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Report

Loss of INCREASED SIZE EXCLUSION LIMIT (ISE)1 or ISE2 Increases the Formation of Secondary Plasmodesmata

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Summary

Plasmodesmata (PD) transport developmentally important nucleic acids and proteins between plant cells [1–3]. Primary PD form during cell division and are simple, linear channels [4, 5]. Secondary PD form in existing cell walls after cell division and are simple, twinned, or branched [4]. PD function undergoes a marked reduction at the mid-torpedo stage of *Arabidopsis* embryogenesis [6]. Two mutants, *increased size exclusion limit* (*ise*)*1* and *ise2,* fail to undergo this transition, and their null mutations are embryonically lethal [7, 8]. We investigated the ultrastructure of PD in early-, mid-, and late-torpedo-stage embryos and in young leaves. Wild-type (WT) embryos contain twinned and branched (T/B) PD at all stages, but *ise1* and *ise2* embryos contain significantly higher proportions of T/B PD than WT embryos. WT T/B PD formation occurs in a stage- and tissue-specific pattern that is reversed in *ise1* embryos. Silencing *ISE1* in *Nicotiana benthamiana* leaves increases the frequency of secondary PD in existing cell walls. Silencing *ISE2* increases the proportion of T/B secondary PD formed. Silenced tissues exhibit increased PD-mediated movement of green fluorescent protein tracers. Thus, silencing of *ISE1* and *ISE2* phenocopies *ise1* and *ise2* mutant embryos: when wild-type ISE1 and ISE2 functions are lost, de novo production of PD occurs, leading to increased intercellular transport.

Results and Discussion

PD Function Correlates with Increased Twinned and Branched PD in *ise1* and *ise2* Embryos

ise1 and *ise2* embryos fail to undergo the mid-torpedo-stage-specific reduction in PD transport [6–8]. To determine whether PD structural changes explain wild-type (WT) or mutant phenotypes, we examined PD of early-, mid-, and late-torpedo-stage WT, *ise1*, and *ise2 Arabidopsis* embryos by transmission electron microscopy (TEM). Figure S1, available online, shows the morphology of the embryos analyzed. Simple PD typically associated with immature tissues were observed in all stages (Figure 1A). We also observed PD with more complex structures, including twinned (T) PD, defined as two PD less than 100 nm apart (Figure 1B), and branched (B) PD (Figures 1C and 1D). For simplicity, we refer to all nonsimple PD as T/B PD.

We scored at least 100 PD in a minimum of two nonserial longitudinal sections of cotyledons or hypocotyls of early-, mid- or late-torpedo-stage WT, *ise1*, and *ise2* embryos (Table S1). Figure S1 exemplifies the quality of the images analyzed. Figure 1E shows first that WT torpedo-staged embryos contain T/B PD. A previous study [7] did not detect T/B PD in WT embryos, presumably because few PD were assayed, whereas here we analyzed 860 WT PD. Second, there is no change in the T/B PD frequency between early- and mid-torpedo stages in WT embryos. Thus, decreased transport in WT mid-torpedo embryos may result from a mechanism for regulating transport that does not detectably alter PD structure. Third, in all stages examined, *ise1* and *ise2* mutants display significantly larger proportions of T/B PD than those of sibling WT embryos. These data suggest that the increased intercellular transport observed in *ise1* and *ise2* is due to increased T/B PD formation.

In WT embryos the hypocotyl contains more T/B PD than the cotyledon in early- and mid-torpedo embryos, but this distribution is reversed in late-torpedo embryos (Figure 1E, black bars). *ise2* mutant embryos show a similar pattern of T/B PD formation (Figure 1E, gray bars). Strikingly, in *ise1* mutant embryos this pattern is exactly reversed (Figure 1E, white bars). Thus, T/B PD formation is under the control of a specific, developmentally regulated program, and loss of *ISE1* critically affects the pathway that determines when and where T/B PD form.

Strategy for Assessing Primary and Secondary PD Formation in Adult Plant Tissues after Silencing *ISE1* and *ISE2*

ise1 and *ise2* mutations are embryonically lethal, precluding the analysis of gene function in adult plant tissues. We therefore adopted virus-induced gene silencing (VIGS) as a strategy for investigating *ISE1* and *ISE2* function in *Nicotiana benthamiana* plants. We determined the full-length sequences of *N. benthamiana ISE1* [8] and *ISE2* (Figure S2) homologs. For VIGS we used *Tobacco rattle virus* (TRV)-based vectors [9, 10] containing fragments of *NbISE1*, *NbISE2*, or both. The efficiency of silencing *ISE1* and *ISE2* transcripts was 80% (Figure S3). *ISE1*- and/or *ISE2*-silenced plants displayed chlorosis but no other developmental or growth abnormalities (Figure S3).

Twinned and branched PD are often intermediates in the formation of new secondary PD close to existing ones [11, 12]. We hypothesized that the T/B PD observed in mutant embryos are secondary and differ in origin from PD in WT embryos. Leaves are a source of accessible cell walls that allow us to investigate the relationship among PD origin, structure, and function. *Nicotiana* leaf epidermal cells result from anticlinal divisions in the L1 layer of the shoot apical meristem [13, 14]. In young sink leaves, PD connecting epidermal cells are mostly primary PD, whereas all PD connecting epidermal cells to their underlying mesophyll cells are secondary PD [15]. Whereas some reports refer to all branched PD as secondary without assessing their origin ([15] and references therein), here we specifically analyze PD of different origins, either primary or secondary.

We used *ISE1*- and/or *ISE2*-silenced *N. benthamiana* plants to measure the distribution of simple or T/B PD in the cell walls connecting epidermal-epidermal or epidermal-mesophyll cells (Figure 2A) in at least 40 cells in three independent experiments (Table S2). Figure S4 shows the quality of the TEM samples. We limited our analysis to epidermal pavement cells because these cells should have similar function. We excluded trichome cells, because they sometimes exhibit periclinal

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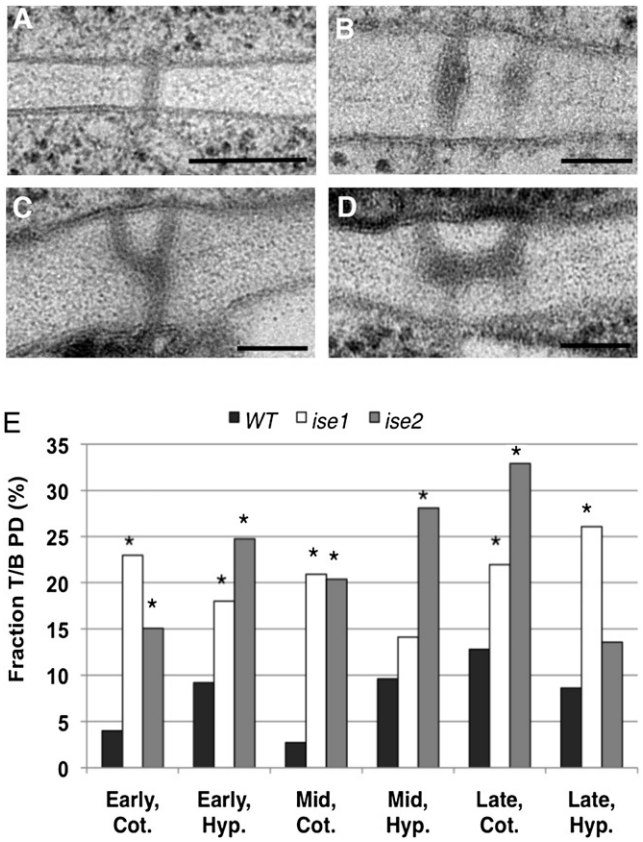

Figure 1. Analysis of PD Structure in Embryonic Cotyledons and Hypocotyls

(A) Simple PD.

(B) Twinned PD.

(C and D) Branched PD may be Y shaped or H shaped.

(E) The fractions of T/B PD were counted in cotyledons and hypocotyls from early-, mid-, and late-torpedo embyos. *p < 0.05 as compared to WT tissues. Refer to Figure S1.

Scale bar represents 200 nm in (A) and 100 nm in (B, C, and D).

divisions, [13] as well as stomatal guard cells, whose function is highly specialized. At 14 dpi, the youngest leaves (Figure S3, Leaf 11) displaying the VIGS phenotype are less than 4 mm long and are photosynthetic sinks [5].

Reduced *ISE1* or *ISE2* Expression Does Not Significantly Affect T/B PD Formation between Epidermal Cells

The frequency of PD, 2.5 to 3.2 μm^{-2}, in the epidermal-epidermal cell wall is virtually unchanged in all silenced plants as compared to nonsilenced controls (Figure 2B). T/B PD formation increases to 31% in *ISE2*-silenced plants, albeit this increase is not significant when compared to the 23% increase in nonsilenced controls (Figure 2C); this increase in T/B formation is consistent with the increase observed in *ise2* mutant embryos (Figure 1 and [7]). *ISE1*-silenced tissues do not display a similar increase in T/B PD formation, and the percentage of T/B PD formed in *ISE1* and *ISE2* double-silenced tissue is more similar to the *ISE1*-silenced samples than to the *ISE2*-silenced ones (Figure 2C).

Reduced *ISE1* or *ISE2* Expression Significantly Increases Secondary PD Formation between Epidermal and Mesophyll Cells

Silencing *ISE1* results in a statistically significant increase in secondary PD frequency, to 3.7 PD μm^{-2}, as compared to

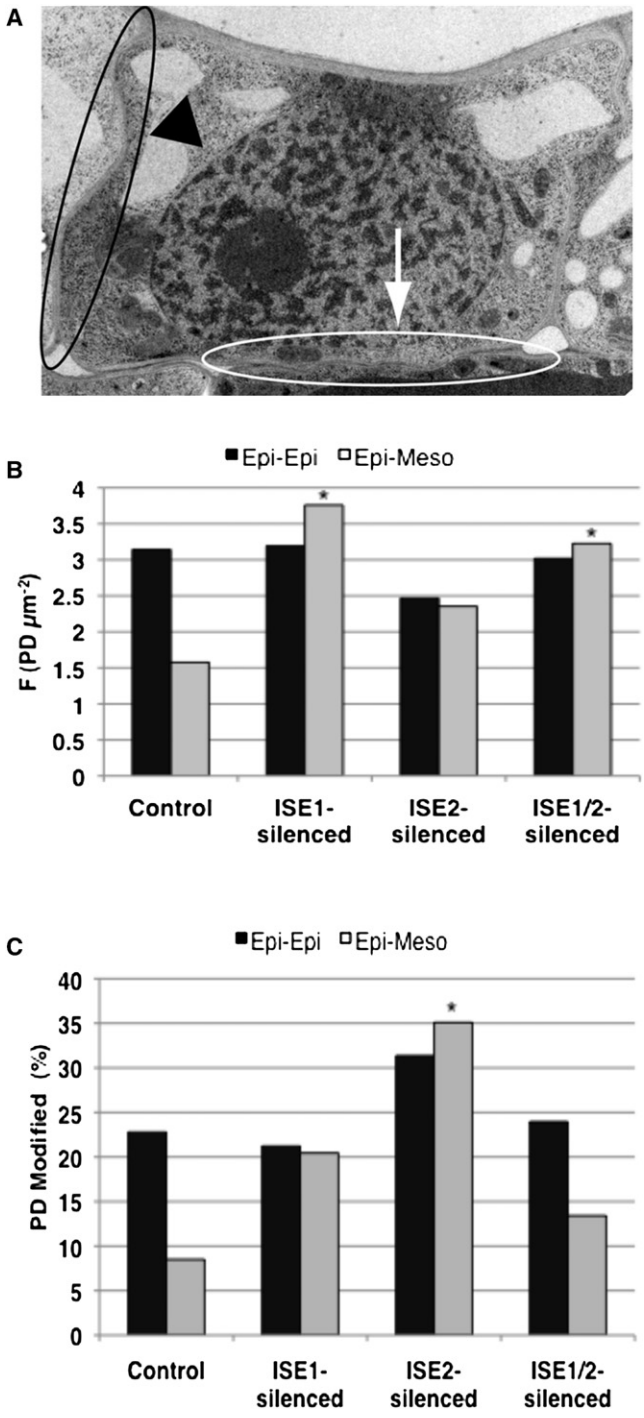

Figure 2. Frequency of PD per um^2 and T/B PD Formed in *N. benthamiana* Cell Walls

(A) Representative TEM image of an epidermal cell from the youngest leaf. Left, epidermal-epidermal cell wall (arrowhead). Bottom, epidermal-mesophyll cell wall (arrow).

(B) Mean PD frequency (um^{-2}) in epidermal-epidermal (black bars) or epidermal-mesophyll (gray bars) cell walls. *p < 0.05 as compared to nonsilenced control leaves.

(C) Proportions of T/B PD in epidermal-epidermal (black bars) and epidermal-mesophyll (gray bars) cell walls. *p < 0.05 as compared to nonsilenced control leaves.