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Identification of immunologically related proteins in sieve-tube exudate collected from monocotyledonous and dicotyledonous plants

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Abstract. The mature, functional sieve-tube system in higher plants is dependent upon protein import from the companion cells to maintain a functional long-distance transport system. Soluble proteins present within the sieve-tube lumen were investigated by analysis of sieve-tube exudates which revealed the presence of distinct sets of polypeptides in seven monocotyledonous and dicotyledonous plant species. Antibodies directed against sieve-tube exudate proteins from *Ricinus communis* L. demonstrated the presence of shared antigens in the phloem sap collected from *Triticum aestivum* L., *Oryza sativa* L., *Yucca filamentosa* L., *Cucurbita maxima* Duch., *Robinia pseudoacacia* L. and *Tilia platyphyllos* L. Specific antibodies were employed to identify major polypeptides. Molecular chaperones related to Rubisco-subunit-binding protein and cyclophilin, as well as ubiquitin and the redox proteins, thioredoxin h and glutaredoxin, were detected in the sieve-tube exudate of all species examined. Actin and profilin, a modulator of actin polymerization, were also present in all analyzed phloem exudates. However, some proteins were highly species-specific, e.g. cystatin, a protease-inhibitor was present in *R. communis* but was not detected in exudates from other species, and orthologs of the well-known squash phloem lectin, phloem protein 2, were only identified in the sieve-tube exudate of *R. communis* and *R. pseudoacacia*. These findings are discussed in terms of the likely roles played by phloem proteins in the maintenance and function of the enucleate sieve-tube system of higher plants.

Key words: Actin filament – Companion cell-sieve element complex – Phloem sap proteins – Plasmodesmal trafficking – Protease inhibitor – Protein folding – Redox regulation

Abbreviations: PP2 = phloem protein 2; RBP = Rubisco-subunit-binding protein; STEP = sieve-tube exudate protein

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Introduction

In plants, photoassimilates, a wide range of micronutrients, plant hormones and various other signaling agents are distributed throughout the plant body within the sieve tubes of the phloem tissue. In higher plants, this system is comprised of files of individual sieve elements that are highly specialized for their long-distance transport function. Governed by a developmental program unique to plants, these cells undergo a remarkable reduction in their protoplasmic constituents. Finally, plasmodesmata connecting contiguous sieve elements undergo transformation to form large openings, or sieve plate pores. Within this enucleate syncytium a functional plasma membrane allows the establishment of osmotic gradients which provide the driving force for pressure-driven mass flow of nutrients and information molecules within the sieve tube (Sjölund 1997). Phloem companion cells – with a dense cytoplasm and abundant ribosomes – are intimately interconnected to the neighboring sieve elements by plasmodesmata. It is via this intercellular pathway that the functions of mature sieve elements are thought to be maintained (Fisher et al. 1992; Lucas et al. 1993; Schobert et al. 1998).

Ultrastructural studies have established the presence of phloem-protein (P-protein) bodies and filaments in mature sieve elements, although the nature of these proteins remained obscure (Cronshaw and Sabnis 1990). Detailed analysis of sieve-tube contents, collected via severed insect stylets from wheat and rice (Fisher et al. 1992; Nakamura et al. 1993), or harvested from the cut hypocotyl of *Ricinus communis* seedlings (Sakuth et al. 1993), revealed the presence of over a 100 different polypeptides within the phloem translocation stream. These proteins were shown to rapidly incorporate [^{35}s] methionine-labeled amino acids, reflecting their constant synthesis and transfer from the companion cells into the enucleate sieve-tube system (Fisher et al. 1992; Sakuth et al. 1993). Further evidence in support of this highly localized synthesis and trafficking of phloem sap proteins was recently gained from studies on the squash phloem protein 2 (PP2; Bostwick et al. 1992), and

thioredoxin h, which is an abundant protein in rice phloem sap (Ishiwatari et al. 1995). Interestingly, glutaredoxin, another thioltransferase, was shown to be abundant in the sieve-tube exudate of *Ricinus* (Szederkényi et al. 1997). Furthermore, ubiquitin and various chaperones were detected in the sieve-tube exudate and it was suggested that the latter may be involved in protein transport through plasmodesmata (Schobert et al. 1995).

It is not presently known whether all of these proteins serve basic functions within the sieve tubes, which would require their presence in the phloem of all species, or if some are carried along in the phloem translocation stream to act as long-distance information molecules (Balachandran et al., 1997; Jorgensen et al., 1998). In the present study, specific antibodies were employed to investigate the composition of the sieve-tube exudate from plant species which allow collection of pure sieve-tube contents. *Ricinus* and *Cucurbita* are well known for their ability to bleed from the phloem, also some tree species like *Robinia* and *Tilia* exude after wounding. It was important to compare these exudates to sieve-tube contents which were obtained via severed insect stylets from rice and wheat, which is a generally accepted method for collection of highly pure contents from functional sieve tubes (Fisher et al. 1992). These studies established that there is a set of constitutive proteins present in the sieve-tube system of monocot and dicot plants, which likely reflects some basic function in the highly specialized phloem long-distance transport system. In addition, we show that, for certain sieve-tube exudate proteins (STEPs), there is wide species-to-species variation in the respective abundance of individual proteins. Furthermore, some phloem sap proteins appear to be species specific. These findings are discussed with respect to the regulation of sieve-tube function.

Materials and methods

Plant material, sieve-tube exudate collection and protein extraction. Seedlings of *Ricinus communis* L. cv Sanguineus were grown as described earlier (Zhong et al. 1996). Sieve-tube exudate protein was collected from 6-d-old seedlings after cutting the hypocotyl (Schobert et al. 1995). Seeds of *Cucurbita maxima* Duch. cv. Big Max (squash) were germinated in wet vermiculite for 10 d at 27 °C. Adult squash plants were grown under previously described greenhouse growth conditions (Balachandran et al. 1997). The hypocotyl (10-d-old seedlings) or stem (1- to 2-month-old plants) was cut and the exuded phloem sap collected using microcapillaries. Collected exudate was transferred to collection buffer (50 mM Tris-HCl, pH 8.2; 5 mM EDTA; 0.5% v/v β-mercaptoethanol), stored on ice and subsequently dialyzed against collection buffer before being stored at −80 °C for later analysis.

Sieve-tube exudate from *Oryza sativa* L. cv. Kantou (rice) was collected via severed brown plant hopper stylets (Nakamura et al. 1993) and proteins therein were precipitated (Ishiwatari et al. 1995). Sieve-tube protein from *Triticum aestivum* L. was obtained via severed aphid stylets as described previously (Fisher et al. 1992).

Yucca filamentosa L. was grown on the University of California, Davis campus. An upper segment of the inflorescence stalk was excised and phloem sap that exuded from the cut surface (attached to plant) was collected, on ice, as described by Baker (1996). Phloem exudate was stored at −80 °C for later analysis.

Robinia pseudoacacia L. trees (approx. age 15–20 years) were grown in the Botanical Garden of the University of Bayreuth, FRG and on the University of California, Davis campus, where 10-year-old *Tilia platyphyllos* L. trees were also grown. Phloem samples were collected during the fall season (October). A pointed knife was used to wound the bark phloem and the droplets of phloem exudate were collected with microcapillaries and stored on ice. Particulate matter was removed by centrifugation (15 000 *g* for 5 min) and protein in the resultant supernatant was precipitated by addition of cold 80% (v/v) acetone.

Protein content of the sieve-tube exudate was routinely determined using a Bio-Rad (Hercules, Calif., USA) micro-assay, with bovine serum albumin being employed as standard. For controls, protein from castor bean, rice and squash tissues was extracted as previously described (Sakuth et al. 1993).

Protein separation, blotting and immunological detection. Proteins were separated by SDS-PAGE (Sakuth et al. 1993), stained with silver using the Gel code kit (Pierce, Rockford, Ill, USA), or blotted, incubated with antibodies and binding visualized by formation of a colored precipitate (Schobert et al. 1995). For enhanced sensitivity, Western blots were incubated with the Renaissance chemiluminescence reagent following the manufacturer's protocol (NEN, Boston, Mass, USA). Pre-immune sera were used to test for non-specific cross-reactivity with sieve-tube exudate samples.

Antisera. Polyclonal antibodies directed against the complement of proteins present in the *Ricinus* sieve-tube exudate (anti-STEP) and specifically against glutaredoxin (anti-GRX) were as described by Szederkényi et al. (1997). Antibodies to cystatin were produced using a His-tagged fusion protein following the protocol detailed for glutaredoxin. A rabbit antiserum to *Ricinus* STEP-20 was generated using purified STEP-20 for immunization. Other polyclonal (PC) and monoclonal (MC) antibodies used in the present study were generous giftsobtained from the following sources: squash anti-PP2 PC antibodies (Bostwick et al., 1992), Dr. Gary Thompson (University of Arizona, Tucson, Ariz., USA); anti-thioredoxin h PC antibodies, Dr. Bob Buchanan (University of Califorina, Berkeley, Calif., USA); anti-profilin PC antibodies prepared using maize pollen profilin, Dr. Chris Staiger (Purdue University, Ind., USA); anti-actin (plant) MC antibody (3H11), Dr. Mandayam Parthasarathy (Cornell University, Ithaca, N.Y. USA); anti-HSP17.6 class I PC antibodies, Dr. Elizabeth Vierling (University of Arizona, Tucson, Ariz., USA); anti-cyclophilin PC antibody, Dr. Charles Gasser (University of California, Davis, Calif., USA); anti-RS21 mcMC antibody, Dr. Richard Sjölund (University of Iowa, Iowa City, IA, USA); anti-ubiquitin PC antibodies, Dr. Anton Bachmair (Universität Wien, Austria); anti-Rubisco-subunit-binding protein (RSBP) PC antibodies, Dr. Anthony Gatenby (Dupont, Wilmington, Del., USA); and anti-β-tubulin MC antibodies were purchased from Sigma Chemical Co. (St. Louis, Mo., USA).

Results

Immunodetection of common proteins within plant sieve-tube exudates. A variety of plants that allowed the collection of sieve-tube contents in quantities sufficient to permit protein analysis were employed in the present study, namely *Triticum aestivum*, *Oryza sativa*, *Yucca filamentosa*, *Ricinus communis*, *Cucurbita maxima*, *Robinia pseudoacacia* and *Tilia platyphyllos*. In general, protein concentration in sieve-tube exudates was low, being in the range of 0.1–1 mg ml^{-1}, which was less than 10% of the total protein content that could be extracted from the supporting hypocotyl or stem tissue (Sakuth

Table 1. Total protein content of sieve-tube exudates collected from various monocot and dicot species. Values calculated to reflect the original protein concentration in the collected samples

Species	Protein content μg μl^{-1}
Triticum sativum	0.1[a]
Oryza sativa	0.2[a]
Tilia platyphyllos	0.1–0.3[b]
Robinia pseudoacacia	0.2–0.4[b]
Ricinus communis	0.2–0.5[b]
Yucca filamentosa	0.5–1.0[b]
Cucurbita maxima	19–30[b]

[a]Content calculated after reconstitution of semi-dry or precipitated material
[b]Content determined immediately after collection of phloem exudate

et al. 1993). Squash phloem sap was exceptional as protein content was at least 20 mg ml^{-1} (Table 1). All phloem exudates contained a complex set of polypeptides, which ranged in size from 5 to 100 kDa. Individual plant species appeared to exhibit a characteristic set of soluble proteins within their sieve-tube exudate (Fig. 1A).

Western blot analysis using an anti-STEP serum raised against all protein present in the *Ricinus* sieve-tube exudate (Szederkényi et al. 1997) revealed that a considerable amount of antigenic epitopes were shared among the proteins present in the assimilate stream (Fig. 1B, Fig. 2). Interestingly, an abundant *Ricinus* sieve-tube exudate 20-kDa protein (STEP-20, Sakuth et al. 1993) was of somewhat larger size in phloem exudates from rice, squash and *Robinia* (Fig. 2).

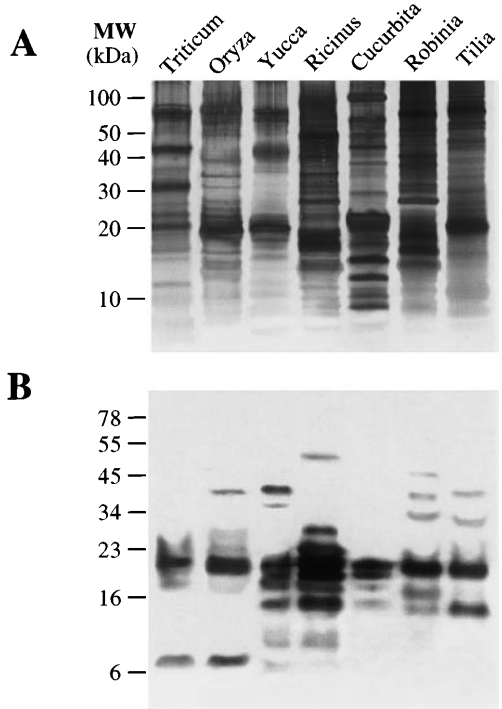

Fig. 1A–B. Comparison of STEP patterns from higher plants and cross-reactivity with anti-STEP serum from *Ricinus communis*. Proteins present in sieve-tube exudate from *Triticum aestivum* L., *Oryza sativa* L., *Yucca filamentosa* L., *Ricinus communis* L., *Cucurbita maxima* Duch., *Robinia pseudoacacia* L. and *Tilia platyphyllos* L. were separated by SDS-PAGE. Phloem proteins were either silver-stained (**A**; 1 μ g protein per lane) or probed with *Ricinus* anti-STEP polyclonal antibodies diluted 1:1000 (**B;** 2 μg protein per lane). Cross-reactive polypeptides were visualized by chemiluminescence detection. Molecular weights were calibrated using a 10-kDa protein ladder (**A**), or were deduced from pre-stained standards (**B**)

Molecular chaperones. Microinjection studies performed on phloem sap proteins obtained from squash and *Ricinus* indicated a general capacity for cell-to-cell transport, in which at least partial protein unfolding appeared to be required for movement through plasmodesmata (Balachandran et al. 1997). Therefore, an appropriate chaperone system would be expected within the sieve-tube exudate to mediate refolding of polypeptides imported from companion cells via plasmodesmata (Schobert et al. 1995). Western blot analysis using anti-RBP and anti-ubiquitin antibodies established the presence of immunologically related polypeptides in phloem exudates from rice, squash and *Robinia*, with signal strengths closely resembling that obtained using *Ricinus* STEP (Fig. 2). Interestingly, a 45-kDa polypeptide in the rice sieve-tube exudate was recognized by the anti-RSBP antibodies, a finding which was unexpected as chaperonins have a conserved size in the range of 60 kDa (Ellis 1990). Phloem exudate proteins from *Ricinus, Robinia* and *Tilia* also gave a specific, but weak, signal when cross-reacted with anti-HSP17.1 antibodies (data not shown).

A number of peptide sequences that were generated from *Ricinus* STEP matched highly conserved sequence motifs present in eukaryotic cyclophilins, which are peptidyl-prolyl *cis-trans* isomerases (Gasser et al. 1990). A polyclonal antibody generated against a cytoplasmic peptidyl-prolyl *cis-trans* isomerase from *Arabidopsis*, ROC1 (Lippuner et al. 1994), identified related chaperones in phloem exudates obtained from wheat, rice, squash, *Ricinus, Robinia, Yucca* and *Tilia* (Fig. 3). It is noteworthy that all phloem saps contained ubiquitin and molecular chaperones such as cyclophilin and RSBP orthologs at fairly constant levels (Fig. 2, Fig. 3).

Redox regulatory proteins. Redox regulatory systems and scavenging agents were proposed to be essential for the longevity of the enucleate sieve elements and therefore should be ubiquitous to all sieve-tube exudate. Consistent with this hypothesis, thioredoxin h, a 13-kDa cytoplasmic oxidoreductase, was recently demonstrated to be a major protein in rice phloem sap (Ishiwatari et al. 1995). Additional support was provided by the finding of an abundant 11-kDa glutaredoxin (glutathione-dependent thiol reductase) within the sieve-tube exudate of *Ricinus* (Szederkényi et al. 1997).

Antibodies directed against thioredoxin h from wheat (Ishiwatari et al. 1995) and glutaredoxin from *Ricinus* (Szederkényi et al. 1997) were used to probe for the presence of similar proteins within the phloem sap from other species. Thioredoxin h appeared to be present in the exudates from most plants, with the highest abundance being present in rice (Fig. 2, Fig. 4). Interestingly,

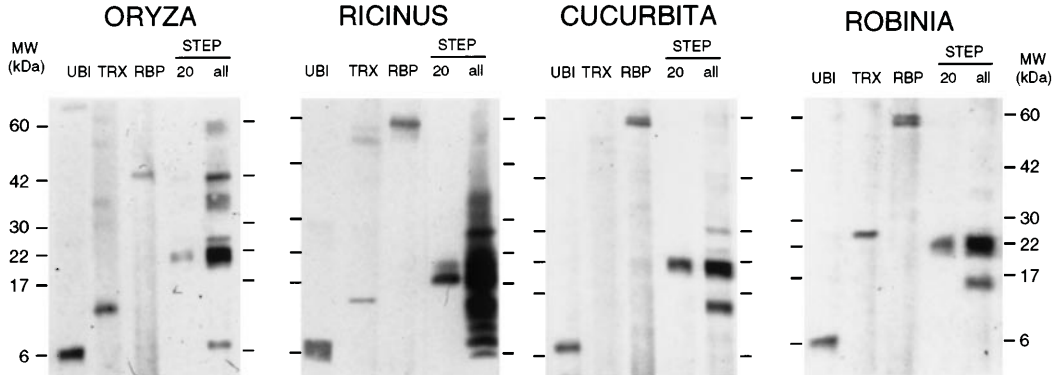

Fig. 2. Cross-reactivity of STEPs from various species detected using antibodies directed against ubiquitin (*UBI*), thioredoxin h (*TRX*), Rubisco-subunit-binding protein (*RBP*), *Ricinus* STEP-20 (*20*) and total *R. communis* STEP (*all*). Sieve-tube proteins (10 μg per lane) from *Oryza sativa*, Ricinus *communis*, *Cucurbita maxima* seedlings and *Robinia pseudoacacia* located at Bayreuth were probed with the indicated antisera at 1:100 dilution; pre-immune sera did not cross-react with phloem proteins. Cross-reactive polypeptides were visualized by detection of colored precipitate. Molecular weight was deduced from pre-stained standards

thioredoxin h was not detected in the phloem sap collected from 12-d-old squash seedlings (Fig. 2), but was detected in adult plants (Fig. 4). Phloem sap collected from *Robinia* trees grown in Davis contained thioredoxin h and an additional cross-reactive band at 22 kDa (Fig. 4), whereas the sap harvested from trees in Bayreuth appeared to contain only the 22-kDa polypeptide (Fig. 2). Finally, thioredoxin h was not detected in the phloem sap collected from *Tilia*.

Western blot analyses indicated that the phloem sap of *Robinia* and *Tilia* trees contained levels of glutaredoxin comparable to those present in *Ricinus*. However, only a low level of glutaredoxin was detected in the phloem sap from *Yucca* and exudates collected from squash, rice and wheat did not cross-react with the anti-glutaredoxin antibodies (Fig. 4). These studies showed that at least one redox regulatory system may operate within the sieve elements of all plants examined.

Cytoskeletal constituents detected in sieve-tube exudate. Some form of a cytoskeletal system must be present in mature sieve tubes to anchor the sieve-element endoplasmic reticulum, plastids and mitochondria to the plasma membrane. Highly specific antibodies directed against plant actin (Andersland et al. 1994) (Andersland et al. 1994), maize profilin (Karakesisoglou et al. 1996) and rat β-tubulin were employed to investigate whether cytoskeletal components are present within the phloem translocation stream. Profilin, a 14-kDa protein presumably involved in actin dynamics (Staiger et al. 1997), was readily detected in the phloem exudate of all species investigated (Fig. 5A). Surprisingly high levels of actin were also found within the sieve-tube exudate of all species tested (Fig. 5B). Analysis of the actin content within a total tissue extract obtained from the *Ricinus*

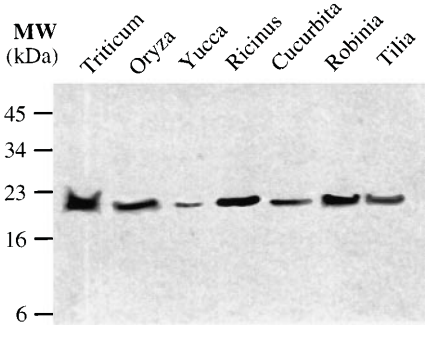

Fig. 3. Presence of cyclophilins in sieve-tube exudates collected from various monocot and dicot species . Sieve-tube proteins (2 μg per lane) from *Triticum aestivum*, *Oryza sativa*, *Yucca filamentosa*, *Ricinus communis*, adult *Cucurbita maxima*, *Robinia pseudoacacia* located at Davis and *Tilia platyphyllos* were probed with anti-ROC1 antibodies (1:500 dilution); cross-reactive polypeptides were visualized by chemiluminescence detection. Molecular weight was deduced from pre-stained standards

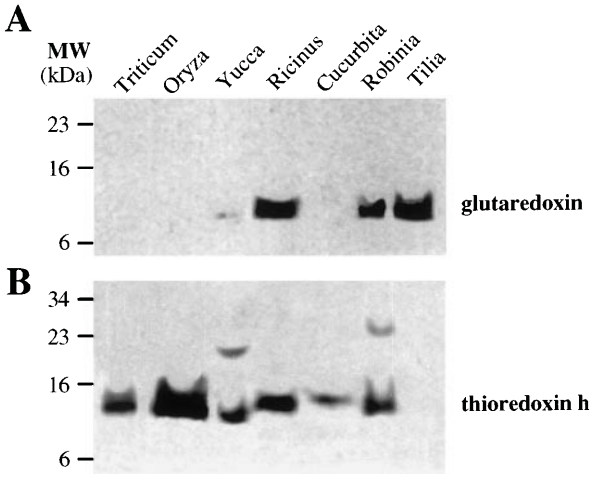

Fig. 4A–B. Presence of glutaredoxin and thioredoxin h in sieve-tube exudates collected from various monocot and dicot species. Sieve-tube proteins (5 μg per lane) from the indicated species (see Fig. 3) were probed with anti- glutaredoxin antibodies (**A**; 1:500 dilution), or anti-thioredoxin h antibodies (**B**; 1:500 dilution). Cross-reactive polypeptides were visualized by chemiluminescence detection. Molecular weight was deduced from pre-stained standards

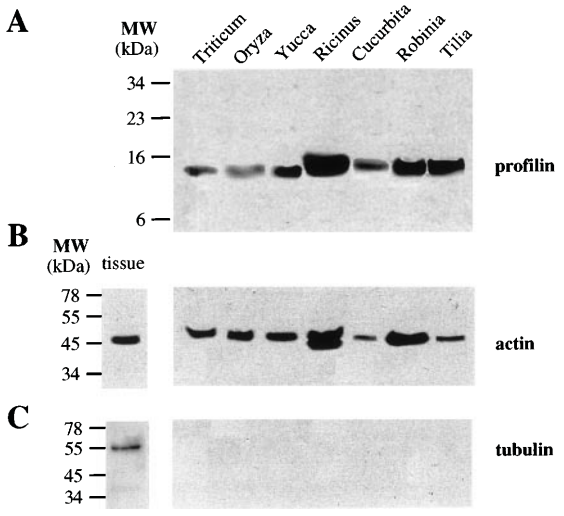

Fig. 5A–C. Presence of profilin and actin and absence of tubulin in sieve-tube exudates collected from various monocot and dicot species. Sieve-tube proteins (5 μg per lane) from the indicated species (see Fig. 3) were probed with anti-profilin polyclonal monoclonal antibodies (**A**; 1:1000 dilution), anti-actin monoclonal antibody (**B**;1:200 dilution), and (**C**) anti-β-tubulin monoclonal antibody (**C**;1:500 dilution). For comparison, proteins were extracted from *R. communis* hypocotyl (**B**; 15 μg) and *C. maxima* stem tissue (**C**; 3 μg) and probed with the appropriate antibodies. Cross-reactive polypeptides were visualized by chemiluminescence detection. Molecular weight was deduced from pre-stained standards

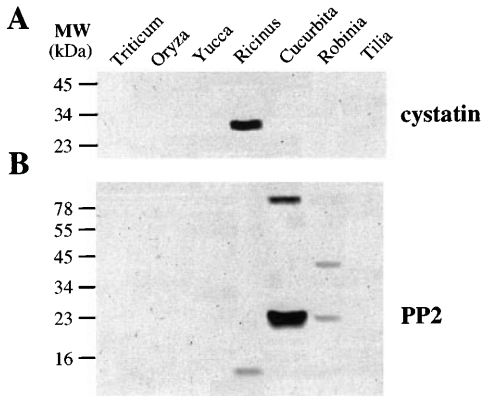

Fig. 6A–B. Presence of cystatin and PP2 in sieve-tube exudates collected from various monocot and dicot species. Sieve-tube proteins (52 μg per lane) from the indicated species (see Fig. 3) were probed with anti-cystatin antibodies (**A**; 1:200 dilution) or anti-PP2 antibodies (**B**;1:500 dilution). Cross-reactive polypeptides were visualized by chemiluminescence detection. Molecular weight was deduced from pre-stained standards

hypocotyl (sample taken from the site of exudate collection) revealed that at least 3 times more protein was needed to give a signal equivalent to that obtained using phloem sap. Finally, whereas the anti-β-tubulin reacted with protein extracted from the *Cucurbita* stem tissue with high sensitivity, a positive reaction was not obtained with sieve-tube exudate collected from the seven plants used in the present study (Fig. 5C). This result is consistent with earlier electron-microscopic studies in which microtubules were not detected within mature sieve elements (Evert 1990; Toth et al. 1994).

Species-specific phloem proteins. A cysteine protease inhibitor protein has been identified in sieve-tube exudate from *Ricinus* (data not shown). An antibody directed against the *Ricinus* cystatin polypeptide established that immunologically related proteins were absent from the phloem sap of the other species examined in the present study (Fig. 6A). Another example for a species-specific phloem protein may be the lectin, PP2, which is highly abundant in squash phloem sap. Antibodies directed against affinity-purified PP2 (Dannenhoffer et al. 1997) recognized the 24-kDa PP2 in the squash phloem sap (Fig. 6B); the cross-reactivity detected at approx. 90 kDa represents phloem protein 1 (PP1). Protein present within the sieve-tube exudate collected from *Ricinus* and *Robinia* also cross-reacted weakly with the anti-PP2 antibodies, whereas no cross-reaction was detected with wheat, rice, *Yucca* or *Tilia* phloem proteins (Fig. 6B).

Discussion

This study provides an extensive immunological survey of individual polypeptides present in sieve-tube exudate collected from plant species that span a wide range of taxa (Table 2). Two criteria were taken as sufficient evidence for the identity of a protein in sieve-tube exudate: (i) the recognized polypeptide from the phloem exudate had to match the molecular weight of the respective protein, and (ii) the identified band should not cross-react with preimmune serum. Pure sieve-tube exudate was obtained from the monocotyledons, *T. aestivum* and *O. sativa*, via severed insect stylets that were directly inserted into the sieve-tube lumen. As this technique avoids a disruptive pressure surge, the soluble proteins in such stylet exudate are considered mobile within the assimilate stream (Fisher et al. 1992; Nakamura et al. 1993). Also, *Ricinus* seedlings exude sieve-tube contents for hours after cutting the hypocotyl without a change in protein pattern (Sakuth et al. 1993; Schobert et al. 1995). Furthermore, in this system, phloem loading has been shown to proceed for several hours during the collection of sieve-tube exudate (Kallarackal et al. 1989; Schobert and Komor 1989).

Trafficking through plasmodesmata within the CC-SE complex requires protein folding. Sieve elements undergo a dramatic differentiation process which removes the nuclei, rough endoplasmic reticulum and other cellular organelles to facilitate pressure-driven assimilate flow (Sjölund 1997). Yet the functional integrity of the plasma membrane must be maintained for active solute loading and to permit osmotically induced water flow. Therefore, it has long been recognized that sieve-element function relies on companion-cell support. The presence and turnover of the proteins within the phloem sap (Fisher et al. 1992; Sakuth et al. 1993), as well as their ability to traffic from cell-to-cell (Balachandran et al. 1997; Ishiwatari et al. 1998), is fully consistent with a

Table 2. Compilation of antisera used and proteins identified in sieve-tube exudates collected from various monocot and dicot species

Antisera employed		Identification in sieve-tube exudate	
Antigen	Source	Species[a]	Comments
Actin	Pea roots	T.a., O.s., Y.f., R.c., C.m., R.p., T.p.	
Cyclophilin (ROC1)	*Arabidopsis*[b]	T.a., O.s., Y.f., R.c., C.m., R.p., T.p.	
Cystatin	*Ricinus*[b]	R. c.	Species-specific
HSP 17.6 class I	Arabidopsis[b]	R.c., R.p., T.p.	Faint signals
Glutaredoxin	*Ricinus*[b]	Y.f., R.c., R.p., T.p.	Faint signal with Y.f.
Profilin (ZmPRO3)	*Zea mays*[b]	T.a., O.s., Y.f., R.c., C.m., R.p., T.p.	Strong signal with R.c.
Phloem protein 2	*Cucurbita maxima*[b]	C.m., R.p.	Faint in R.p.[c]
RBP	Pea	R.c., C.m., R.p.	Different MW in O.s.
RS21	*Streptanthus* phloem		No signal on Western blot
STEP	*Ricinus* phloem sap	T.a., O.s., Y.f., R.c., C.m., R.p., T.p.	Dominant signals at 20 kDa
STEP-20	*Ricinus* phloem sap	O.s., R.c., C.m., R.p.	
Thioredoxin h	Wheat	T.a., O.s., Y.f., R.c., C.m., R.p.	Strong signal with O.s., not presented in T.p.
β-tubulin	Rat		Not detected in exudate
Ubiquitin	Yeast	O.s., R.c., C.m. R.p.	Strong signal in all exudates

[a] *Triticum aestivum*, T.a.; *Oryza sativa*, O.s.; *Yucca filamentosa*, Y.f.; *Ricinus communis*, R.c.; *Cucurbita maxima*, C.m.; *Robinia pseudoacacia*, R.p.; *Tilia platyphyllos*, T.p.
[b] Raised against recombinant protein overexpressed in *Escherichia coli*
[c] Additional bands with different molecular weights were detected in R.c. and R.p.

dynamic exchange of proteins between the companion cells and the sieve-tube members. Based on microinjection studies, it would appear that protein unfolding is essential during the process of protein import into the sieve tube (Schobert et al. 1995; Balachandran et al. 1997; Kempers and Van Bel 1997). The results from the present study support this hypothesis, as chaperones involved in protein folding, such as cyclophilin, are constitutive and abundant in sieve-tube exudate harvested from all investigated plants, which ranged in development from seedlings to adult plants.

Redox regulatory systems are ubiquitous in sieve elements. The detection of the small redox regulatory proteins, thioredoxin h and/or glutaredoxin, in sieve-tube exudates from all investigated plants is consistent with the hypothesis that redox buffer systems are essential for the maintenance of the functional sieve-tube (Ishiwatari et al. 1995; Szederkényi et al. 1997). Furthermore, oxidative damage should also be attenuated by these redox proteins. Most of the monocot species tested appear to utilize thioredoxin h, whereas for dicot species glutaredoxin is also present in the phloem sap. Although the basis for this difference remains to be elucidated, it is interesting to note that thioredoxin h was detected in the sieve-tube saps from all investigated plant species except for *Tilia*. As this tree sap was harvested late in the season, the absence of thioredoxin h may be related to the deposition of dormancy callose onto sieve pores (see below). Finally, compared to the other species examined, the level of thioredoxin h present within the squash phloem sap was low, and in seedlings, if present, this protein was below the detection limit of the immunoassay.

The role of cytoskeletal elements in mature sieve tubes. Prior to the present study, only limited information was available with respect to the presence of cytoskeletal elements within the mature sieve elements. Ultrastructural studies had earlier indicated that microtubules were not detectable within mature sieve elements (Toth et al. 1994), and the presence of microfilaments was difficult to establish (Evert 1990). Our data show that actin represents a relatively high portion of the total soluble protein present within the sieve-tube exudate. This suggests that actin may serve more functions within the sieve tube, in addition to the anchorage of organelles. In any event, profilin would be expected to be involved in the regulation of actin filament polymerization (Staiger et al. 1997). It is tempting to speculate that formation of micro filaments might be involved as an initial step in wound-induced sealing of the sieve-pores. Finally, the high profilin levels detected in *Ricinus, Robinia* and *Tilia* sieve-tube exudate (Fig. 5) may attenuate wound-induced actin polymerization yielding a phloem system with a propensity to bleeding. Further characterization of profilin properties and actin dynamics, within sieve elements, is needed to test this hypothesis.

Phloem protein involvement in long-distance signaling. The functioning of higher plants is controlled by complex signaling networks. Regulatory cascades that likely impact photoassimilate translocation may include (i) changes during the sink-to-source transition, (ii) the synthesis and operation of sucrose carriers, proton-ATPases and ion channels within the companion cell-sieve element (CC-SE) complex (Schobert et al. 1998), and (iii) the control of sieve-tube conductance (Zhong et al. 1995). Some of the proteins identified in the present study may well participate in such regulatory networks. Cyclophilin is well known for its potential interaction with protein phosphatases (Luan et al. 1993). As protein phosphorylation occurs in sieve elements (Nakamura et al. 1993, 1996), control of protein phosphatase activity may be an additional aspect of

cyclophilin function within the CC-SE complex. Thioredoxin h, as well as glutaredoxin, may transduce changes in redox potential within the CC-SE complex, via either membrane-bound and/or soluble redox sensitive proteins, to adjust the activity of transport systems within the sieve-tube plasma membrane. In tree species, changes in redox state may be related to seasonal changes in sieve-tube physiology, e.g. in the deposition of dormancy callose on the sieve plates (Aloni and Peterson 1997).

In addition to acting at their site(s) of synthesis and entry into the sieve-tube system, proteins within the phloem sap may play a role in long-distance signaling. Experiments with *Cucurbita* species heterografts revealed that proteins may move long distance within the sieve tubes from the stock to the scion (Tiedemann and Carstens-Behrens 1994). Therefore, it seems plausible that sieve-tube proteins, synthesized at the site of loading in a leaf, may be delivered to distant sink organs, such as the shoot and/or root apex, where they can act on cellular processes in the companion cells and beyond (Lucas et al. 1996; Mezitt and Lucas 1996; Balachandran et al. 1997). Current experiments are aimed at identifying such long-distance signaling molecules that move within the phloem translocation system of the plant.

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