SHORT COMMUNICATION

An abundant TIP expressed in mature highly vacuolated cells

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Summary
Aquaporins are water channel proteins found in vacuolar membranes and plasma membranes, and belong to the major intrinsic protein (MIP) family of proteins. In the present study, we purified a 75 kDa MIP protein from a crude fraction of spinach leaf intracellular membranes. Upon urea/SDS-PAGE, the 75 kDa protein appeared as a 21 kDa polypeptide, and the 75 kDa species therefore probably represents a tetramer. The corresponding cDNA was obtained by PCR cloning and had an open reading frame encoding a 25.1 kDa protein. The protein, So-TIP, was most homologous to the tonoplast intrinsic protein (TIP) subfamily of plant MIPs. Using affinity-purified So-TIP-specific peptide antibodies, we investigated the subcellular and tissue distribution of So-TIP. So-TIP was specifically located in the vacuolar membrane. It was abundant in most vacuolated cells in all vegetative organs, but was excluded from the leaf epidermis as well as from the root phloem parenchyma and meristem. In spite of the high sequence homology between TIPs of spinach, Arabidopsis, sunflower and radish, their expression patterns were totally different. However, a comparison of the expression pattern of So-TIP with that of more distantly related TIPs showed similarities with Arabidopsis γ-TIP, which is expressed in zones of cell elongation/differentiation but excluded from meristematic tissues. Meristematic cells are characterized by many small vacuoles as opposed to elongating and mature cells, which generally harbour a single, large vacuole. Our results indicate that the expression of So-TIP may be induced when the large vacuole is formed.

Introduction
At the organism level, plant water balance is maintained by the transpiration stream, which distributes water throughout the whole organism. Within tissues and organs, water movement is also guided by osmotic gradients, e.g. due to the loading of solutes (sucrose, ions) into the phloem and xylem, thereby causing water to enter as well. At the cellular level, water balance is characterized by the fluxes of water necessary to fulfill the needs of each individual cell. The transpiration stream in plants involves the absorption of water by the roots and the escape of water through the stomatal pores of the leaves. Long-distance water transport is carried out in the vascular tissues, by the xylem and the phloem, where water is transported by bulk flow and membrane barriers are in most cases absent. However, in order for water to reach vascular tissues a transport of water through non-vascular tissues is necessary. The movement of water through non-vascular tissues frequently necessitates transport across membranes (reviewed in Johansson et al., 2000; Kjellbom et al., 1999). Results consistent with the presence of protein-facilitated transmembrane water flow in plant cells (Wayne and Tazawa, 1990) and the identification of major plant membrane proteins with high sequence homology to the animal AQP1 and MIP (Preston and Agre, 1991) led to the identification of plant aquaporins (Maurel et al., 1993). Plant cells contain aquaporins both in the tonoplast and in the
plasma membrane. One aquaporin, NOD26, has been localized to the peribacteroid membrane of soybean nodules (Rivers et al., 1997). In *Arabidopsis*, there are at least 30 expressed members of the major intrinsic protein (MIP) family (U. Johanson et al., unpublished data). According to amino acid sequence similarities, these MIPs either belong to the MIPs of the tonoplast, i.e. are tonoplast intrinsic proteins (TIPs), or to the MIPs of the plasma membrane, i.e. are plasma membrane intrinsic proteins (PIPs), except for the NLMs (NOD26-like MIPs), which seem to form a group of their own. Most plant MIP homologues that have been tested for water transport activity have been found to be water channels. It is reasonable to assume that the majority of the remaining *Arabidopsis* MIP will be aquaporins too. Thus, plants seem to harbour a large number of aquaporins, about half localized in the tonoplast and half in the plasma membrane. The physiological importance of aquaporins in plants is implicated by the many genes coding for aquaporins, and their widespread occurrence in animals, fungi and bacteria. It is probable that a majority of the plant genes coding for MIP homologues are expressed only in certain cell types and tissues and/or at specific developmental stages.

Mature living plant cells are characterized by a large single vacuole, occupying most of the intracellular space, and a thin layer of cytosol between the cell membrane and the vacuolar membrane. To maintain optimal conditions for the various metabolic activities taking place in the cytosol, the composition of the cytosol has to be controlled tightly. Aquaporins in the vacuolar and plasma membrane are likely to be crucial in the regulation of the cytosolic osmolarity. In the event of a sudden change in apoplastic osmolarity, regulation of water flow through aquaporins may present a rapid response to avoid drastic osmotic perturbations in the cytosol. In the present work, we have characterized a highly abundant spinach TIP, So-\(\delta\)TIP. By using So-\(\delta\)TIP-specific antibodies and immunoelectron microscopy, we show that So-\(\delta\)TIP is specifically located in the vacuolar membrane and expressed in most cell types in leaves, petioles and roots, with the exception of cells of the epidermis and meristematic tissues.

**Results and Discussion**

**Purification of So-\(\delta\)TIP and cloning of So-\(\delta\)tip**

Some aquaporins are highly abundant proteins. The spinach TIP, So-\(\delta\)TIP, cloned and characterized in this study, is expressed at high levels, can be purified easily from intracellular membranes by MonoQ anion exchange chromatography, and appears as a protein band of 75 kDa after SDS–PAGE (Figure 1a). As part of a proteomics project aimed at identifying major membrane proteins, the N-terminal amino acid sequence of the 75 kDa protein band was determined and found to be homologous to the TIPs of the MIP family of proteins. Degenerated primers based on the N-terminal sequence and on the highly conserved NPA amino acid motif, characteristic for all MIPs, were used for RT–PCR. The resulting 253-bp PCR product was used as a probe to screen a spinach leaf cDNA library, and a full-length clone was isolated (EMBL accession number AJ245953). The open reading frame encodes a 247-amino acid protein with a calculated molecular mass of 25.1 kDa.

The deduced amino acid sequence of So-\(\delta\)TIP, and the postulated amino acid sequences of most TIPs found in the databases, together with some representative PIPs and other MIPs, were aligned and the result is shown as a phylogenetic tree (Figure 2a). So-\(\delta\)TIP falls in a cluster of \(\delta\)-TIP homologues, which is why we have chosen the name So-\(\delta\)TIP, where So stands for *Spinacia oleracea*. For sequence comparisons (Figure 2b), So-\(\delta\)TIP was aligned with At-\(\delta\)TIP (79% identity; Daniels et al., 1996) and with *Arabidopsis* TIPs, representing each major branch of the phylogenetic tree, as well as with the spinach plasma membrane aquaporin PM28A (Johansson et al., 1996, 1998).

The 75 kDa band represents an oligomer of a polypeptide migrating at 21 kDa

The C-terminal region is the least conserved part of the homologues aligned in Figure 2(b). A polyclonal antiserum was raised against a synthetic peptide corresponding to the last 11 amino acids (QDDAPLSSVEY) in the C-terminus of So-\(\delta\)TIP, and peptide-specific antibodies were purified by affinity chromatography using the synthetic peptide. When these antibodies were used to label protein gel blots of MonoQ fractions enriched in the 75 kDa band, they recognized the 75 kDa band but also bands at 39 and 21–23 kDa (Figure 1b, lane 2). The N-terminal amino acid sequence of the 21–23 kDa band was determined and found to be identical to that of the 75 kDa band. In order to understand the origin of the 75 and 39 kDa forms, intracellular membranes were subjected to urea/SDS–PAGE followed by immunoblotting using the So-\(\delta\)TIP-specific antibodies. Upon urea/SDS–PAGE, only the band at 21 kDa remained, indicating that the 75 and 39 kDa bands represent oligomeric forms of the 21 kDa polypeptide (Figure 1b, lanes 3 and 4). According to recent electron microscopy data for AQP1 (reviewed in Heymann et al., 1998), the native structure of AQP1 is as tetramers, although individual monomers have water transport activity. Thus, the high molecular weight form observed for So-\(\delta\)TIP at about 75 kDa might represent the native tetramer rather than non-specific aggregation of the hydrophobic protein.

**Organ distribution of So-\(\delta\)tip transcript and protein**

Knowledge of the expression patterns of different aquaporins is essential to provide clues to the function of
aquaporins at the whole plant level. The expression patterns may suggest in which cells rapid transmembrane water transport is especially important. To investigate the organ distribution of So-δTIP, the protein and transcript levels were determined. The transcript was abundant in all organs and the levels seemed to be the same in leaves and roots but higher in petioles (data not shown). The corresponding amounts of So-δTIP protein were similar in all organs when quantified by immunoblotting using the affinity-purified antibodies (data not shown). In contrast, At-δTIP, the Arabidopsis orthologue of So-δTIP, is predominantly located in the shoot and almost undetectable in the root (Daniels et al., 1996; Weig et al., 1997).

As we used an antiserum raised against a synthetic peptide corresponding to the C-terminal part of the protein, where the homology between So-δTIP and its closest relatives is low (Figure 2b), the antiserum was probably specific for So-δTIP. However, we cannot exclude cross-reactivity to other, not yet identified, homologues. The observed differences between transcript levels and protein amounts could reflect different transcript stability and/or protein turnover in the different organs.

Tissue and subcellular distribution of So-δTIP

To estimate the expression levels of So-δTIP, membrane fractions were prepared from spinach leaves using free-flow electrophoresis as described by Auderset et al. (1986). So-δTIP was present in the vacuolar membrane fraction as revealed by SDS-PAGE and Western blotting (data not shown). The relative amount of So-δTIP was estimated to be 10–15% in this fraction based on the Coomassie brilliant blue R-250 staining, using the software ImageQuaNT™ version 4.0 (Molecular Dynamics, Sunnyvale, CA, USA).

Figure 1. Purification of So-δTIP.
(a) Fractionation by anion exchange chromatography and SDS-PAGE. Spinach leaf intracellular membranes were solubilized with dodecyl-β-D-maltoside and loaded on a MonoQ HR 5/5 column. Proteins were eluted with a 0–0.5 M NaCl gradient. A part of the chromatogram is shown (top), with the polypeptide pattern of fractions 16–29 inserted below. The gel was stained with Coomassie brilliant blue R-250. The 75 kDa band (diamonds) peaking in fractions 24 and 25 was subjected to N-terminal amino acid sequencing. The sequence obtained was AIAFRGFD-SFSWSAKAYI.
(b) The 75 kDa protein band is a multimer of So-δTIP. The polypeptides of the So-δTIP-enriched fraction obtained after anion exchange chromatography (Figure 1a, fraction 24) were separated by SDS-PAGE, and either stained with Coomassie brilliant blue R-250 (lane 1) or electropholated to a PVDF membrane, and immunodecorated with So-δTIP-specific antibodies (lane 2). Lanes 3 and 4 show immunoblots using total intracellular membranes from spinach leaves rather than partially purified So-δTIP. In lane 3 polypeptides were separated by conventional SDS–PAGE, whereas in lane 4 the gel also contained 4 M urea to better resolve protein complexes. Note that the 75 kDa band seen in lanes 2 and 3 disappears in lane 4, where only a single band at 21 kDa is observed. The positions of the molecular weight markers are indicated to the left.

To discern the expression pattern of So-\(\delta\)TIP in more detail, immunolocalization experiments were performed using ultrathin sections of leaves, petioles and roots. Antibody labelling of So-\(\delta\)TIP was visualized by electron and light microscopy using gold-conjugated secondary antibodies followed by silver enhancement. High density of gold particles could be observed in the vacuolar membrane with virtually no labelling in other cellular structures (Figure 3). This unequivocally demonstrated the subcellular location of So-\(\delta\)TIP in the vacuolar membrane. In agreement with the results obtained from immunoblotting, So-\(\delta\)TIP could be detected at similar levels in leaf, petiole and root (Figure 3a–d). In leaves, a high density of gold particles could be detected in the vacuolar membrane of the mesophyll cells (Figure 3a). In contrast, the epidermal cells and the guard cells were totally devoid of labelling (Figure 3e,f). Thus, So-\(\delta\)TIP is expressed in the leaf palisade parenchyma cells and in the spongy parenchyma cells, but not in epidermal cells nor in guard cells. In mature roots (Figure 3g), labelling is strong in the endodermal cell layer, weaker but evident in xylem parenchyma cells, but absent in phloem parenchyma cells. Furthermore, meristematic cells of the root tip show no labelling (Figure 3h).

From this analysis, we conclude that So-\(\delta\)TIP is abundant in most cell types in all vegetative organs, but excluded from leaf epidermal and root phloem parenchyma and meristematic cells. The tissue-specific expression has also been determined for \(\delta\)-TIP in Arabidopsis, sunflower and radish. In Arabidopsis, the \(\delta\)-TIP promoter was fused to the GUS (\(\beta\)-glucuronidase) reporter gene and the construct was expressed in tobacco. The GUS signal could only be detected in the shoot, and preferentially in association with developing vascular tissues (Daniels et al., 1996). The expression of the sunflower \(\delta\)-TIPS, SunTIP7 and SunTIP20, in leaves was examined using in situ hybridization.

tion, which showed expression exclusively in the guard cells (Sarda et al., 1997). However, these transcripts were also present in roots according to Northern hybridization analysis, and the clones were initially isolated from a root cDNA library. Rs-SVM23, a recently characterized δ-TIP homologue from radish, is expressed in tap roots, petioles and young leaves. It is expressed in cells of leaf veins, but not in leaf mesophyll cells nor in young root cells (Higuchi et al., 1998). Thus, in spite of the high sequence homology between the δ-TIPs in spinach, Arabidopsis, sunflower and radish, their expression patterns in the plants are totally different. It appears as if expression patterns, and consequently the physiological roles, of different TIPs cannot be extrapolated from one species to another based exclusively on sequence homologies. The C-terminal region of TIPs is the region most distinct to PIPs and NLMs, and peptides corresponding to this region have therefore been used for raising TIP-specific antisera. However, as this region is only weakly conserved between δ-TIPs of different species it is of limited use for raising antisera recognizing δ-TIP orthologues. This may question the validity of data obtained using antibodies raised against a δ-TIP of one species in another species (e.g. Jauh et al., 1999).

A comparison of the expression pattern of So-δTIP with that of more distantly related TIPs shows some similarities to Arabidopsis γ-TIP, which is expressed in zones of cell elongation/differentiation but is excluded from mesistematic tissues (Ludevid et al., 1992). In contrast, ZmTIP1, a γ-TIP homologue in maize, is abundant in meristematic and epidermal tissues, as well as in zones of cell elongation (Barrieu et al., 1998; Chaumont et al., 1998).

In conclusion, we have shown that the spinach δ-TIP homologue So-δTIP is expressed at comparatively high protein levels in most vacuolated cells of both leaves, petioles and roots, but in neither leaf epidermal and guard cells nor in meristematic cells of root. Meristematic cells are characterized by many small vacuoles as opposed to elongating and mature cells, which generally harbour a single large vacuole. Our results indicate that the expression of So-δTIP may be induced at the time point when the large vacuole is formed. Other spinach TIP isoforms are likely to be present in the meristematic cells.

**Experimental procedures**

**Isolation of intracellular membranes**

Intracellular membranes were isolated from spinach leaves, petioles and roots using aqueous two-phase partitioning (Kjellbom and Larsson, 1984).

**Purification of So-δTIP**

Total intracellular membranes were diluted to a protein concentration of 5 mg ml⁻¹ in 250 mM sucrose, 10 mM HEPES–KOH, pH 7.5, 1 mM PMSF, 2 mM DTT. Four milliliters of this membrane suspension were mixed with an equal volume of 50 mM Tris–HCl, pH 7.2, 50 mM sucrose (buffer A), also containing 2 mM EDTA, 2 mM EGTA and 1 mg ml⁻¹ Brij 58, and centrifuged for 30 min at 140 000 g. The pellet was resuspended in 4 ml of buffer A and membrane proteins solubilized by adding 4 ml of buffer A containing 10 mg ml⁻¹ dodecyl-β-D-maltoside. The detergent solution was added dropwise and with continuous stirring. After 30 min at room temperature, unsolubilized material was pelleted at 125 000 g for 30 min. A FPLC system (Pharmacia, Uppsala, Sweden) equipped with a MonoQ HR 5/5 anion-exchange column was used to resolve the solubilized membrane proteins. The column was equilibrated at room temperature with 20 mM histidine–HCl, pH 6.5, 0.5 mg ml⁻¹ dodecyl-β-D-maltoside, and the proteins were eluted with a linear NaCl gradient (0–0.5 M). Fractions of 1.0 ml were collected and analysed by SDS–PAGE.

**Figure 2.** Sequence comparisons.

(a) Phylogenetic sequence comparisons. The amino acid sequences of most TIPs were compared, together with representative plasma membrane intrinsic proteins, NOD26-like-MIPs, and the Escherichia coli aquaporin. The analysed sequences with accession numbers and species names are listed: Am-TIP (P33560) from Antirrhinum majus (garden snapdragon); At-NLM1 (Y07625), At-PIP1a (P34285), At-PIP1c (S44083), At-PIP2a (P43298), At-PIP3 (U78297), At-RD28 (D13254) At-γTIP (P26587), β-γTIP (U39485), α-γTIP2 (Z97343), α-γTIP (P25818) and γ-γTIP (AFO51737) from Arabidopsis thaliana; BoβTIP26-1 (U92651) from Brassica oleracea (cauliflower); Cs-γMP (45077) and Cs-αMP (45078) from Cucurbita sp. (pumpkin); Dc-TIP (AB005060) from Daucus carota (carrot); Ec-AQP2 (P48388) from Escherichia coli; Gh-TIP (U62778) from Gossypium hirsutum (cotton); Gm-NOD26 (P89995), Gm-SCPC1 (JQ2287) and Gm-SCPC2 (JQ2288) from Glycine max (soybean); Ha-Sun7βT (X95953), Ha-Sun7αT (X95950), Ha-Sun7αT8 (X95951) and Ha-Sun7αT8 (X95952) from Helianthus annuus (sunflower); Hv-γTIP (X92666) from Hordeum vulgare (barley); Le-γTIP (U95008) from Lycopersicon esculentum (tomato); Mc-TIP (U43291) from Mesembryanthemum crystallinum (common ice plant); Ms-MCP1 (AFO27903) from Medicago sativa (alfalfa); Nl-AQ1 (AFO24511), Nl-TIP1 (A127751) and Nl-TipR (S45046) from Nicotiana tabacum (tobacco); Os-γTIP (P05156) from Oryza sativa (rice); Pa-TIP (A105078) from Picea abies (norway spruce); Pp-αTIP (P23958) from Phaseolus vulgaris (kidney bean); Ps-γ1 (AB010416) and Ps-γ2 (D84669) from Raphanus sativus (radish); So-Pm28a (L77969), So-Pm28b (A1249384) and So-γTIP (A124953) from Spinacia oleracea (spinach); St-PoγTIP (U67500) from Solanum tuberosum (potato); Ta-TIP (U68763) and Ta-αTIP (U68762) from Tritium aestivum (wheat); Th-TIP (X95650) from Tulipa gesneriana (tulip); Tr-TIP (Z29946) from Trifolium repens (white clover); Vf-TIP (AFO47173) from Vernicia fordii (tung tree); Zm-TIP1 (AFO37081) and Zm-TIP2 (AFO57182) from Zea mays (maize). The sequences were aligned using PileUp (Wisconsin Package Version 10.0, Genetics Computer Group [GCC], Madison, WI), the tree was constructed using Pau (Swofford, 1993) and the numbers next to the nodes are bootstrap values from 100 replicates. The homologues used for sequence comparisons in Figure 2b) are in bold.

(b) Alignment of the deduced amino acid sequences for So-δTIP, some representative TIPs, and the spinach plasma membrane aquaporin So-Pm28a. At-αTIP (P26587), At-γTIP (U39485), β-γTIP (P25818) and At-NLM1 (Y07625) are Arabidopsis thaliana MIPs representing each major branch of the phylogenetic tree in Figure 2a). The spinach plasma membrane aquaporin So-Pm28a (L77968) was included to show the relatively low homology of So-δTIP to spinach PIPs. The sole transporting NTI-TIPs (A127751; Gerbeau et al., 1998) from tobacco is also included as it represents a separate branch of the tree. The percentage of amino acid sequence identity of the aligned sequences to that of So-δTIP is shown. NPA boxes and putative transmembrane domains are underlined. Amino acids conserved in at least four out of seven proteins are boxed. Note the low homology at the C-terminal end of the aligned sequences. Thus, a peptide corresponding to the last 11 amino acids of So-δTIP (ODDHAPLSNEY, depicted in bold) was used to produce homologue-specific antibodies.

SDS–PAGE

SDS–PAGE was performed according to Laemmli (1970), with minor modifications. A 5% stacking gel and a 12% separating gel were used and the samples were solubilized at 20°C for 20 min in standard sample buffer. Urea/SDS–PAGE gels contained 4 M urea in 9–15% gradient polyacrylamide gels. Samples were solubilized at 20°C for 20 min in standard sample buffer containing 4 M urea.

Amino acid sequencing

The MonoQ fractions enriched in the 75 kDa band were pooled and polypeptides separated by SDS–PAGE, electrophoresed to a polyvinylidene difluoride (PVDF) membrane, and stained with Coomassie brilliant blue R-250. The 75 kDa (and later also the 21 kDa band) was cut out and N-terminally sequenced using an ABI 476A sequencer (Applied Biosystems/Perkin-Elmer, Foster City, CA, USA).

RT–PCR cloning, cDNA library screening and sequencing of So-§TIP

The following degenerated oligonucleotides were used as primers for PCR. The forward primer 5’-CCGCGTAGATCT/C TACGATGCACTGTA-3’ corresponds to the sequence IAFCRFD in the N-terminus of So-§TIP. The reverse primer 5’- CGGAGATTCA/G/TA/G/TGTNACA/G/TGCA/G/TGAT/ TGA/GTTNAC/T-3’ corresponds to a conserved MIP motif, HI/NI/ VNPVFT. To facilitate cloning, a XhoI site was included at the 5’ end of the forward primer and an EcoRI site at the 5’ end of the reverse primer. First-strand cDNA was synthesized from total RNA by reverse transcription. The PCR product was cloned into pBluescript II SK+ (Stratagene, La Jolla, CA, USA) by using standard methods (Sambrook et al., 1989), and used as a probe for screening of a spinach leaf cDNA library constructed in Uni- ZAP XR (Stratagene). Sequencing was performed on an ABI Prism 310 Genetic Analyser using a Taq FS sequencing Kit (Perkin-Elmer). Homology searches were done using the computer program BLAST (Altschul et al., 1997).

Figure 3. Intracellular and tissue distribution. (a–d) Intracellular localization of So-§TIP. Immunogold labelling using affinity-purified antibodies raised against a peptide representing the C-terminal end of So-§TIP localizes So-§TIP to the vacuolar membrane in leaf mesophyll cells (a) and in cells surrounding the xylem (b). So-§TIP is also located in the vacuolar membrane in cells of the petiole (c) and the root (d). Arrowheads point to the labelled vacuolar membrane. Ch, chloroplast; N, nucleus; *, secondary thickenings of xylem walls. The scale bars are 2 μm.

(e–h) Tissue distribution of So-§TIP. In leaves (e) So-§TIP is expressed in palisade parenchyma cells and in spongy parenchyma cells, but not in the upper or lower epidermis. Higher magnification (f) of the lower epidermis and the mesophyll cells in (e) confirms the lack of labelling in epidermal cells including the stomatal cells. Transverse section of a mature root (g) showing labelling in endodermal cells, in all vacuolated cells surrounding the xylem vessels, but not in the outer cortical layer and not in phloem parenchyma cells. Transverse section of a root tip (h) demonstrating that So-§TIP is not expressed in the apical root meristem. The scale bars in images (e), (g) and (h) are 100 μm, and in image (f) 40 μm.
**Free-flow electrophoresis**

Fractionation of a spinach microsomal fraction using free-flow electrophoresis was performed as in Auderset et al. (1996), using an Elphor Vap22 unit (Bender and Hobein, Munich, Germany).

**Antibodies**

A peptide, CODDHAPLSNEY, representing the C-terminal end (except for the N-terminal C added to facilitate linking; see below) of the predicted amino acid sequence of So-δTIP was synthesized. The peptide was linked to keyhole limpet haemocyanin via its N-terminal cysteine, and the conjugate used to produce a rabbit antiserum. An IgG fraction was obtained by chromatography on a Poros Protein A (20A) column, equilibrated with 0.1 M Tris–HCl, pH 8.0, 0.15 M NaCl, and mounted on a BIOCAD (Perkin-Elmer). The IgG fraction was eluted with 0.1 M glycine, pH 3.0. The synthetic peptide was used to affinity purify antibodies from the IgG fraction using a SulfoLink™ Kit (Pierce, Rockford, IL, USA).

**Immunoblotting**

Polypeptides were separated by SDS–PAGE (± urea) and transferred onto PVDF membranes. So-δTIP was detected using the affinity-purified antibodies diluted 1:2000. Goat anti-rabbit IgG conjugated to alkaline phosphatase was used as secondary antibody.

**Immunogold labelling and tissue preparation**

Pieces of leaves, petioles and roots were sampled from well-watered spinach plants and infiltrated with 2.5% (v/v) glutaraldehyde (EM grade), 0.05 M sodium cacodylate buffer, pH 7.2, under vacuum (0.1 Torr) and then processed for low temperature L.R. White resin embedding as described previously (Bush and McCann, 1999). Resin sections were cut and immunolabelled according to Bush and McCann (1999) with the following modifications. Sections were incubated with 10% (v/v) sheep serum in PBS and 0.1% Tween-20 to block non-specific labelling. Affinity-purified anti-So-δTIP IgG was diluted 1:5 in the same sheep serum–PBS–Tween solution and applied to sections overnight at 4°C. The sections were washed with PBS and incubated overnight with an anti-rabbit IgG 5 nm gold conjugate (British BioCell International) diluted 1:30 in sheep serum–PBS. Sections were washed in PBS, treated with 1.0% glutaraldehyde in PBS to cross-link antibody complexes, and washed with distilled water prior to silver enhancing and counterstaining. Silver enhancement was for 2 and 10 min for electron and light microscopy, respectively, using a silver enhancement kit (British BioCell International). Control sections were treated in parallel but with the omission of anti-So-δTIP.

**Figure 3(e–h).**
Microscopy

For light microscopy, gold-labelled silver-enhanced sections were counterstained with basic fuchsin and examined with either a Nikon Eclipse E800 light microscope or a Leica TCS laser scanning confocal microscope, set up for reflection imaging using excitation and emission wavelengths of 488 nm and 515 nm, respectively, and an RT30/70 mirror. Fuchsin autofluorescence and light epi-reflected from the silver-enhanced gold were recorded digitally as separate images and merged using Confocal Assistant 4.02 software. A Jeol 1200EX transmission electron microscope was used to examine ultrathin sections counterstained with uranyl acetate and lead citrate.

Acknowledgements

We thank Adine Karlsson for excellent technical assistance, Josette Güçlü for help with the free-flow electrophoresis and Bengt Widgren for help with sequence comparisons. Grants from the Swedish Council for Forestry and Agricultural Research, the Swedish Natural Science Research Council, the EU-Biotech program (BIO4-CT98-0024) and the Swedish Strategic Network for Plant Biotechnology are gratefully acknowledged.

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EMBL accession number AJ245953 (So-αtip).