Class VIII Myosins Are Required for Plasmodesmatal Localization of a Closterovirus Hsp70 Homolog

Dror Avisar, Alexey I. Prokhnevsky, and Valerian V. Dolja*

Department of Botany and Plant Pathology and Center for Genome Research and Biocomputing, Oregon State University, Corvallis, Oregon 97331

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The Hsp70 homolog (Hsp70h) of Beet yellows virus (BYV) functions in virion assembly and cell-to-cell movement and is autonomously targeted to plasmodesmata in association with the actomyosin motility system (A. I. Prokhnevsky, V. V. Peremyshl, and V. V. Dolja, J. Virol. 79:14421–14428, 2005). Myosins are a diverse category of molecular motors that possess a motor domain and a tail domain involved in cargo binding. Plants have two classes of myosins, VIII and XI, whose specific functions are poorly understood. We used dominant negative inhibition to identify myosins required for Hsp70h localization to plasmodesmata. Six full-length myosin cDNAs from the BYV host plant Nicotiana benthamiana were sequenced and shown to encode apparent orthologs of the Arabidopsis thaliana myosins VIII-1, VIII-2, VIII-B, XI-2, XI-F, and XI-K. We found that the ectopic expression of the tail domains of each of the class VIII, but not the class XI, myosins inhibited the plasmodesmatal localization of Hsp70h. In contrast, the overexpression of the motor domains or the entire molecules of the class VIII myosins did not affect Hsp70h targeting. Further mapping revealed that the minimal cargo-binding part of the myosin VIII tails was both essential and sufficient for the inhibition of the proper Hsp70h localization. Interestingly, plasmodesmatal localization of the Tobacco mosaic virus (TMV) movement protein and Arabidopsis (true) protein RGP2 was not affected by myosin VIII tail overexpression. Collectively, our data implicate class VIII myosins in protein delivery to plasmodesmata and suggest that more than one mechanism of such delivery exist in plants.

It is well established that plant virus movement from cell to cell occurs via plasmodesmata, the plant-specific organelles that mediate the translocation of proteins, RNAs, and viruses between adjacent cells (3, 11, 21). All nondefective plant viruses encode one or more proteins that are required for the cell-to-cell movement. Many of these movement-associated proteins share an ability for plasmodesmatal localization in the absence of other virus-encoded products, indicating that their targeting relies on endogenous cellular pathways (5, 21). Despite extensive effort, the mechanisms of such autonomous plasmodesmatal targeting are poorly understood. One recurring theme in studies of plasmodesmatal targeting is the potential role of the endoplasmic reticulum (ER) and secretory or endocytic vesicles (12, 15, 17). Another, perhaps complementary theme is the involvement of microtubular and actomyosin motility systems in the delivery of plasmodesma-associated proteins to their destination (6, 12, 15, 17, 42). Most data supporting the role of cytoskeletal pathways in protein targeting to plasmodesmata are obtained using drugs that affect the integrity of microtubules and actin microfilaments or inhibit the cytoskeleton-associated molecular motors. We also used these techniques to reveal that inhibitors of actomyosin, but not microtubular transport machineries, abolish the plasmodesmatal targeting of the Hsp70 homolog (Hsp70h), a movement-associated protein encoded by Beet yellows virus (BYV) (30).

The two principal components of actomyosin machinery are actin, which forms long actin microfilaments, and myosin, a molecular motor that is capable of moving along the microfilaments (40). The energy for the physical translocation of myosin and its cargo is provided by ATP hydrolysis catalyzed by the N-terminal motor domain that is also responsible for binding microfilaments. This domain is conserved among all known myosins (10). The C-terminal parts of myosins are more variable and contain (sub)domains required for dimerization, the binding of regulatory proteins (myosin light chains), and the movement of cargo by myosins. Myosins are panneukaryotic proteins; at least 24 distinct myosin classes have been identified, with functions varying from muscle contraction to organelle transport to cell motility to host cell invasion by parasites (10). Higher plants typically possess 10 to 20 individual myosins that belong to classes VIII and XI, each of which is evolutionarily related to animal and fungal class V myosins (10, 13, 33). Although the biophysical properties of the class XI myosins suggest their involvement in extremely rapid and unceasing intracellular trafficking of plant organelles (37, 39), the specific functions of individual myosin genes have not been established. Likewise, subcellular localization studies have implicated class VIII myosins in cell plate formation and plasmodesmatal function (32, 34), but no experimental support of this hypothesis has been provided so far. It is also not known which, if any, myosins are required for the plasmodesmatal targeting of viruses or their movement-associated proteins.

BYV is a filamentous virus with a 15.5-kb, positive-strand RNA genome that has emerged recently as an attractive model for investigating the mechanisms of viral transport (9). The cell-to-cell movement machinery of BYV involves five proteins, among which only one, an ER-localized transmembrane
protein, p6, fits the definition of a dedicated movement protein (MP) (1, 28, 29). The remaining four movement-associated proteins are integral virion components. Three of them, including Hsp70h, form a narrow tail at the virion end that encapsidates the 5′ extremity of the genome (27). Yet an additional tail protein, p20, is specifically required for viral long-distance transport via the plant vascular system (31). Because tail formation is dispensable for genome protection but is essential for virus transport between cells and organs of the infected plant, the tail can be considered a specialized transport device (2, 27, 31). Interestingly, Hsp70h is the only BYV movement-associated protein that was found in plasmodesmata upon ectopic expression or virus infection (22, 30). As indicated above, autonomous plasmodesmal targeting of Hsp70h requires the actomyosin motility system (30).

In this work, we show that interference with the cargo-binding activity of the class VIII but not the class XI myosins abolishes the plasmodesmal localization of Hsp70h. Our results suggest that class VIII myosins function in protein delivery to plasmodesmata and can be used by viruses for the needs of their intracellular and perhaps intercellular translocation.

MATERIALS AND METHODS

Isolation, nucleotide sequencing, and bioinformatic analyses of myosin cDNAs of *Nicotiana benthamiana*. A conserved region in myosin mRNAs was reverse transcribed and PCR amplified using degenerate primers as described previously (4). The resulting PCR products were cloned into pGEM-T vector (Promega). Nucleotide sequencing of the 300 individual clones yielded six distinct variants with more than 10 identical clones for each of these variants (2a). The cDNA cloning of the 5′- and 3′-terminal regions of the corresponding mRNAs to obtain complete myosin cDNAs was done using the FirstChoice RNA ligase-mediated rapid amplification of cDNA ends kit (Ambion). Once again, at least 10 individual clones were sequenced to obtain the consensus nucleotide sequence for each fragment. The nucleotide and amino acid sequences corresponding to each of the six complete myosins of *N. benthamiana* were used as a query to find the highest-scoring BLASTN and BLASTP hits among *Arabidopsis thaliana* myosins to determine likely orthologous relationships. Myosin domain maps (see Fig. 1) were obtained using the program SMART 5 (18).

Ecotropic protein expression and confocal imaging. The cDNAs encoding complete or partial variants of each myosin were amplified by reverse transcription-PCR and cloned into binary vector PCB302, modified to accommodate a protein expression cassette (26). A sequence encoding a triple hemagglutinin (HA) tag has been inserted into this cassette using NcoI and AvrII restriction endonuclease sites, while the myosin-encoding cDNAs were inserted downstream from the HA coding sequence using AvrII and XbaI sites (primer sequences are available upon request). The resulting clones were sequenced to confirm the lack of mutations introduced during the cloning. The exact borders of the coding sequences for each of the 18 myosin variants used in this work are shown in Table 1.

*Agrobacterium tumefaciens* strain C58 GV2260 cells were transformed with each of the binary expression vectors, and the resulting bacteria at an optical density of 600 nm of 0.2 to 0.5 were used for *N. benthamiana* leaf infiltrations (30). The expression of the full-size and truncated myosin variants was assayed by immunoblottting using a rat anti-HA monoclonal antibody (Roche) ~24 h postinfiltration. A mouse monoclonal green fluorescent protein (GFP)-specific antibody (Roche) was used for immunoblot detection of the GFP-tagged proteins. The binary vectors for the ectopic expression of GFP- or monomeric red fluorescent protein (mRFP)-tagged variants of BYV Hsp70h, as well as Hsc70 of *A. thaliana* and Tobacco mosaic virus (TMV) MP-GFP variants, were generated as previously described (30). The GFP-tagged *A. thaliana* protein RGP2 was expressed using binary vector pBlmARTGPP2GFP, described previously (35). To coexpress these fluorophore-tagged proteins with myosin variants, the corresponding bacterial strains were mixed prior to agroinfiltration.

Confocal laser scanning microscopy was done using a Zeiss LSM 510 META microscope fitted with the following configurations of excitation and emission filters, respectively: 488 and 508 nm for GFP, 558 and 583 nm for mRFP, and 513 and 527 nm for yellow fluorescent protein.

The GenBank accession numbers for the sequences encoding the *N. benthamiana* myosins described herein are as follows: XI-2, DQ875135; XI-F, DQ875136; XI-K, DQ875137; VIII-1, DQ875138; VIII-2, DQ875139; and VIII-B, DQ875140.

### RESULTS

Molecular cloning of myosin cDNAs from *N. benthamiana*. Because *N. benthamiana* is a convenient experimental host for BYV (8) that also provides a facile system for transient protein expression, we set out to clone and sequence myosin cDNAs from this plant species. Altogether, six complete myosin cDNAs were characterized (2a). Amino acid sequence comparisons revealed that the isolated cDNAs encoded three class XI and three class VIII myosins. Database searches identified apparently orthologous relationships between *N. benthamiana* myosins and *A. thaliana* class XI myosins previously named MYA2, XI-F, and XI-K, as well as class VIII myosins designated ATM1, ATM2, and VIII-B (33). In an attempt to make the myosin nomenclature more systematic yet related to that between class V and XI myosins (10, 20, 25).

**TABLE 1.** Myosin variants that were transiently expressed in *N. benthamiana* leaves

<table>
<thead>
<tr>
<th>Myosin variant</th>
<th>Coding region nucleotides</th>
<th>Amino acid residues</th>
<th>Molecular mass with HA tag (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete VIII-1</td>
<td>1–3453</td>
<td>1–1150</td>
<td>142</td>
</tr>
<tr>
<td>VIII-1 IQ to C terminus</td>
<td>2479–3453</td>
<td>827–1150</td>
<td>41</td>
</tr>
<tr>
<td>VIII-1 CC to C terminus</td>
<td>2830–3453</td>
<td>944–1150</td>
<td>28</td>
</tr>
<tr>
<td>VIII-1 GTD</td>
<td>2983–3453</td>
<td>995–1150</td>
<td>21</td>
</tr>
<tr>
<td>VIII-1 motor domain</td>
<td>1–2478</td>
<td>1–826</td>
<td>96</td>
</tr>
<tr>
<td>VIII-1 motor domain to CC</td>
<td>1–2982</td>
<td>1–994</td>
<td>116</td>
</tr>
<tr>
<td>Complete VIII-2</td>
<td>1–3591</td>
<td>1–1196</td>
<td>140</td>
</tr>
<tr>
<td>VIII-2 IQ to C terminus</td>
<td>2659–3591</td>
<td>887–1196</td>
<td>39</td>
</tr>
<tr>
<td>VIII-2 CC to C terminus</td>
<td>2944–3591</td>
<td>982–1196</td>
<td>28</td>
</tr>
<tr>
<td>VIII-2 GTD</td>
<td>3100–3591</td>
<td>1034–1196</td>
<td>22</td>
</tr>
<tr>
<td>VIII-2 motor domain</td>
<td>1–2592</td>
<td>1–864</td>
<td>102</td>
</tr>
<tr>
<td>VIII-2 motor domain to CC</td>
<td>1–3099</td>
<td>1–1033</td>
<td>121</td>
</tr>
<tr>
<td>VIII-B IQ to C terminus</td>
<td>2491–3471</td>
<td>831–1156</td>
<td>40</td>
</tr>
<tr>
<td>VIII-B CC to C terminus</td>
<td>2830–3471</td>
<td>944–1156</td>
<td>28</td>
</tr>
<tr>
<td>VIII-B GTD</td>
<td>2971–3471</td>
<td>991–1156</td>
<td>22</td>
</tr>
<tr>
<td>XI-2 IQ to C terminus</td>
<td>2203–4539</td>
<td>735–1512</td>
<td>92</td>
</tr>
<tr>
<td>XI-F IQ to C terminus</td>
<td>2197–4710</td>
<td>733–1569</td>
<td>100</td>
</tr>
<tr>
<td>XI-K IQ to C terminus</td>
<td>2197–4590</td>
<td>733–1529</td>
<td>102</td>
</tr>
</tbody>
</table>

* a Nucleotide numbers start at the first nucleotide of the translation initiation codon according to the nucleotide sequences deposited in GenBank.

* Excluding the triple HA tag.

* IQ, the IQ motif; all IQ motifs in corresponding myosins, starting with the most N-terminal motifs, were included (Fig. 1).

* CC, the coiled-coil motif.

Class VIII myosin tails interfere with Hsp70h targeting to plasmodesmata. Because the recognition and attachment of the cargo are the functions of myosin tails (20, 25), the overexpression of the tails should saturate the tail-binding capacities of the matching cargoes and inhibit their translocation by Hsp70h.
the endogenous myosin motors. In addition, free tails may interact with the heads of cognate myosins, thus reducing the motor activity (19). This tail overexpression-based dominant negative inhibition strategy was employed to determine the potential roles of each of the six *N. benthamiana* myosins in the delivery of BYV Hsp70h to plasmodesmata. The myosin tails that encompassed IQ motifs, coiled-coil motifs, and GTDs (Fig. 1) were tagged with HA epitopes and ectopically expressed at similar levels in the leaves (Fig. 2C).

The localization patterns of Hsp70h in the cells overexpressing myosin tails were monitored using the Hsp70h-GFP fusion and confocal laser scanning microscopy. As described previously (30), in the absence of myosin tails, Hsp70h-GFP was localized to paired punctate bodies identified as plasmodesma-rich pit fields (Fig. 2A, top panels). Similar localization was observed when the 70-kDa heat shock cognate protein (Hsc70) from *A. thaliana* was coexpressed with Hsp70h-GFP (Fig. 2B, top panels), indicating that the ectopic expression of the recombinant protein per se does not perturb Hsp70h-GFP distribution. Furthermore, the overexpression of each of the class XI myosin tails (Fig. 2A, three bottom rows) did not interfere with the plasmodesmal targeting of Hsp70h-GFP.

Strikingly, the ectopic expression of the tails of myosins VIII-1, VIII-2, and VIII-B resulted in a pattern of Hsp70h-GFP distribution dramatically different from that occurring in the absence of myosin tails. Instead of localized punctate bodies, Hsp70h exhibited virtually uniform distribution at the cell peripheries (Fig. 2B, three bottom rows). It should be stressed that such a pattern was observed in 100% of the cells that showed microscopically detectable levels of Hsp70h-GFP expression. In fact, an identical Hsp70h-GFP distribution pattern in the presence of the microfilament-disassembling drugs latrunculin and cytochalasin D and the generic myosin inhibitor 2,3-butanedione monoxime was described previously (30).

To ensure that the observed inhibition of the proper Hsp70h-GFP targeting was due to the ectopic expression of the myosin VIII tails as proteins rather than to RNA interference triggered by corresponding mRNA (3), Hsp70h-GFP was coexpressed with myosin VIII-B tails and helper component protease (HC-Pro), a potent potyviral suppressor of RNA interference that acts via the nonspecific binding of small interfering RNAs (14, 16). Because HC-Pro did not restore the plasmodesmatal localization of Hsp70h-GFP (data not shown), we concluded that the inhibitory effect of the myosin VIII tails was protein mediated. It is also important to emphasize that the ectopic expression of the myosin VIII tails did not affect the architecture of the actin cytoskeleton in any detectable way (Fig. 3).

These results were interpreted to indicate that the class VIII myosins are required for either delivering or anchoring Hsp70h to plasmodesmata. Because each of the three tested class VIII myosin tails had similar effects, it can be concluded that these myosins are at least in part functionally redundant.

**Mapping the minimal myosin domain that interferes with Hsp70h targeting.** To determine if the inhibitory effect of the class VIII myosin tails on Hsp70h is indeed dependent on the interference with cargo binding rather than on some fortuitous disturbance of myosin function, we systematically tested the effects of a series of six distinct domain combinations (Fig. 1). As expected, the ectopic expression of the full-size myosin

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**FIG. 1.** Diagram showing the domain architecture of the *N. benthamiana* myosins characterized in this work. Color-coded dotted lines correspond to the truncated myosin variants used for overexpression. The exact nucleotide sequences of these variants are presented in Table 1. DIL-motif, dilute motif.
VIII-1 (Fig. 4C, lane F) did not affect Hsp70h-GFP targeting to plasmodesmata (Fig. 4A, top panels). Likewise, the overexpression of the motor domain (Fig. 4C, lane M) did not interfere with Hsp70h-GFP localization (Fig. 4A, middle panels). Furthermore, the extended configuration that included the motor domain along with IQ and coiled-coil motifs (Fig. 4C, lane MC) also had no detectable effect on Hsp70h-GFP targeting to plasmodesmata (Fig. 4A, bottom panels).

In addition to the entire myosin tails encompassing IQ and coiled-coil motifs and the GTD (Fig. 4D, lane IQC), we examined the effects of shorter configurations that included the coiled-coil motif and the GTD (Fig. 4D, lane CCC) and the GTD only (Fig. 4D, lane GTD). The ectopic expression of each of these three domain configurations resulted in apparently complete abolishment of the plasmodesmatal localization of Hsp70h-GFP (Fig. 4B). It should be emphasized that mapping experiments were also done using full-size myosin VIII-2, as well as its five truncated variants corresponding to those described above for myosin VIII-1. Once again, only the three GTD-containing tail variants, but not the full-size myosin VIII-2 or its motor domain, interfered with Hsp70h localization (Fig. 5).

Taken together, these results demonstrated that the GTD of the class VIII myosins is both essential and sufficient for the inhibition of Hsp70h-GFP delivery to plasmodesmata. Because the principal function of the GTD is recognizing and binding the cognate cargo, we concluded that the observed mislocalization of Hsp70h-GFP in the presence of the class VIII myosin GTD was likely due to the inhibition of the direct or indirect association of viral protein with the proper molecular motor.

Plasmodesmatal targeting of TMV MP and A. thaliana RGP2 is not affected by myosin VIII tails. To determine if myosin VIII-mediated targeting is a common mechanism whereby diverse plasmodesmal proteins are delivered to their subcellular destination, we employed GFP fusion forms of two well-characterized proteins capable of plasmodesmal localization, TMV MP (24, 38) and A. thaliana RGP2 (35). As shown in Fig. 6 A and C, the coexpression of either of these proteins with the myosin VIII-2 tails resulted in the proper plasmodesmal localization. In fact, this result was not unexpected since we have previously found that none of the microfilament-disrupting drugs affect TMV MP localization to plasmodesmas (30).

To ensure that the same myosin VIII tail-expressing N. benthamiana cells were competent for the plasmodesmal localization of MP-GFP or RGP2-GFP but incompetent for the delivery of Hsp70h-GFP to plasmodesmata, we coexpressed...
the Hsp70h-mRFP fusion with myosin VIII-2 tails and either MP-GFP or RGP2-GFP. Although both MP-GFP and RGP2-GFP retained their characteristic plasmodesmatal localization, Hsp70h-mRFP was uniformly distributed at the cell peripheries (Fig. 6B and D). These coexpression experiments were also done using tails of myosins VIII-1 and VIII-B (data not shown); the corresponding protein distribution patterns were identical to those shown in Fig. 6 for myosin VIII-2 tails.

Collectively, these data suggest that the myosin VIII-mediated pathway of plasmodesmatal protein targeting is not universal and that at least one pathway that does not involve these myosins exists in the plant cells. It should also be emphasized that the ability of MP-GFP and RGP2-GFP to reach plasmodesmata in these experiments indicates that the expression of myosin VIII tails does not disrupt plasmodesmatal structure in a way that would inhibit an alternative pathway of protein targeting to these organelles.

DISCUSSION

The evolutionary conservation and proliferation of two plant-specific classes of myosin genes in flowering plants (2a, 10, 33) points to the functional importance of the myosin molecular motors in plant physiology. It is also well accepted that the actomyosin motility system plays a paramount role in the interior dynamics of the plant cells. Based primarily on indirect data, it was proposed previously that class XI myosins are responsible for rapid trafficking of plant organelles such as Golgi stacks, mitochondria, and peroxisomes and slower movements of the nucleus and chloroplasts (37, 41). However, the experimental data on class XI myosin functions are limited so
far to the identification of the role of myosin XI-K in root hair elongation (23). Virtually no information on class VIII myosins is available, although the functional association of both actin and class VIII myosin with plasmodesmata was suggested previously (7, 34). In addition, the actomyosin system was implicated in the targeting of certain viral MPs to plasmodesmata (12, 42), as well as in the cell-to-cell movement of TMV (15).

Here, we employed dominant negative inhibition of myosin function based on the overexpression of the myosin cargo-binding domains to identify the myosins involved in the local-

FIG. 5. Mapping the minimal myosin VIII domain required for dominant negative inhibition of Hsp70h-GFP localization to plasmodesmata. (A) Coexpression of Hsp70h-GFP with the full-size myosin VIII-2, its motor domain, or the motor domain with the coiled-coil motif. (B) Coexpression of Hsp70h-GFP with the truncated myosin VIII-2 variants as indicated on the panels. The scale bars in the left columns represent 50 μm. The images in the right columns are close-ups of the areas in white boxes in the corresponding left panels; scale bars represent 10 μm. Abbreviations are the same as those in Fig. 4.

FIG. 6. The coexpression of the myosin VIII-2 tails does not affect the plasmodesmal localization of TMV MP-GFP (A and B) or A. thaliana RGP2-GFP (C and D) but does abolish the plasmodesmal localization of Hsp70h-mRFP (B and D). (C) GFP-tagged TMV-MV-p30 coexpression with both mRFP-tagged Hsp70h and the myosin VIII tail. (D) GFP-tagged A. thaliana RGP2 coexpression with both mRFP-tagged Hsp70h and the myosin VIII tail. The scale bars in the left images in panels A to D represent 50 μm. The right images in panels A to D are close-ups of the areas in white boxes in the corresponding left panels; scale bars represent 10 μm.
ization of the BYV Hsp70 to plasmodesmata. Our data dem-
strate unequivocally that it is class VIII, but not class XI, myosin tails that interfere with proper Hsp70 targeting. Fur-
thermore, we showed that the minimal, very C-terminal GTD of class VIII myosins was able to abolish Hsp70 localization.
Because the primary function of the GTD is binding the cogen-
tate cargo, we assume that the inhibitory effects of GTDs were
due to the interference of the GTDs with the direct or indirect asso-
ciation between class VIII myosins and Hsp70-GFP. Our pilot
experiments aimed at the communoprecipitation of the
Hsp70 and the GTD suggested that their interaction may be
indirect and/or transient.

Interestingly, class VIII myosin-mediated targeting appears
not to be the only mechanism for protein delivery to plasmo-
desmata. Indeed, the overexpression of the class VIII myosin
tails did not disrupt the plasmodesmatal localization of either
TMV MP or the A. thaliana protein RG2P. Based on the tight
association of TMV MP with the ER (15, 42), it seems possible
that this protein can migrate along ER tubules and reach the
desmotubule, a modified outfit of the continuous ER network
(21). Because RG2P is also found in the Golgi apparatus (35),
it can be assumed that this protein can arrive at the plasmodes-
mata via a specialized branch of a secretion route.

The mechanism by which class VIII myosins mediate Hsp70 localization to plasmodesmata is yet to be determined. One
possibility is that class VIII myosin motors are specifically
associated with and move along the subpopulation of the actin
microfilaments anchored in the plasmodesmal vicinity. An
alternative scenario is the diffusion of Hsp70 into the cytosol,
followed by class VIII myosin-assisted anchoring to plasmodes-
mata. This scenario would be in line with the previous suggestion of a tight association of the class VIII myosins with
plasmodesmata (34). A daunting extension of this possibility
would be the actual movement of myosin VIII motors along the
actin microfilaments that transit through plasmodesmata and
interconnect adjacent cells.

In addition to unraveling the mechanism by which class VIII
myosins act to assist Hsp70 localization to plasmodesmata,
the potential role of these myosins in the cell-to-cell movement
of BYV and other viruses and also plant proteins and RNAs
that traffic between cells via plasmodesmata needs to be inves-
tigated. However, the data presented herein support the
involvement of plant-specific, class VIII myosins in processes of
intercellular transport and communication that occur via plant-
specific organelles, the plasmodesmata.

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