Plant Physiol. (1996) 110: 511–520

Molecular Cloning, Immunochemical Localization to the Vacuole, and Expression in Transgenic Yeast and Tobacco of a Putative Sugar Transporter from Sugar Beet

Tzyy-Jen Chiou and Daniel R. Bush*

Photosynthesis Research Unit, United States Department of Agriculture-Agricultural Research Service (D.R.B.), and Department of Plant Biology (T.-J.C., D.R.B.), 196 Edward R. Madigan Laboratories, 1201 West Gregory, University of Illinois, Urbana, Illinois 61801

Several plant genes have been cloned that encode members of the sugar transporter subgroup of the major facilitator superfamily of transporters. Here we report the cloning, expression, and membrane localization of one of these porters found in sugar beet (*Beta vulgaris* L.). This clone, cDNA-1, codes for a protein with 490 amino acids and an estimated molecular mass of 54 kD. The predicted membrane topology and sequence homology suggest that cDNA-1 is a member of the sugar transporter family. RNA gel blot analysis revealed that this putative sugar transporter is expressed in all vegetative tissues and expression increases with development in leaves. DNA gel blot analysis indicated that multiple gene copies exist for this putative sugar transporter in the sugar beet genome. Antibodies directed against small peptides representing the N- and C-terminal domains of the cDNA1 protein identified a 40-kD polypeptide in microsomes isolated from cDNA-1-transformed yeast (*Saccharomyces cerevisiae*). Moreover, the same protein was identified in sugar beet and transgenic tobacco (*Nicotiana tobacum* L.) membrane fractions. Detailed analysis of the transporter's distribution across linear sucrose gradients and flotation centrifugations showed that it co-migrates with tonoplast membrane markers. We conclude that this carrier is located on the tonoplast membrane and that it may mediate sugar partitioning between the vacuole and cytoplasmic compartments.

Although plants are photoautotrophic organisms, they are composed of many heterotrophic tissue systems, such as roots, flowers, seeds, and developing leaves, that must import organic nutrients to support growth and development. These nutrient-dependent cells are nonphotosynthetic and, therefore, must import previously assimilated carbon, usually as sugars, from photosynthetic tissues. This redistribution process between the photosynthetic (source) and heterotrophic (sink) tissues is known as assimilate partitioning, and it is a fundamental activity in plants as multicellular organisms. We are interested in plant sugar transporters because they are key players in this essential resource redistribution system.

There are many sugar transporters in higher plants that mediate carbon distribution within cells and between organs. They are differentiated by physiological contributions, transport properties (substrate specificity, thermodynamics, transport direction, and reaction mechanism), expression patterns, and membrane location (Fig. 1). For example, there may be as many as six independent Suc transport systems that function in the plant. These carriers mediate Suc partitioning within cells and between sources and sinks. Of these, only the proton-Suc symport has been described biochemically (Bush, 1992, 1993), cloned (Riesmeier et al., 1992; Sauer and Stolz, 1994), and examined in transgenic plants (Riesmeier et al., 1994). In addition to the Suc transporters, there is similar complexity in the number and activity of hexose transporters and, likewise, only a few have been well described. Although significant advances have been achieved in cloning plant sugar transporters, little is known about the regulation and function of most carrier systems that contribute to assimilate partitioning in the plant.

A gene family of sugar transport proteins was recently identified based on functional similarity, sequence homology, and predicted topology in the membrane (Maiden et al., 1987; Baldwin and Henderson, 1989; Griffith et al., 1992). Members of this family contain 12 putative transmembrane domains and a central hydrophilic region containing 60 to 65 amino acid residues. This is an interesting group of transporters because members transport a variety of mono- and disaccharides, they include active (ion-coupled) and passive carriers, and examples have been found in both prokaryotic and eukaryotic organisms (Henderson, 1990; Griffith et al., 1992). Indeed, these sugar carriers constitute a subgroup of a large gene family, currently termed the MFS (Marger and Saier, 1993), that appears to have been derived from a common primordial gene.

The existence of an ancient family of sugar transporters suggests the possibility that many of the plant sugar porters described above are members of this extended gene family (Bush, 1993). The Arabidopsis Glc carriers, for example, are members of this gene family (Sauer and Tanner, 1993). In addition, as many as 31 PCR fragments that encode putative members of this family have been identi-

* Corresponding author; e-mail d-bush1@uiuc.edu; fax 1–217–244–4419.

Abbreviations: BiP, binding protein; DM, dense membrane; KLH, keyhole limpet hemacyanine; LM, light membrane; MFS, major facilitator superfamily; r_{max}, maximum radius; TBST, Tris-buffered saline plus Tween 20; TIP, tonoplast intrinsic protein.

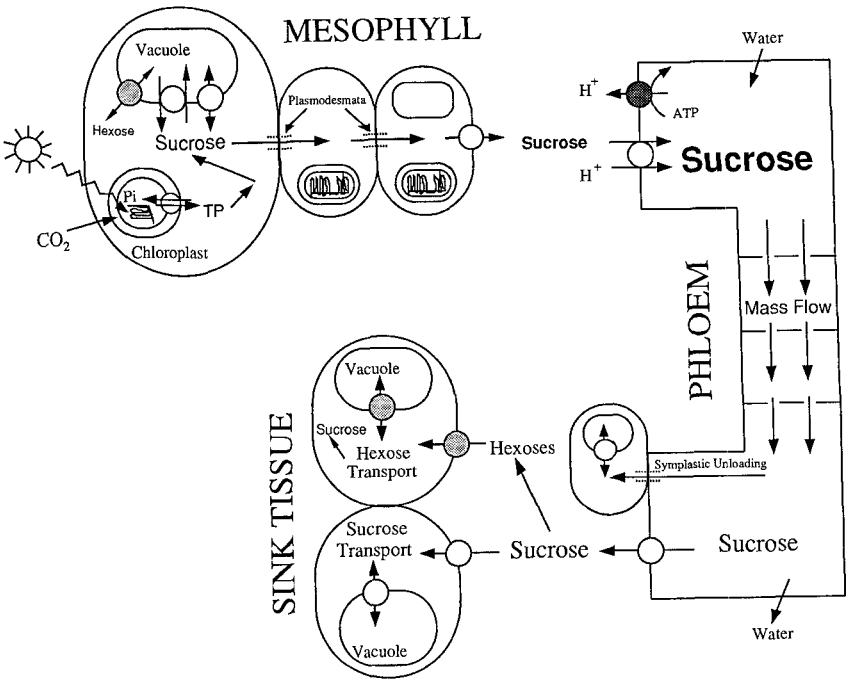

Figure 1. Diagrammatic representation of a plant illustrating the heterogeneity in sugar carriers involved in carbon partitioning. Open circles represent Suc transporters and shaded circles represent hexose carriers. TP, Triose-phosphate.

fied in Arabidopsis (Caspari et al., 1994), castor bean (Weig et al., 1994), *Chenopodium* (Roitsch and Tanner, 1994), and sugar beet (*Beta vulgaris* L.; this paper).

Several cDNAs that encode putative members of the sugar transporter gene family have been cloned from sugar beet in our laboratory (T.-J. Chiou and D.R. Bush, unpublished data). Each contains conserved sequence motifs associated with the family, and hydropathy analysis predicts a typical six-loop-six membrane topology. Significantly, each clone exhibits both tissue-specific and developmentally regulated expression patterns. Although several plant transport systems have been functionally described when heterologously expressed in yeast (*Saccharomyces cerevisiae*; Sauer et al., 1990; Riesmeier et al., 1992; Hsu et al., 1993), our clones have resisted this analysis. Indeed, of the many (>30) putative MFS carriers cloned from higher plant tissue, only 3 of them have been successfully expressed and studied in yeast (Sauer et al., 1990; Sauer and Stadler, 1993; Weig et al., 1994). To characterize the function of the putative sugar transporters that we have identified, polyclonal antibodies were generated against synthetic peptides whose sequences match the deduced amino acid sequence of the N- and C-terminal domains of our clones. In addition, our cDNA clones were introduced into yeast and tobacco (*Nicotiana tobacum* L.) for protein identification and membrane localization. In this report, we focus on our results with one clone, cDNA-1, that is expressed in leaf, root, and storage tissue.

MATERIALS AND METHODS

Plant Material

Sugar beet (*Beta vulgaris* L. cv Great Western) plants were grown in a hydroponic system as previously described (Bush, 1990). Leaves from 2- to 6-month-old plants were used to isolate membranes and RNA. Tobacco (*Nicotiana tobacum* L.) plants of about 4 to 6 weeks of age were harvested for leaf disc transformation.

Reverse Transcription-PCR

Partially expanded leaves (30% of fully expanded size) were harvested, and total RNA was isolated by the hot borate method (Hall et al., 1978). Poly(A)$^+$ RNA was purified from total RNA with the PolyATtract mRNA isolation system (Promega). First-strand cDNA was reverse transcribed from poly(A)$^+$ RNA primed with random hexamers and subsequently amplified with PESPR(W/Y/F)L and (V/L)PETKG degenerate primers. The degeneracy of both primers was 1024. Amplification was achieved with the following conditions: beginning at 95°C for 2 min; 35 cycles of denaturing at 95°C for 2 min, annealing at 37 or 45°C for 2 min and elongation at 72°C for 2 min; ending the program at 72°C for 5 min. The PCR product was analyzed by agarose gel electrophoresis and cloned into pBluescript II SK (Stratagene). Cloned PCR fragments were re-amplified with an internal primer [Q(L/F)(T/S)GIN] and one of the original primers. Promising clones were sequenced and analyzed further. Unless stated otherwise, standard molecular techniques were performed according to the methods of Sambrook et al. (1989).

Construction and Screening of a cDNA Library

First-strand cDNA was synthesized with SuperScript reverse transcriptase (BRL) by priming poly(A)$^+$ RNA with oligo(dT) (Pharmacia), and then RNase H and DNA polymerase I (BRL) were added for second-strand synthesis (Gubler and Hoffman, 1983). The cDNA was blunted with T4 DNA polymerase (BRL) and *Eco*RI-*Not*I adaptors (In-

vitrogen, San Diego, CA) were then ligated to both ends of the cDNA. Excess adaptors and cDNA smaller than 1 kb were removed by Chroma Spin-1000 column (Clontech, Palo Alto, CA) and with gel electrophoresis. Recovered cDNA was cloned into phosphorylated pBluescript II KS(+) plasmid vector through *Eco*RI sites with T4 DNA ligase (Promega) and subsequently transformed into MC1061 *Escherichia coli* [araD139, Δ(ara, leu)7697, $\Delta(lac)_{x74}$, galU, galK, hsdR2, strA, mcrA, mcrB1] by electroporation (Bio-Rad) (Dower et al., 1988). Transformants were screened with ^{32}P-labeled fragments of PCR-amplified product. Approximately 4×10^6 transformants were screened. Positive clones with inserts of approximately 2 kb were chosen for sequence analysis.

DNA Sequencing

Plasmids containing cDNA fragments of interest were purified from *E. coli* with the SDS-alkaline lysis method and Qiagen column chromatography (Qiagen, Chatsworth, CA). Insert fragments were sequenced by Sequenase T7 DNA polymerase (United States Biochemical) with T3 and T7 primers or gene-specific primers with the dideoxy-mediated chain-termination method (Sanger et al., 1977). DNA sequences were analyzed with DNA Strider software (Commissariat à l'Energie Atomique, Saclay, France) and the derived amino acid sequences were analyzed for homology with other polypeptides in the data bases using the BLAST program (Altschul et al., 1990).

RNA Gel Blot Analysis

Total RNA was isolated from leaves of different developmental stages and from storage tissue and true roots. Total RNA (25 μg) for each tissue was run on formaldehyde RNA gels (Zielinski, 1987) and then transferred to Nylon membranes (Nytran, Schleicher & Schuell) with 10× SSC for 12 h. The membrane was fixed by UV cross-linking and then hybridized at 65°C with cDNA-1 ^{32}P-probe, which was labeled by random-primed DNA synthesis (Boehringer Mannheim). Hybridization was performed according to the manufacturer's recommendations. After a typical overnight hybridization, membranes were washed twice with 2× SSC and 0.1% SDS at room temperature for 10 min each and in 0.2× SSC and 0.1% SDS three times at 65°C for 20 min each. Autoradiography was performed after the final wash. Membranes were stripped and reprobed with an 18S rRNA gene (Goldsbrough and Cullis, 1981) to evaluate equal loading.

DNA Gel Blot Analysis

Genomic DNA was extracted from mature leaves according to the method of Dellaporta et al. (1993). Different sets of restriction enzymes were used to digest 40 μg of DNA. Digested DNA was separated on 0.8% agarose gels. After depurination in 0.25 M HCl for 10 min, DNA was transferred overnight to a Hybond N^+ membrane (Amersham) with 0.4 N NaOH. The membrane was briefly rinsed in 2× SSC and then hybridized with ^{32}P-labeled full-length cDNA-1 clones in a manner similar to that described for northern analysis.

Antibody Production

Synthetic peptides were prepared using sequence information from the N- and C-terminals of cDNA-1 protein as the antigens for antibody production. They are peptide 4N (CR^{17}KPFLHTGSWYR^{28}) and peptide 4C (CL^{481}EEIQWSFRR^{490}). An extra Cys residue was added at the N terminus for coupling to a carrier protein. The peptides were coupled to the KLH carrier with glutaraldehyde, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide, and *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (Harlow and Lane, 1988). After the peptides were coupled, 330 μg of each KLH-peptide coupling reaction were combined and mixed with an equal volume (approximately 1 mL) of complete Freund's adjuvant (Freund, 1956) and then injected into rabbits by subcutaneous and intramuscular injections. Two rabbits were used for each antigen. Rabbits were given booster injections after 3 weeks with the same amount of antigen and Freund's incomplete adjuvant. Blood was collected and analyzed with a western dot blot. Serum with the strongest signal was affinity purified through a Sepharose 4B column conjugated with BSA-peptide (synthetic peptide coupled to BSA rather than KLH) (Harlow and Lane, 1988). Purified antibodies were used for western analysis.

Yeast Transformation

cDNA-1 was inserted into a yeast expression vector, NEV-E (Sauer and Stolz, 1994), which has a 2μ replication origin. Expression was driven by the yeast H^+-ATPase gene promoter (PMA1) (Serrano et al., 1986). Plasmid vectors with or without the cDNA-1 insert were transformed into *Saccharomyces cerevisiae* (DBY 2617, a, his 4–539am, lys 2–801am, ura 3–52, suc 2–438) (Kaiser and Botstein, 1986) by electroporation (Becker and Guarente, 1990). Transformants were selected on synthetic complete medium (Sherman et al., 1986) minus uracil.

Tobacco Transformation

pCGN-1547 binary vector (McBride and Summerfelt, 1990) was engineered by introducing the 35S cauliflower mosaic virus promoter in the T-DNA region. cDNA-1 was subsequently inserted in the sense direction under the 35S cauliflower mosaic virus promoter. This construct was transformed into a "disarmed" strain of *Agrobacterium tumefaciens* (PC2760::pAL4404Ti) (An et al., 1985). Tobacco leaves were infected with agrobacteria by a standard leaf-disc transformation method (Horsch et al., 1985), except agrobacteria were precultured for 24 h in a medium containing 5 mM octopine and 100 μM acetosyringone before infection. Tobacco plants were regenerated under kanamycin selection. Total RNA from transformed tobacco leaves was isolated and cDNA-1 expression was analyzed by northern slot blots probed with cDNA-1. Transformed to-

bacco plants with strong cDNA-1 signals were used for membrane isolation and western analysis.

Membrane Isolation and Membrane Marker Analysis

Yeast transformants with or without cDNA-1 inserts were grown in Glc medium and harvested at mid-logarithmic phase. The yeast cells were broken with glass beads, and total microsome membranes were isolated using standard protocols (Serrano, 1988).

Microsome membrane vesicles from control tobacco, transgenic tobacco, and sugar beet leaves were purified according to the method of Bush (1989). Microsomes were separated further on a 34 and 55% (w/w) Suc step gradient by centrifugation at 131,000*g* (r_{max}) for 2 to 4 h. Membrane vesicles were collected from the two interfaces. These were designated LM and DM fractions. The LM fractions from sugar beet and transgenic tobacco were further separated on linear Suc gradients (20–45%, w/w) with an overnight centrifugation at 119,000*g* (r_{max}) (Bush, 1989). One-milliliter fractions were collected, and the Suc concentration for each fraction was determined by refractometry. Each fraction was diluted with resuspension buffer (250 mM Suc, 10 mM KCl, 2 mM Hepes/1,3-bis[tris(hydroxymethyl)methylamino] propane, pH 7.4, 1 mM DTT) and pelleted. Membrane vesicles from each fraction were resuspended in 200 μL of resuspension buffer.

Microsome vesicles from transgenic tobacco and sugar beet leaves were also separated by flotation centrifugation (see "Membrane Fractionation and Localization" in "Results") (Gibeaut and Carpita, 1990). Microsomes were first separated on a five-step Suc gradient (12, 19, 27, 37, and 51% [w/v]) with a 40-min centrifugation at 131,000*g* (r_{max}). Membrane vesicles were collected from each interface and then brought to 47% Suc (w/v). They were further purified by layering on top of 5 mL of a 55% Suc step and then overlaid with four additional Suc steps (40, 34, 20, and 19%). Membrane vesicles were then separated by flotation centrifugation at 131,000*g* (r_{max}) for 2 h.

Membrane proteins associated with each fraction collected from the linear Suc gradient and flotation centrifugation were separated on a 10% SDS-PAGE for western analysis. In addition, each fraction was analyzed for enzyme activities that serve as markers for various plant membranes, including the plasma membrane, tonoplast, Golgi, and ER (Bush, 1989). Specific markers include the VO_4^--sensitive P-type ATPase activity (plasma membrane), NO_3^--sensitive V-type ATPase activity (tonoplast), Mg^{+2}-IDPase (Golgi), and antimycin A-insensitive NADH-dependent Cyt *c* reductase (ER).

Protein Gel Blot Analysis

Proteins from different membrane fractions were separated on 10% SDS-PAGE (Laemmli, 1970) and transferred to a polyvinylidene difluoride membrane (Millipore) with a semidry blotter. The membrane was blocked with 3% BSA in TBST (100 mM Tris, pH 8, 150 mM NaCl, 0.05% Tween 20) at room temperature for 12 h and then washed with TBST three times for 10 min each. The blots were then incubated for 2 h at room temperature with affinity-purified antibodies against cDNA-1 (1000-fold dilution of anti-4N or 2000-fold dilution of anti-4C) or antibodies directed against peptides associated with specific membranes, e.g. the tonoplast H^+-pyrophosphatase (kindly provided by Phil Rea, University of Pennsylvania, Philadelphia) or anti-BiP as an ER marker (Walker et al., 1993; Anderson et al., 1994). The membranes were washed three times with TBST and then incubated with secondary antibodies (alkaline phosphatase-conjugated goat anti-rabbit antibody, 3000-fold dilution, Bio-Rad) for 1 to 2 h at room temperature. After a final TBST wash, the membrane was incubated in bromochloroindoyl phosphate/nitroblue tetrazolium solution for color development. The reaction was stopped by rinsing the membrane several times with water.

RESULTS

Molecular Cloning of cDNA-1

Recently, more than 50 polypeptides have been identified to be members of the sugar transporter subgroup in the MFS (Marger and Saier, 1993; Sauer and Tanner, 1993; Caspari et al., 1994; Maloney, 1994). Each member is predicted to contain 12 transmembrane domains and a central hydrophilic loop 60 to 65 amino acid residues long. Several regions of conserved amino acids have been identified that appear to be hallmark motifs of sugar carriers in the MFS (Griffith et al., 1992). Amino acid sequences at the ends of the 6th and 12th transmembrane domains, PESPR and PETKG, respectively, are examples of conserved sequences among members of the sugar family. These two sequences were chosen as templates to design degenerate PCR primers. They should amplify a fragment of approximately 800 bp. We also identified a less conserved sequence, located in the 7th transmembrane domain, Q(L/F)(T/S)GIN, that we used as an internal PCR primer to test the identity of the primary PCR products by re-amplification.

cDNA used as the template for PCR was synthesized from poly(A)$^+$ RNA isolated from expanding leaf tissue. After amplification, a band of the predicted size was observed on the agarose gel. All fragments from this PCR reaction were cloned into pBluescript II SK, and several clones containing inserts of 750 to 850 bp were obtained. One of them (PCR4) was analyzed further and will be discussed in this paper. PCR4 was re-amplified with the internal primers and either one of the original primers, yielding fragments of approximately 250 and 550 bp, respectively.

Sequence data showed that PCR4 had significant sequence homology with other sugar transporters in the MFS. In addition, hydropathy analysis of the deduced amino acid sequence (Kyte and Doolittle, 1982) revealed a profile similar to those of the sugar transporter gene family. This suggested that the PCR4 fragment represents a portion of an MFS transporter in sugar beet.

To obtain a full-length clone, a cDNA library from mature leaf tissue was constructed and screened with the PCR4 clone. After 4×10^6 transformants were screened, 58 positive colonies were identified. Positive transformants

with inserts of approximately 2 kb were selected, and cDNA-1 was chosen for detailed analysis. It contains 2020 bp with an open reading frame that encodes a protein containing 490 amino acids with an estimated molecular mass of 54 kD (Fig. 2A). Hydropathy analysis of the deduced amino acid sequence (Fig. 2B) indicates that cDNA-1 contains 12 membrane-spanning domains and a central hydrophilic region. No sites of potential glycosylation are present. Searching the GenBank data base revealed that cDNA-1 has homology with other sugar transporters, ranging from 20 to 30% identity and 60 to 70% similarity in amino acid sequence. In addition, consensus sequences that are hallmark motifs in the sugar transporter subgroup of the MFS are present in this clone. For example, (R/K)XGR(R/K) (X can be any amino acid) is found between the second and third and also the eighth and ninth transmembrane helices (Griffith et al., 1992). We conclude that cDNA-1 encodes a putative sugar transport protein.

Expression Pattern and Genomic Organization of cDNA-1 in Sugar Beet

The expression pattern of this putative sugar transporter was investigated with RNA blot analysis. Blots of sugar beet leaf-derived RNA probed with cDNA-1 identified a message of approximately 2 kb (Fig. 3A). Total RNA from leaves of different developmental stages and from different tissues, such as hypocotyl (storage organ) and true root, was also investigated to explore tissue- and development-specific expression patterns. cDNA-1 was expressed in all tissues (Fig. 3A). The signal increased with the transition from very young to mature leaves. This was intriguing because Suc transport activity has previously been shown to increase in a similar pattern (Lemoine et al., 1992; D.R. Bush, unpublished data).

Gel blot analysis of genomic DNA digested with restriction enzymes that have different cutting patterns in the cDNA-1 clone (*Eco*RI and *Hin*dIII, no cut; *Pvu*II and *Sca*I, one cut; *Bgl*II and *Nco*I, two cuts) yielded multiple bands (Fig. 3B). This is consistent with the existence of multiple copies of cDNA-1 or very closely related genes in the sugar beet genome.

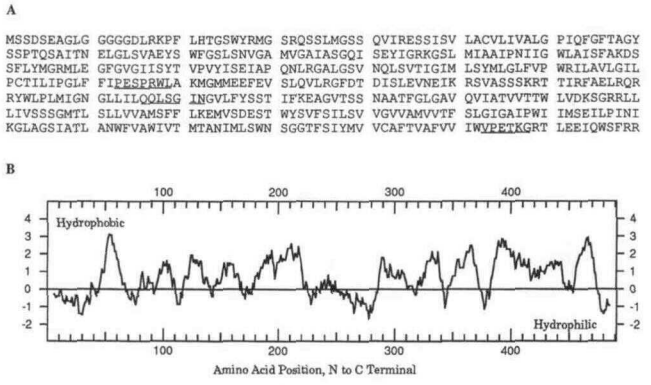

Figure 2. Deduced amino acid sequences (A) and hydropathy profile (B) of cDNA-1. cDNA-1 encodes a protein containing 490 amino acids with an estimated molecular mass of 54 kD. The PCR primers are underlined.

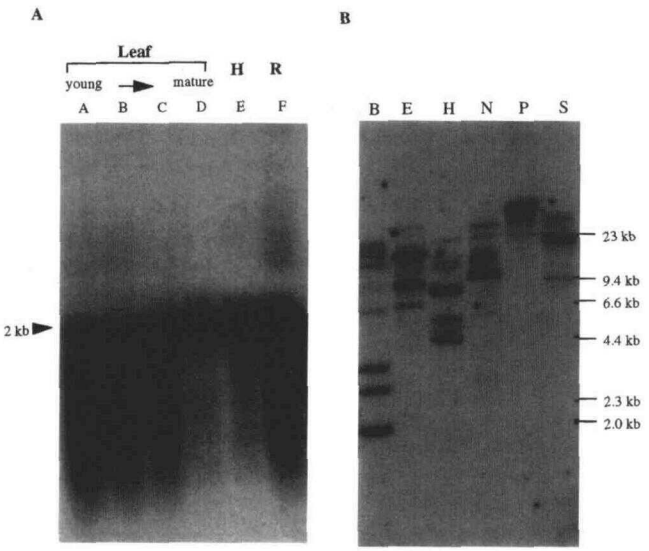

Figure 3. RNA and DNA blot of cDNA-1 in sugar beet. A, RNA blot analysis. Total RNA was isolated from leaves of different developmental stages (lanes A–D, from young to mature; lane A, approximately 3 cm long; lane B, approximately 5 cm long; lane C, approximately 9 cm long; lane D, approximately 25 cm long) and from storage tissue (lane E) and true root (lane F). Total RNA (25 μg) was loaded in each lane. The membrane was hybridized with ^{32}P-labeled cDNA-1. After the blots were stripped and re-probed with the 18S rRNA gene, they all showed equal loading in each lane (data not shown). B, DNA blot analysis. Sugar beet genomic DNA (40 μg) was digested with various restriction enzymes (B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; N, *Nco*I; P, *Pvu*II; S, *Sca*I) and hybridized with cDNA-1 ^{32}P-labeled probe.

Heterologous Expression in Yeast

cDNA-1 was heterologously expressed in yeast as a strategy for overexpression and to examine transport function. Microsome membrane vesicles were isolated from yeast that were transformed with insert-free vector or cDNA-1. Western analysis with antibodies directed against N- and C-terminal peptides detected cDNA-1 protein in the isolated microsomes (Fig. 4). A smeared band at approximately 40 kD was observed in the cDNA-1 transformant (lane B) but not in the insert-free control (lane A). Moreover, this was the only protein detected by both anti-4C and anti-4N antibodies. This is good evidence that we have identified the cDNA-1 protein in yeast. Poor resolution on SDS-PAGE and migration as a peptide smaller than the predicted molecular mass (54 kD) is a common observation for hydrophobic membrane proteins (Maddy, 1976). Unfortunately, no transport activity of any one of a variety of sugar substrates was observed in the transgenic yeast (data not shown). Likewise, protein expression levels remained below the detection limit of Coomassie blue.

Expression of cDNA-1 in Transgenic Tobacco

Transgenic tobacco plants expressing cDNA-1 were generated by *Agrobacterium*-mediated transformation. The expression of cDNA-1 in 53 positive transformants was examined by RNA slot blot analysis (data not shown). Under

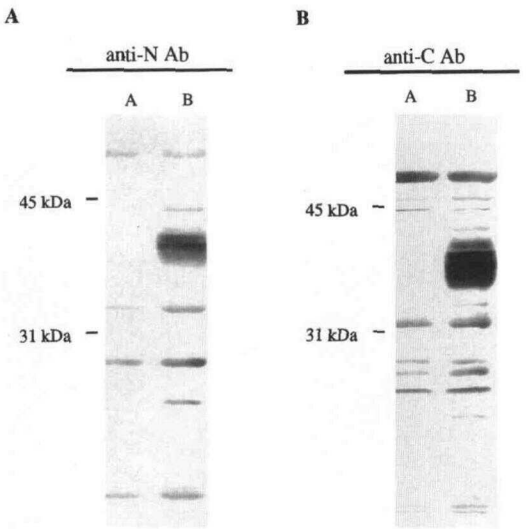

Figure 4. Protein gel blot analysis of cDNA-1 expression in yeast. Total microsome membranes were isolated from yeast transformants and separated with SDS-PAGE. Protein blots were probed with antibodies (Ab) directed against N-terminal (A) or C-terminal (B) peptide of cDNA-1. An additional band at approximately 40 kD was observed in the cDNA-1 transformant (lane B) but not the insert-free control (lane A). This was the only protein recognized by both antibodies.

high-stringency hybridization conditions, no signal was found in nontransformed tobacco leaf tissue. Sugar beet leaf and root tissues were used as the positive controls. The expression levels of cDNA-1 varied widely. Those with the strongest signals, usually exceeding the positive controls, were used for further analysis.

Microsome membrane vesicles for gel blot analysis were isolated from leaf tissue of nontransformed and transgenic tobacco. These membrane vesicles were further separated on a two-step Suc gradient (34 and 55% [w/w]). Membrane vesicles collected from the interfaces were designated LM (collected on top of the 34% step) and DM (removed from the top of the 55% step). Protein gel blot analysis identified a strong signal in the LM of the transformed plants (Fig. 5A). The same experiment was repeated for microsome membrane vesicles isolated from sugar beet and a similar band at approximately 40 kD was also observed in the LM fraction (Fig. 5B). These blots were probed with anti-4C antibody. When anti-4N antibody was used, the same 40-kD protein was also observed in the LM fraction of both sugar beet and transgenic tobacco (data not shown). As was observed in transgenic yeast, other proteins cross-reacted with these antibodies. However, the 40-kD band was the only protein that reacted with both sets of polyclonal antibodies. We conclude that this represents the protein encoded by cDNA-1 and that the other signals originate from unspecific interactions or from partially degraded and/or processed protein. It is noteworthy that the migration pattern of this protein on SDS-PAGE was virtually identical in a variety of expression systems (i.e. in vitro [data not shown], in transformed yeast, in transgenic tobacco, and as native peptide from sugar beet).

The apparent localization of the cDNA-1 protein in the LM fraction was completely unexpected. This fraction is composed primarily of endomembrane vesicles derived from the ER, Golgi, and tonoplast. The DM fraction is enriched in plasma membrane vesicles. We expected the strongest signal from cDNA-1 protein in the DMs because porters in this gene family are found almost exclusively in the plasma membrane.

Membrane Fractionation and Localization

To understand better the membrane localization of cDNA-1 protein, the LM fractions from both transgenic tobacco and sugar beet were further separated on linear Suc gradients (20–45% [w/w]) (Quail, 1979; Bush, 1989). In transgenic tobacco, cDNA-1 protein peaked between 28 and 32% Suc (Fig. 6,B and C). This signal co-migrated with

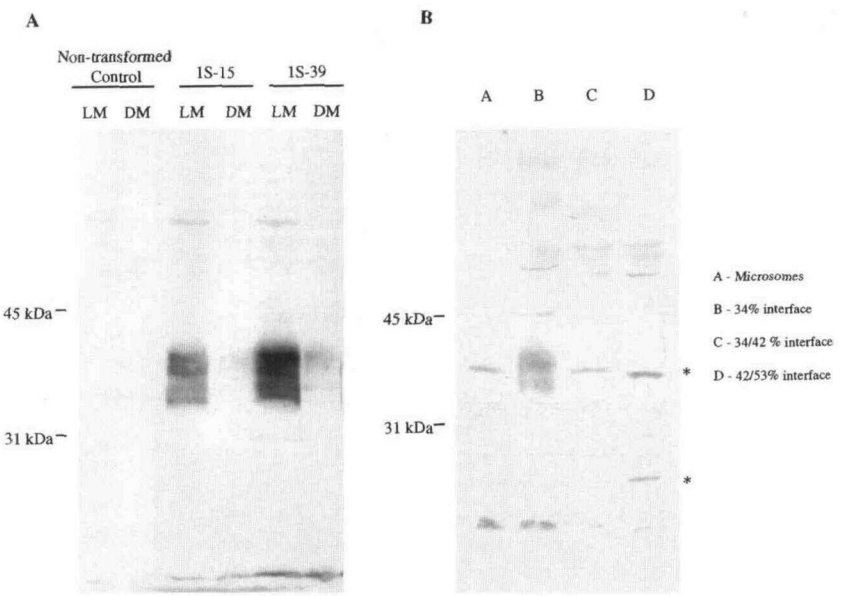

Figure 5. Protein gel blot analysis of cDNA-1 protein in tobacco and sugar beet. Total microsome membranes were isolated from tobacco (A) and sugar beet (B) leaf tissue. Membranes were separated on Suc step gradients. Antibody directed against the C-terminal peptide recognized protein in the LM fraction (<34% Suc [w/w]) in both sugar beet and transgenic tobacco (1S-15 and 1S-39, respectively). LM, <34% Suc (w/w); DM, >34% Suc (w/w). Asterisk (*), Nonspecific cross-reaction, not found on blots probed with the antibody against the N-terminal peptide.

two tonoplast markers, the pyrophosphatase protein using protein blot analysis (Fig. 6B) or NO_3^--sensitive ATPase activity (Fig. 6A). When sugar beet was examined, the cDNA-1 protein peaked between 30 and 34% Suc and also co-migrated with the tonoplast markers (Fig. 7). However, the marker distributions for the ER and Golgi membranes partially overlapped with tonoplast markers. Thus, an unequivocal localization to the tonoplast could not be made.

To provide a better separation between the tonoplast and other endomembranes, flotation centrifugation was performed (Gibeaut and Carpita, 1990). The procedure is described in Figure 8. The numbers (1, 2, 3, and 4) are the fractions from first downward centrifugation and the letters (A, B, C, D, and E) are the fractions removed from the second flotation centrifugation. Protein gel blot analysis and enzyme marker activities were determined for each fraction. The results shown in Figure 9 are for transgenic tobacco. Based on these data, we were able to separate Golgi and ER from tonoplast. The majority of ER and Golgi membranes were distributed in the dense fractions (3C, 3D, and 4D), whereas the tonoplast was enriched in lighter fractions. Tonoplast marker activity (vacuole-ATPase) was the strongest in fractions 2B and 2C, and cDNA-1 protein was also most abundant in these fractions. Tonoplast marker activity and cDNA-1 protein also co-migrated in sugar beet (data not shown).

The signals of cDNA-1 protein for both plants consistently co-migrated with the tonoplast marker activity. We conclude from these results that the porter encoded by cDNA-1 is localized in the tonoplast membrane. cDNA-1 signals associated with the ER and Golgi may represent intermediate states of processing or trafficking to the tonoplast or, perhaps, some level of localization to these membranes in addition to the tonoplast. Moreover, protein gel

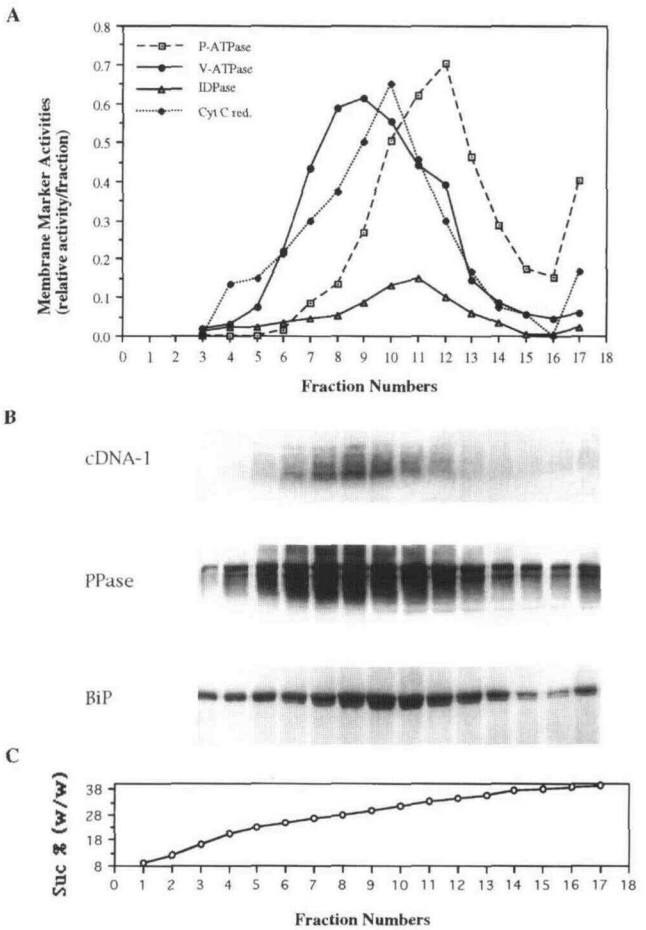

Figure 6. Gradient distribution of enzyme markers and cDNA-1 protein in transgenic tobacco membranes. The LM fraction was further separated on linear Suc gradients (20–40% [w/w]). Membrane marker activities and cDNA-1 protein were detected for each fraction. A, Distributions of membrane marker activity: P-ATPase, VO_4^--sensitive P-type H^+-ATPase, a marker for plasma membrane, and ER; V-ATPase, NO_3^--sensitive V-type H^+-ATPase, the marker for tonoplast; IDPase, Triton-stimulated IDPase, the marker for Golgi membrane; Cyt C red., NADH-dependent, antimycin A-insensitive Cyt *c* reductase, the marker for ER. B, Protein gel blot analysis of cDNA-1 protein (anti-4C), pyrophosphatase (anti-PPase), and BiP (anti-BiP). C, Suc concentration across the fractions.

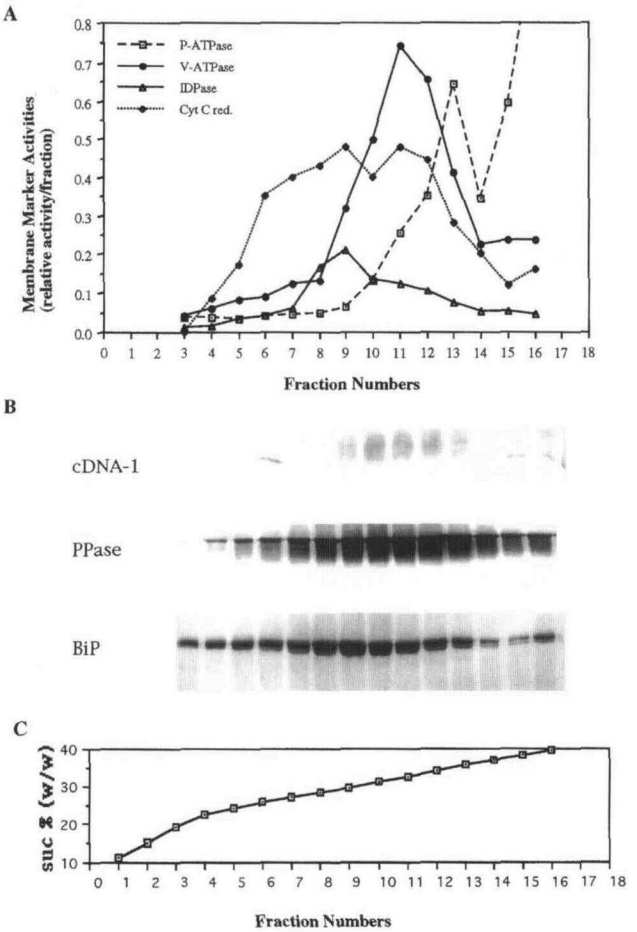

Figure 7. Gradient distribution of enzyme markers and cDNA-1 protein in sugar beet membranes. Sugar beet membranes were separated, and membrane marker activities and cDNA-1 protein were determined for each fraction as described in Figure 8. A, Distributions of membrane marker activities. B, Protein gel blot analysis of cDNA-1 protein (anti-4C), pyrophosphatase (anti-PPase), and BiP (anti-BiP). C, Suc concentration across the fractions. Other abbreviations are as in legend to Figure 6.

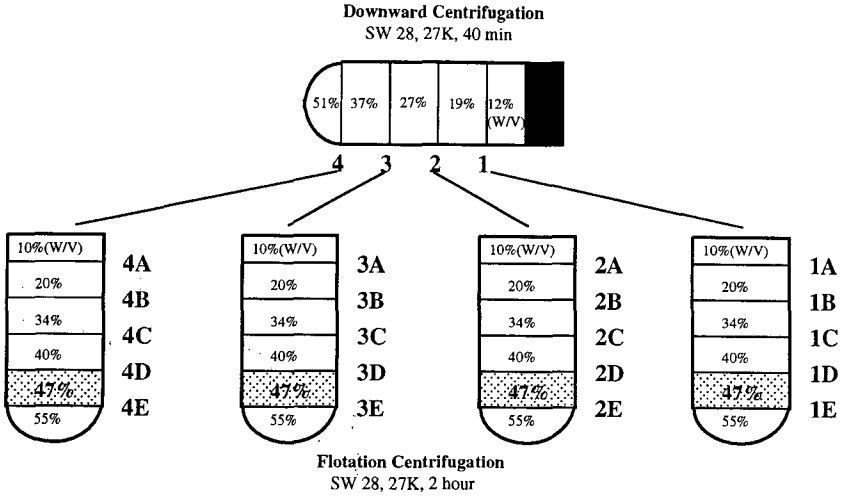

Figure 8. Schematic diagram of flotation centrifugation (Gibeaut and Carpita, 1990).

blot analysis of purified plasma membrane vesicles (Bush, 1989) failed to detect cDNA-1 protein (data not shown). It is clear from these combined data that cDNA-1 is not localized to the plasma membrane and is targeted primarily to the tonoplast.

DISCUSSION

The cDNA clone described here belongs to the sugar transporter subgroup of the MFS based on sequence similarity, conserved motifs, and predicted membrane topology. The amino acid sequence of this clone exhibited 20 to 30% identity and 60 to 70% similarity to other members of this family, including the carriers transporting Ara (Henderson et al., 1992), Gal (Szkutnicka et al., 1989; Kruckeberg and Bisson, 1990), Glc (Sauer and Tanner, 1989; Gould and Bell, 1990; Sauer et al., 1990), maltose (Cheng and Michels, 1989), and Xyl (Henderson et al., 1992) in both prokaryotes and eukaryotes. In addition to placing this clone in the MFS, sequence comparisons provided additional clues to the function of this transport system. For example, cDNA-1 may encode a proton-coupled porter because it contains a conserved basic amino acid residue (Arg or Lys) at the beginning of the sixth transmembrane domain. All transporters in the MFS that are known to catalyze proton-coupled transport contain one of these basic amino acid residues (Griffith et al., 1992).

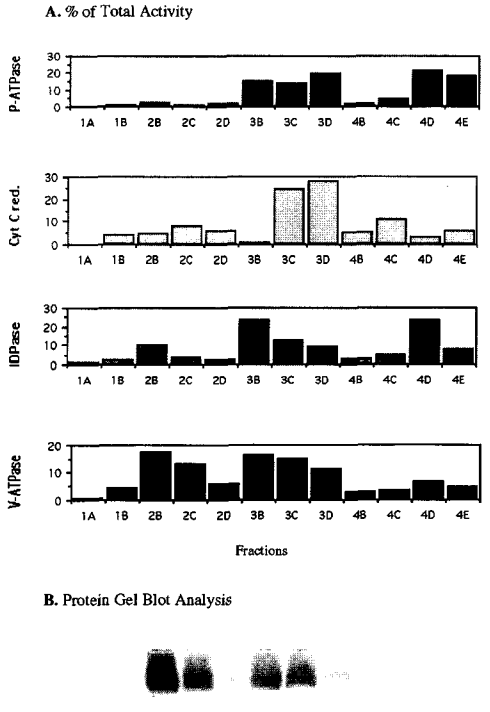

Figure 9. Distribution of membrane markers and cDNA-1 protein after flotation centrifugation for transgenic tobacco. A, Distributions of membrane marker activity. B, Protein gel blot analysis of cDNA-1 protein. Abbreviations are as in legend to Figure 6.

The putative sugar carrier encoded by cDNA-1 was expressed successfully in both transgenic yeast and tobacco. The same protein was detected by antibodies directed against either the N- or C-terminal portions of the peptide. The protein appeared as a smeared band on SDS-PAGE and it migrated as a peptide (about 40 kD) smaller than the predicted molecular mass (54 kD). This is not unusual for membrane proteins because of their high hydrophobicity (Maddy, 1976). Significantly, cDNA-1 protein expressed from in vitro transcription and translation (in the presence or absence of microsome membranes) or in transgenic yeast and tobacco exhibited the same peptide migration pattern in SDS-PAGE as the protein from sugar beet. This suggests that this protein does not undergo significant posttranslational modification, such as cleavage of a signal peptide or protein glycosylation. Indeed, there was no typical signal peptide or sites of potential glycosylation in the deduced amino acid sequence of cDNA-1. The protein expressed in yeast and tobacco can be detected by antibodies; however, it was not visible in gels stained with Coomassie blue, even when placed under the regulation of a strong, constitutive promoter. We believe this result suggests that these eukaryotic cells sometimes regulate the amount of protein placed in a given membrane, regardless of the abundance

or expression level of the encoding message. However, in spite of testing several vectors, insert constructs, and cell lines, we cannot rule out irregular translational or processing steps as the cause for low expression levels.

cDNA-1 protein in sugar beet and transgenic tobacco exhibited similar patterns of membrane distribution. The protein was observed in the LM fractions (<34% Suc [w/w]) of both plants (Fig. 5). This indicated that this sugar transporter is located on an intracellular membrane, such as the ER, Golgi, or tonoplast. Further analysis by linear Suc gradients and flotation centrifugation showed that the distribution of cDNA-1 protein across these gradients corresponded to that of tonoplast markers (Figs. 6, 7, and 9). In addition, this protein could not be detected in plasma membrane vesicles purified with the aqueous phase partitioning method (data not shown). These results clearly demonstrate that the cDNA-1 gene product is found in the tonoplast, although additional targeting to other membranes cannot be ruled out. So far, all of the members in the sugar transport gene family are located in the plasma membrane except one of the human Glc porters (GLUT 7), which was reported to remain in the ER membrane because of a consensus motif (KKMKND) at the C terminus (Waddell et al., 1992). To our knowledge, the cDNA-1 protein we identified here is the first member of MFS targeted to the tonoplast.

Many soluble vacuolar proteins are synthesized as propeptides that are cleaved during or after transport of the proteins to the vacuole. Recently, sorting signals have been identified in the N- or C-terminal propeptides of several vacuolar proteins (Chrispeels and Raikhel, 1992; Nakamura and Matsuoka, 1993). However, very little is known about the sorting of vacuolar membrane proteins. The only information is from TIP. It was reported that the last transmembrane domain of TIP may be involved in targeting to the tonoplast (Höfte and Chrispeels, 1992). cDNA-1 protein does not contain any identified sorting signals of vacuolar proteins and does not contain amino acid sequences that are similar to the targeting domain of TIP. This indicates that the targeting mechanism of cDNA-1 may be different from those of vacuolar proteins and TIP.

Many examples of functionally active plant carriers expressed in transgenic yeast have been reported (Sauer et al., 1990; Riesmeier et al., 1992; Hsu et al., 1993; Sauer and Stolz, 1994). Therefore, we also isolated intact vacuoles from transgenic yeast to look for cDNA-1 porter function. Although we were able to show that the isolated vacuoles were transport competent by demonstrating proton pumping and proton-coupled Lys transport (data not shown; Sato et al., 1984), we were unable to identify the transport substrate for the cDNA-1 porter. This was in spite of testing many potential substrates and also exploring both import and export activities. The absence of a measurable activity may be due to nonfunctional insertion of the cDNA-1 protein or it may reflect our inability to predict substrate specificity. Further experiments aimed at identifying the transported substrate are underway.

Received October 11, 1995; accepted October 31, 1995.
Copyright Clearance Center: 0032–0889/96/110/0511/10.
The GenBank accession number for the sequence reported in this article is U43629.

LITERATURE CITED

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol **215:** 403–410

An G, Watson BD, Stachel S, Gordon MP, Nester EW (1985) New cloning vehicles for transformation of higher plants. EMBO J **4:** 277–204

Anderson JV, Li Q-B, Haskell DW, Guy CL (1994) Structure organization of the spinach endoplasmic reticulum-luminal 70-kilodalton heat-shock cognate gene and expression of 70-kilodalton heat-shock genes during cold acclimation. Plant Physiol **104:** 1359–1370

Baldwin B, Henderson P (1989) Homologies between sugar transporters from eukaryotes and prokaryotes. Annu Rev Physiol **51:** 459–471

Becker DM, Guarente L (1990) High efficiency transformation of yeast by electroporation. Methods Enzymol **194:** 182–187

Bush DR (1989) Proton-coupled sucrose transport in plasmalemma vesicles isolated from sugar beet leaves. Plant Physiol **89:** 1318–1323

Bush DR (1990) Electrogenicity, pH-dependence, and stoichiometry of the proton-sucrose symport. Plant Physiol **93:** 1590–1596

Bush DR (1992) The proton-sucrose symport. Photosynth Res **32:** 155–165

Bush DR (1993) Proton-coupled sugar and amino acid transporters in plants. Annu Rev Plant Physiol Plant Mol Biol **44:** 513–542

Caspari T, Will A, Opekarova M, Sauer N, Tanner W (1994) Hexose/H^+ symporters in lower and higher plants. J Exp Biol **196:** 483–491

Cheng Q, Michels CA (1989) The maltose permease encoded by the MAL61 gene of *Saccharomyces cerevisiae* exhibits both sequence and structural homology to other sugar transporters. Genetics **123:** 477–484

Chrispeels MJ, Raikhel NV (1992) Short peptide domains target proteins to plant vacuoles. Cell **68:** 613–616

Dellaporta ST, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep **1:** 19–21

Dower WJ, Miller JF, Ragsdale CW (1988) High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res **16:** 6127–6145

Freund J (1956) The mode of action of immunologic adjuvants. Adv Tuberc Res **7:** 130–148

Gibeaut DM, Carpita NC (1990) Separation of membranes by flotation centrifugation for in vitro synthesis of plant cell wall polysaccharides. Protoplasma **156:** 82–93

Goldsbrough PB, Cullis CA (1981) Characterization of the genes for ribosomal RNA in flax. Nucleic Acids Res **9:** 1301–1309

Gould GW, Bell GI (1990) Facilitative glucose transporters: an expanding family. Trends Biochem Sci **15:** 18–23

Griffith JK, Baker ME, Rouch DA, Page MGP, Skurray RA, Paulsen IT, Chater KF, Baldwin SA, Henderson PJF (1992) Membrane transport proteins: implications of sequence comparisons. Curr Opin Cell Biol **4:** 684–695

Gubler U, Hoffman BJ (1983) A simple and very efficient method for generating cDNA libraries. Gene **25:** 263–269

Hall TC, Ma Y, Buchbinder BU, Pyne JW, Sun SM, Bliss FA (1978) Messenger RNA for G1 protein of French bean seeds: cell-free translation and product characterization. Proc Natl Acad Sci USA **75:** 3196–3200

Harlow E, Lane D (1988) Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Henderson PJF (1990) The homologous glucose transport proteins of prokaryotes and eukaryotes. Res Microbiol **141:** 316–328

Henderson PJF, Baldwin SA, Cairns MT, Charalambous B, Dent HC (1992) Sugar-cation symport system in bacteria. Int Rev Cytol **137:** 149–208

Höfte H, Chrispeels MJ (1992) Protein sorting to the vacuolar membrane. Plant Cell **4:** 995–1004

Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science **227:** 1229–1231

Hsu L-C, Chiou T-J, Chen L, Bush DR (1993) Cloning a plant amino acid transporter by functional complementation of a yeast amino acid transport mutant. Proc Natl Acad Sci USA **90:** 7441–7445

Kaiser CA, Botstein D (1986) Secretion-defective mutants in the signal sequence for *Saccharomyces cerevisiae* invertase. Mol Cell Biol **6:** 2382–2391

Kruckeberg AL, Bisson LF (1990) The HXT2 gene of *Saccharomyces cerevisiae* is required for high-affinity glucose transport. Mol Cell Biol **10:** 5903–5913

Kyte J, Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. J Mol Biol **157:** 105–132

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Lemoine R, Gallet O, Gaillard C, Frommer W, Delrot S (1992) Plasma membrane vesicles from source and sink leaves. Changes in solute transport and polypeptide composition. Plant Physiol **100:** 1150–1156

Maddy AH (1976) A critical evaluation of the analysis of membrane proteins by polyacrylamide gel electrophoresis in the presence of dodecyl sulphate. J Theor Biol **62:** 315–326

Maiden M, Davis EO, Baldwin S, Moore D, Henderson P (1987) Mammalian and bacterial sugar porters are homologous. Nature **325:** 641–643

Maloney PC (1994) Bacterial transporters. Curr Opin Cell Biol **6:** 571–582

Marger MD, Saier JMH (1993) A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport. Trends Biochem Sci **18:** 13–20

McBride KE, Summerfelt KR (1990) Improved binary vector for Agrobacterium-mediated plant transformation. Plant Mol Biol **14:** 269–276

Nakamura K, Matsuoka K (1993) Protein targeting to the vacuole in plant cells. Plant Physiol **101:** 1–5

Quail PH (1979) Plant cell fractionation. Annu Rev Plant Physiol **30:** 425–484

Riesmeier JW, Willmitzer L, Frommer WB (1992) Isolation and characterization of a sucrose carrier cDNA from spinach by functional expression in yeast. EMBO J **11:** 4705–4713

Riesmeier JW, Willmitzer L, Frommer WB (1994) Evidence for essential role of the sucrose transporter in phloem loading and assimilate partitioning. EMBO J **13:** 1–7

Roitsch T, Tanner W (1994) Expression of a sugar-transporter gene family in a photoautotrophic suspension culture of *Chenopodium rubrum* L. Planta **193:** 365–371

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-termination inhibitors. Proc Natl Acad Sci USA **74:** 5463–5467

Sato T, Ohsumi Y, Anraku Y (1984) Substrate specificities of active transport systems for amino acids in vacuolar-membrane vesicles of *Saccharomyces cerevisiae*. J Biol Chem **259:** 11505–11508

Sauer N, Friedlander K, Graml-Wicke U (1990) Primary structure, genomic organization and heterologous expression of a glucose transporter from *Arabidopsis thaliana*. EMBO J **9:** 3045–3050

Sauer N, Stadler R (1993) A sink-specific H^+/monosaccharide co-transporter from *Nicotiana tabacum*: cloning and heterologous expression in baker's yeast. Plant J **4:** 601–610

Sauer N, Stolz J (1994) SUC1 and SUC2: two sucrose transporters from *Arabidopsis thaliana*; expression and characterization in baker's yeast and identification of the histidine-tagged protein. Plant J **6:** 67–77

Sauer N, Tanner W (1989) The hexose carrier from *Chlorella* cDNA cloning of a eucaryotic H^+-cotransporter. FEBS Lett **259:** 43–46

Sauer N, Tanner W (1993) Molecular biology of sugar transporters in plants. Bot Acta **106:** 277–286

Serrano R (1988) H^+-ATPase from plasma membrane of *Saccharomyces cerevisiae* and *Avena sativa* roots: purification and reconstitution. Methods Enzymol **157:** 533–544

Serrano R, Kielland-Brandt MC, Fink GR (1986) Yeast plasma membrane ATPase is essential for growth and has homology with (Na^+, K^+), K^+-and Ca^{++}-ATPase. Nature **319:** 689–693

Sherman F, Fink GR, Hicks JB (1986) Laboratory Course Manual for Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 164–165

Szkutnicka K, Tschopp JF, Andrews L, Cirillo VP (1989) Sequence and structure of the yeast galactose transporter. J Bacteriol **171:** 4486–4493

Waddell ID, Zomerschoe AG, Voice MW, Burchell A (1992) Cloning and expression of a hepatic microsomal glucose transport protein. Comparison with liver plasma-membrane glucose-transport protein GLUT 2. Biochem J **286:** 173–177

Walker RP, Waterworth WM, Hooley R (1993) Preparation and polypeptide composition of plasma membrane and other subcellular fractions from wild oat (*Avena fatua*) aleurone. Physiol Plant **89:** 388–398

Weig A, Franz J, Sauer N, Komor E (1994) Isolation of a family of cDNA clones from *Ricinus communis* L. with close homology to the hexose carriers. J Plant Physiol **143:** 178–183

Zielinski RE (1987) Calmodulin mRNA in barley (*Hordeum vulgare* L.). Apparent regulation by cell proliferation and light. Plant Physiol **84:** 937–943