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Sieve-tube exudate from *Ricinus communis* L. seedlings contains ubiquitin and chaperones

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Abstract. The cut hypocotyl of *Ricinus communis* L. seedlings exudes phloem sap which contains a characteristic set of proteins (Sakuth et al. 1993, Planta **191**, 207–213). These sieve-tube exudate proteins were probed with antibodies to highly conserved proteins, namely ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco), Rubisco-subunit-binding protein, heat-shock protein (HSP 70), chaperonin GroEL and ubiquitin. Homologous proteins in the sieve-tube exudate were identified with antisera to HSP 70, Rubisco-subunit-binding protein and ubiquitin. Ribulose-1,5-bisphosphate carboxylase-oxygenase, which was present in the tissue, was not detected. Of all the cross-reactive proteins detected, ubiquitin was special because the ubiquitin-to-protein ratio in the sieve-tube exudate was higher than in both the surrounding hypocotyl and in the cotyledonary tissues. Therefore, ubiquitin features properties which favour its transfer into the sieve tubes and which might rely on efficient transport through plasmodesmata. It is assumed that chaperones and ubiquitin are needed for the maintenance of sieve-tube function, e.g. to ensure correct folding of proteins. Their possible involvement in protein translocation through plasmodesmata from companion cells to sieve tubes is discussed.

Key words: Euphorbiaceae – Heat shock protein – Phloem protein – Plasmodesmata – *Ricinus* – Sieve-tube exudate protein

Introduction

The sieve tubes in the phloem are the main route for nutrient allocation to growing and storage tissues in the plant. Sieve tubes are highly specialized, enabling them to cope with the pressure-driven flow of the concentrated assimilate solution. During differentiation the individual sieve-tube members loose nucleus, tonoplast, dictyosomes, microtubules, microfilaments and ribosomes. Only the plasmalemma, a parietal endoplasmatic reticulum, plastids and mitochondria persist in the functional sieve tube (Evert 1990). Consequently, the mature sieve tube is confronted with serious preservation problems during its entire life-span regarding the constant strain caused by the assimilate flow (Raven 1991). The most obvious is connected to protein turnover: though the protein-synthesizing machinery is apparently absent in mature sieve tubes, soluble sieve-tube proteins were found to be continuously present in the stylet exudate of rice (Nakamura et al. 1993) and wheat (Fisher et al. 1992), as well as in the sieve-tube exudate collected from the cut hypocotyl of *Ricinus* seedlings (Sakuth et al. 1993). The particular functions of these sieve-tube exudate proteins (STEPs) are unknown but it is envisaged that some of them are involved in basic "housekeeping" reactions needed to maintain active sieve tubes.

Therefore, we tested whether proteins engaged in basic cellular functions occur in sieve-tube sap by using antibodies directed against highly conserved proteins of eukaryotes. Antibodies against ubiquitin and chaperones were considered as first choice since, on the one hand, these proteins are assumed to occur in all cells because of their fundamental role in correct protein folding and protein turnover and, on the other hand, because the absence of protein synthesis in sieve tubes necessitates a protein-import device from the companion cell where chaperone-like action is required. Heat-shock proteins, which are involved in the folding, assembly, rearrangement or degradation of proteins (Ang et al. 1991), are highly conserved and ubiquitous proteins in higher eukaryotes and plants (Vierling 1991). Analogous functions are met by the chaperonin GroEL and the ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco)-subunit-binding protein in prokaryotes and plastids (Hemmingsen et al. 1988). Ubiquitin is a small polypeptide of 8.5 kDa which is highly conserved among yeast, animals

Abbreviations: HSP = heat-shock protein; Rubisco = ribulose-1,5-bisphosphate carboxylase-oxygenase; RBP = Rubisco-subunit-binding protein; STEP = sieve-tube exudate protein

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and plants. It is involved in diverse, essential reactions in the eukaryotic cell when conjugated to target proteins. As shown for yeast, a main function of ubiquitin is to tag proteins for degradation by the multicatalytic protease, the 26S proteasome (Heinemeyer et al. 1991; Seufert and Jentsch 1992). All components of the ubiquitin-dependent proteolytic pathway have also been identified in plants (Viestra 1993).

It was possible to demonstrate in the sieve-tube exudate the presence of ubiquitin and of proteins cross-reacting with antibodies to HSP 70, GroEL and Rubisco-subunit-binding protein. The physiological significance of these findings is discussed.

Material and methods

Growth of seedlings. Seeds of *Ricinus communis* cv. Carmencita and cv. Sanguineus (obtained from Jelitto Staudensamen, Schwarmstedt, Germany) were soaked in water overnight. The next day, fungus-free seeds were surface-sterilized with 0.3% chinosol (8-hydroxy-chinolin-sulfate), washed three times in sterile water and then transferred either to areated liquid culture (Schobert and Komor 1987) or to autoclaved vermiculite saturated with water (first day of growth). Seedlings were used for experiments at day 5 or 6 of growth.

Collection of STEPs. Seedlings were cut at the hypocotyl hook (Kallarackal et al. 1989) and incubated at 27° C with the endosperm embedded in wet vermiculite above a waterbath under a Plexiglas chamber which maintained high air humidity (>80%). The emerging droplets of sieve-tube exudate were collected at 20-min intervals with 50-µl microcapillaries inserted through holes in the incubation chamber. Sieve-tube exudate from adult plants was obtained at 10-min intervals from cut inflorescences of six-month-old-plants grown in a greenhouse The exudate samples were immediately pooled on ice and stored frozen at −20° C. Sieve-tube exudate proteins were concentrated with Centricon 3 (Amicon, Beverly, Mass., USA).

Heat-shock treatment. Six-day-old seedlings grown in liquid culture were transferred in the aerated culture vessel to 42° C for 2 h before collection of STEPs at 42° C.

Protein extraction from tissue. After freezing plant material in liquid nitrogen, tissues were powdered on solid CO_2 with mortar and pestle. The fine powder (1 g FW · ml^{-1}) was vortexed for 1 min in extraction buffer [50 mM Tris, 50 mM N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine (Tricine), pH 8.25; 0.05% SDS; 1 mg · ml^{-1} dithiothreitol (DTT); 0.2 mg · ml^{-1} each of EDTA, EGTA and 6-amino-caproic acid; 0.1 mg · ml^{-1} each of phenylmethylsulfonyl fluoride (PMSF) and benzamidine]. Insoluble material was pelleted at maximum speed for 10 min in an Eppendorf centrifuge. The supernatant was removed and stored in aliquots at −20° C. Protein contents were determined with a micro protein assay (Bio-Rad, München, Germany).

Gel electrophoresis, blotting and immunological detection. Proteins were separated by SDS-PAGE in 10% acrylamide (Schägger and von Jagow 1987) in a Bio-Rad minigel system and blotted onto Immobilon membranes (Millipore, Bedford, Mass., USA) under semidry conditions in a Multiphor II setup (Pharmacia, Freiburg, Germany). Blots were blocked with 5% low-fat dry milk powder in Tris-buffered saline (TBS; 0.9% NaCl, 20 mM Tris-HCl pH 7.4) for 2 h and incubated with the first antibody (rabbit sera) usually diluted 1:100 for 1 h. Antibody binding was visualized after incubation with ^{125}I-iodinated protein A and exposure to X-ray film, or after incubation with a second antibody directed against rabbit immunoglobulin G (IgG) coupled to horseradish peroxidase (Sigma, Deisenhofen, Germany). The ubiquitin content was estimated for the sieve-tube exudate and the respective seedling tissue by comparing signal strength of the anti-ubiquitin cross-reactive material to the signal strength of known amounts of ubiquitin blotted next to the samples. Colour development by the horseradish peroxidase coupled to rabbit IgG was stopped when a clear signal was obtained with STEP. Protein molecular-weight standards were obtained from Biomol (Hamburg, Germany), Serva (Heidelberg, Germany), and Bio-Rad. Ubiquitin from bakers yeast was obtained from Sigma (Deisenhofen, Germany).

Antibodies used in this study were generously supplied by A. Bachmair, Institut für Botanik, Universität Wien, Austria (anti-Ubiquitin); D. Wolf, Institut für Biochemie, Universität Stuttgart, Germany (anti-ubiquitin-conjugates); S. Jentsch, Friedrich-Miescher Laboratorium, MPI, Tübingen, Germany (anti-ubiquitin conjugating enzyme: UBC 4); U. Kull, Biologisches Institut, Universität Stuttgart, Germany (anti-potato proteasome); T. Gatenby, Dupont, Wilmington, Del. USA (anti-GroEL; anti-pea Rubisco-subunit-binding protein: RBP).

Results

Heat-shock protein (HSP 70) and Rubisco. The sieve-tube exudate from *Ricinus* seedlings contains a characteristic set of proteins (STEPs) at a constant concentration (Sakuth et al. 1993) but their function has not previously been determined.

Therefore, we used antibodies to HSP 70 to detect cross-reactive proteins in the sieve-tube exudate and, for comparison, also in the cotyledon and the hypocotyl. All protein sources, sieve-tube exudate, cotyledon and hypocotyl contained a protein of molecular weight 70 kDa, which cross-reacted with the antibody to HSP 70 (Fig. 1). From the binding intensity it appears that sieve-tube exudate contained (on a protein basis) less HSP 70 than cotyledonary and hypocotyl tissues. A heat-shock treatment (temperature shift to 42° C) did not further in-

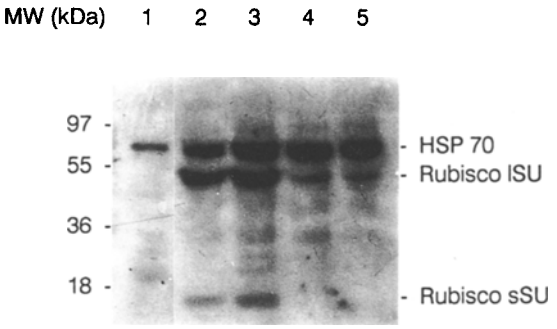

Fig. 1. Proteins which cross-react with antibodies to HSP 70 and Rubisco in the sieve-tube exudate, the cotyledon and the hypocotyl of *Ricinus* seedlings. Sieve tube exudate proteins (*1*) and proteins extracted from the cotyledon (*2, 3*) and the hypocotyl (*4, 5*) were probed with antibodies to HSP 70 and to Rubisco large (*lSU*) and small subunits (*sSU*), respectively. Protein from control seedlings was loaded in *lanes 1, 2* and *4* (each 30 µg), protein obtained from seedlings after a heat-shock treatment (2 h, 42° C) was loaded in *lanes 3* and *5* (30 µg). The positions of the respective cross-reactive materials are marked on the right. The molecular weights were deduced from the position of stained standards (18 kDa: β-lactoglobin; 36 kDa: lactate dehydrogenase; 55 kDa: glutamic dehydrogenase; 97 kDa: phosphorylase B)

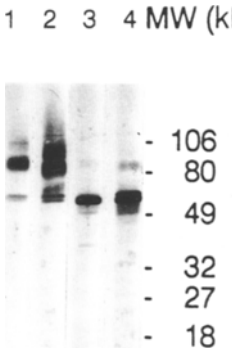

Fig. 2. Proteins which cross-react with antibodies to GroEL and RBP in the sieve-tube exudate and the hypocotyl of *Ricinus* seedlings. Sieve tube exudate proteins (*1, 3*) and protein extracted from the hypocotyl (*2, 4*) was probed with antibodies to GroEL (*1, 2*) or Rubisco subunit binding protein (*3, 4*). Ten micrograms of protein was loaded to each lane; molecular weights were deduced from the position of stained standards (18 kDa: lysozyme; 27 kDa: soybean trypsin inhibitor; 32 kDa: carbonic anhydrase: 49 kDa: ovalbumin; 80 kDa: bovine serum albumin (BSA); 106 kDa: phosphorylase B)

crease the constitutively high HSP 70 content in the seedlings (Fig. 1, lanes 3 and 5).

An antiserum to Rubisco was used to check for contamination from the cut surface of the hypocotyl. The large and the small subunits of Rubisco were easily detected in the cotyledon (Fig. 1, lanes 2 and 3) and the large subunit was clearly present in the hypocotyl (Fig. 1, lanes 4 and 5) but no trace could be detected in the sieve-tube exudate (Fig. 1, lane 1). Therefore, the HSP 70-type protein is definitely carried along in the sieve tubes and is not a contamination from the cut surface of the hypocotyl.

GroEL and RBP. Besides HSP 70, which resides in the cytosol, HSP 60-type chaperones are also engaged in protein folding. The antiserum to GroEL cross-reacted with proteins in the sieve-tube exudate of *Ricinus* seedlings (Fig. 2, lane 1) predominantly at 90 kDa; however, a clear but relatively faint signal was obtained at 60 kDa which is the typical molecular weight for GroEL. In the hypocotyl extract, too, several proteins at 80–100 kDa cross-reacted, and at 60 kDa the signal was weaker (Fig. 2, lane 2). At present it is not certain whether the major reacting proteins in *Ricinus* seedlings represent GroEL-related protein. However, the situation with RBP is clearcut. The antibodies detected a dominant band at 60 kDa as expected, both in sieve-tube exudate and in the hypocotyl (Fig. 2, lanes 3, 4). In the hypocotyl extract the RBP-related material appeared as a double band.

Ubiquitin. Because the sieve tubes undergo profound proteolysis during maturation, we tested whether components of the ubiquitin-dependent protein-degradation system are present in the sieve-tube exudate of *Ricinus* seedlings, e.g. ubiquitin, ubiquitin-conjugating enzymes and the proteasome.

Antibodies to ubiquitin yielded an extremely strong signal with polypeptides in the sieve-tube exudate (Fig. 3,

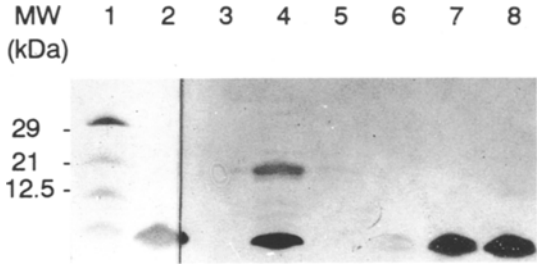

Fig. 3. Identification of ubiquitin-related protein in the sieve-tube exudate of *Ricinus* seedlings. Proteins extracted from the cotyledon (*3*) and the hypocotyl (*5*) and STEPs (*4*) – 10 µg each – were separated by SDS-PAGE and blotted to Immobilon. For comparison ubiquitin from baker's yeast was loaded at 5 µg (*2*), 2 µg (*8*), 1 µg (*7*) and 0.2 µg (*6*). For estimation of molecular weight, dalton-marker proteins (12.5 kDa: cytochrome *c*; 21 kDa: trypsin inhibitor; 29 kDa: carbonic anhydrase) were used (*1*). *Lanes 1* and *2* were stained with amido black, *lanes 2* to *8* were probed with an antibody to ubiquitin diluted 1:200

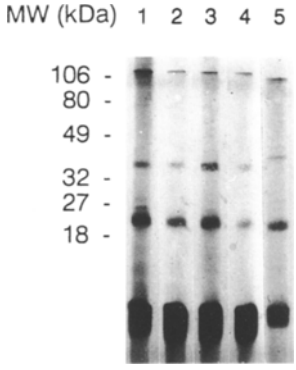

Fig. 4. Time course of ubiquitin-related proteins in the sieve-tube exudate *Ricinus* seedlings and comparison with sieve-tube exudate from adult plants. Exudate was collected for 8 h after cutting the hypocotyl. Sieve-tube exudate proteins exuded in the first 60 min (*1*), from 60 to 150 min (*2*), from 150 to 240 min (*3*), from 240 to 480 min (*4*) and STEPs exuded from cut inflorescences of six-months-old *Ricinus* (*5*) at 10 µg each were probed with an antibody to ubiquitin. Molecular weights were deduced from the position of stained standards (18 kDa: lysozyme; 27 kDa: soybean trypsin inhibitor; 32 kDa: carbonic anhydrase; 49 kDa: ovalbumin; 80 kDa: BSA; 106 kDa: phosphorylase B)

lane 4). It is concluded that this represents the free ubiquitin pool present in the sieve tubes because the ubiquitin-related material was smaller than 12 kDa and co-migrated with commercially available mono ubiquitin from yeast (Fig. 3, lanes 2, 6, 7, 8). Because this blot was only developed for a short time to allow semi-quantitative information for the ubiquitin content in the sieve-tube exudate, no signal was obtained with identical amounts of protein extracted from the cotyledon and the hypocotyl (Fig. 3, lanes 3 and 5). Based on the signal strength obtained with the various amounts of ubiquitin from yeast it can be estimated that ubiquitin represents up to 10% of the total STEP. On the other hand, the proportion of ubiquitin in tissue extracts must be far below 2%. Ubiquitin was present at high level – up to 8 h – as long as sieve-tube exudate was harvested (Fig. 4). A similar pattern of ubiquitin-cross-reactive material was detected

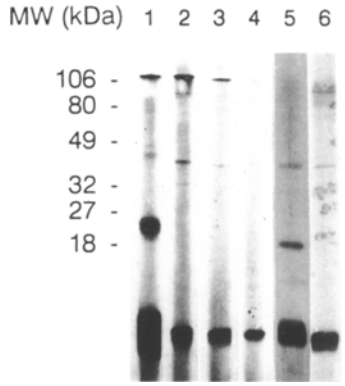

Fig. 5. Ubiquitin-related proteins in the sieve-tube exudate, the hypocotyl, the cotyledon and the endosperm of *Ricinus* seedlings. Sieve-tube exudate proteins (*1, 5*) and protein extracted from the hypocotyl (*2, 6*), the cotyledon (*3*) and the endosperm (*4*) at 15 µg each were probed with an antibody to ubiquitin (*1, 2, 3, 4*) or an antibody to ubiquitin conjugates (*5, 6*). Molecular weights were deduced from the position of stained standards (18 kDa: lysozyme; 27 kDa: soybean trypsin inhibitor; 32 kDa: carbonic anhydrase; 49 kDa: ovalbumin; 80 kDa: BSA; 106 kDa: phosphorylase B)

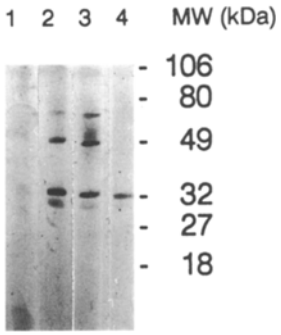

Fig. 6. Proteasome-related proteins in the sieve-tube exudate, the hypocotyl, the cotyledon and the endosperm of *Ricinus* seedlings. Sieve-tube exudate proteins (*1*) and protein extracted from the hypocotyl (*2*), the cotyledon (*3*) and the endosperm (*4*) at 15 µg each were probed with an antiserum to potato proteasome. Molecular weights were deduced from the position of stained standards (18 kDa: lysozyme; 27 kDa: soybean trypsin inhibitor; 32 kDa: carbonic anhydrase; 49 kDa: ovalbumin; 80 kDa: BSA; 106 kDa: phosphorylase B)

with STEPs obtained from adult plants (Fig. 4, lane 5). The comparison with proteins from various tissues of the *Ricinus* seedlings showed that, in addition to mono ubiquitin, a cross-reacting protein at about 20 kDa, probably a ubiquitin conjugate, was present in sieve-tube exudate (Figs. 3–5). This conjugate was present in the exudate in variable amounts (Fig. 4) but was not found in other tissues of the *Ricinus* seedlings (Fig. 5). No additional ubiquitin-related material was detected with an antibody raised to ubiquitin conjugates (Simeon et al. 1992), either in sieve-tube exudate, or in other seedling tissue (Fig. 5, lanes 5 and 6).

Antibodies directed against ubiquitin-conjugating enzyme and the proteasome were employed to address the question of whether components necessary for a functional ubiquitin-dependent degradation pathway are present in sieve-tube exudate. Antibodies to the UBC 4 protein, a ubiquitin-conjugating enzyme specifically associated with the ubiquitin-dependent degradation pathway in yeast (Seufert and Jentsch 1990), did not cross-react with protein extracts from *Ricinus* (data not shown). Antibodies raised against the proteasome from potato detected no cross-reactive protein in sieve-tube exudate. On the other hand, cross-reactivity was demonstrated between the proteasome antiserum and a protein at a molecular weight of about 30 kDa (Fig. 6) in the cotyledon, the hypocotyl and the endosperm. This corresponds well to the 29 kDa reported for the proteosome of other plant species (Schliephacke et al. 1991). The absence of proteasome-related proteins from the sieve-tube exudate suggests that the ubiquitin-dependent, proteasome-mediated proteolytic pathway is not functional in sieve tubes.

Discussion

For the first time, antisera raised against highly conserved chaperones (Ellis 1990) and ubiquitin (Vierling 1991) have been shown to cross-react with proteins from sieve-tube exudate. In the case of ubiquitin, HSP 70 and RBP, the cross-reactive proteins exhibited the expected molecular weight, so that the identity of these proteins can be assumed. All of these chaperones seem to be continually carried along in the assimilate stream, as unequivocally shown here for ubiquitin and shown earlier for overall STEPs (Sakuth et al. 1993). In some cases, this is surprising, because RBP is assigned to plastids and GroEL-related proteins are usually found in the mitochondria (Hemmingsen et al. 1988). But because of their vital function it was proposed that HSP 60 – e.g. GroEL-related proteins – may also occur in the cytosol (Ellis 1990). Indeed, GroEL-related protein was detected in the cytosol of oat cells (Grimm et al. 1991). Our data are ambiguous only in the case of GroEl-related protein in the sieve-tube exudate. Also, this study does not aim to characterize in detail the high-molecular-weight ubiquitin-conjugates in the seedling tissue of *Ricinus*.

At present it is not possible to test directly the specific functions of ubiquitin, HSP 70 and RBP in the sieve tubes of *Ricinus*. However, several functions are envisaged in which HSP 70- and RBP-related proteins might be involved in enabling and maintaining sieve-tube function. These are: (i) to ensure the correct folding of soluble proteins present in the sieve tubes; (ii) to enable protein import into the plastids (Ellis 1990) and the mitochondria (Pfanner and Neupert 1990) which are still present in mature sieve tubes (Evert 1990); (iii) to help in assembly and integration of membrane proteins, e.g. H^+-ATPase, into the sieve-tube plasmalemma; (iv) to mediate protein import into sieve tubes through plasmodesmata.

An important function for ubiquitin in phloem tissue has already been demonstrated in tobacco transformed with ubiquitin R-48 (Bachmair et al. 1990) where substitution of lysine by arginine prevented the formation of multi-ubiquitin chains on proteins, a process which is supposed to be a prerequisite for protein degradation via

the proteasome (Seufert and Jentsch 1992). In consequence, disorders of the vascular tissue were observed. However, because the proteasome was not detected in the sieve-tube exudate, the relevance of ubiquitin is probably not to tag sieve-tube proteins for proteolytic degradation in the sieve tube proper. Also, the engagement in other known functions, e.g. the involvement of ubiquitin in DNA-repair mechanisms and progression through the cell cycle (Jentsch et al. 1990), as well as ribosome biogenesis (Finley et al. 1989), will require ubiquitin to abscond from the sieve tubes again. Further studies are necessary in order to reveal the particular functions of the ubiquitin pool in the sieve tubes.

Though it has to be kept in mind that the soluble protein content in the sieve tube of *Ricinus* is low, 0.2 mg · ml^{-1}, compared with, for example, hypocotyl tissue where about 10 mg · $(g\ FW)^{-1}$, is extractable (Sakuth et al. 1993), our data show a higher ubiquitin-to-protein ratio in the sieve tube than in tissue extracts (Figs. 3, 5). Therefore, ubiquitin exhibits properties which make it more suitable for transfer in the sieve tubes than other proteins. At present it is not known whether this preference merely relies on the small size of ubiquitin or if sequential or structural properties cause this phenomenon.

Consideration of the site of synthesis and the functional relevance of soluble proteins in the sieve tubes raises complex questions (Fisher et al. 1992; Sakuth et al. 1993). Because the sieve tubes lack a nucleus and ribosomes (Cronshaw 1981) these rapidly synthesized proteins probably originate from the companion cells and therefore have to pass through plasmodesmata to gain entrance to the sieve tubes. Injection of fluorescent probes into the extrafascicular phloem of *Cucurbita maxima* established that passage of molecules upto a size of 3 kDa is possible through plasmodesmata from companion cells to sieve elements (Kempers et al. 1993). On the other hand, recent experiments have demonstrated the trafficking through plasmodesmata of viral movement proteins with a molecular weight of 35 kDa (Fujiwara et al. 1993). It is tempting to speculate that the viral movement proteins use an endogenous transport pathway for macromolecules through plant plasmodesmata (Citovsky 1993; Lucas et al. 1993). The earlier observations on P-protein from the *Cucurbitaceae* agree with these ideas: whereas the mRNA encoding the phloem lectin gene PP2 was localized exclusively in companion cells (Bostwick et al. 1992), PP2 protein was detected both in companion cells and in sieve tubes (Smith et al. 1987). The proposed protein translocation through plasmodesmata might require specific recognition sites displayed by the transported proteins (Fisher et al. 1992) as well as machinery to unfold the proteins for passage through plasmodesmata and to refold these proteins into a functional form in the sieve tubes. In analogy to intracellular protein transport such as protein import into mitochondria (Pfanner and Neupert 1990), the HSP 70- and HSP 60-related proteins in the sieve tubes could be involved in the postulated transport process through plasmodesmata.

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