54 kDa and 81 kDa, as 2XGFP but not 3XGFP moves from the hypocotyl to the root (Fig. 2.1f, g). The boundary between the hypocotyl and shoot apex domains has a SEL less than 54 kDa as evidenced in the MSG2 line where 2XGFP moves from its site of synthesis at the SAM to surrounding cells but it fails to move downward into the hypocotyl (Fig. 2.1d, e). Movement within the hypocotyl is only observed when GFP expression occurs within the hypocotyl itself (just above the root) under the control of the *STM* promoter; in this case, 2X and 3XGFP move upward from their site of synthesis (Fig. 2.1b, c, and e).

Thus, groups of cells with similar developmental fates carry PD with similar apertures. Differential PD connectivity likely is critical during organ formation, providing a means whereby groups of cells can exchange factors essential for common developmental programming. PD connectivity also varies in adult plants, being highest in young organs and more restricted in mature tissues (reviewed in Burch-Smith et al. 2011).

2.1 Further Refinement of Localized Embryonic Symplastic Sub-domains

Additional embryonic symplastic sub-domains were defined by promoter-specific expression of 1XGFP in the protodermis or stele of the embryo (Stadler et al. 2005). The *GLABRA 2* promoter drives expression in the protodermis, and when 1XGFP was expressed in the outermost protodermal layer of the heart-stage embryonic hypocotyl, it moved inward to internal ground tissues and to neighboring protodermal

cells in cotyledons. Such movement was reduced at the torpedo stage (Stadler et al. 2005). When 1XGFP was expressed under the *SUCROSE TRANSPORTER3 (AtSUC3)* promoter it moves freely between cells of the hypocotyl in earlier stages (Stadler et al. 2005), but becomes restricted to the stele in the mid-torpedo stage (Fig. 2.3b). Thus, cell-to-cell transport inward from the protodermis and outward from the stele is regulated during the torpedo stage of embryogenesis.

Significantly, the above data underscore that the extent of cell-to-cell movement is dramatically affected by the location of the initial site of GFP synthesis. 1XGFP freely moves to every cell in embryos following expression in the SAM, (Kim et al. 2005a, b), but its movement is limited when expressed from epidermal cells (Stadler et al. 2005). Potentially, PD in and around the SAM are more active, or have larger apertures, than those in the epidermis as meristems are likely the source of morphological signals to enable patterning during embryogenesis.

The above studies provoke many questions. What factors regulate the establishment of embryonic symplastic domains? Do such factors bind directly to PD to regulate their aperture? Do different factors differentially regulate PD at boundaries between symplastic domains? Are PD in boundary cells more tightly regulated than PD within symplastic domains?

2.2 Maternal Influences on PD Function Just Before and During Embryogenesis

While this review focuses on PD function and regulation during embryogenesis, we also alert the reader to studies of PD function during the developmental period immediately prior to embryogenesis. A recent study with stunning images documents PD and phloem transport during ovule development, and provides evidence for two switches in transport capacity (Werner et al. 2011). Ovules initiate their development in stage 11 flowers during which time they are symplastically connected to the maternal phloem. However, when the female gametophyte is produced during stage 12, phloem unloading is switched off. Phloem unloading is resumed in developing embryos of stage 13 flowers when fertilization occurs, though the resumption of symplastic unloading is not fertilization-dependent.

2.3 Embryo Suspensor Cell-to-Cell Communication

After fertilization the zygote undergoes polar cell division. The upper apical cell will become the embryo and lower basal cell forms the suspensor. We previously reviewed (Kim and Zambryski 2005) GFP transport from the embryo to the suspensor and *vice versa*, but it is important to highlight here too. The suspensor is a terminally differentiated linear file of embryonic cells that connects the embryo

to maternal tissues and provides a direct route for nutrient transport from material tissues to the early embryo. In globular embryos, GFP can move in both directions, implying that the embryo and suspensor together form a single symplast at the globular stage. However, as the embryo makes the transition from radial to bilateral symmetry at the heart stage, PD mediated movement from the suspensor to the embryo restricts maternal input.

2.4 Embryo-Seed Coat Cell-to-Cell Transport

Finally, GFP moves differentially in the five cell layers of the *Arabidopsis* seed coat consisting of the innermost endothelial layer, followed by two cell layers each of inner and outer integuments. GFP expressed in the outer integument cannot move to the inner integument layers and *vice versa*. Thus, the inner and outer integuments are separate symplastic domains. Even small tracers such as HPTS (0.5 kDa) are not transported across the boundary between the outer and the inner integuments (Stadler et al. 2005).

The above studies highlight both dynamic and highly regulated plant intercellular transport during ovule development, early embryo development, stage specific embryogenesis, and seed coat development.

3 Isolation of Mutants with Altered PD Transport During Embryogenesis

Genetics is an obvious classical approach to identify PD-specific components. However, alterations in components that affect PD structure or function are likely to have severe effects on plant growth and development. Indeed, PD defects are expected to manifest at the first stages of plant growth, during embryogenesis, and thereby exhibit a defective or even lethal phenotype. However, embryos make an especially excellent system for genetic screens because embryo-lethal/defective mutants can be propagated as heterozygote plants, and segregating defective embryos can be detected in seedpods. This is especially true for Arabidopsis because their seedpods, siliques, contain approximately 40 embryos in a linear file that are easily observed by simple light microscopy. Furthermore, because embryogenesis is a vitally important phase in plant development where basic tissues and anatomy of a plant are established, one might expect to see regulation of PD function during this time frame.

As mentioned above, we used exogenously added 10-kDa F-tracers to determine that PD aperture is reduced significantly during the mid-torpedo stage of embryogenesis. We then used this time frame to screen 5,000 embryo-lethal mutants for alterations in cell-to-cell transport of fluorescent tracers (Kim et al. 2002); see Sect. 2.1

above for description of the F-dextran loading assay. Fifteen mutants that continued to traffic 10 kDa F-dextran at the torpedo stage were identified and called *increased size exclusion limit (ise)*. In a separate study we screened for the opposite phenotype, reduced transport of the low molecular weight tracer HTPS, and identified a single mutant called *decreased size exclusion limit* (*dse*) (XM, unpublished).

To date we have cloned and characterized three mutants, *ise1*, *ise2*, and *dse*. All three mutations were induced by ethane methane sulfonate (EMS) that causes alterations in single nucleotides. This less severe mutagenesis strategy was in fact critical to our successful identification of mutants with altered PD function. After each mutant gene was identified, we could then obtain the relevant T-DNA insertion alleles. In all cases, T-DNA-induced null alleles of the identified genes are severely retarded; *ise1* null mutants form only a large mass of cells, and *ise2* and *dse* null mutants do not develop beyond the torpedo stage.

Contrary to our expectations, none of the encoded products of *ISE1*, *ISE2*, or *DSE*, localize to PD. The *ISE1* gene encodes a DEAD-box RNA helicase that localizes to mitochondria (Stonebloom et al. 2009). *ISE2* encodes a DEVH-box RNA helicase first shown to localize to the cytoplasm and chloroplasts (Kobayashi et al. 2007). Recent higher resolution microscopy reveals that ISE2 localizes entirely within chloroplasts Burch-Smith et al. 2011.

3.1 Mutants with Altered PD Function Increase De Novo Formation of PD

Significantly, *ise1* and *ise2* increase PD formation (Burch-Smith and Zambryski 2010), as would be expected from their effects on increasing PD transport. PD can have several different morphologies: simple linear channels, twinned PD (simple PD within 100 nm of each other) (Faulkner et al. 2008), moderately branched, or highly branched. The latter highly branched structures are found in mature tissues such as source leaves. PD that arise during cell division are called primary PD, and those that arise post division are called secondary PD. As the overall morphology of *ise1*, *ise2*, or *dse* torpedo embryos are similar to wild type torpedo embryos, these mutations do not affect cell division patterns and therefore are unlikely to effect primary PD formation. Instead we predicted that mutations in *ise1, ise2*, or *dse1* might affect the *de novo* formation of secondary PD.

Figure 2.2a illustrates the frequencies of twinned and branched PD in hypocotyls of late-staged torpedo embryos in three mutant lines for which published data is available. Wild type embryos contain ~9% twinned and branched PD and *ise1* (26%) and *ise2* (14%) mutant embryos have significantly increased frequencies of twinned and branched PD. Twinned PD are likely the result of duplication of a simple PD (Faulkner et al. 2008); by definition then twinned PD are secondary. Branched PD may arise from both primary and secondary simple PD, so it is impossible to know the origin of branched PD from their structure.

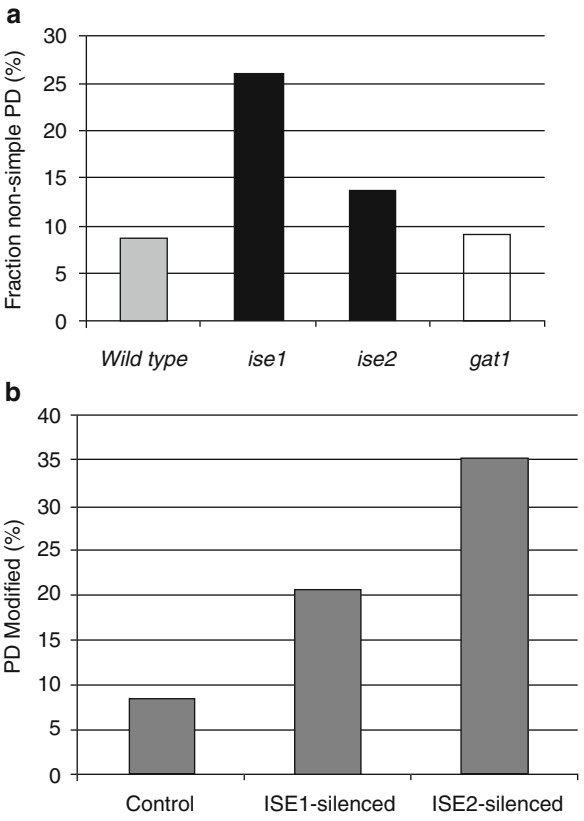

Fig. 2.2 *Fraction of total PD that are not simple*. (**a**) The fraction of non-simple PD is plotted as a percentage of the total PD. Data for wild type (*grey bar*), *ise1* and *ise2* embryos (*black bars*) are from Burch-Smith and Zambryski 2010. Data for *gat1* seedlings are from Benitez-Alfonso et al. 2009. (**b**) Compares the frequency of de novo secondary PD formation in non-silenced leaves compared to *ISE1* or *ISE2* silenced leaves. Secondary PD were defined as PD in the cell walls between the epidermal and mesophyll layer as in Fig. 2.3b

However, secondary twinned or branched PD can be unambiguously identified if they occur in cell walls that are explicitly non-division walls. Non-division walls are easily identified in longitudinal cell files, such as those of the expansion zone of the root (Scheres et al. 2002). In addition, leaf epidermis-mesophyll cell interfaces do not result from cell division, and provide a ready supply of tissue for specific analyses of secondary PD (Burch-Smith and Zambryski 2010). Thus, we determined if the increased or decreased numbers of branched PD we observed in mutant embryos reflect increased or decreased branching of secondary PD by an independent approach that specifically examined unambiguous secondary PD in the cell walls at epidermal-mesophyll interfaces of the leaf. To induce the loss of ISE1, or ISE2 in leaves, we performed virus induced gene silencing (VIGS) (Burch-Smith and Zambryski 2010) of *ISE1*, and *ISE2*. As VIGS is easy and efficient in *Nicotiana benthamiana* (Burch-Smith et al. 2004) we first identified homologs of *ISE1*, and *ISE2* in *N. benthamiana*. Following VIGS of *ISE1* and *ISE2*, we then performed ultrastructural analyses to monitor the frequency of *de novo* secondary PD formation at the cell wall interfaces between epidermal and mesophyll cells of young leaves.

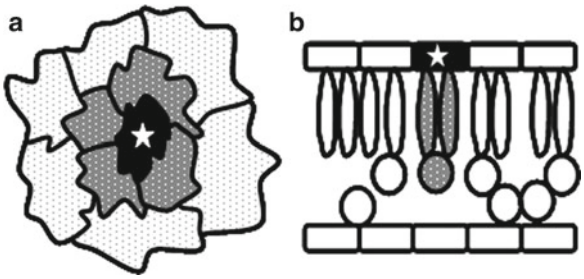

Fig. 2.3 *Movement assay in leaf cells*. Agrobacterium carrying constructs to express GFP fused to TMV MP were infiltrated into leaf epidermal cells at low dilution to ensure transfection of single cells. An initially transfected cell is indicated as a black cell with a white star. (**a**) Depicts movement to two rings of cells away from the transfected cell as *grey* and *white* cells respectively. (**b**) Depicts movement from a single epidermal cell down into the mesophyll layer (*grey cells*). Palisade mesophyll cells are elongated and spongy mesophyll cells are shown as *circles*

Our results were dramatic. Control non-silenced wild-type leaves contained 8% twinned and branched PD in the cell walls between the leaf epidermis and the underlying mesophyll cells that specifically contain secondary PD. Silencing of *ISE1* or *ISE2* lead to 20% and 35% twinned and branched PD respectively (Fig. 2.2b). These results lead us to extrapolate that the increased twinned and branched PD observed in embryos are also likely to be secondary.

3.2 Gene Silencing of ISE1 or ISE2 in Leaves Increases PD Cell-to-Cell Transport

In addition, we used our gene silencing strategy to demonstrate that silencing of *ISE1* or *ISE2* increases cell-to-cell transport in leaves, reiterating the effects of *ise1*, and *ise2* mutants during embryogenesis. Leaves silenced for *ISE1* or *ISE2* were assayed for the movement of tobacco mosaic virus (TMV) movement protein (MP) P30 fused to GFP. To more sensitively detect potential increased movement in *ISE1* or *ISE2* silenced leaves we monitored 2XGFP fused to P30 (P30-2XGFP). P30-2XGFP-fusion proteins were detected following *Agrobacterium*-mediated transient expression. The results obtained were exactly as predicted, and demonstrated that loss of *ISE1* or *ISE2* also impacts intercellular movement via PD in mature leaf cells. Figure 2.3 diagrams the movement assay. Movement was quantified first as the number of rings of cells to which the P30-2XGFP moved away from the initially transfected single cell (Fig. 2.3a). Thus, leaves silenced for *ISE1* or *ISE2* resulted in more rings of P30-2XGFP movement than control non-silenced leaves (Burch-Smith and Zambryski 2010). Besides horizontal movement (which includes primary and secondary PD-mediated transport) we also specifically assayed for movement via secondary PD, assessed as movement from the epidermal cell

layer into the mesophyll layer (Fig. 2.3b). Movement via secondary PD was increased in *ISE1* and *ISE2*-silenced leaves (Burch-Smith and Zambryski 2010). Importantly, in the above studies we infiltrated leaves with Agrobacterium (carrying the P30-2XGFP construct) at very low dilutions to ensure that foci observed and analyzed represent single transfection events.

3.3 Additional Mutants with Altered PD Transport

In an independent study Benitez-Alfonso et al. (Benitez-Alfonso et al. 2009) mutagenized transgenic seedlings expressing GFP in phloem-specific cells that leads to GFP movement cell-to-cell into surrounding tissues. They screened for mutants, called *GFP arrested trafficking* (*gat*) that exhibit reduced PD function by failing to unload GFP from the phloem in seedling roots. *GAT1* encodes a plastid thioredoxin and *gat1* mutants increase callose production (Benitez-Alfonso et al. 2009) (discussed further below). Benitez-Alfonso et al. (2009) also analyzed PD frequency and structure in *gat1* seedling roots. They found no increase in PD frequency but suggested there was an increase in branched PD. Figure 2.2 shows that the frequency of PD branching (9%) observed in *gat1* seedling roots is identical to the frequency of twinned and branched PD observed in wild type late torpedo hypocotyls. We also demonstrated that early and mid-torpedo hyocotyls contain ~9% twinned and branched PD (Burch-Smith and Zambryski 2010), but here we specifically illustrate our results for the late torpedo stage hypocotyls as they are most comparable to early seedling roots analyzed by Benitez-Alfonso et al. (2009).

Finally, we continue to analyse mutants that lead to increased cell-to-cell movement at the mid-torpedo stage of embryogenesis identified in our original screen (Kim et al. 2002), and are currently mapping the mutant loci to identify their affected genes. We are most far along with the characterization of the *dse* mutant line; in terms of the ultrastructual and functional assays mentioned above for *ise1* and *ise2*.

4 Surprising Novel Results from Studies of PD Transport in Embryos

4.1 Twinned and Branched PD Occur in Wild Type Embryos and Young Tissues

We (Kobayashi et al. 2007), and others (Benitez-Alfonso et al. 2009), previously assumed that embryos or seedling root meristems only contain simple unbranched PD; both reports mention the presence of branched PD specifically as a feature of the

mutant (*ise2* or *gat1*) phenotype. However, quantitative and statistically significant measurements of PD structures during embryogenesis now reveal that wild type embryonic hypocotyls contain 9% twinned and branched PD between early and late-torpedo stages of development (Burch-Smith and Zambryski 2010). Thus, the 9% branched PD reported in seedling roots (Benitez-Alfonso et al. 2009) is not significantly different from the frequency observed in wild type embryos; a quantitative study of simple, versus twinned and branched PD remains to be performed in wild type root seedlings. While Kobayashi et al. (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 sufficiently large number of PD in both experimental **and** wild type tissues. Thus, we missed the existence of twinned and branched PD in our earlier report as we counted too few PD in wild type tissues.

A recent review re-evaluates the evidence for different types of PD in diverse tissues, emphasizing that, contrary to previous assumptions, young tissues do not contain "only" simple unmodified PD, but also contain twinned and branched PD, and proposing a re-evaluation of some of the older data with regard to the nomenclature of the different PD forms (Burch-Smith et al. 2011).

4.2 Plant Viral Movement Proteins as Probes to Study PD During Embryogenesis

The movement protein (MP) of TMV is a veritable workhorse and remarkably useful tool for studying intercellular transport via PD. Besides its ability to move extensively from cell to cell, it targets to PD so that GFP fusions to TMV MP mark PD as puncta in the cell perimeter. Besides moving via PD TMV MP also "gates" PD aperture allowing large F-tracers to move cell-to-cell in trans (Wolf et al. 1989). Proteins that potentially localize to PD are assayed by their co-localization with TMV MP. Finally a transgenic line constitutively expressing TMV MP has lead to important insights in distinguishing PD transport in sink and source leaves. However, these data lead 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 subsequently asserted that viral MPs preferentially localize to "secondary" PD in mature tissues (e.g. Epel 1994; Hofius et al. 2001) and TMV MP was used to distinguish "secondary" PD from primary PD (Roberts et al. 2001).

During our studies to characterize the movement of different sized GFP tracers (1X, 2X, and 3XGFP) during embryogenesis (described above) we also performed studies using the same *STM* promoter to drive expression of 1X and 2XGFP fused to TMV MP (Kim et al. 2005b). We embarked on these studies to determine if TMV MP could also "gate" PD aperture in embryos to allow more extensive cell-to-cell transport than similarly sized soluble GFP. Comparison of the movement of 2XGFP (54 kDa) versus MP-GFP (57 kDa) revealed that MP-GFP moved more extensively

down into the root tip and within the hypocotyl than 2xGFP (Kim et al. 2005b). Thus, MP can gate PD in embryos to facilitate its own transport.

Importantly, MP-GFP targets to PD in embryos and forms the classic punctate pattern previously seen for its PD localization in more mature tissues. As more than 90% of embryonic PD are simple (Burch-Smith and Zambryski 2010) these data unambiguously demonstrate that MP also localizes to simple PD. We further confirmed that MP-GFP localizes to primary PD in the differentiation zone just above the root meristem (Burch-Smith et al. 2011).

In summary, studies to monitor cell-to-cell transport in embryos have revealed new properties of TMV MP, to target to and gate simple PD, and thereby increase the utility of TMV MP to now investigate PD structure and function in developing tissues.

4.3 Reactive Oxygen Species Production During Embryogenesis Alters PD Transport

As mentioned, embryo-defective *ise1* mutants increase PD transport and exhibit delayed development (Kim et al. 2002). *ISE1* encodes a nuclear-encoded mitochondria localized RNA helicase that is likely essential for mitochondrial mRNA processing, and *ise1* mutants are defective in the formation of mitochondrial electron transport gradients necessary for ATP production (Stonebloom et al. 2009). In addition, *ise1* mutants produce increased levels of reactive oxygen species (ROS). The *gat1* mutant produces increased levels of ROS as well (Benitez-Alfonso et al. 2009). *GAT1* encodes a plastid thioredoxin and *gat1* mutants increase callose production, leading to PD closure (Benitez-Alfonso et al. 2009). ROS are thought to act as critical signalling molecules mediating cross-talk between mitochondria, plastids and the nucleus. However, there is no "one size fits all" for the role of ROS in PD transport as *ise1* and *gat1* mutants have the opposite effect on intercellular trafficking (Benitez-Alfonso et al. 2009; Stonebloom et al. 2009). Thus, we proposed (Stonebloom et al. 2009) that the site of ROS production, mitochondria versus plastids, differentially regulates cellular processes and associated specific targets that in turn affect PD function. We recently performed a systematic study to chemically induce ROS in mitochondria versus chloroplasts and the results support our hypothesis (Stonebloom et al. 2011). Thus, oxidized mitochondria lead to increased cell-to-cell transport and oxidized plastids lead to reduced cell-to-cell transport. In the same study, we assayed the redox state of the mitochondria versus plastids following gene silencing of *ISE1* or *ISE2*. Loss of ISE1 leads to mitochondrial oxidation as expected. However, loss of ISE2 leads to reduction of plastid redox state, which supports that loss of ISE2 has the opposite effect on PD transport compared to *gat1* (which is expected to have oxidized plastids). In addition, we proposed (Stonebloom et al. 2009) that relative amount of ROS may differentially affect PD function, as the levels of ROS produced in *gat1* likely are higher than those produced in *ise1*. Indeed, a recent study showed that treatment of *Arabidopsis* roots with low concentrations of H_2O_2 drastically increased PD permeability while high concentrations induced PD occlusion (Rutschow et al. 2011).

5 New Concept: Cellular Homeostasis Affects PD Formation and PD Function

The data revealing *de novo* formation of secondary PD in *ise1* and *ise2* mutants and following gene silencing of *ISE1* or *ISE2* are the only demonstration to date of genetic pathways that regulates PD biogenesis. When we embarked on our mutant screen for PD transport alterations, we expected to uncover genes whose products localized to PD. In hindsight this was a naïve expectation. However, our findings are not disappointing. Instead they reveal that cellular homeostasis and cross talk between important cellular organelles (mitochondria for ISE1 and chloroplasts for ISE2) are vital to establishing the degree of intercellular transport via PD and the *de novo* formation of PD. Studies with the *gat1* mutant reinforce this idea and reveal that interfering with plastid homeostasis by altering redox state via thioredoxin and ROS levels negatively regulates PD function.

We are currently evaluating the transcriptomes of *ise1* and *ise2* embryos compared to wild type embryos. Since both mutants have similar overall morphology and similar effects on PD (increased PD transport and increased secondary PD formation) yet their gene products reside in different organelles, mitochondria and chloroplasts, we expect to uncover genes that are commonly effected in both mutants that will allow us to dissect their respective roles in PD formation/function. It is important to note that ISE1 and ISE2 are both RNA helicases. RNA helicases are essential in all stages of mRNA production and turnover. Thus, defects in ISE1 and ISE2 are expected to affect mRNA levels in either mitochondria or chloroplasts. Thus, acute defects resulting from loss of mitochondrial or chloroplast function should allow systematic investigation of their downstream effects on PD.

Our analyses of gene expression in *ise1* and *ise2* embryos found that the most significantly affected class of transcripts in both mutants encodes products targeting to and enabling plastid function (Burch-Smith et al. 2011). Indeed, ultrastructural analyses show that plastid development is defective in both mutants (Burch-Smith et al. 2011). While defective plastids were expected following loss of plastid-localized ISE2, the defective plastids formed following loss of mitochondrial ISE1 were surprising. These results reinforce the importance of plastid-mitochondria-nucleus crosstalk, and PD as critical players in the plant cell communication network. Thus, they illuminate a new signaling pathway we dub organelle-nucleus-plasmodesmata signaling (ONPS).

6 Conclusions and Perspectives

Arabidopsis embryogenesis occurs relatively rapidly. Torpedo embryos are formed within 4 days post fertilization, and different stages of embryogenesis can be easily visualized in independent siliques along the inflorescence axis. *Arabidopsis* embryogenesis is an especially attractive system to study genes critical for development as severely defective mutants can nevertheless be propagated in heterozygote plants

that carry both wild type and mutant embryos segregating in the same silique for direct comparison.

Surprisingly, while mutants such as *ise1* and *ise2* cause significant alterations in PD formation and function, their gene products do not localize to PD. Similarly, a seedling defective mutant with altered PD transport, *gat1*, also does not localize to PD. These data underscore how little we know about the intricacies of PD synthesis and modes of action, but nevertheless open the door to investigate more global cellular processes to uncover their impact on PD synthesis and regulation. These studies are extremely important given the remarkable ability of PD to transport a range of developmentally critical factors, from small growth regulators and nutrients, to macromolecules, such as transcription factors, gene silencing RNAs and microRNAs.

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