Sink Plasmodesmata as Gateways for Phloem Unloading. Myosin VIII and Calreticulin as Molecular Determinants of Sink Strength?[1]

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Phloem-mediated movement of photoassimilates is one of the most critical processes in plants. Photosynthetically active leaves (source) produce an excess of photoassimilates that are exported, via sieve elements of the phloem, into photosynthetically inactive tissues (sink). For instance, growing root apices are heterotrophic sink organs that are dependent on the continuous supply of photoassimilates from the above-ground source organs. Thus, root apices represent an ideal object to study mechanisms of the phloem unloading of photoassimilates and of the sink strength. The simplest definition of sink strength considers the competitive ability of heterotrophic organs to import, process, and store photoassimilates (Herbers and Sonnenwald, 1998). It is unfortunate that both factors and molecules that determine the sink strength remain controversial. Nevertheless, general agreement exists that plasmodesmata, which interconnect most cells of higher plants into a symplasmic continuum, substantially contribute to phloem unloading into sink tissues. This has been shown for root apices and for several other sink tissues such as potato (*Solanum tuberosum*) tubers and *Agrobacterium tumefaciens* sp. tumors (Fisher and Oparka, 1996; Pradel et al., 1999; Oparka and Santa Cruz, 2000). Plasmodesmata also participate in Suc export from leaves (Stitt, 1996), highlighting their prime importance for source-sink interactions.

Maize (*Zea mays*) and Arabidopsis root apices serve as excellent model objects to study the role of plasmodesmata in unloading of photoassimilates from phloem elements into sink tissues. Although a symplasmic pathway for phloem unloading is accepted for root apices, the number of plasmodesmata that have been calculated to be present is not sufficient to support the extensive carbon demand of rapidly growing root apices (Bret-Harte and Silk, 1994). Thus, these authors concluded that either plasmodesmata permeability is actively regulated or that alternative transport mechanisms exist for Suc transport to the apical meristem. A possible explanation for this paradox, in favor of the first possibility, comes from the recent studies on plasmodesmata that reveal that these complex cell wall "tunnels" are gateable (van Bel and Kesteren, 1999; Jackson, 2000; Zambryski and Crawford, 2000).

PLASMODESMATA AS GATEABLE AND CONTRACTILE STRUCTURES

Plasmodesmata are plasma membrane-lined cytoplasmic "bridges" that span cell walls throughout plant tissues, providing higher plants with their unique supracellular nature (Lucas et al., 1993). Primary plasmodesmata are formed during the culmination of plant-specific cytokinesis by entrapment of endoplasmic reticulum (ER) elements within cytokinetic cell plates (Hepler, 1982). Later, when the cytokinetic cell plates transform into young cell walls (Samuels et al., 1995), plasmodesmata retain their juvenile callosic nature (Baluška et al., 2000a), whereas ER elements become tightly appressed to form the so-called central rod or desmotubule (for a model, see Overall and Blackman, 1996). The latter element of plasmodesmata not only stabilizes their internal structure but it also limits their lumen and porosity. This is due to the fact that both the plasma membrane and desmotubule are densely covered with globular particles that are interlinked with spoke-like elements providing the dense sieve-like character of plasmodesmata. The molecular nature of plasmodesmata proteins remains unclear even after many years of devoted studies. Nevertheless, recent advances in immunofluorescence techniques allow identification of proteins that can be enriched at plasmodesmata. These proteins include actin, myosins, ER-based calreticulin, centrin, and calcium-dependent protein kinase (White et al., 1994; Yahalom et al., 1998; Baluška et al., 1999, 2000b; Blackman et al., 1999; Reichelt et al., 1999; Overall et al., 2000). Noninvasive

[1] This work was supported by the Deutsches Zentrum für Luft- und Raumfahrt, Bonn (to F.B. and D.V.) and by the Czech Ministry of Education Program, Czech Republic (no. J13/98:113100003 to F.C.). F.B. is partially supported by the Slovak Academy of Sciences, Grant Agency Vega, Bratislava, Slovakia (grant no. 6030).

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transfection studies using green fluorescent protein reporters have shown that plasmodesmata exist in three basic conformations: closed, open, and dilated (Crawford and Zambryski, 2000; Zambryski and Crawford, 2000). All this strongly suggests that plasmodesmata are contractile "organelles" that fluctuate between these three structural states and that actomyosin- and centrin-based forces are in a position to participate in the gating of plasmodesmata via calcium-sensitive pathways.

CALCIUM-RELATED SIGNALING PATHWAYS CONTROL PLASMODESMAL PERMEABILITY

Intracellular calcium waves regulate plasmodesmal permeabilities within a few seconds; even slight increases in cytoplasmic calcium cause immediate closure of plasmodesmata (Holdaway-Clarke et al., 2000). Mastoparan-mediated activation of heterotrimeric G-proteins induces cytoplasmic calcium waves that rapidly, but transiently, close plasmodesmata (Tucker and Boss, 1996). Similar calcium waves might be implicated in plasmodesmata gating in response to environmental signals such as light (Epel and Erlanger, 1991). Besides calcium, polyphosphoinositols (IP_2 and IP_3) inhibit cell-to-cell transport in staminal hairs of *Setcreasea purpurea*, and IP_3 itself can be transported rapidly through plasmodesmata (Tucker, 1988). Because, at least in other plant systems, a Ca^{2+}-dependent signaling pathway may be controlled by IP_3 (Franklin-Tong et al., 1996), these phenomena seem to be closely interlinked.

SPECIALIZED CELL WALL MICRODOMAINS: POSSIBLE ROLES IN PLASMODESMAL GATING

It is well known that plasmodesmata are firmly embedded within cell walls, a feature that has hindered biochemical analysis of these structures. It is important that cell walls show a unique composition around plasmodesmata in which cellulose is depleted, whereas callose is abundant in wall microdomains that surround plasmodesmata (e.g. Radford et al., 1998). Two recent experimental studies showed that callose participates in the gating of plasmodesmata in vivo. First, callose inhibited symplasmic transport in wheat root apices exposed to aluminum toxicity (Sivaguru et al., 2000). Second, high depositions of callose in transgenic tobacco (*Nicotiana tabacum*) plants deficient in β-1,3-glucanase reduced the size exclusion limit (SEL) of their plasmodesmata (Iglesias and Meins, 2000).

Like callose, pectins belong to "juvenile" cell wall components that are abundant within cytokinetic cell plates and at plasmodesmata (Baluška et al., 2000a). Several studies reported that cell wall microdomains around plasmodesmata are characterized by a unique composition of pectins (e.g. Orfila and Knox, 2000). The cell wall enzyme pectin methylesterase, which is responsible for de-esterification of secreted pectins, localizes preferentially around plasmodesmata (Morvan et al., 1998). For many years, the relevance of plasmodesmata-associated pectin-based cell wall microdomains to plasmodesmata permeability has been unclear. However, recently two papers have reported that the viral movement protein of the tobacco mosaic virus interacts directly with pectin methylesterase, and that this interaction is essential for the dilating of plasmodesmata via this movement protein (Dorokhov et al., 1999; Chen et al., 2000). Pectin methylesterase might rapidly change the structural state of cell wall pectins around plasmodesmata, especially at the cell wall-plasma membrane interface, which could affect directly the architecture of plasmodesmata (see Fig. 2 in Zambryski and Crawford, 2000).

IMPACT OF MOVEMENT PROTEINS ON PLASMODESMAL GATING IN SOURCE-SINK INTERACTIONS

The current boom of functional data on plasmodesmata (for the latest reviews, see Jackson, 2000; Zambryski and Crawford, 2000) is closely related to the ability of plant viruses to dilate plasmodesmata transiently using their movement proteins (Wolf et al., 1989). Viral movement proteins associate with the cytoplasmic face of cortical ER elements (Heinlein et al., 1998) and with the cytoskeleton (McLean and Zambryski, 2000). Both of these features might be related to the passage of viral particles through plasmodesmata even when their sizes clearly exceed the diameter of the plasmodesmal microchannels. It is intriguing that recent data implicate the movement protein of tobacco mosaic virus in conferring cold stability on microtubules, perhaps via lateral contacts with microtubule protofilaments and interactions with microtubule nucleation sites (Boyko et al., 2000). These unique properties of viral movement proteins may be crucial for effective targeting of viral particles toward plasmodesmata where interactions with ER and actin cytoskeleton may be implicated in the gating of plasmodesmata.

The movement protein of tobacco mosaic virus impacts on the regulation of carbon partitioning in transgenic tobacco seedlings (Lucas and Wolf, 1999). In particular, constitutive expression of movement protein increases assimilate levels in leaves (Olesinski et al., 1995) and reduces root biomass (Balachandran et al., 1995). The capacity of viral movement proteins to induce changes in photoassimilate allocation (Lucas and Wolf, 1999) supports the importance of plasmodesmal gating for root-shoot communication networks.

UNIQUE STATUS OF SINK PLASMODESMATA

Plasmodesmata conductivity was originally reported to be 0.8 to 1 kD, suggesting that the diameter

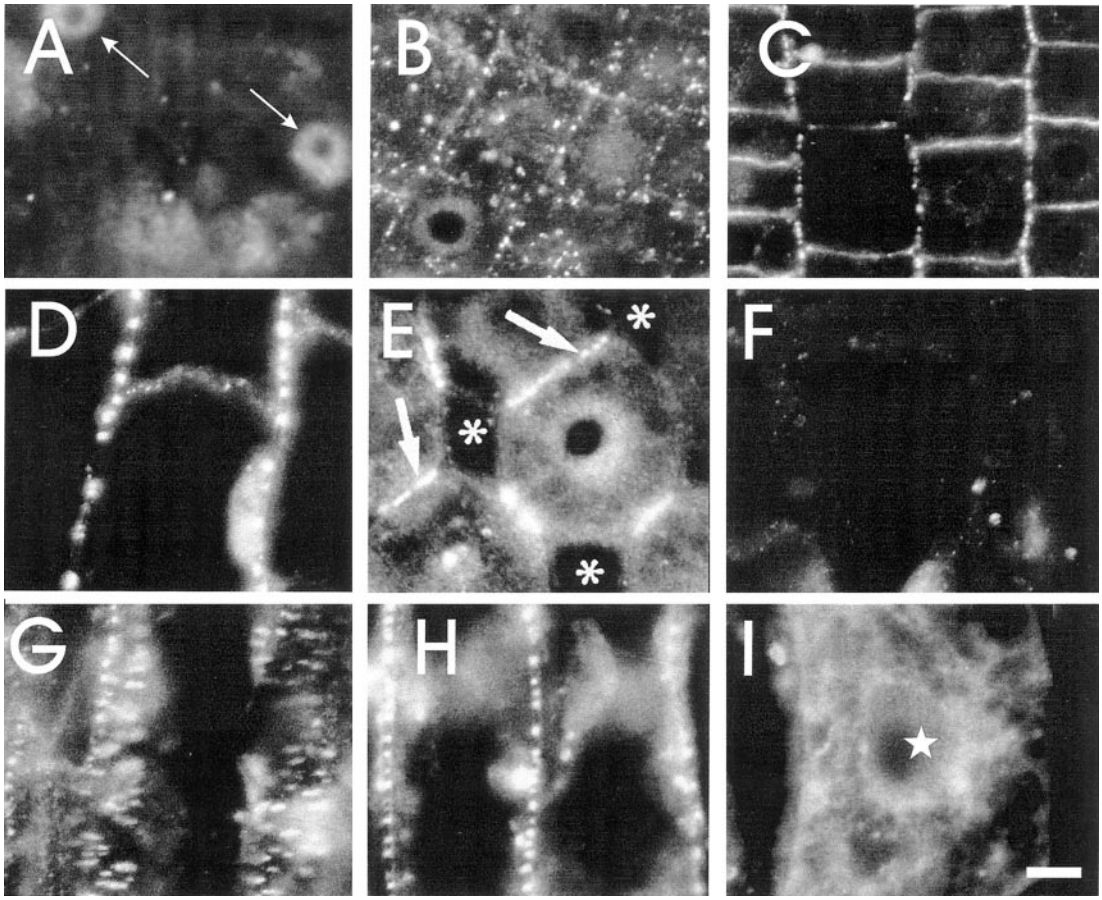

Figure 1. Myosin VIII and calreticulin distributions in maize root apices. A, Myosin VIII distributes diffusely in root cap cells showing enrichment in nuclei (arrows). B, In the distal part of the apical meristem, fine spots (representing plasmodesmata; see Reichelt et al., 1999) appear at cell peripheries while the nuclear signal gets fainter. C and D, In cells of the inner cortex, accumulation of myosin VIII at plasmodesmata grouped into pit fields is much more prominent in the distal part of the transition zone (C) and in the distal part of the elongation region where the signal corresponds to plasmodesmata clustered into pit fields (D). E, Viewing cross sections of root apices, myosin VIII accumulates at cell-to-cell contacts (arrows) whereas cell periphery domains facing intercellular spaces (asterisks) are depleted in myosin VIII. F, In post-mitotic cells of the outer cortex, myosin VIII shows faint signal and it does not localize to cellular peripheries. G, H, and I, Calreticulin localizes abundantly to plasmodesmata grouped into pit fields in the inner cortex of the transition zone. G, Paradermal section revealing the typical lens-shaped form of pit fields, whereas H shows cross-sectioned pit fields (compare with D for myosin VIII). I, Epidermis cell from the same root region localizes ER-based calreticulin to perinuclear networks, whereas cell peripheries do not show accumulation of calreticulin (nucleus is marked by a star). For details on the immunofluorescence technique, see Baluška et al. (1999). Bar = 10 μm.

of their microchannels is about 3 nm. However, later studies showed that this SEL is not universally valid and many plasmodesmata naturally exist in a dilated state allowing passage of larger molecules. For instance, tobacco leaf trichome plasmodesmata have a basal SEL of around 7 kD (Waigmann and Zambryski, 1995). Plasmodesmata between sieve elements and companion cells permit passage of 3- to 10-kD fluorescent probes (Kempers and van Bel, 1997).

Recent data reveal that sink plasmodesmata are in a dilated configuration, and we suggest that this feature might be directly related to sink strength. In Arabidopsis root apices, 3-kD fluorescent probes were reported to move freely in the post-phloem pathways (K. Oparka and D. Prior, unpublished data; Fisher and Oparka 1996). Dilated plasmodesmata similarly were reported for tissues of developing wheat grains (Wang and Fisher, 1994). In sink leaves, 27-kD green fluorescent protein and fusion proteins up to about 50 kD were shown to move freely in Arabidopsis (Imlau et al., 1999) and in tobacco (Oparka et al., 1999). More recently, these surprising findings also have been extended to other plants and organs (Itaya et al., 2000). Even more dramatic alterations to plasmodesmata architecture were found in clover (*Trifolium incarnatum*) and tomato (*Lycopersicon esculentum*) root cortical cells parasitized by the nematode *Criconemella xenoplax*. Here, plasmodesmal microchannels enlarged and, eventually, the central ER-based desmotubule disappeared (Hussey et al.,

1992). In some respects, these structurally modified plasmodesmata resemble sieve pores of phloem elements.

MYOSIN VIII AND CALRETICULIN ARE ENRICHED AT SINK PLASMODESMATA

Unconventional myosin VIII is a plant-specific myosin (Hodge and Cope, 2000; Reichelt and Kendrick-Jones, 2000), whereas calreticulin is a conserved ER-based protein that sequesters calcium. Our immunofluorescence data document that these molecules accumulate at plasmodesmata grouped into pit fields in the transition zone of root apices (Fig. 1; Baluška et al., 1999, 2000b), which are active in transport (Oparka et al., 1994). Moreover, F-actin also accumulates at these pit fields (Fig. 2, C and D; Baluška et al., 2000b). Myosin VIII, calreticulin, and actin are enriched especially at the outer portions of plasmodesmata grouped into pit fields (Fig. 2, A–C) where their

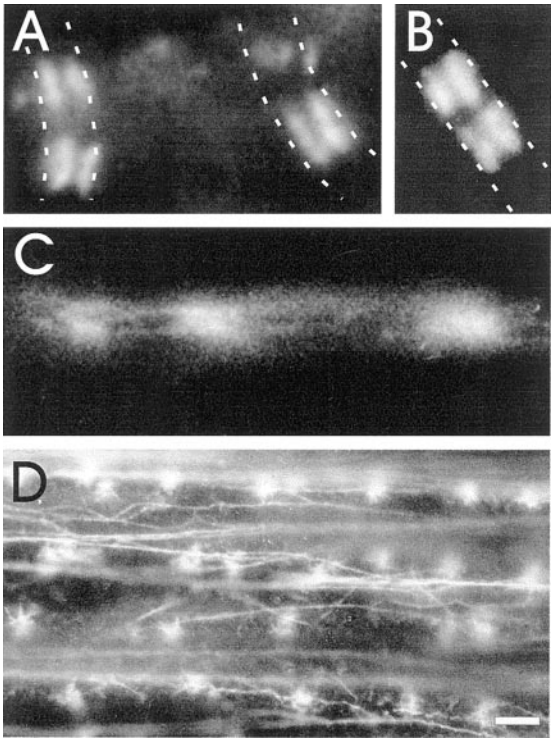

Figure 2. Myosin VIII, calreticulin, and actin at pit fields of the inner cortex. A, B, and C, In the inner cortex, myosin VIII (A), calreticulin (B), and actin (C) all are abundant at the peripheral part of plasmodesmata grouped into pit fields, whereas their central parts show lower signal for these proteins (pit fields are cross sectioned in these images). Outlines of the cell wall-cytoplasm interface in A and B are enhanced with dashed lines. D, Paradermal section showing F-actin meshworks accumulated at inner cortex pit fields in the transition zone of maize root apices. F-actin-enriched pit fields are star shaped, indicating that adjacent cortical ER elements also accumulate F-actin, whereas individual pit fields are interlinked with longitudinal (aligned parallel with the root axis) F-actin bundles. For details on the immunofluorescence technique, see Baluška et al. (1999). Bar = 1.5 μm for A, B, and C and 8 μm for D.

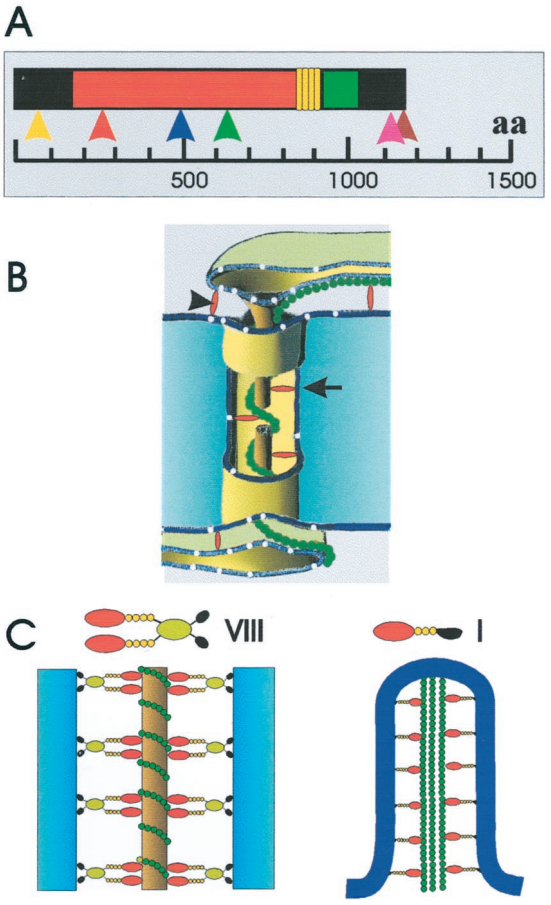

Figure 3. Schematic view of the AtATM1 molecule, plasmodesmata, and microvilli. A, Sequence analysis of the ATM1 myosin VIII molecule (composed of 1,166 amino acids) showing five main domains: N terminus (left, black), motor domain (red), four IQ motifs (yellow), coiled-coil domain (green), and C terminus (right, black). Arrowheads (from left to right) indicate positions of known motifs (yellow, PEST sequence; red, GESGAGKT motif conserved for all myosin heads; blue, RDALAK motif characteristic for myosin I; green, RGD motif; and brown, TRY microbody targeting sequence). Purple arrowhead indicates the position of phosphorylation site (Thr) for protein kinase A (see also Fig. 4). Coiled-coil domain is responsible for the dimerization of myosin VIII molecules. B, Schematic model (adapted from Overall and Blackman, 1996) of a plasmodesma showing the modified ER element, in the form of the desmotubule (brown rod), which traverses the cell wall (pale blue), associates with F-actin (green pearls), and is interlinked with the plasma membrane (dark blue line) via myosin VIII and centrin (red spokes, arrow). Myosin VIII and centrin (see Fig. 4 in Blackman et al., 1999) may also anchor cortical ER elements (shown in pale green), associated with F-actin (green pearls) at the plasma membrane outside of plasmodesmata (red spokes, arrowhead). C, Left side, hypothetical view of myosin VIII-based linkages between the plasma membrane and the actin-associated ER-based desmotubule. Note that myosin VIII molecules are predicted to form dimers via their coiled-coil domains. C, Right side, a similar structural (but not necessarily functional) principle is known for the brush border myosin I, monomers of which form spokes interlinking the central F-actin bundles (green pearls) with the plasma membrane in microvilli (for a model, see Fig. 2 in Osherov and May, 2000).

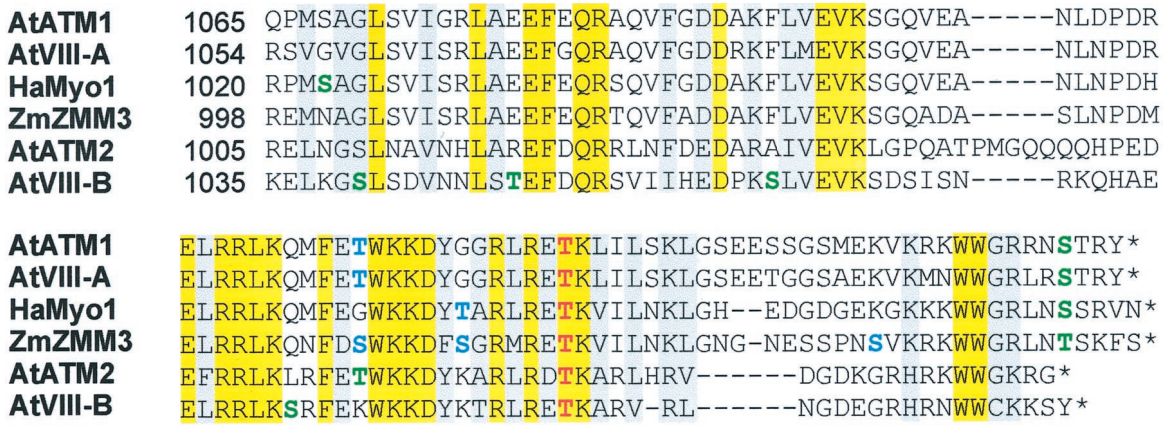

Figure 4. Comparison of C termini domains conserved for myosin VIII sequences. Sequence analysis of C termini of available myosin VIII sequences that are unique for this class of unconventional myosins. Sequences are aligned using the MACAW software (Schuler et al., 1991) with the BLOSUM62 scoring matrix, considering only similarity blocks with $P < 10^{-10}$. Amino acids conserved among all sequences are highlighted in yellow and positions with conserved residue types are shown on gray background. Putative phosphorylation sites were determined using the PhosphoBase patterns (Kreegipuu et al., 1999). They are shown in red for protein kinase A, blue for protein kinase C, and green for other and/or multiple kinases. The conserved sequence motif containing a putative phosphorylated Thr (red color) is present at the C termini of all myosin VIII sequences except HaMyo3 (which is not included in this figure). Numbers denote the position of the first amino acid shown.

sphincter-like necks (Olesen, 1980; Radford et al., 1998) are clustered together and associate with cortical ER elements. It is intriguing that the architecture of plasmodesmata necks seems to be F-actin dependent, being sensitive toward treatment with cytochalasin D (White et al., 1994).

In contrast, root cap plasmodesmata do not accumulate actin, myosin VIII, and calreticulin at their plasmodesmata/pit fields (Baluška et al., 1999, 2000b; Fig. 1A). Moreover, they lack sphincter-like necks (Radford et al., 1998) and are symplasmically isolated (U. Tirlapur, K. König, F. Baluška, and D. Volkmann, unpublished data). Actin, myosin VIII, and calreticulin similarly do not accumulate at plasmodesmata of postmitotic cells of the root epidermis (Baluška et al., 1999, 2000b; Fig. 1I), which are also symplasmically isolated (Duckett et al., 1994; Tirlapur and König, 1999).

MYOSIN VIII AND CALRETICULIN AS POSSIBLE DETERMINANTS OF SINK STRENGTH?

Three basic processes determine the strengths of diverse plant sinks competing among each other for available photoassimilates: unloading, utilization, and storage of photoassimilates (Herbers and Sonnenwald, 1998). Symplasmic phloem unloading, using sink plasmodesmata as cell-to-cell gateways, is typical for diverse sinks such as root apices, potato tubers, and *A. tumefaciens*-induced tumors (Fisher and Oparka, 1996; Pradel et al., 1999; Oparka and Santa Cruz, 2000). Thus, the sink strength is expected to be mediated via gateable sink plasmodesmata representing some kind of "bottleneck" for the sink strength.

Participation of coordinated actions of actin and myosin in opening/dilating/closing of plasmodesmata (for hypothetical model, see Fig. 3, B and C) is supported by findings that depolymerization of F-actin dilates plasmodesmata (Ding et al., 1996). Moreover, inhibition of myosin ATPases constricts plasmodesmal necks (Radford and White, 1998) and dilates ER elements near plasmodesmata (Šamaj et al., 2000). It is important that both constriction and maintenance of constricted plasmodesmata could turn out to be an ATP-dependent process because ATP depletion opens plasmodesmata (Cleland et al., 1994).

PERSPECTIVES

Myosin VIII (Figs. 3A and 4) comprises a unique class of unconventional myosins found only in plants (Knight and Kendrick-Jones, 1993; Hodge and Cope, 2000; Reichelt and Kendrick-Jones, 2000; Liu et al., 2001), suggesting that these myosins could perform some functions specific for plants. In support of this notion, myosin VIII localizes to plasmodesmata, which are plant-specific structures. An attractive possibility is that plant myosin VIII, by analogy to the better known brush border myosin I that mechanically stabilizes microvilli of intestinal epithelial cells (e.g. Osherov and May, 2000), regulates the architecture of plasmodesmata via formation of radial spoke-like linkages between the central desmotubules and the plasma membrane (Fig. 3, B and C). This would fit into the emerging scheme that unconventional myosins are more important for generation of tension at the plasma membrane than for generation of cytoplasmic motilities (for myosin I, see Osherov and

May, 2000). It is interesting in this respect that myosin VIII contains the RDALAK motif in its head domain (Fig. 3A), which was proposed earlier to be conserved for myosin I (Knight and Kendrick-Jones, 1993).

In the current database, sequences of seven myosin VIII proteins are available (Hodge and Cope, 2000; Reichelt and Kendrick-Jones, 2000). The most characteristic feature of myosin VIII sequences is their unique C terminus that contains several predicted phosphorylation sites for protein kinases A and C (Fig. 4). Moreover, the presence of four calmodulin-binding IQ motifs (Fig. 3A) implies regulation with both calmodulin and calcium (Reichelt and Kendrick-Jones, 2000), suggesting that ER-based calreticulin may regulate the architecture of plasmodesmata by its calcium-buffering capacity (Baluška et al., 1999). Our testable working hypothesis predicts that specialized sink plasmodesmata are actively maintained in open configuration by local calreticulin-mediated regulation of cytoplasmic calcium levels that sensitively modulate actomyosin- and centrin-based contractilities. In conclusion, myosin VIII, calreticulin, and centrin emerge as prime candidates for molecules that participate in modulation of the sink strength via gating of sink plasmodesmata.

ACKNOWLEDGMENTS

We thank Nigel Chaffey and Helena Lipavská for their useful comments on the manuscript.

Received December 29, 2000; accepted February 20, 2001.

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