SUT2, a Putative Sucrose Sensor in Sieve Elements

Laurence Barker, Christina Kühn, Andreas Weise, Alexander Schulz, Christiane Gebhardt, Brigitte Hirner, Hanjo Hellmann, Waltraud Schulze, John M. Ward, and Wolf B. Frommer

In leaves, sucrose uptake kinetics involve high- and low-affinity components. A family of low- and high-affinity sucrose transporters (SUT) was identified. SUT1 serves as a high-affinity transporter essential for phloem loading and long-distance transport in solanaceous species. SUT4 is a low-affinity transporter with an expression pattern overlapping that of SUT1. Both SUT1 and SUT4 localize to enucleate sieve elements of tomato. New sucrose transporter–like proteins, named SUT2, from tomato and Arabidopsis contain extended cytoplasmic domains, thus structurally resembling the yeast sugar sensors SNF3 and RGT2. Features common to these sensors are low codon bias, environment of the start codon, low expression, and lack of detectable transport activity. In contrast to LeSUT1, which is induced during the sink-to-source transition of leaves, SUT2 is more highly expressed in sink than in source leaves and is inducible by sucrose. LeSUT2 protein colocalizes with the low- and high-affinity sucrose transporters in sieve elements of tomato petioles, indicating that multiple SUT mRNAs or proteins travel from companion cells to enucleate sieve elements. The SUT2 gene maps on chromosome V of potato and is linked to a major quantitative trait locus for tuber starch content and yield. Thus, the putative sugar sensor identified colocalizes with two other sucrose transporters, differs from them in kinetic properties, and potentially regulates the relative activity of low- and high-affinity sucrose transport into sieve elements.

INTRODUCTION

Sucrose, the major product of photosynthesis in mature leaves, is loaded into the vascular tissue for translocation to heterotrophic tissues to support their growth. In solanaceous plants, SUT1 is essential for phloem loading into sieve elements (Riesmeier et al., 1994; Kühn et al., 1996; Bürkle et al., 1998). SUT1 serves as a high-affinity transporter for sucrose ($K_m \approx 1$ mM; Riesmeier et al., 1993), whereas SUT4, with a $K_m$ of $\approx 11$ mM, is a low-affinity sucrose transporter (Weise et al., 2000). Both proteins colocalize in sieve elements (Kühn et al., 1997; Weise et al., 2000).

Localization of SUT1 protein in sieve elements and SUT1 mRNA at the orifices of plasmodesmata interconnecting companion cells and sieve elements, together with the high turnover of both SUT1 mRNA and protein, indicate that trafficking of mRNA or protein occurs from companion cells into enucleate sieve elements by way of plasmodesmata (Kühn et al., 1997).

Sugar transport is highly regulated, and sucrose-specific signaling pathways are involved in controlling transport activity (Chiou and Bush, 1998), potentially by using protein phosphorylation (Roblin et al., 1998). Overexpression of pyruvate decarboxylase in potato leads to a 10-fold increase in sugar export, demonstrating the capacity to regulate sugar export from leaves within a wide dynamic range (Tadege et al., 1998). This poses the question of how regulation is coordinated between sieve elements that contain the transporters and companion cells in which transcription takes place (Lalonde et al., 1999).

Yeast, for which the study of transporter regulation is much further advanced, may provide hints as to the general mechanism of regulation of sugar transport. Complex regulatory networks control uptake of nutrients in response to intracellular needs and a rapidly changing external environment. For metabolism of sugars, yeast has developed a two-step regulatory system to ensure coordination between the external availability of sugars and the enzymatic machinery inside cells: (1) the extracellular concentration of sugars is sensed, and sugar transport activity is regulated accordingly; and (2) sugar transport activity determines the flux of sugars into the cell, subsequently generating intracellular signals for adapting metabolism (Lalonde et al., 1999).
The 18 yeast monosaccharide transporters found thus far span a wide range of affinities, and thus, depending on the external sugar availability, the kinetic parameters of uptake can be adapted accordingly, for instance, by changing from low to high affinity if sugar availability drops (Lalonde et al., 1999; Wieczorke et al., 1999). HXT2 and HXT7, for example, which serve as high-affinity glucose transporters, are induced by low concentrations of glucose but are repressed at high concentrations, whereas the low-affinity transporter HXT1 is induced by high concentrations of glucose (Özcan and Johnston, 1995; Boles and Hollenberg, 1997). Consequently, the sensors for extracellular glucose must respond not only to the kind of carbon source but also to its concentration.

Glucose is sensed by SNF3 and RGT2, which trigger the induction of hexose transporter genes based on the glucose concentration (Liang and Gaber, 1996; Özcan et al., 1996b). These two proteins resemble glucose transporters but, in addition, possess unusually long C-terminal domains that reside in the cytoplasm (Özcan et al., 1996a). The large C-terminal extension of SNF3 (303 amino acids) contains two nearly identical repeats of 25 amino acids. One of these repeats is also present in RGT2. The C-terminal domains of SNF3 and RGT2 mediate the signaling function of the proteins (Özcan et al., 1998; Vagnoli et al., 1998). Deletion of these extensions leads to a loss of sensor function. Attachment of the SNF3 C terminus to glucose transporters HXT1 and HXT2 confers the ability to signal glucose availability. Apparently, SNF3 is a sensor of low glucose concentrations that mainly regulates expression of high-affinity glucose transporters, whereas RGT2 is responsible for sensing high glucose concentrations and regulating expression of low-affinity transporters. Consequently, induction of the high-affinity transporter HXT2 by low concentrations of glucose is absent in snf3 mutants, and induction of the low-affinity transporter HXT1 by high concentrations of glucose is strongly reduced in rgt2 mutants. In addition, SNF3 is required for repression of the high-affinity transporters HXT2, -6, and -7 at high glucose concentrations (Liang and Gaber, 1996; Vagnoli et al., 1998). Despite the high homology of SNF3 and RGT2 to glucose permeases, neither appears to be able to mediate substantial glucose transport (Liang and Gaber, 1996; Özcan et al., 1998).

Little is known about sugar sensing and signaling with respect to transport of sucrose and glucose in plants (Hellmann et al., 2000). Yeast thus may serve as a model to improve our understanding of the role of sugar transporters in sugar sensing. The existence of plasma membrane–bound sensing pathways has been suggested from physiologic analyses using nonmetabolizable glucose analogs (Martin et al., 1997; Roitsch, 1999). Furthermore, a genetic approach has led to identification of sugar-regulated signaling cascades that modulate sugar uptake (Hellmann et al., 2000).

To investigate whether plants contain multiple sucrose transporters present in sieve elements and to identify putative sugar sensors, we initiated a search for additional sucrose transporter-like genes. A new sucrose transporter-like protein, SUT2, was identified that colocalizes with low- and high-affinity sucrose transporters in enucleate sieve elements of tomato and shares features with yeast sugar sensors. Most importantly, SUT2 is a candidate gene for a major quantitative trait locus (QTL) for tuber starch and yield in potato, the genome of which is colinear with that of tomato (Gebhardt et al., 1991).

RESULTS

Isolation of Sucrose Transporter cDNAs

Two novel SUT homologs were identified from tomato by low-stringency hybridization and reverse transcription-polymerase chain reaction (PCR). LeSUT1 is highly similar to StSUT1 from potato (94% similarity of amino acid sequences) and appears to be the ortholog of StSUT1 from potato (Kühn et al., 1997). LeSUT2, with a cDNA of 2144 bp, encodes a 65-kD protein with unique features not reported previously for SUT homologs. SUT2 has an N-terminal domain ~30 amino acids longer than the sucrose transporters already identified (see Lalonde et al., 1999), and its extended central cytoplasmic loop is ~50 amino acids longer (Figure 1A). According to high-stringency DNA gel blot analysis, LeSUT2 does not have a closely related paralog in the tomato genome (Figure 2B). Screening bacterial artificial chromosome and expressed sequence tag databases led to the identification of a putative ortholog of LeSUT2 in Arabidopsis, AtSUT2, that contains the same two extended domains (Figure 1B). The SUT2s are 82% similar (68% identical), and the genes are structurally related, that is, the intron–exon borders and the number of introns are conserved (Figure 2A). Besides the domains that are conserved in all sucrose transporter–like proteins, LeSUT2 and AtSUT2 share highly conserved sequence motifs within the extended central loop, suggesting a functional similarity between AtSUT2 and LeSUT2 as well (Figures 1B and 1C).

Similar to the yeast hexose sensors, both SUT2 proteins are characterized by low codon bias and low homology with Kozak consensus (Kozak, 1996), indicating a low translation efficiency (Iraqui et al., 1999; J.M. Ward, unpublished results).

Expression of SUT Genes in Yeast

LeSUT2 and AtSUT2 were cloned into the yeast expression vector pDR195 (LeSUT2) and pDR196 (AtSUT2) for functional characterization (Rentsch et al., 1995). LeSUT1 complemented the yeast mutant SUSY7/ura3 (Riesmeier et al., 1992), consistent with being the ortholog of SUT1 (Figure 3A), whereas LeSUT2 and AtSUT2 were unable to comple-
ment the mutant. Similar results were obtained when 5' and 3' untranslated regions of LeSUT2 were removed and a different vector (YEpLac112A1NE) was used (Riesmeier et al., 1992). For all constructs, a variety of complementation conditions was tested, including sucrose concentrations between 0.5 and 2% and the pH of the medium ranging from 4.0 to 7.0.

To verify expression of LeSUT2, we raised polyclonal antibodies against synthetic peptides of LeSUT2 and used them for protein gel blot analysis. Specificity of the antisera was verified on membrane proteins isolated from yeast cells expressing LeSUT2. LeSUT2 migrates as a larger protein than LeSUT1, as their sequences would predict, and appears as a doublet at ~65/67 kD, possibly because of post-translational modifications in yeast (Figure 3B). No signals were detected in yeast transformed with the empty vector. Antibodies raised against SISUT1 located LeSUT1 in plasma membranes that had been purified from tomato leaves and showed it to migrate at an apparent molecular mass of 46 kD (Figure 3B). Sequences chosen for antisera production were unique. Lack of cross-reactivity with known LeSUTs was confirmed by immunolocalization in yeast (Figure 4).

**Expression of Sucrose Transporter Genes in Tomato**

RNA gel blot analysis was performed to investigate organ-specific expression of LeSUT1 and LeSUT2. LeSUT1 was most highly expressed in source leaves, similar to its ortholog in potato (Riesmeier et al., 1993), with lower expression
in all other organs investigated (Figure 5A). Expression of SUT2 was low in general but was higher in sink leaves and stems than in source leaves.

Because sucrose transporter activity has been shown to be regulated by sugars in sugar beet (Chiou and Bush, 1998), the regulatory effect of sugars on sucrose transporter expression was investigated in tomato and potato. SUT2 was induced in the presence of 100 mM sucrose in source leaves from potato. SUT1 expression remained unaltered, whereas patatin (a storage protein expressed in potato tubers) expression was induced primarily by sucrose and to a lesser extent by glucose (Figure 5B). In tomato, the sucrose induction of LeSUT2 was seen in sink leaves but not in source leaves (data not shown). Again, SUT1 expression was unaltered in the presence of sucrose.

Colocalization of SUT1 and SUT2 in Tomato Petioles and Stems

For localization of LeSUT1 and LeSUT2 in tomato, antisera were affinity purified and used for immunocytochemistry. Both transporters were localized exclusively in sieve elements, as was LeSUT4 (Weise et al., 2000). The presence of sieve plates, together with the absence of nuclei, allowed unambiguous identification of sieve elements (Figures 6A, 6B, and 6D). LeSUT1 was present in sieve elements of source-leaf minor veins (Figure 6H), whereas SUT2 was detected in the midrib of sink and source leaves but not in minor veins (Figures 6F and 6G). All three transporters—LeSUT1, LeSUT2 (Figures 6A and 6B), and LeSUT4—colocalized in mature sieve elements of stem and petiole. Elec-
putative sucrose sensor 1157

Tron microscopic immunogold labeling clearly showed that LeSUT2 is present at the plasma membrane (Figure 6J). Pre-immune serum, purified on protein A-Sepharose and used at the same protein concentration as LeSUT2, caused weak cytoplasmic background labeling, although not at plasma membranes (Figure 6K).

A series of control experiments was performed to exclude any bias from nonspecific binding. This was necessary because of the preferential binding of immunoglobulins to sieve elements at high concentrations, which was detectable after long exposures. No immunofluorescence was detectable with IgG-enriched purified preimmune serum (Figure 6E), nor after incubation with the secondary antibody alone (data not shown). Confirmation that purified antisera were specific resulted from immunolocalization on spheroplasted yeast cells transformed with transporter genes or an empty expression vector (Figure 4). Localization with a second antisera against different domains of SUT1 and SUT2 further confirmed results obtained with the first antisera. Similar results were obtained when tissue was embedded in acrylic resins after conventional fixation or when tissue was prepared by high-pressure freezing (data not shown). Antibody-peptide competition experiments also were performed to exclude nonspecific antisera binding. Fluorescent signals resulting from SUT2 detection were strongly reduced or abolished after preincubating the SUT2 antisera with its corresponding peptide (Figure 6C). However, preincubation with a peptide corresponding to another epitope of the same transporter or to a control peptide had no effect on SUT2 signals (Figure 6D).

AtSUT2 Expression in Transgenic Plants

Transgenic Arabidopsis plants expressing the reporter gene for β-glucuronidase (GUS) under control of a 1.2-kb AtSUT2 promoter fragment showed GUS staining in the vascular tissue of the petiole and in the major and second-order veins of source leaves. Among 10 hygromycin-resistant transformant lines, nine showed the same GUS expression. In a few cases, weak staining of third-order veins was detectable. Staining also was observed in trichomes of source leaves and in hydathodes (Figure 6I). For the longer 2.2-kb promoter-GUS construct, only two transformants were analyzed, both showing the same GUS expression pattern as seen for the shorter construct.

Map Positions of SUT1, SUT4, and SUT2 Genes in Potato

When used as restriction fragment length polymorphism (RFLP) markers on the potato molecular map, the three cDNA clones each mapped to a separate locus. StSUT1 identified a locus (SUT1) on linkage group XI, whereas LeSUT4 detected an unlinked locus (SUT4) on linkage group V (Figure 7).

**DISCUSSION**

The mechanisms for regulation of sucrose transport during loading and unloading are not fully understood. Sucrose uptake kinetics determined in plasma membrane vesicles are multiphasic, suggesting the existence of multiple carrier systems (Lemoine et al., 1996). Theory also predicts additional carriers in several different cell types, including sucrose.
import and export systems (Lalonde et al., 1999). In addition to SUT1, three other sucrose transporters have been identified in solanaceous species: LeSUT4, which appears to play a role in low-affinity sucrose transport (Weise et al., 2000); NtSUT3, a carrier involved in pollen nutrition (Lemoine et al., 1999); and SUT2, a putative sucrose sensor. All sucrose transporters that have been isolated have a predicted topology of 12 transmembrane domains. SUT2 has not only this predicted structure but also two additional features: extended N-terminal and central loop domains that average 30 and 50 amino acids longer, respectively, than those of other SUT family members. According to hydrophobicity analysis and experimental evidence for PmSUC2 (Stolz et al., 1999), the N terminus and central loop are localized intracellularly, which allows these extended domains to interact with proteins in the cytoplasm. Another characteristic of SUT2 is the low similarity to the Kozak consensus for high rates of translation initiation (Kozak, 1996) and a low codon bias, indicating low translatability.

In contrast to other members of the SUT family, SUT2 does not complement the yeast mutant that is deficient in sucrose uptake, and its expression in yeast appears to cause toxicity. Even in a more sensitive selection system, one deficient in hexose uptake, which thus leads to reduced background growth, SUT2 is still nonfunctional. Although an apparently full-length SUT2 protein can be detected by protein gel blot analysis, the data do not exclude the possibility that nonfunctionality is the result of insufficient targeting to yeast plasma membranes or that the large central loop acts as an autoinhibitory domain.

Putative Sensor Function of SUT2

In tomato, SUT2 was localized to sieve elements, in which it colocalizes with the high- and low-affinity sucrose transporters SUT1 and SUT4, respectively. SUT2 is regulated by sucrose, adding to other evidence for the sugar regulation of sucrose transporters at the plasma membrane level (Chiou and Bush, 1998). In general, SUT2, which shows many features similar to the yeast sugar sensors SNF3 and RGT2, may be involved directly in controlling expression, activity, and turnover of the other two sucrose transporters SUT1 and SUT4 and thereby may act to control sucrose fluxes across the plasma membrane of sieve elements.

Interestingly, the hexose transporter family from Arabidopsis also contains several transporter-like members that contain extended cytosolic loops, which thus represent potential candidates for hexose sensors (Lalonde et al., 1999). An emerging mechanism found in many organisms for various substrates is the presence of transporters or transporter-like proteins involved in sensing and regulating the

Figure 4. Specificity of the LeSUT2 Antiserum Shown by Yeast Immunofluorescence.

LeSUT1, LeSUT2, or the empty vector pDR195 was expressed in SUSY7/ura3, the cells were fixed, and SUT proteins were detected with affinity-purified antisera. No cross-reactivity of the SUT1 antiserum on SUT2-expressing yeast or SUT2 antiserum on SUT1-expressing yeast was detected. No signals were detected on yeast expressing pDR195.

Figure 5. Expression of SUT2.

(A) Organ-specific expression of LeSUT1 and LeSUT2 (20 μg/lane) in stringent hybridization conditions. Probes were the full-length cDNA of LeSUT1 and a 1.3-kb fragment of LeSUT2.

(B) Comparison of SUT1, SUT2, and patatin expression under different treatments in potato. SUT2 was specifically induced after treatment with sucrose (100 mM), whereas SUT1 expression remained unchanged. Both sucrose and to a lesser extent glucose (100 mM) induced patatin expression. Sorbitol also was used as an osmotic control at a concentration of 100 mM.
Figure 6. Immunocytochemical Localization of SUT Proteins.

(A) Immunofluorescent detection of SUT1 in enucleate sieve elements of tomato stems, colocalized with the aniline blue-stained callose in the sieve plate. DAPI-stained nuclei are visible in neighboring phloem cells.

(B) SUT2 colocalized with aniline blue-stained sieve plate callose in enucleate sieve elements of stem.

(C) and (D) Loss of LeSUT2 signal after incubation of SUT2 antiserum with its corresponding peptide (C). No reduction in signal after incubation of SUT2 antiserum with a control peptide (D).

(E) No signals detected with preimmune serum; aniline blue-stained sieve plate callose indicates presence of sieve element.

(F) No immunofluorescence labeling detectable in source-leaf minor veins of tomato plants treated with anti-LeSUT2 antiserum.

(G) Bright-field view of the same section as in (F), showing minor vein anatomy.

(H) LeSUT1 detectable in source-leaf minor veins on a serial section as in (F).

(I) Source leaf of a transgenic Arabidopsis plant transformed with the 1.2-kb AtSUT2 promoter-GUS fusion construct. GUS staining is detectable in first-, second-, and third-order veins but not in minor veins.

(J) Immunogold labeling of the sieve element plasma membrane in a petiole cross-section by using anti-LeSUT2 antibodies.

(K) Control treated with IgG-enriched preimmune serum on a serial section as in (J).

Bar in (A) to (H) = 40 μm; bar in (J) and (K) = 500 nm. n, nucleus; pp, phloem parenchyma cell; se, sieve element; sp, sieve plate; w, cell wall.
activity of carriers according to substrate requirement, such as the bacterial iron/citrate transporter/sensor FecA, the yeast amino acid sensor Ssy1, and the putative plant metal sensor EIN2 (Boles and Hollenberg, 1997; Alonso et al., 1999; Braun and Killmann, 1999; Iraqui et al., 1999).

High conservation of central sequence motifs within the extended cytoplasmic loops of SUT2 proteins (CCB1 and CCB2) suggests an important role for SUT2 function. Extended cytoplasmic domains could represent domains for effector binding or signal transduction. For example, the CCB2 domain contains a serine residue at a highly conserved position, which is predicted to be the target for regulation by phosphorylation by a serine/threonine kinase. Sucrose transport already has been described to be regulated by phosphorylation (Roblin et al., 1998).

**SUT2 Trafficking into Sieve Elements**

The present study of SUT2 localization together with a parallel analysis of SUT4 has shown that three SUT proteins, falling into three clusters, colocalize in sieve elements (Figure 1C; Weise et al., 2000). SUT1 is likely to be transported from the companion cell to the sieve element, probably in the form of mRNA, given that both its mRNA and protein turn over rapidly (Kühn et al., 1997). Previous studies showing that SUT1 mRNA localizes to the orifices of plasmodesmata both in companion cells and in sieve elements lead to the suggestion that all three SUTs use similar trafficking mechanisms for their mRNA. The mechanism may involve receptor-mediated transport of RNA, similar to that occurring in Drosophila oocytes (St. Johnston, 1995) possibly involving a CmPP16 homolog that has been shown to increase SUT1 mRNA mobility between mesophyll cells (Xoconostle-Cázares et al., 1999). Although the structural requirements of the mRNA that allow for plasmodesmal movement have not been identified, investigation into the involvement of cis elements or a common RNA structure in the three SUT genes may help reveal such a mechanism.

**SUT2 Is Expressed in the Transport but Not in the Loading Phloem**

Expression of the *AtSUT2* promoter reporter gene fusion was found in the vascular tissue of leaf major veins in trichomes and in hydathodes but not in minor veins, the places of phloem loading. These data are consistent with the immunolocalization data of LeSUT2. Different parts of the phloem were classified according to their different functions into the collection phloem, the transport phloem, and the delivery phloem (van Bel, 1996). Anatomic characteristics reflect the different tasks of the phloem (i.e., the size relationship between the companion cells and the sieve elements: sieve elements of the transport phloem, to which a retrieval function was assigned, are bigger than the neighboring companion cells). Thus, differences in the localization of SUT1, which is strongly expressed in source-leaf minor veins (Riesmeier et al., 1993; Kühn et al., 1997), and SUT2, which is localized in the transport phloem, indicate a strikingly different function of the two sucrose transporter-like proteins.

**Linkage of SUT Genes to QTLs for Tuber Starch Content and Tuber Yield**

Transport of sucrose from source leaves to sink tubers is essential for starch accumulation in developing tubers.
Genetic variability of the genes controlling sucrose transport therefore may have a quantitative effect on the starch content of mature tubers. QTLs for tuber starch content and tuber yield have been positioned on the potato RFLP map. QTLs for tuber yield and tuber starch content are linked, suggesting that at least some factors controlling tuber starch content have pleiotropic effects on tuber yield (Schäfer-Pregl et al., 1998). Given their essential physiologic role, genes controlling sucrose transport are candidates for QTLs controlling tuber starch content. If alleles at one or more transporter or sensor loci indeed are responsible for a QTL effect on tuber starch content, tight genetic linkage will be observed between the QTL and the candidate gene locus. Therefore, the positions of SUT loci were compared with the positions of QTL for starch content and tuber yield.

Three unlinked SUT loci were identified in the potato molecular map: SUT4 on linkage group IV, SUT1 on linkage group XI, and SUT2 on linkage group V. On linkage group IV, QTLs for tuber starch content are located in the interval flanked by RFLP loci GP180(a) and GP261(a) and are closely linked to the branching enzyme locus BE (Schäfer-Pregl et al., 1998). SUT4, however, maps in between the two QTLs (Figure 7). Similarly, a minor QTL for tuber starch content is linked to marker loci GP125 and UGPase (UDP-glucose pyrophosphorylase) on linkage group XI (Schäfer-Pregl et al., 1998), whereas SUT1 occupies a more proximal position on the same linkage group (Figure 7). On linkage group V, a major QTL for tuber starch content and tuber yield is linked to markers GP179 and GP291(a) (Schäfer-Pregl et al., 1998). The SUT2 locus is tightly linked to the same markers (Figure 7) and therefore to this QTL. This linkage suggests that (1) SUT2 alleles may play an important role in controlling tuber starch content in the field and (2) SUT2 alleles may have pleiotropic effects on tuber starch content and tuber yield. Further work is required to confirm this important role, because linkage analysis cannot exclude the possibility that the gene controlling the QTL is linked, but is functionally unrelated to, the candidate gene locus SUT2.

METHODS

LeSUT Genes and Expression Analysis

Screening a flower cDNA library from Lycopersicon esculentum cv UC82b with an NISUT3 genomic fragment (Lemoine et al., 1999) under low stringency led to the isolation of 15 LeSUT1 cDNA clones. The partial genomic sequence of LeSUT2 was isolated by screening a genomic library from L. esculentum cv VF88 in EMBL-3 (Clontech, Palo Alto, CA). By hybridization with SISUT1 under nonstringent conditions, 11 positive λ phages were obtained. One of the more weakly hybridizing λ phages (17/2) represented LeSUT2. A 2.1-kb EcoRI fragment was used to identify the LeSUT2 cDNA from a leaf cDNA library from L. esculentum VC82b, in λ-ZAPXR (Stratagene).

The partial genomic LeSUT2 clone was characterized further by using a 2.1-kb XhoI fragment, overlapping the LeSUT2 cDNA from positions 274 to 604 upstream of the ATG, and a 4-kb genomic XhoI fragment, containing sequences from position 802 to the end of the LeSUT2 DNA. The gap in the genomic sequence between the two fragments was filled by polymerase chain reaction (PCR) of the DNA of the λ-phage isolate 17/2 with the use of appropriate primers.

RNA was isolated from different organs of greenhouse grown L. esculentum Moneymaker, as described (Schwacke et al., 1999). RNA gel blots were hybridized in 50% formamide at 42°C and washed with 0.2 × SSC/0.1% SDS at 65°C (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate). For DNA gel blot analysis, genomic DNA from L. esculentum was digested with restriction enzymes, resolved on 1% agarose gels, and blotted to Hybond N+ (Amersham Pharmacia Biotech). Hybridization was performed with 32P-labeled probes under high stringency, followed by washing at 65°C in 0.2 × SSC/0.1% SDS. The LeSUT2 sequence was deposited in GenBank under the accession number AF166498.

ATSUT2 Analysis

The cDNA of ATSUT2 was cloned by reverse transcription-PCR from Arabidopsis thaliana Columbia (Col-0) ecotype leaves. After reverse transcription of 5 μg of total leaf RNA, PCR was performed by using specific primers with the cloning sites EcoRI (f) and XhoI (r), and the product was cloned into the yeast expression vector pDR196. The genomic sequence of ATSUT2 is in GenBank under the accession number AC004138.

The ATSUT2 promoter fragment was isolated by PCR on A. thaliana Col-0 ecotype genomic DNA by using Pfu-Polymerase (Stratagene). A transcriptional fusion was generated by cloning 2.2- and 1.2-kb promoter fragments into pGPTV-HPT (Becker et al., 1992). Arabidopsis was transformed by vacuum infiltration of transformed Agrobacterium GV2260 in 0.5× Murashige and Skoog medium (Murashige and Skoog, 1962) containing 5% sucrose, 0.005% Silwet, and 0.044 μM benzylaminopurine (Clough and Bent, 1998). Selection was performed on Murashige and Skoog medium containing 50 μg/L hygromycin. Plant material was infiltrated under vacuum with 2 mM 5-bromo-4-chloro-3-indolyl-glucuronide, 50 mM sodium phosphate buffer, pH 7.2, and 0.5% Triton X-100 and was incubated at 37°C for 12 to 16 hr (Jefferson et al., 1987). Destaining was performed in 70% ethanol.

Sugar Induction

Plants were grown in the greenhouse with a 16-hr-light/8-hr-dark cycle, and leaves were harvested from 3-month-old plants. Petioles of detached leaves were re-cut while submerged in water, EDTA (2.5 mM) was added to inhibit callose formation, and the cut petioles were transferred to solutions containing 100 mM sugar, where they were kept for 24 hr under greenhouse conditions. All experiments were repeated independently.

Yeast Strain Construction

To construct SUSY7/ura3, we digested Yep24 (Biolabs, Schwabach, Germany) with Ncol and Apai (which deleted an internal 137-bp fragment of the URA3 gene), and then Yep24 was treated with T4 polymerase and blunt-ligated, which yielded pURA3 (W.N. Fischer and W.B. Frommer, unpublished results). The truncated URA3 gene
was excised with EcoRI and SmaI, and the linearized Dura3 fragment was used for transformation of SUSY7 (Riesmeier et al., 1992). The Dura3 mutants were selected on 5-fluoroorotic acid. Deletion of the internal URA3 fragment was controlled by PCR (data not shown).

Membrane Isolation and Protein Gel Blot Analysis

Yeast cells were grown in minimal medium (200 mL) to an OD600 of ^1.1. Cells were harvested at 1000g, washed in distilled water, and then washed in 25 mM Tris-HCl, pH 7.5, containing 5 mM EDTA. Cells were resuspended in 100 mL of the same buffer. In a small glass tube, 1 g of glass beads (0.5 mm in diameter) was added, mixed with 3 mL of protease inhibitor mix (0.1 M phenylmethylsulfonyl fluoride and 0.25 M p-aminobenzamidine in DMSO), and vortex-mixed four times for 30 sec each. The cell homogenate was centrifuged for 5 min at 1000g, and the membranes were pelleted from the supernatant by ultracentrifugation at 100,000g for 60 min. The sediment was resuspended in 480 mL of a solution of 100 mM sodium phosphate, pH 8.0, 50 mM NaCl, 2.5% β-dodecylmaltoside containing 1:100 (w/v) protease inhibitor mix, and the membranes were solubilized for 5 min on ice. Plant plasma membranes were purified as described (Larsson, 1985). SDS-PAGE and protein gel blotting have been described previously (Lemoine et al., 1996). Immunodetection was by electrochemiluminescence (Amersham).

Immunocytochemistry

Rabbits were immunized with synthetic peptides coupled to keyhole limpet hemocyanin corresponding to the N terminus or central loop of LeSUT2 (N terminus: MDAVSRVPYKLKC; central loop: PKN-EEQPRDKDQGDS). Affinity purification was performed as described (Lemoine et al., 1996). Preimmune serum was purified as above, except that protein A-Sepharose (Bio-Rad) was used instead of peptide affinity. Antiserum for detection of LeSUT1 prepared against StSUT1 cross-reacts with tomato (Kühn et al., 1997).

Yeast cells prepared for immunolabeling were grown to OD600 ^0.75 and fixed in 3.7% formaldehyde for 15 min at 25°C. Cultures (2 mL) were harvested and resuspended for 30 min at 28°C in 1 mL of 0.1 M potassium phosphate, pH 6.4, containing 3.7% formaldehyde. Cells were washed three times in 0.1 M potassium phosphate, pH 6.4, and once in 1.2 M sorbitol in 0.1 M phosphate citrate buffer, pH 5.9. Cells were incubated in 200 μL of 1.2 M sorbitol/phosphate citrate with 7 μL of zymolase 100T (ICN Biomedicals Research, Eschwege, Germany) (3 mg/mL in 10% glucose) for 60 min at 28°C, which was followed by washing in 1.2 M sorbitol/phosphate citrate, resuspension in 300 μL of 1% BSA in PBS (100 mM sodium phosphate, pH 7.5, and 100 mM NaCl), and incubated overnight at 4°C with primary antibody on an orbital shaker. After three washes in PBS-BSA, Cy3-coupled secondary antibody diluted 1:100 was added in BSA-BSA, and the mixture was incubated for 1 hr on an orbital shaker in darkness. After four washes in BSA-BSA, cells were analyzed by confocal laser scanning microscopy TCP-SP (Leica, Bensheim, Germany).

Fluorescent immunodetection of LeSUTs was performed with modifications according to Kühn et al. (1997). Hand-cut fragments (1 mm²) from tomato leaves, stems, and petioles were fixed overnight under light vacuum in MOPS buffer (50 mM MOPS/NaOH, pH 6.9, 5 mM EGTA, and 2 mM MgCl2) containing 0.1% glutaraldehyde and 6% formaldehyde. After three washes with MOPS buffer on ice, fragments were dehydrated in an ethanol series. After overnight incubation in an equal volume solution of ethanol and methacrylate (75% [v/v] butylmethacrylate, 25% [v/v] methacrylamide, 0.5% benzoine ethyl ether, and 10 mM DTT), the material was embedded in 100% methacrylate. Polymerization took place overnight under UV light (365 nm) at 4°C. Semithin sections (1 μm thick) were mounted on Histobond slides (Camon, Weisbaden, Germany) and dried at 50°C. To remove the methacrylate, we incubated the slides for 30 sec in acetone; they were then rehydrated by an ethanol series and blocked for 1 hr with 2% BSA in PBS. After overnight incubation with affinity-purified antibodies, the slides were washed twice in PBS containing 0.1% Tween (PBS-T) and once with PBS, followed by 1 hr incubation with anti-rabbit IgG–fluorescein isothiocyanate conjugate. After three sequential washes with PBS-T, PBS, and distilled water, photographs were taken with a fluorescence microscope (Axiopt, Zeiss) using fluorescein isothiocyanate filters. For double-staining of transporters and nuclei with 4,6-diamidino-2-phenylindole (DAPI), sections were treated as described above. After three washes, slides were incubated for 1 hr in 0.2 μg/mL DAPI. DAPI fluorescence was detected at 365 nm. For double staining of transporters and sieve plate calllose with aniline blue, slides were incubated for 10 min in 0.05% aniline blue in 50 mM sodium phosphate, pH 7.2. Aniline blue fluorescence was detected at 365 nm. For antibody-peptide competition experiments, affinity-purified antiserum was incubated, before immunolocalization, for ~16 hr at 4°C, either with its corresponding peptide or with a peptide mimicking another epitope of the same transporter or control peptide (CAFGDIPDSKLRS), at 10× molar excess.

For electron microscopic immunogold staining, petiole material of tomato plants was fixed under vacuum for 12 hr with 4% formaldehyde/0.1% glutaraldehyde in 100 mM Pipes, pH 7.2. After rinsing in 100 mM Pipes, tissue was dehydrated in ethanol and embedded in acrylic resins (Uniloc, Plano, Wetzlas, Germany; or LR White; London Resin Co., Berkshire, UK). Polymerization took place at 60°C over 2 days. Ultrathin sections (100 nm) were stretched with chloroform and mounted on gold grids (Gilder-Grids 400 Hex; Plano), after which the grids were incubated for 10 min in 0.1 M HCl and washed with distilled water. After blocking for 1 hr in 2% (v/v) BSA in PBS, grids were incubated with affinity-purified LeSUT2 antibodies diluted 1:4 in PBS in a humid chamber at 4°C overnight. Sections were washed with PBS-T and PBS and were incubated for 1 hr with 10-nm gold-coupled goat anti-rabbit IgG (Amersham) diluted 1:50 in PBS. After three washes in PBS-T and PBS, the sections were postfixed for 10 min in 2% glutaraldehyde in PBS and rinsed with distilled water. Ultrathin sections were poststained with uranyl acetate and lead citrate for better tissue contrast. Gold labeling was detected with a Philips CM 100 microscope (Fellbach, Germany).

Mapping

Restriction fragment length polymorphism (RFLP) analysis and mapping of cDNA clones StSUT1, LeSUT4, and LeSUT2 were performed in the diploid potato mapping population BC916² of 67 individuals as described (Gebhardt et al., 1991).

ACKNOWLEDGMENTS

We thank W.N. Fischer for pDura and SUSY7/ura3 and Lukas Bürkle for help with LeSUT characterization. We are very grateful to Nicole


Received February 10, 2000; accepted April 28, 2000.

REFERENCES


