MINI-REVIEW

Plant Signaling & Behavior 9, e27899; February; © 2014 Landes Bioscience

Plasmodesmata-associated proteins

Can we see the whole elephant?

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Keywords: Plasmodesmata, size exclusion limit, symplastic transport, callose, reactive oxygen species

Abbreviations: Pd, plasmodesmata; SEL, size exclusion limit; TMV, *Tobacco mosaic virus*; CMV, *Cucumber mosaic virus*; GSL, glucan synthase-like; BG, β glucanase; PDCB, plasmodesma callose binding proteins; PDLP, plasmodesma-located proteins; ^{C1}RGP, class 1 reversibly glycosylated polypeptides; GnTL, glucosaminyltransferase-like; ROS, reactive oxygen species; TRX-m3, thoredoxin-m3; ISE, increased size exclusion

Encased in rigid cell walls, plant cells have evolved unique channel structures, plasmodesma (Pd), to create a pathway for molecular exchange between adjacent cells. Pd are basically cytoplasmic channels through the cell wall, which are lined by plasma membrane, and contain a modified strand of ER that spans them. These structures provide cytoplasmic and membrane continuity between connected cells, and that continuity is utilized for short and long distance molecular trafficking. Pd sphincters, made from constricting the Pd openings by outer layers of callose, together with the ER strand that occludes the Pd lumen set the upper limit for the size of molecules that can freely diffuse through the cytoplasmic component of the Pd channel. This limit, called the size exclusion limit (SEL), is a major factor that restricts macromolecular transport through Pd.

Pd have been implicated in transport of diverse macromolecules, including nucleic acids and proteins, both endogenous and pathogen-derived, and regulation of this Pd-mediated intercellular transport represents an important aspect of plant physiology and development. Recent studies have identified several Pd-associated proteins involved in Pd transport. Furthermore, emerging evidence indicates that even factors that reside in other cellular organelles, such as mitochondria and chloroplasts, can play roles in regulation of traffic through Pd channels. In this minireview, we summarize the current knowledge regarding proteins that associate with Pd and other cellular organelles and likely participate in regulation of Pd transport.

Overview of Pd structure and composition

Pd have been implicated in cell-to-cell transport of plant-encoded protein regulators of tissue specification and development,[1] small RNA molecules, such as siRNA an micro RNA,[2-5] plant viruses,[6,7] and even plastids.[8] Transport through Pd is intimately connected with the structure of these channels. The ultrastructure of Pd has been well characterized at the electron microscopic level;[9,10] they represent membrane-lined cytoplasmic bridges, the central region of which is occupied by the trans-Pd ER that forms a continuum between the adjacent cells. The Pd channel has an outer diameter of approximately 50 nm,[9] and at its orifice in the cell wall, accumulate deposits of callose, i.e., 1,3-β-D-glucan, that form collar structures. These collars are thought to function as molecular sphincters: increasing callose deposits at the sphincter decreases Pd SEL whereas reducing these deposits increases SEL and facilitates Pd transport.[6]

The space between the trans-Pd ER and the plasma membrane that bounds the Pd structure contains cytoplasmic channels through which soluble molecules move from cell to cell. Electron microscopy studies revealed that, inside the Pd cavity, are located fine thread-like structures, linking the plasma membrane to the proteins on the surface of desmotubule (for review, see 9-11); the molecular composition of these structures are yet to be identified. Actin filaments are observed near and within the Pd channel, and local actin polymerization status determines Pd permeability. Many viral movement proteins (MPs) that mediate cell-to-cell spread of plant viruses are known to increase Pd SEL, and those of *Tobacco mosaic virus* (TMV) and *Cucumber mosaic virus* (CMV) have been suggested to depolymerize actin associated with Pd.[12] Thus, there are at least 2 independent modes to control Pd transport: callose sphincters and actin filaments.

A more comprehensive insight into the protein composition of Pd was obtained from a proteomic study of *Arabidopsis* cell walls enriched with Pd. This study revealed that large proportion of proteins contained in this fraction are membrane-associated proteins, which include a substantial number of receptor-like proteins.[13-15] Consistently, at least 6 receptor-like kinase-like proteins associating with Pd were also identified in rice,[16] indicating that the ER and plasma membrane inside the Pd cavity are rich in receptor-like proteins. The functions of

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Submitted: 12/24/2013; Revised: 01/17/2014; Accepted: 01/17/2014; Published Online: 02/11/2014
Citation: Ueki S, Citovsky V. Plasmodesmata-associated proteins: Can we see the whole elephant?. Plant Signaling & Behavior 2014; 9:e27899; PMID: 24518352; http://dx.doi.org/10.4161/psb.27899

Table 1. Summary of major Pd-associated proteins involved in regulation of Pd permeability.

Name	Reference	Description
PdBG1 PdBG2 PdBG3	23	PdBG1 and PdBG2 are involved in development of lateral root primordia. The biological function of PdBG3 is still unknown.
BG_pap	24	Loss of function leads to high constitutive accumulation of callose at Pd due to reduced callose degradation, and limited Pd-mediated i diffusion of cytoplasmic GFP.
GSL12/CalS3	22	Involved in regulation of root development by controlling callose levels at Pd.
PDCBs	25	Contain GPI-anchor motif with X-8 domain. Bind callose in vitro; likely bind and stabilize callose in vivo.
^{C1}RGPs	31, 32	Overexpression increases callose accumulation in source leaves and restricts viral spread.
PDLPs	26, 30	Type I transmembrane proteins. Negatively regulate Pd-mediated macromolecular diffusion. Serve as anchoring points for viral MP-containing tubule formation.
PDLP5	28, 29	Also known as HWI1. Expression levels positively correlate with callose accumulation.
GnTL	37	Interacts with calreticulin, most likely localizes at Pd orifice.
Remorin	33	Associates with membrane raft-like structures. Expression levels negatively correlate with local and systemic movement of PVX.

these proteins in regulation of Pd-mediated trafficking remain to be discovered.

Pd-associated proteins: gate-keepers for the channel

Although proteomic approaches have identified a relatively large number of potential Pd components, only few Pd-associated proteins have been experimentally shown to participate in or regulate Pd transport (**Table 1**). One of the best known mechanisms to regulate Pd permeability is through callose deposits, which—with some specific exceptions, such as wounded cells, sieve pores in phloem, and pollen tubes—are found specifically at Pd.[6] Thus, it is not surprising that proteins involved in callose metabolism also localize to Pd and are often involved in Pd transport. Callose accumulation levels are directly determined by 3 classes of cellular factors: callose synthases that produce callose, 1,3-β-D-glucanases that degrade callose, and callose retaining proteins that associate with callose to stabilize it.

To date, 12 putative callose synthases (CalS, or more often termed glucan-synthase-like, GSL, proteins) have been identified in *Arabidopsis* based on sequence homology.[17] Among them, GSL7,[18-20] GSL8,[21] and GSL12[22] have been demonstrated to be involved in regulation of callose at Pd, while the actual Pd localization was only demonstrated for GSL12.[22]

On the other hand, the *Arabidopsis* family of callose hydrolyzing enzymes, 1,3-β-D-glucanases, comprises more than 50 members, and some of them, including PdBG1/2/3,[23] have been shown to target to Pd. In addition, another Pd-associating 1,3-β-D-glucanase with a GPI anchor, BG_pap, was shown to be involved in Pd regulation;[24] its loss leads to high constitutive accumulation of callose at Pd, due to reduced degradation, and restricts Pd-mediated intercellular diffusion of cytoplasmic GFP.[24]

Besides callose-hydrolyzing enzymes, other Pd-targeted proteins are linked to callose regulation. For example, a group of proteins with a GPI-anchor, which bind to and likely stabilize callose, have been demonstrated to be targeted to Pd, and, therefore, were designated Pd callose binding proteins (PDCBs).[25] Pd-located proteins (PDLPs) represent another example of Pd-associated cellular factors[26]; these belong to the family of type I transmembrane proteins with the transmembrane domain proposed to contain a Pd targeting sequence. Among 11 *Arabidopsis* homologs identified based on the protein sequence similarity, 8 were shown to localize at Pd. Presumably, because of the redundant expression of several PDLPs in leaf tissues,[26,27] single mutants in individual PDLPs did not affect symplastic transport. On the other hand, GFP cell-to-cell diffusion in leaf tissue was markedly increased in double knockout lines that carried deletions in 2 of the 3 PDLPs which are predominantly expressed in the wild type leaves, suggesting that at least some, if not all, PDLPs may act as negative regulators for macromolecular diffusion through Pd. This notion is also supported by the observation that overexpression of PDLP1 leads to a striking decrease in GFP diffusion via Pd. A later study provided additional insights into the PDLP action, showing that overexpression of one of leaf PDLPs, PDLP5 (also known as HWI1[28]), leads to callose accumulation at Pd, while its suppression reduces Pd callose levels, suggesting that PDLPs function by elevating callose deposits, which, in turn, restrict macromolecular diffusion through Pd.[29] Interestingly, PDLPs also play a crucial role in movement of *Grape fan leaf virus* (GFLV) and *Cauliflower mosaic virus* (CaMV).[30] MPs of these viruses form tubular structures that span Pd, and all 8 PDLPs interact with GFLV MP.[30] Thus, these viruses may utilize PDLPs as anchor points for initiation of the tubule assembly, while inhibiting the activity of PDLPs as callose upregulators, to facilitate tubule formation through the channel.

Class 1 reversibly glycosylated polypeptides (^{C1}RGPs) are yet another type of Pd-targeted proteins that are delivered to the plasma membrane of Pd through the ER/Golgi pathway.[31] In ^{C1}RGP2 overexpressing tobacco plants, callose accumulation is increased in source leaves while the flow of photoassymilates and viral spread are impaired.[32] These observations implicate ^{C1}RGPs in callose regulation, and, in turn, in Pd-mediated macromolecular traffic between cells. The molecular mechanism by which ^{C1}RGP2 achieves these effects, however, remains unknown.

Another protein that resides in the plasma membrane in close proximity to Pd is remorin.[33] Remorin molecules cluster in a specific membrane domain, similar to lipid rafts, within the cytosolic leaflet of the plasma membrane.[33] Whether this

protein participates in regulation of Pd is still unclear; however, its expression levels negatively correlate with cell-to-cell movement of *Potato virus X* (PVX) and with phloem unloading in the inoculated leaves, suggesting involvement of remorin in the process of viral movement between cells.[33]

Several other membrane-associated proteins have been found to target to Pd, but their functions in Pd are still obscure. For example, calreticulin, an ER resident protein, has been shown to accumulate at Pd,[34] most likely within the trans-Pd ER. In the cellular ER, calreticulin is involved mainly in Ca^{2+} sequestration and signaling and in chaperone activity,[35] whereas the role that calreticulin might play at Pd awaits further studies. Interestingly and consistent with its Pd accumulation, calreticulin was shown to interact with TMV MP,[36] suggesting that it may be involved in viral movement through Pd. Recent data indicate that, in *Arabidopsis*, calreticulin also interacts with a glucosaminyl transferase-like (GnTL), which itself associates with Pd.[37] Interestingly, GnTL colocalized with PDCB, but not with TMV MP. Within Pd, TMV MP often accumulates in the central cavity[38] whereas PDCB colocalizes with callose at the neck, sphincter region of Pd,[25] suggesting that GnTL also localizes at Pd collar region, for as yet unknown function.

Non-Pd regulators of pd

Traditionally, studies of Pd regulation have focused on factors associated with these channels or located in their vicinity. Defying this apparent logic, several factors that associate with cell organelles distant from Pd have been suggested to participate in regulation of Pd transport.[39-42] One of such non-Pd factors is a chloroplastic protein thioredoxin-m3 (TRX-m3) that may participate in modulation of callose deposits by regulating intracellular levels of reactive oxygen species (ROS).[42] A loss of function mutation in the TRX-m3 gene, originally designated *gfp arrested trafficking 1* (*gat1*), leads to reduced diffusion of GFP through Pd from the companion cells in the root phloem; the mutant lines accumulate high levels of ROS, and, importantly, exhibit higher levels of callose in their root tips, indicating a functional link between ROS and callose at Pd.[42]

The effect of ROS on Pd permeability is complex because, in another instance, higher ROS levels in fact increase the Pd-mediated macromolecular diffusion. Specifically, mutations in the *Arabidopsis* DEAD-box RNA helicase *INCREASED SIZE EXCLUSION* (*ISE1*) gene elevated ROS levels and increased Pd permeability in embryos at the mid-torpedo stage.[43,44] ISE1 is a mitochondrial protein, and silencing its expression results in more oxidized mitochondria, but more reduced plastids, as determined using redox-sensitive GFP reporter targeted to the mitochondria or plastids.[45] ISE2, later identified as a putative DEVH-box RNA helicase, is another example of a non-Pd protein that modulates Pd permeability.[46] ISE2 is a chloroplast protein,[47] and similar to *ISE1*-silenced tissues, silencing of *ISE2* resulted in more reduced plastids and elevated Pd permeability.[45] Furthermore, pharmacological studies revealed that, when oxidative shift in plastids was induced, Pd permeability was also reduced even when the same treatment induced oxidization of mitochondria, suggesting that oxidative shift in plastids overrides the positive effect of oxidized mitochondria on Pd permeability. Collectively, these studies indicated that oxidization status of plastids and mitochondria and their crosstalk affect Pd permeability, potentially acting as regulators of intercellular transport. One of the possible mechanisms of this regulatory effect could be via structural modification of Pd due to the altered redox status during Pd development. Increased diffusion through Pd in the *ise1* or *ise2* mid-torpedo embryos was accompanied by increased incidence of morphologically altered Pd, specifically, twinned and branched channels.[43,47,48] Transcriptome analysis revealed that expression levels of callose metabolism-related genes are altered in these mutants, however, these changes did not parallel changes in Pd permeability; thus, ISE1 and ISE2 may exert their regulatory effects on Pd permeability by a callose sphincter-independent mechanism. However, the same study revealed that expression of many other genes belonging to different functional categories was also altered in the mutant lines, suggesting that the mutations in these genes significantly affected the plant homeostasis. Thus, the precise mechanism of regulation of Pd by ISE1 and ISE2 remains undefined.

Concluding Remarks

Identification of Pd-associated proteins has always been one of the major stumbling blocks to the studies of Pd function and regulation. Development of sensitive proteomic approaches along with refined Pd fractionation techniques allowed identification of increasing numbers of Pd-associated factors.[13,14] Many of them are annotated as membrane-associated proteins, including receptor-like proteins. In many cases, however, these breakthroughs in Pd protein discovery have not been followed by equally successful insights into the detailed functions of the discovered factors within Pd yet. From those factors the function of which is known, most act in the callose-mediated Pd regulation pathway. In contrast, the protein machinery of the actin-mediated Pd regulatory pathway, the second major regulatory mechanism of Pd transport, is virtually unknown; this pathway will likely represent a focus for future studies, especially since the highly dynamic nature of actin microfilaments makes it a good candidate for fine tuning of and quick changes in Pd permeability.

In addition to these Pd-associated regulatory machineries, several lines of evidence demonstrated the existence of a non-Pd system, originating from plastids and mitochondria, that regulates Pd permeability. Because organized cell-to-cell communication is essential to orchestrate the whole-body physiology of higher plants, the existence of complex crosstalk regulatory pathways for Pd permeability to respond to environmental and developmental stimuli makes biological sense. Thus, most likely we have not yet discovered the entire complement of Pd components, structural or functional. Identification of these components and understanding their activity will represent the focus of the future research.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The V.C. group is supported by grants from USDA/NIFA, NIH, NSF, BARD, and BSF, and the S.U. group is supported by grants from Nagase Science and Technology Foundation, Casio Science Promotion Foundation, and Ohara Foundation for Agricultural Research.

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