Immunolocalization of Solanaceous SUT1 Proteins in Companion Cells and Xylem Parenchyma: New Perspectives for Phloem Loading and Transport

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Leaf sucrose (Suc) transporters are essential for phloem loading and long-distance partitioning of assimilates in plants that load their phloem from the apoplast. Suc loading into the phloem is indispensable for the generation of the osmotic potential difference that drives phloem bulk flow and is central for the long-distance movement of phloem sap compounds, including hormones and signaling molecules. In previous analyses, solanaceous SUT1 Suc transporters from tobacco (Nicotiana tabacum), potato (Solanum tuberosum), and tomato (Solanum lycopersicum) were immunolocalized in plasma membranes of enucleate sieve elements. Here, we present data that identify solanaceous SUT1 proteins with high specificity in phloem companion cells. Moreover, comparisons of SUT1 localization in the abaxial and adaxial phloem revealed higher levels of SUT1 protein in the abaxial phloem of all three solanaceous species, suggesting different physiological roles for these two types of phloem. Finally, SUT1 proteins were identified in files of xylem parenchyma cells, mainly in the bicollateral veins. Together, our data provide new insight into the role of SUT1 proteins in solanaceous species.

Unlike in animals, vascular flow in higher plants is not powered by a single heart-like organ, but rather by numerous molecular pumps in the plasma membranes of specialized cells (apoplastic loaders) or by a different, not yet fully understood mechanism (symplastic loaders). In the phloem of apoplastic loaders, the molecular pumps generate intracellular, osmotic driving forces that represent the basis for the mass flow from fully developed source leaves to all kinds of sink tissues and thus for long-distance allocation of photosynthates and for long-distance signaling. In many higher plants, including Brassicaceae (e.g. Arabidopsis [Arabidopsis thaliana]) and Solanaceae (e.g. tomato [Solanum lycopersicum; former name: Lycopersicon esculentum], tobacco [Nicotiana tabacum], and potato [Solanum tuberosum]), Suc is the main product of photosynthesis and, therefore, used as the primary osmoticum to generate and maintain this driving force. In other plant families, Suc is allocated together with polyols (e.g. mannitol in Apiaceae or sorbitol in Plantaginaceae) or with raffinose family oligosaccharides, such as raffinose, stachyose, or verbascose (e.g. in Lamiaceae and Cucurbitaceae).

In Suc and Suc-/polyol-translocating plants (apoplastic loaders), these assimilates enter the sieve element (SE)-companion cell (CC) complexes via plasma membrane-localized transporters (Stadler et al., 1985; Stadler and Sauer, 1996; Kühn et al., 1997; Ramsperger-Gleixner et al., 2004). In Suc-/raffinose family oligosaccharide-translocating plants, phloem loading appears to be entirely symplastic (Turgeon et al., 1993, 2001; Schulz, 2005; Turgeon, 2006).

In apoplastic loaders, the driving force for phloem bulk flow is well understood. Fueled by H⁺-ATPases (Giaquinta, 1979; Delrot, 1981; Parets-Soler et al., 1990; DeWitt and Sussman, 1995) and regulated by K⁺-channels (Deeken et al., 2000, 2002; Lacombe et al., 2000; Philippart et al., 2003), plasma membrane-localized transporters load their substrates into the cytoplasm of the SE-CC complex. This loading generates high intracellular solute concentrations (Moing et al., 1992; Lohaus et al., 2000; Lohaus and Fischer, 2002) and thus a water potential gradient between the SE-CC complex and the surrounding tissues. This gradient drives water into the sieve tube system and increases turgor pressure. The differential between Suc loading and unloading in source and sink regions drives the bulk flow through the sieve tube system and assimilate allocation (Münch, 1930).

The importance of the involved solute transporters became obvious in plants with reduced mRNA levels (Riesmeier et al., 1994; Bürkle et al., 1998) or with blocked expression of Suc transporter genes (Kühn et al., 1996; Gottwald et al., 2000). In these mutants,
leaves accumulated soluble carbohydrates and starch and showed reduced chlorophyll content and eventually chlorotic lesions.

Phloem bulk flow is also required for long-distance signaling by phytohormones or macromolecular factors. Biosynthetic enzymes for abscisic acid (Koizumi et al., 2004) or jasmonic acid (Hause et al., 2003) or for enzymes involved in day-length perception and flowering time regulation (An et al., 2004; Corbesier et al., 2007; Lin et al., 2007) were identified in or shown to act from phloem CCs. Moreover, different types of RNAs were shown to move through the phloem (Jorgensen et al., 1998; Yoo et al., 2004; Haywood et al., 2005; Banerjee et al., 2006; Roney et al., 2007), and plant viruses utilize phloem mass flow for the rapid spread between different plant organs (Ding et al., 1996; Horns and Jeske, 1991; Cruz et al., 1998; Lucas and Wolf, 1999).

Immunohistochemical studies on the cell specificity of transporters involved in phloem loading yielded different results for different dicot families. Whereas the SUC2 proteins of Arabidopsis and Plantago were identified in CCs (Stadler et al., 1995; Stadler and Sauer, 1996), solanaceous transporters from tomato (LeSUT1), potato (StSUT1), and tobacco (NtSUT1) were found exclusively in SEs (Kühn et al., 1997). Because mature SEs are enucleate and devoid of ribosomes (Esau, 1969; Evert, 1990), it was hypothesized that solanaceous SUT1 proteins may be synthesized either within the CCs and then targeted to the SEs, or within the SEs by a so far undetected mechanism after targeted cell-to-cell trafficking of SUT1 mRNAs (Kühn et al., 1997; Lalonde et al., 2003).

Since then, three more articles have been published (Barker et al., 2000; Kühn et al., 2003; Hackel et al., 2006) supporting this SE-specific localization of solanaceous SUT1 proteins. Additional evidence came from the identification of Suc transporter mRNAs in the phloem sap of potato (Kühn et al., 1997), barley (Hordeum vulgare; Doering-Saad et al., 2002), and pumpkin (Cucurbita maxima; Ruiz-Medrano et al., 1999; Roney et al., 2007) and in nematode-induced syncytia of Arabidopsis roots (Jürgensen et al., 2003). These syncytia are linked symplastically to phloem SEs, and the connecting plasmodesmata were shown to have size exclusion limits allowing macromolecular cell-to-cell movement (Hoth et al., 2005).

To further investigate the reasons for this discrepancy between the results and models of different groups, we raised an antiserum against a 43-amino-acid peptide from the highly conserved loop-region in the center of the predicted cytoplasmic loop between transmembrane helices VI and VII (amino acid residues 239–280 in the published sequence; Fig. 1A). This second PCR introduced a stop codon at the 3′ end of the fragment that was fused to the 3′ end of the ORF of the Escherichia coli maltose-binding protein (MBP). The fusion was used to immunize two rabbits. In previous publications (Lemoine et al., 1996; Kühn et al., 1997), shorter peptides from the same region (Fig. 1A) were used to raise antisera that identified solanaceous SUT1 proteins in protein fractions from SUT1-expressing yeast cells and in immunohistochemical analyses of plant tissue.

The initially obtained NISUT1 ORF differed slightly (two additional plus seven different amino acids) from the published NISUT1a sequence (Bürkle et al., 1998; Supplemental Fig. S1). Most of these differences were conserved in tomato and potato SUT1 proteins (Supplemental Fig. S1). The corresponding gene was named NISUT1x (x for Xanthii) and deposited in the EMBL database (accession no. AM491605).

To test if the observed differences are cultivar-specific (Xanthii [this article] versus Samsun [Bürkle et al., 1998]), we amplified and sequenced the complete NISUT1 ORF also from Samsun. However, several independently analyzed sequences from Samsun turned out also to be NISUT1x (accession no. FM164640; 99.02% identity on the amino acid level with NISUT1x from Xanthii; Fig. 1A; Supplemental Fig. S1).

During further attempts to find the published NISUT1 sequence in the tobacco cultivars Xanthii and Samsun, a second NISUT1 sequence was identified in both cultivars (97.8% [Xanthii] and 98.2% [Samsun] identity on the amino acid level with the NISUT1x protein from the same cultivar). These sequences encode 100% identical proteins in both cultivars and were named NISUT1y (accession no. for NISUT1y from Xanthii, FM164638; accession no. for NISUT1y from Samsun, FM164639). Under no conditions, even with primers that were designed to amplify specifically the published NISUT1a sequence (Bürkle et al., 1998), were we able to find NISUT1a sequences.

RESULTS

Production and Test of a New Antiserum

A complete NISUT1 open reading frame (ORF) was amplified from RNA of tobacco leaves (Xanthii) with primers designed according to the published NISUT1a sequence (accession no. X82276; Bürkle et al., 1998). A second pair of NISUT1a-derived primers was used to amplify a truncated, internal fragment encoding the predicted cytoplasmic loop between transmembrane helices VI and VII (amino acid residues 239–280 in the published sequence; Fig. 1A). This second PCR introduced a stop codon at the 3′ end of the fragment that was fused to the 3′ end of the ORF of the Escherichia coli maltose-binding protein (MBP). The fusion was used to immunize two rabbits. In previous publications (Lemoine et al., 1996; Kühn et al., 1997), shorter peptides from the same region (Fig. 1A) were used to raise antisera that identified solanaceous SUT1 proteins in protein fractions from SUT1-expressing yeast cells and in immunohistochemical analyses of plant tissue.

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Based on these observations and after intensive controls, we conclude that solanaceous species execute their phloem loading and retrieval process(es) from the CCs. Moreover, differences in the amount of SUT1 proteins identified in the abaxial and adaxial phloem of Solanaceae provide new insight into the different physiological roles of these two types of phloem.
The 43-amino-acid, NtSUT1x-derived peptide that was eventually used to raise new antiserum shared 93.0% identity with the corresponding peptides of the published NtSUT1a and the newly identified NtSUT1y sequences, and 88.4% identity with the corresponding peptides from LeSUT1 (X82275) and StSUT1 (X69165).

After affinity purification of the new anti-solanaceous SUT1 antiserum (αSolSUT1), it was tested on western blots of plasma membrane proteins from StSUT1-expressing yeast cells. αSolSUT1 recognized a single, 47-kD polypeptide but no band in controls (Fig. 1B). This demonstrated that αSolSUT1 that had been raised against an NtSUT1 peptide recognized the highly related StSUT1 protein.

Immunolocalization Analyses with αSolSUT1 in Tobacco

To confirm the specificity of affinity-purified αSolSUT1 antiserum also in fixed and embedded plant tissue, immunofluorescence analyses were performed with tobacco source leaves. Tobacco leaves possess five vein classes (Avery, 1933), with class I veins representing the midrib, class II the veins arising as single branches from this midrib, and class III the veins that form “islands” between adjacent class II veins. The smallest veins found within these “islands” are called minor veins or, depending on their developmental stage, class IV and class V veins. Minor veins and class III veins are built from collateral vascular bundles with a single, so-called abaxial phloem below their xylem. Class I and class II veins possess an additional adaxial phloem that is located above the xylem vessels in the leaves and inside the xylem ring of the stem.

As expected, αSolSUT1-derived fluorescence was detected in the abaxial and adaxial phloem of midribs from tobacco source leaves (Fig. 2, A and B). Unexpectedly, however, and in contrast to published data (Kühn et al., 1997), the fluorescence in the adaxial phloem was much weaker. The identity of the labeled cells was determined by simultaneous staining with αSolSUT1/second antibody and 4',6'-diamidino-
2-phenyl-indole (DAPI). Based on previous publications (Kühn et al., 1997) and because mature SEs are enucleate, αSolSUT1-dependent fluorescence (green) and DAPI-derived fluorescence (blue) were expected in different cells. However, in cross (Fig. 2C) and longitudinal sections (Fig. 2D) of tobacco source leaf phloem, many of the αSolSUT1-labeled cells were nucleate. This finding, the shape of the labeled cells (long with small diameter), and the fact that the αSolSUT1-labeled cells typically ended next to sieve plates (Fig. 2D) suggested that αSolSUT1 labels CCs and not SEs.

This was confirmed by immunolocalization analyses with cross sections of tobacco minor veins (Fig. 2, E and F). Again, the αSolSUT1-labeled cells were unambiguously identified as CCs due to their large size and their well-conserved positions. None of the analyzed sections showed fluorescence in the tiny SEs in the center (Fig. 2, E and F). This minor vein-typical ratio in cell diameters (CCs larger than SEs) that is inverse in midribs (CCs smaller than SEs) has been described (Esau, 1969). In fact, in tobacco midribs, αSolSUT1 labeled only the smallest cells, which again identified these cells as CCs (Fig. 2G). Tobacco sink leaves showed no αSolSUT1 labeling (Fig. 2H). This complies with previous reports on very low SUT1 mRNA levels in sink leaves (Riesmeier et al., 1993; Bürkle et al., 1998) and with analyses of LeSUT1-promoter/GUS plants that demonstrated that the activity of the LeSUT1 promoter follows the sink-to-source transition (Kühn et al., 2003).

αSolSUT1-specific localization of NtSUT1 was observed in all vein classes connecting the midrib and the minor veins. Figure 2, I and K, shows labeling of CCs in class II and class III veins of a tobacco source leaf. Typically, class II veins (Fig. 2I) are already bicollateral, whereas class III veins (Fig. 2K) are still collateral and do not yet possess adaxial phloem. As in class I veins (Fig. 2B), αSolSUT1 treatment showed no or only weak labeling of CCs in the adaxial phloem of class II veins. In contrast, CCs were nicely labeled in the abaxial phloem of class II and III veins.

Finally, NiSUT1 localization was studied in tobacco stem sections (Fig. 2, L and M). Typically, these sections showed more or less equal labeling of the CCs in the two types of phloem (Fig. 2M). In sections of younger stem regions, however, that were from near the top of the respective plant, labeling was occasionally seen preferentially in the abaxial phloem (Fig. 2L).

To our surprise, αSolSUT1-specific fluorescence was not restricted to the phloem. Mainly stem sections (Fig. 2M), but with decreasing intensity, also leaf-derived sections of class I (Fig. 2B), class II (Fig. 2I), and class III veins (Fig. 2K), showed a clear αSolSUT1-dependent labeling of xylem parenchyma cells. This labeling was observed primarily in parenchyma cells showing direct contact to lignified xylem vessels (Fig. 2, B, K, I, M, and N). However, less intense staining was also seen in cell files extending into the younger, not yet or hardly lignified xylem (Fig. 2M). In none of the previous reports on SUT1 localization in Solanaceae was labeling of xylem parenchyma observed.

Immunolocalization Analyses with αSolSUT1 in Tomato and Potato

So far, our analyses have demonstrated that αSolSUT1, which was raised against a region conserved in solanaceous SUT1 proteins, labels potato StSUT1 on western blots (Fig. 1B) and tobacco NiSUT1 in immunohistochemical analyses. In contrast to previous reports, αSolSUT1 localized NiSUT1 to phloem CCs and not to phloem SEs, αSolSUT1 labeling differed between abaxial and adaxial phloem, and αSolSUT1 labeling was observed also in xylem parenchyma.

For further analyses of the cell specificity of SUT1 proteins, similar analyses were performed with tomato (Fig. 3, A–C) and potato (Fig. 3, D–F). As in tobacco, αSolSUT1 labeled individual cells in the phloem of tomato (Fig. 3A) and potato midribs (Fig. 3F), and again the labeling was stronger in the abaxial phloem. As in tobacco, the labeled cells were characterized as CCs based on the presence of nuclei (Fig. 3D), on their small diameter in midribs (Fig. 3C), and their large diameter and position in minor veins (Fig. 3, B and E). No labeling was detected in the tiny, central SEs of minor veins (Fig. 3, B and E) or in the larger SEs of midrib phloem (Fig. 3C). Finally, αSolSUT1 labeled cells of the xylem parenchyma both in tomato (Fig. 3A) and, although only weakly, in potato (Fig. 3F).

Immunolocalization Controls

So far, immunohistochemical analyses have been presented only for solanaceous species known to share high degrees of sequence homology (about 90% identity) in those parts of their SUT1 proteins that correspond to the NtSUT1 peptide used for immunization. To test the possibility of unspecific binding of αSolSUT1 to an unknown epitope, we performed immunolocalizations also with less closely related solanaceous species. In none of these (habanero chili [Capsicum chinense; Fig. 4D], bell pepper [Capsicum annuum; not shown], petunia [Petunia hybrida; not shown], physalis [Physalis peruviana; Fig. 4C], or blue potato bush [Lycianthes rantoncelli; not shown]) has SUT1 sequences been determined. As further controls, we included non-solanaceous species with known Suc transporter sequences (plantain [Plantago major; Fig. 4B] and Arabidopsis (Fig. 4A)). The sequence identity between the tobacco SUT1 peptide used to raise αSolSUT1 (Fig. 1A) and the corresponding peptides from the plantain PmSuc2 protein (accession no. X75764) or the Arabidopsis AtSuc2 protein (accession no. Q39231) are only 44.2% and 46.5%, and immunodetection of AtSuc2 or PmSuc2 by αSolSUT1 should not occur.

In none of these species were αSolSUT1-specific signals detected, although fluorescence labeling was obtained in parallel experiments with tobacco (not shown). This makes it unlikely that affinity-purified αSolSUT1 binds unspecifically to non-SUT1 epitopes in CCs or xylem parenchyma.
Figure 2. Localization of NtSUT1 in tobacco by fluorescence detection with αSolSUT1. Yellow and orange staining shows autofluorescence of phenolics in lignified cells; green staining shows NtSUT1 localization. White light images and confocal images were superimposed in A, C to E, G to L, and N. A, transverse section of the midrib (class I vein) of a source leaf. B, Transverse section of the midrib of a source leaf. C and D, Histochemical staining of nuclei with DAPI (0.2 μg mL⁻¹) in cross (C) and longitudinal (D) sections of the midrib of a source leaf. E, NtSUT1 protein in a transverse section of a source leaf minor vein (class V). F, Schematic drawing of image E highlighting the regular arrangement of cells. G, Localization of NtSUT1 in the smallest cells of the abaxial phloem in a midrib; transverse section of a source leaf. H, Absence of NtSUT1 from sink leaves (transverse section). I, Localization of NtSUT1 in the CCs of the abaxial phloem in a class II vein. No labeling is seen in the adaxial phloem. J, Localization of NtSUT1 in the CCs of the abaxial phloem in a class III vein. K, Localization of NtSUT1 in the CCs of the abaxial phloem in a class III vein. L, Localization of NtSUT1 in the...
Controls for αSolSUT1 Purification and Comparative Controls with αSolSUT1 and P1-Anti-StSUT1

Another explanation for the difference between our and the published localization data might be that the observed αSolSUT1-dependent fluorescence (Figs. 2 and 3) does not result from αSolSUT1 binding to SUT1 proteins at all but rather from potentially coenriched antibodies that bind to an unknown, non-SUT1 epitope. This copurification might result from unspecific binding of antibodies to components of the blocking buffer (e.g. milk proteins) or to the MBP portion of the MBP-NISUT1x fusion protein during the purification procedure. To exclude this possibility, two sets of controls were performed.

For the first control, two parallel affinity purifications of raw αSolSUT1 antiserum were performed. One was done with the MBP-NtSUT1x fusion protein, the other with unfused MBP. These two purifications differed only in the presence or absence of the 43 amino acids of NISUT1x, and potentially unspecifically bound antibodies should be purified in both cases. The obtained, affinity-purified antisera (αSolSUT1 from MBP-NISUT1x and MBP-αSolSUT1 from MBP) were analyzed in parallel, and images were taken under identical conditions. Whereas αSolSUT1 yielded the expected CC-specific signals (Fig. 5B), no fluorescence was seen with MBP-αSolSUT1 (Fig. 5A). For the second control, tobacco sections were treated with affinity-purified preimmune serum. Again, these analyses showed no labeling (Supplemental Fig. S2).

The peptides used to raise StSUT1-specific antisera in previous analyses (Kühn et al., 1997) were 13 or 15 amino acids long and represented fragments of the 43-amino-acid peptide used to raise αSolSUT1 (Fig. 1A). In principle, all of these sera should recognize the same epitope, and, consequently, immunohistochemical analyses should yield the identical localization.

Therefore, comparative immunohistochemical studies were performed in yeast cells (StSUT1-expressing and control strains) with αSolSUT1 and with a published anti-StSUT1 antiserum (P1; Kühn et al., 1997). P1-anti-StSUT1 was used to successfully label SEs in potato and tobacco and was raised against the StSUT1 peptide RENELPEKDEQEIDE of the predicted central loop of SUT1 proteins (Fig. 1A). The yeast strains were identical to those that had already been used for the western blot shown in Figure 1B. Cells were fixed, embedded, and sectioned using the identical protocol that had been applied for all plant tissues shown so far. Figure 5, D and F, demonstrates that fluorescence was detected with both antisera exclusively in sections of the SUT1-expressing strain. No fluorescence was seen in the strain harboring the empty vector (Fig. 5, C and E).

This result demonstrated that αSolSUT1 and P1-anti-StSUT1 recognize the same protein. Therefore, we repeated the immunolocalization of NtSUT1 and StSUT1 (Kühn et al., 1997) in parallel with αSolSUT1 and P1-anti-StSUT1. With αSolSUT1, we obtained the already-described labeling of CCs in all sections (not shown). With P1-anti-StSUT1, however, we obtained two totally unexpected results. First, in our hands, purified P1-anti-StSUT1 did not label SEs, and second, at sufficiently high concentration (1:50–1:250 dilution), P1-anti-StSUT1 antiserum decorated clearly and specifically CCs of tobacco (Fig. 5G) and potato source leaves (Fig. 5, H and I). Moreover, we observed P1-anti-StSUT1-dependent fluorescence in xylem parenchyma cells (data not shown). Interestingly, the fluorescence signals obtained in potato were much brighter than the signal obtained in tobacco, which might result from a minor difference in the NISUT1 sequence and the peptide originally used to raise P1-anti-StSUT1. When we used the P1-anti-StSUT1 antiserum at higher dilutions (up to 1:5,000), no fluorescence was detected (not shown).

In previous articles that used P1-anti-StSUT1 antiserum for immunodetection of SUT1 proteins, tissue fixation was typically performed with different chemicals (0.1% glutaraldehyde and 6% formaldehyde; Barker et al., 2000; Kühn et al., 2003; Hackel et al., 2006). To test the potential influence of this fixation technique, we also used the glutaraldehyde/formaldehyde fixation protocol. In our hands, no or only very weak signals were detected with this fixation protocol, but these weak signals were also restricted to CCs (not shown). This suggested that the glutaraldehyde/formaldehyde fixation destroys or masks at least part of the antigenic epitope of the SUT1 proteins analyzed.

What Is the Reason for the Observed Discrepancy in SUT1 Localization?

The results obtained in our comparative analyses with αSolSUT1 (this article) and the previously published P1-anti-StSUT1 (Kühn et al., 1997) were both clarifying and puzzling. They were clarifying, because two antisera that were raised using different approaches (peptide coupled to a carrier protein [Kühn et al., 1997] versus C-terminal fusion protein [this
article]) against slightly different peptides of a conserved region of plant Suc transporters and that were affinity-purified with different techniques (peptide attached to a column [Kuhn et al., 1997] versus fusion protein bound to nitrocellulose [this article]) labeled the same protein on western blots and in immunohistochemical analyses of tissue sections. They were puzzling, however, because the very same antiserum labeled SEs and not CCs when used by different groups. To find a possible answer for this discrepancy, we performed additional tests.

First, immunohistochemical analyses were performed with unpurified αSolSUT1 serum. We observed strong labeling of SEs in all sections from tobacco (Fig. 6, A and B), potato, and tomato (not shown) with unpurified αSolSUT1. No or only weak labeling was seen in CCs under these conditions. However, in several independently performed affinity purifications of raw αSolSUT1 serum and after enrichment of the affinity-purified antiserum (see “Materials and Methods”), the strong labeling of SE was removed, most of the label in the adaxial phloem disappeared, and specific labeling of CCs was obtained (Figs. 2 and 3).

Second, control analyses were performed with the preimmune serum of the rabbit that had been used to raise the αSolSUT1 antiserum. As with unpurified αSolSUT1, this labeling yielded strong and specific fluorescence of tobacco (Fig. 6, C and D), potato, or tomato (not shown) SEs, but no labeling of CCs. Moreover, the preimmune serum showed the previously described (Kuhn et al., 1997) equal labeling of abaxial and adaxial phloem (Fig. 6C).

Based on these results, we analyzed preimmune sera of almost 50 rabbits on cross sections of tobacco midribs (not shown). With about 80% of these preimmune sera, we observed selective labeling of SEs. This finding suggested that antibodies directed against an unknown, SE-specific epitope should be found with a reasonably high frequency in antisera raised against non-SE proteins. In fact, when we used a serum raised by another group against the Arabidopsis chloroplast protein AtCSP41B (At1g09340; Raab et al., 2006), we could label SEs of solanaceous plants (Fig. 6E). Similar results were obtained with other antisera (not shown).

In summary, these results suggest that the previous localization of solanaceous SUT1 proteins in SEs (Kuhn et al., 1997, 2003; Barker et al., 2000; Hackel et al., 2006) may have resulted from an inherent background of SE-recognizing antibodies frequently present in rabbit sera. We suppose that preimmune sera controls were performed in all previous reports on SUT1 immunolocalization (Kuhn et al., 1997; Kuhn et al., 2003; Lalonde et al., 2003).
DISCUSSION

CCs Mediate Phloem Loading Also in Solanaceous Species

The presented data immunolocalize the well-characterized solanaceous Suc transporters LeSUT1, StSUT1, and NtSUT1 to the CCs of tomato, potato, and tobacco. This contradicts previous reports on the immunolocalization of these proteins in SEs (Kühn et al., 1997, 2003; Barker et al., 2000; Hackel et al., 2006). It is in agreement with localization data published for Suc transporters of other dicot families, such as Arabidopsis (Brassicaceae; Stadler and Sauer, 1996) and Plantago (Plantaginaceae; Stadler et al., 1995), where the respective proteins were localized to CCs. In summary, these data suggest that Solanaceae, and potentially all apoplastic loading dicots, execute their loading and retrieval process(es) from the CCs and that species-specific differences for this essential step may not exist.

In several control experiments, we were able to demonstrate that SE-specific antibodies are frequently found in rabbit preimmune sera (Fig. 6, C and D) and that SE-specific labeling can be obtained with antisera raised against non-SE proteins (Fig. 6E). This may contribute to the previously published SE-specific localization of SUT1 proteins. Another reason for the observed discrepancy may be the use of different fixation protocols. In our hands, the previously published P1-anti-StSUT1 antiserum (Kühn et al., 1997) labels CCs but not SEs (Fig. 5, G–I) after tissue fixation with ethanol/acetic acid. On glutaraldehyde/formaldehyde-fixed tissue (Kühn et al., 1997), however, the signals detected with P1-anti-StSUT1 in CCs were only very weak. This may indicate that in contrast to the fixation with ethanol/acetic acid, glutaraldehyde/formaldehyde fixation reduces the antigenicity of SUT1 proteins.

Indirect evidence for a possible CC-specific localization of solanaceous SUT1 was obtained in previous analyses with tobacco plants expressing a LeSUT1/GFP fusion under the control of the Roci promoter from Agrobacterium (Lalonde et al., 2003). In these analyses, the LeSUT1-GFP fusion was detected only in CCs, but, based on the reported localization of SUT1 in SEs, it was concluded that the C-terminal GFP fusion blocks cell-to-cell movement of LeSUT1.

SE-specific localization (Barth et al., 2003, PmSUC3 in Plantago major; Meyer et al., 2004; AtSUC3 in Arabidopsis) or dual localization in SEs plus CCs (Knop et al., 2004; AmSUT1 in Alonsoa meridionalis) has also been described for Suc transporters of other dicot species. However, AtSUC3 and PmSUC3 belong to a different subgroup of Suc transporters that does not mediate phloem loading (Barth et al., 2003; Hackel et al., 2006), and for A. meridionalis, sequence information about potentially cross-reacting SUC3-type Suc transporters is lacking. Similarly, SE-localized Suc transporters were described in wheat (Triticum aestivum, TaSUT1; Aoki et al., 2004) and rice (Oryza sativa, OsSUT1; Scofield et al., 2007b). However, in both species, the identity of the labeled protein is not absolutely clear, because cross-reactions of the used antiserum with other Suc transporters could not be excluded (Furbank et al., 2001; Scofield et al., 2007a). Obviously, these data demonstrate the presence of SE Suc transporters, but most of these transporters are discussed to be involved either in the release of Suc from SEs or in Suc retrieval.

Tobacco Has Two NtSUT1 Genes

In both tobacco cultivars that were used to amplify NtSUT1 cDNAs (Xanthii and Samsun), we found two almost-identical SUT1 sequences that were named NtSUT1x and NtSUT1y. Under no condition and with no set of primers tested (see “Materials and Methods”) were we able to amplify the previously published NtSUT1a sequence (Bürkle et al., 1998). NtSUT1x and NtSUT1y are likely to represent orthologous SUT1 genes and to be encoded by the two subgenomes of the allotetraploid species N. tabacum that is known to have formed from the diploid progenitors Nicotiana sylvestris and Nicotiana tomentosiformis (Kenton et al., 1997).
1993; Kitamura et al., 2000). Comparison of NtSUT1a (Bürkle et al., 1998), NtSUT1x, and NtSUT1y (this article) protein sequences and of a partial tobacco SUT1 sequence from a tobacco hybrid (Nicotiana langsdorffii × Nicotiana sanderae; NlxsSUT1; assembled from accession nos. ABF06450 and ABF06446; Supplemental Fig. S1) demonstrates that the NtSUT1a sequence quite likely was obtained from an NtSUT1x mRNA but contains sequencing errors.

Obviously, one could speculate that αSolSUT1 might discriminate between the two NtSUT1 proteins and label only NtSUT1x, which was used to raise the antiserum, but not NtSUT1y. This can be excluded. Between the 43-amino-acid peptide used to raise αSolSUT1 and the respective peptide in NtSUT1x, there are only two differences: His-250 in NtSUT1x is Gln-250 in NtSUT1y, and Gly-257 in NtSUT1x is Ala-257 in NtSUT1y. In StSUT1 (Fig. 1A), the respective residues (Glu-256 and Ala-263) are identical to those in NtSUT1y. Nevertheless, αSolSUT1 labels StSUT1 both on western blots (Fig. 1B) and in fixed leaf sections from potato (Fig. 3, D–F).

**Different Abundance of SUT1 Proteins in Abaxial and Adaxial Phloem Suggests Physiological Roles for These Tissues**

The abaxial phloem of the different vein classes in Solanaceae corresponds to the sole phloem in Brassicaceae and other plant families. In contrast to Kühn et al. (1997), who described equal labeling in both the abaxial and the adaxial phloem, we always observed stronger αSolSUT1 signals in the abaxial than in the adaxial phloem of all vein classes with bicollateral phloem (Figs. 2, B and I, and 3, A and F). This may be taken as novel molecular evidence for different physiological roles of these two types of phloem. In contrast to the primary phloem loading, i.e. the loading of newly synthesized Suc that has just been released from the mesophyll that is generally accepted to occur in the single (abaxial) phloem of minor veins (class V), I, P1-anti-StSUT1 on a minor vein (class IV) from a potato source leaf with two minor veins (class V). I, P1-anti-StSUT1 on a minor vein (class IV) from a potato source leaf. Abbreviations: abp, abaxial phloem; adp, adaxial phloem; ue, upper epidermis; xp, xylem parenchyma; xv, xylem vessel(s); xy, xylem. Antibody detection was by anti-rabbit IgG-Cy2 conjugate on 1-μm microtome sections in C to F, on hand-cut sections A, B, and G, or on 5-μm microtome sections in H and I. Bars = 2 μm (C–F), 10 μm (I), 25 μm (G), 50 μm (H), and 100 μm (A and B).
SUT1 or SUT1-Type Transporters Are Present in Xylem Parenchyma

Unexpectedly, our analyses with αSolSUT1 identified SUT1 proteins also in the xylem parenchyma of bicollateral leaf veins and with higher intensity in stem sections (Figs. 2, B and I, and 3, A and F). Again, this result disagreed with previous analyses from Solanaceae. However, it is consistent with reports from other species.

Decourteix et al. (2006) identified a SUT1-type Suc transporter, JrSUT1, in walnut (Juglans regia) xylem parenchyma, which is thought to regulate changes in Suc concentrations during winter (Amélio et al., 2004). Moreover, a SUT1-type Suc transporter (DcSUT2) was found in the xylem parenchyma of carrot (Daucus carota) tap roots (Shakya and Sturm, 1998). It was speculated that Suc might be the primary carbon source for the xylem parenchyma cells and possibly to cells involved in cellulose production and lignification. Alternatively, the identified Suc transporters may have retrieval functions similar to those discussed for Suc transporters in the phloem (Maynard and Lucas, 1982; Stadler et al., 1995; Hafke et al., 2005), and their physiological function might be to keep the xylem Suc free. A direct release of 14C-labeled assimilates from the abaxial or adaxial phloem into the xylem sap has not been observed in previous analyses (Bonnemain, 1968).

What Are the Consequences of These New Localization Data?

Based on the initial report on the localization of SUT1 proteins in enucleate SEs (Kühn et al., 1997), a cell-to-cell transport mechanism from CCs into SEs had to be postulated either for SUT1 proteins or for SUT1 mRNAs. In fact, evidence for mRNA trafficking was provided by in situ localization analyses that revealed accumulation of StSUT1 mRNA at the orifices of the plasmodesmata between CCs and SEs and that detected StSUT1 mRNA within potato SEs. However, it was shown before (Anderson and Cronshaw, 1969) that sequestration of vascular tissue frequently results in pressure release and in an increased hydrostatic flow that...
sweeps ruptured organelles, starch grains, or proteins into the sieve pores of SEs. Most likely, this pressure drop extends into the intimately connected CCs, and their cellular content, including SUT1 mRNAs, may therefore be forced toward the connecting plasmodesmata. This may explain the accumulation of SUT1 mRNA at the orifices of the plasmodesmata connecting CCs and SEs (Kühn et al., 1997).

Clearly, the absence of SUT1 proteins from SEs renders discussions on SUT1 mRNA translation in ribosome-free SEs unnecessary. Nevertheless, Suc transporter mRNAs were found in the phloem sap of several plants (Ruiz-Medrano et al., 1999; Doering-Saad et al., 2002; Roney et al., 2007) and in symplastically linked, nematode-induced syncytia of Arabidopsis (AtSUC2; Jürgensen et al., 2003; Hoth et al., 2005). However, despite this presence of AtSUC2 mRNA, AtSUC2 protein has been found neither in Arabidopsis SEs nor in nematode-induced Arabidopsis syncytia (Stadler and Sauer, 1996; Hoth et al., 2005). Nevertheless, the finding of SUC2 and SUT1 mRNAs in phloem sap plus the identification of various other types of RNAs in the phloem sap (Jørgensen et al., 1998; Yoo et al., 2004; Haywood et al., 2005; Banerjee et al., 2006) show that CC-synthesized mRNAs and other types of RNAs do enter the SEs. It has been shown repeatedly that phloem movement of RNAs is part of a so far not fully understood signaling cascade (Ruiz-Medrano et al., 1999; Yoo et al., 2004).

After the localization of solanaceous SUT1 proteins in the plasma membranes of SEs (Kühn et al., 1997), Suc transporters of the SUT2 (Barker et al., 2000) and SUT4 type (Weise et al., 2000) were also localized in SE plasma membranes of solanaceous species and SUT4 was even discussed as a low affinity/high capacity phloem loader. This colocalization (Reinders et al., 2002a) initiated studies on the physical interaction of these three types of proteins. In fact, coexpression in yeast seemed to support this hypothesis (Reinders et al., 2002a, 2002b; Schulze et al., 2003). However, after the localization of SUT1-type proteins in CCs (this article) and after the recent localization of SUT4-type transporters in vacuoles (Endler et al., 2006), the model of regulatory SUT1, SUT2, and SUT4 interactions is quite unlikely.

Finally, the identification of SE-specific antibodies in numerous rabbit preimmune sera demonstrates that special care has to be taken in immunolabelings of higher plant vasculature. For results suggesting SE-specific localization of a protein, careful controls will be needed.

MATERIALS AND METHODS

Strains and Growth Conditions

Tomato (Solanum lycopersicum) Moneymaker, potato (Solanum tuberosum) Solara, and tobacco (Nicotiana tabacum) Xanthii and Samsun plants were grown in potting soil in the greenhouse under ambient conditions. Leaf material of other species (habanero chili [Capsicum chinense], bell pepper [Capsicum annuum], petunia [Petunia hybridra], physalis [Physalis peruviana], and Solanum rantonnetii) was from field-grown plants. Cloning was performed in Escherichia coli strain DH5α (Hanahan, 1983).

Fusion Protein Isolation and Immunohistochemistry

cDNA sequences representing complete NISUT1 coding sequences were amplified from cDNAs derived from total RNA from Xanthii or Samsun leaf tissue using the following primers: (NISUT1-5′) GAA TTC AAT GGT GTA AAA GAA ATC GAG AAT GGT ACC AAA AAA CTT and (NISUT1-3′) GAA TTC AAT GAT GCT CAT GGT GAT GGA AAC CGC CCA TTC TGG TCG GTT TGG CA. The fragment encoding the NiSUT1x 43-amino-acid peptide was amplified with primers (NiSUT1x 5′) GAA TTC CGC GAA AAC GAG CTC CCC GAA and (NiSUT1x 3′) GGA TCA ACA ACA GCC CCA AA that should amplify NiSUT1x, NiSUT1y, and NiSUT1z, and the PCR products were sequenced directly. To enhance amplification of NiSUT1x, PCRs were eventually performed with the primers NiSUT1xREF889 (GCG TCA TTC CCG GCG AAA AGC G) and NiSUT1xREF770 (AAG AAC GGG ACT TTT GAT TTT CCG G). The 3′ ends of these primers were designed to span the predicted gaps after positions 168 and 256 in the NiSUT1x protein sequence (Supplemental Fig. S1).

For affinity purification of a NiSUT1x nitrocellulose filters soaked with MBP-NiSUT1x fusion peptide (1 mg peptide mL−1) and blocked with skim milk-containing buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 1% skim milk powder) were incubated in raw antiserum for at least 60 min at 4°C, washed, and bound antibodies were released as described (Sauer and Stadler, 1993). Antibodies against the MBP were removed from this solution in a second round using nitrocellulose filters that had been soaked with unfused MBP.

Preparation of Yeast Plasma Membranes, Gel Electrophoresis, and Western Blots

Plasma membrane preparation and protein extraction was as described (Sizol et al., 1994) and separated proteins (Laemmli, 1970) were transferred to nitrocellulose filters as published (Dunn, 1986).

Tissue Sectioning, Fixation, and Embedding

Immunolocalizations in free-hand sections of nonembellished leaf tissue or in microtome sections of methacrylate-embedded tissue as well as DAPI stainings were performed as described (Stadler and Sauer, 1996) using ethanolo:acetic acid (3:1) for fixation. For selected controls, fixations were performed with 0.1% glutaraldehyde and 4% formaldehyde instead. Antibody detection was by anti-rabbit IgG-Cy3 (red fluorescence; Dianova) or by IgG-Cy2 conjugates (green fluorescence; Dianova) on microtome and hand-cut sections.

Confocal Microscopy

Antibody-decorated sections were imaged using a confocal laser scanning microscope (Leica TCS SP II; Leica Microsystems). CY2 antibody conjugate was excited by 488-nm light, and fluorescence was observed using a detection window from 495 to 530 nm. CY3 antibody conjugate was excited by 543-nm light, and fluorescence was observed in a detection window from 552 to 617 nm. Cell wall autofluorescence was excited by 488-nm light and detected in a window of 560 to 640 nm. DAPI fluorescence was excited using a 405-nm diode and observed using a detection window of 425 to 475 nm.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AM491605 for NISUT1x (Xanthii), FM164640 for NISUT1x (Samsun), FM164638 for NISUT1y (Xanthii), and FM164639 for NISUT1z (Samsun).

Supplemental Data

The following materials are available in the online version of this article.
Supplemental Figure S1. Alignment of tobacco SUT1-type sequences and comparison with selected residues in SUT1 proteins from other Solanaceae.

Supplemental Figure S2. Control analyses with affinity-purified preimmune serum.

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This article is dedicated to Prof. Widmar Tanner on the occasion of his 70th birthday.

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LITERATURE CITED


**Figure S1.** Alignment of tobacco SUT1-type sequences and comparison with selected residues in SUT1 proteins from other Solanaceae.
Comparison of the NtSUT1x-type sucrose transporter sequences from *N. tabacum* cv. Xanthii (NtSUT1x-X) and *N. tabacum* cv. Samsun (NtSUT1x-S) with the NtSUT1y sequences from the same cultivars (NtSUT1y-X and NtSUT1y-S), and with the partial sequence from a tobacco hybrid (*N. langsdorffii* x *N. sanderae*; NlxsSUT1).

Sequences were aligned with the program BESTFIT of the Wisconsin Package Version 10.2 (Genetics Computer Group, Madison, Wisconsin, USA). The fragment of the NtSUT1x protein from *N. tabacum* cv. Xanthii that was used to raise αSolSUT1 is highlighted in yellow, predicted transmembrane regions of the SUT1 proteins are shown in grey. Differences in NtSUT1a and all other tobacco SUT1 proteins are highlighted in green, amino acid residues of the respective positions in LeSUT1 and in StSUT1 are shown. Residues allowing a clear discrimination between NtSUT1x and NtSUT1y are shown in red and characterize NtSUT1a as an NtSUT1x transporter and NlxsSUT1 as an NtSUT1y transporter. N-terminal and C-terminal residues that were forced by the NtSUT1a-derived PCR primers are shown in blue.
**Figure S2.** Confocal sections obtained from incubation of free-hand sections, A and B, or microtome sections, C and D, from tobacco source leaves with affinity-purified pre-immune serum (= αPIS), A and C, or with affinity-purified αSolSUT1, B and D. Photographs of αPIS-treated and αSolSUT1-treated section were taken under identical condition.


To exclude the possibility that labeling of companion cells (CCs) with affinity-purified αSolSUT1 antiserum results from antibodies that (i) are already present in
the preimmune serum, that (ii) recognize an unknown, non-SUT1 epitope in CCs and that (iii) are enriched during affinity purification, an identical affinity-purification was performed with the preimmune serum (PIS). Whereas αSolSUT1-treated sections showed the expected labeling of the abaxial phloem and the xylem parenchyma, no signals were detected in αPIS-treated sections.