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# Isolation and characterization of a sucrose carrier cDNA from spinach by functional expression in yeast

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**Active loading of the phloem with sucrose in leaves is an essential part of the process of supplying non-photosynthetic tissues with carbon and energy. The transport is protein mediated and coupled to proton-symport, but so far no sucrose carrier gene has been identified. Using an engineered *Saccharomyces cerevisiae* strain, a cDNA from spinach encoding a sucrose carrier was identified by functional expression. Yeast strains that allow the phenotypic recognition of a sucrose carrier activity were constructed by expressing a cytoplasmic invertase from yeast, or the potato sucrose synthase gene, in a strain unable to transport or grow on sucrose due to a deletion in the *SUC2* gene. A spinach cDNA expression library established from the poly(A)$^+$ RNA from source leaves of spinach and cloned in a yeast expression vector yielded transformed yeast clones which were able to grow on media containing sucrose as the sole carbon source. This ability was strictly linked to the presence of the spinach cDNA clone pS21. Analysis of the sucrose uptake process in yeast strains transformed with this plasmid show a pH-dependent uptake of sucrose with a $K_m$ of 1.5 mM, which can be inhibited by maltose, α-phenylglucoside, carbonyl cyanide *m*-chlorophenylhydrazone and *p*-chloromercuribenzenesulfonic acid. These data are in accordance with measurements using both leaf discs and plasma membrane vesicles from leaves of higher plants. DNA sequence analysis of the pS21 clone reveals the presence of an open reading frame encoding a protein with a molecular mass of 55 kDa. The predicted protein contains several hydrophobic regions which could be assigned to 12 membrane-spanning regions. Thus, the functional, biochemical and structural analyses are in agreement with the interpretation that the spinach cDNA clone pS21 encodes a sucrose carrier.**

*Key words:* proton cotransport/*Saccharomyces cerevisiae*/spinach/sucrose transport

## Introduction

Photosynthesis represents the major source of energy required for biological processes. As a primary product of photosynthesis carbohydrates are formed in photosynthetically active cells. In higher plants leaves, and to a certain extent also other parts of the plant, e.g. stem tissue, represent the primary sites for photosynthesis. In contrast other parts of the plant e.g. roots, seeds or tubers, do not contribute significantly to the energy gained by photosynthesis but rather are dependent on carbon dioxide fixed in photosynthetically active parts of the plant. Thus, there is a net flow of energy and carbon from photosynthetically active tissues representing the sources (net exporters of fixed carbon) to photosynthetically inactive tissues of the plant representing the sinks (importers of fixed carbon) (cf. Ho, 1988 for a review).

Primary products of photosynthesis are exported from the chloroplast via the triosephosphate translocator. The cDNA for this metabolite transporter has been cloned (Flügge *et al.*, 1989). In the cytosol of photosynthetically active mesophyll cells the exported triosephosphates are used to synthesize sucrose. Sucrose represents the principal chemical form used by the majority of higher plants for long-distance transport and the distribution of photoassimilates. Ultrastructural analysis of the movement of injected fluorescent dyes into mesophyll cells suggests that large areas of leaves are connected via densely populated plasmodesmata. Therefore sucrose flow takes place through these plasmodesmata thus being essentially symplastic (cf. Robards and Lucas, 1990 for a review).

Sucrose which is going to be exported from source leaves has to be loaded into the sieve element/companion cell complex (SECCC). Despite many reports in the literature it is still a matter of debate whether loading of the SECCC occurs via the symplasm or involves an apoplastic step. Based on the density of plasmodesmata connecting the companion cells and the mesophyll cells and furthermore based on the fact that the concentration of sucrose present in the sieve elements is ~8- to 10-fold higher (800-1000 mM) than its concentration in mesophyll cells, a physical separation of the SECCC from the mesophyll cells seems to be very likely (Giaquinta, 1983; Turgeon, 1989). Direct evidence for the existence of an apoplastic loading step of sucrose in source leaves has been obtained by the expression of an invertase gene from yeast in the apoplast of leaves from tobacco, potato and *Arabidopsis* (von Schaewen *et al.*, 1990; Sonnewald *et al.*, 1991; Heineke *et al.*, 1992). According to the Münch hypothesis, transport of solutes within the phloem is mainly regulated by mass flow and is driven by (e.g. osmotic) gradients created by loading (in the case of source tissue) or unloading (in the case of sink tissue) processes.

As the final step in the partitioning of sucrose, unloading of the phloem can occur either symplastically as in the case of sink leaves (Turgeon, 1989), or apoplastically via sucrose transport or involving the apoplastic cleavage of sucrose and subsequent import of the hexoses e.g. in the case of storage organs (Thorne, 1985; Wright and Oparka, 1989). The gene for a hexose transporter has been isolated from higher plants (Sauer *et al.*, 1990).

Due to the large difference between the sucrose concentration present in the mesophyll cells compared to that found in the phloem, a carrier has to be postulated which actively

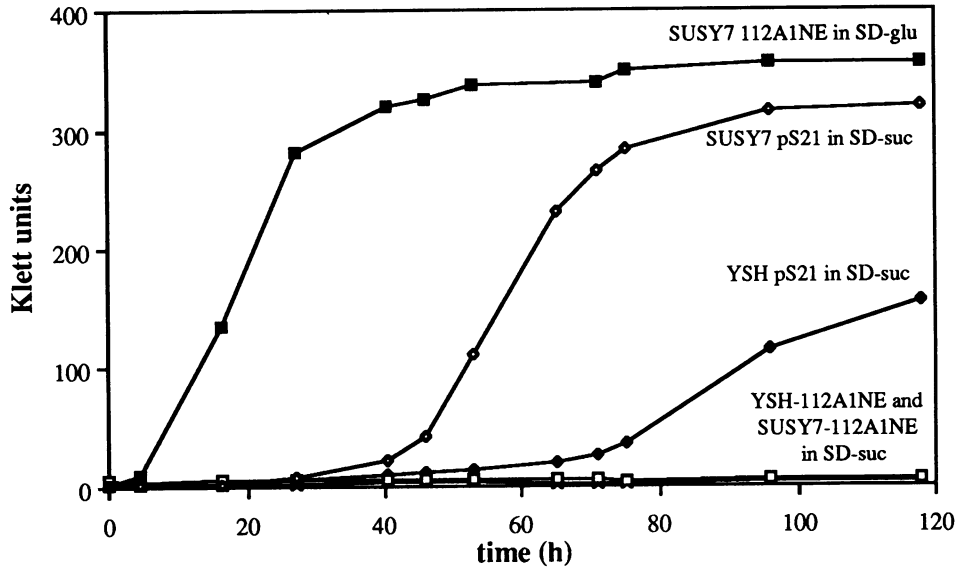


**Fig. 1.** Growth of several different yeast strains either devoid or expressing the sucrose carrier gene in minimal medium supplemented with either 2% glucose (SD-glu) or 2% sucrose. (SD-suc). YSH-112A1NE, YSH containing the expression plasmid YEP112A1NE; SUSY7-112A1NE, SUSY7 containing the expression plasmid YEP 112A1NE; SUSY7-pS21, SUSY7 containing the sucrose carrier encoding cDNA pS21; YSH-pS21, YSH containing the sucrose carrier encoding cDNA pS21.

loads sucrose from the mesophyll cells of source leaves into the sieve elements.

Numerous authors have identified such an activity in various plants. This activity has been described as a sucrose proton cotransporter (Giaquinta, 1977, 1983; Delrot, 1981; Buckhout, 1989; Bush, 1989, 1990; Williams *et al.*, 1992), the activity of which is dependent on energization of the membrane (Lemoine and Delrot, 1989). It has a high specificity for sucrose with an estimated $K_m$ of ~1 mM (Hitz *et al.*, 1986; Williams *et al.*, 1992), is highly sensitive to thiol-modifying agents such as *p*-chloromercuribenzenesulfonic acid (PCMBS) and *N*-ethylmaleimide (NEM) (Giaquinta, 1976; Delrot *et al.*, 1980) and there is circumstantial evidence for an apparent molecular mass of 42 kDa (Lemoine *et al.*, 1989).

Due to its postulated central role with respect to partitioning of energy and carbon in the form of sucrose throughout the plant, the sucrose carrier has been the target of many attempts aimed at identifying and purifying this protein at the molecular level. These experiments were mainly biochemical approaches involving gain of function such as vesicle-based reconstitution experiments, loss of function via antibodies (Delrot *et al.*, 1991; Li *et al.*, 1991) or photoaffinity-labeling with sucrose derivatives (Ripp *et al.*, 1988). Though a sucrose binding protein with a molecular mass of 62 kDa has been identified in this way (Ripp *et al.*, 1988), to date none of these approaches has led to the functional and molecular identification of the sucrose carrier.

In order to understand better the role and function of the sucrose carrier we set out to identify and clone the corresponding gene. In view of the fact that most biochemical approaches had failed up to now, we decided to follow a genetic approach based on the functional expression in a heterologous host. A unicellular organism would be suitable for this, that is devoid of sucrose uptake and is enabled to grow on sucrose as the sole carbon source by functional expression of a plant gene. An organism which has successfully been used to identify plant genes by complementation of mutants is *Saccharomyces cerevisiae* (Anderson *et al.*, 1992; Minet *et al.*, 1992; Sentenac *et al.*, 1992). We have modified a yeast strain in such a way that it does not efficiently grow on sucrose, but is able to metabolize internalized sucrose. We describe the molecular identification and characterization of a cDNA from spinach that fulfils the characteristics previously described for the sucrose carrier from higher plants.

## Results

Initial studies were performed to characterize the ability of yeast to take up and metabolize sucrose. Wild type *Saccharomyces* strains grow efficiently on sucrose due to a secreted invertase that hydrolyses the sucrose extracellularly and the subsequent uptake of hexoses by hexose transport systems. In addition several reports suggest that yeast contains endogenous sucrose transport systems (Khan *et al.*, 1973; Santos *et al.*, 1982). As maltose is structurally very similar to sucrose, the uptake could be mediated by the maltose permease. Therefore we decided to test the strain YSH 2 64-1A (YSH; Gozalbo and Hohmann, 1990) which is deficient in both invertase ($suc^-$) and maltose utilization (mal0) for its ability to transport and metabolize sucrose. This was first done by analysing the growth characteristics on media containing sucrose as the sole carbon source. YSH is unable to grow on media containing sucrose as the sole carbon source (Figure 1). Direct uptake measurements with [$^{14}$C]sucrose showed a slow, saturable uptake which was due to trace-contamination of the radiolabeled sucrose with hexoses as could be shown by TLC. After purification of the sucrose no uptake was measurable, which demonstrates that YSH contains no endogenous sucrose permease. Furthermore in agreement with the description of this strain no intra- or extracellular invertase activity was detectable. Maltose uptake experiments showed that the mal0 phenotype is due to the absence of a maltose transport system (data not shown).

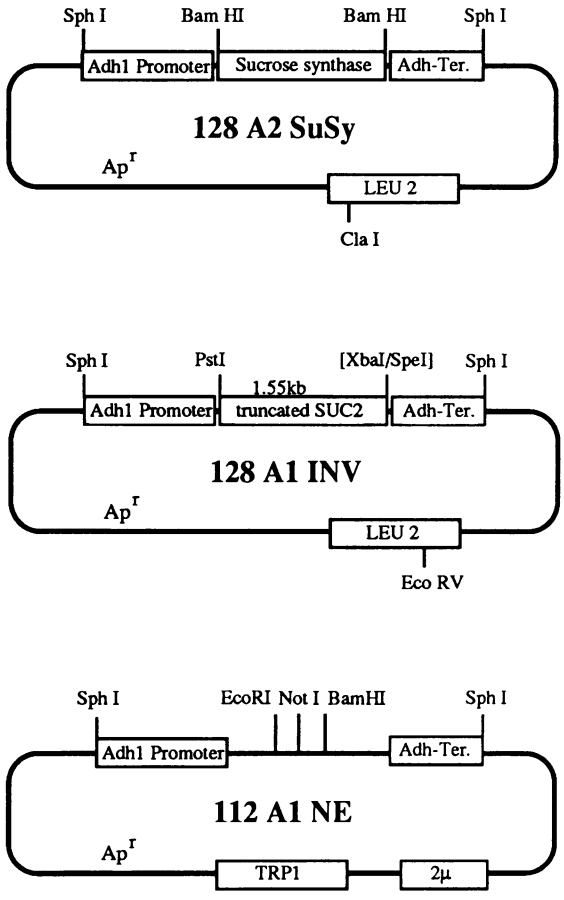


**Fig. 2.** Structure of the different plasmids used for either integration of the sucrose synthase (128A2SUSY) or the cytosolic invertase (128A1INV) into the genome of YSH or for construction of the cDNA expression library (112A1NE).

### *Construction of a yeast strain able to metabolize intracellular but not extracellular sucrose*

As shown above YSH is devoid of a sucrose transport activity thus allowing the functional identification of a plant homologue, provided the strain is enabled to metabolize sucrose entering the cell. Thus for the construction of an artificial complementation system a new sucrose hydrolysing activity restricted to the interior of the yeast has to be introduced. Two approaches were taken in this respect. First, a truncated form of the yeast-derived invertase was expressed in YSH. To this end the vectors YIP 128A1 and 128A2 were constructed that allow the expression of genes under control of the Adh1 promoter after integration into the yeast genome (Figure 2). A truncated yeast invertase gene lacking the signal peptide was cloned in YIP 128A1 and was stably integrated into the *Leu2* gene of YSH. Several transformants showed a cytosolic invertase activity. The strain with the highest activity (INV8; 68 mU/mg protein) was used for further analysis. INV8 grows slightly better on sucrose than YSH, but is strongly retarded compared to the wild-type strain S288C. Under the assumption that the remaining growth might be due to leakage of invertase, the expression of a protein that needs a cofactor for sucrose hydrolysis might be preferable. A candidate protein for this is sucrose synthase. Sucrose synthase needs UDP as cosubstrate for the cleavage of sucrose. Furthermore, due to the fact that it shows no homology to endogenous yeast sequences, possible problems due to recombination events could be excluded. Therefore, the cDNA of a potato sucrose synthase gene (Salanoubat and Belliard, 1987) was cloned in YIP 128A2 and stably integrated into the *Leu2* gene of YSH. Several clones showed sucrose synthase activity, and one transformant (SUSY7; 25 mU/mg protein) was further characterized. The growth behaviour of SUSY7 is indistinguishable from YSH. Thus both strains fulfil the criteria necessary for being used as hosts in an artificial complementation system allowing the recognition of the expression of a protein that mediates sucrose transport across the plasma membrane.

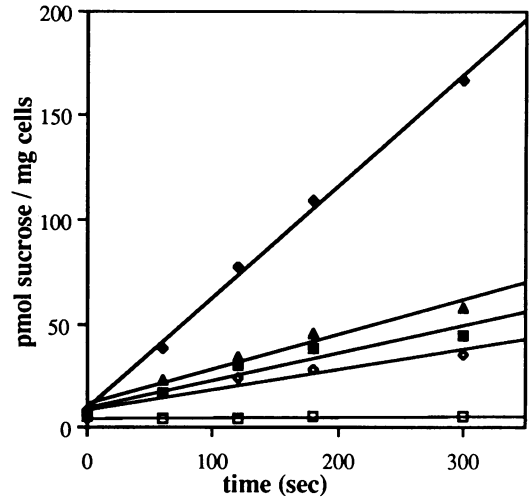


**Fig. 3.** Influence of glucose, CCCP and PCMBS on sucrose uptake by the strain SUSY7-S21. SUSY7-S21 cells were grown in SD medium containing 2% sucrose. In order to test the influence of glucose cells were preincubated in 10 mM glucose containing medium for 5 min prior to the sucrose uptake experiments (◆) or left in carbohydrate-free medium (△). Inhibition by 500 μM PCMBS (■) and 2.5 μM CCCP (◇) was determined by exposing the cells for 30 s to the inhibitor. These cells had been activated for sucrose uptake by prior incubation for 5 min in 10 mM glucose. Uptake of sucrose into yeast cells devoid of the plasmid pS21 (i.e. YSH) is shown for comparison (□).

### *Construction of a cDNA library from spinach leaves in a yeast expression vector*

In order to identify a sucrose carrier from higher plants a cDNA library established from poly(A)$^+$ RNA of spinach leaves was cloned in the newly constructed yeast expression vector YEP 112A1NE which is an episomal vector and contains TRP1 as a selectable marker (Figure 2). The inserts of a cDNA library from spinach source leaves first established in λ ZAPII (Sonnewald *et al.*, 1992) were excised and two fractions (less than and greater than 1.5 kb) were cloned into the yeast expression vector. The library was amplified in *Escherichia coli* and shown to contain a high proportion of recombinant clones (data not shown).

### *Transformation of INV8 and SUSY7 and selection for growth on sucrose*

The yeast strains INV8 and SUSY7 were transformed with a cDNA expression library, and as a first step selected on glucose-containing minimal medium in order to identify transformants complemented for tryptophan auxotrophy. This step yielded ~ $10^5$ primary transformants. In a second step the cells were washed from the plates and selected for growth on minimal medium containing sucrose as the sole

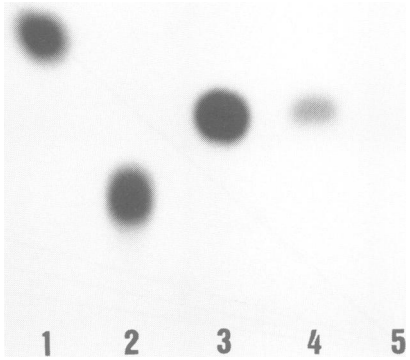


**Fig. 4.** TLC showing the presence of intact sucrose in yeast strains expressing the sucrose carrier (SUSY7-S21) but not in cells devoid of the plasmid pS21. SUSY7-S21 cells (expressing the sucrose carrier) and YSH.264-1A cells containing the expression plasmid 112A1NE were incubated under sucrose uptake conditions and after washing extracted for soluble carbohydrates using 80% ethanol. The figure shows the autoradiography of a thin layer chromatographic separation of the extracted sugars. Lane 1, 2 and 3 are references containing glucose, maltose and sucrose; lane 4 contains the sugar extracted from the strain SUSY7-S21 and lane 5 shows the autoradiogram of the sugars extracted from the control strain YSH.264.1A containing the empty expression plasmid 112A1NE.

carbon source. Fifty-four clones that showed significantly better growth under these conditions were isolated and reselected in liquid medium. Plasmid DNA was extracted from the yeast transformants, amplified in *E.coli* and analysed. Seven clones deriving from transformants of both SUSY7 and INV8 showed an identical restriction pattern with an insert size of 1.95 kb. Transformation of SUSY7 with these seven plasmids proved that growth on sucrose is dependent upon the recombinant plasmid (Figure 3). Due to the fact that all seven inserts showed the same restriction pattern and resulted in the same change in phenotype, further analysis was restricted to one transformant named SUSY7-S21 which contains the plasmid pS21 containing the supposed sucrose carrier cDNA.

### *pS21 encodes a true transport activity and leads to intracellular accumulation of sucrose*

The fact that the strain SUSY7-S21 grows on sucrose does not necessarily mean that the cDNA clone encodes a sucrose carrier activity. An alternative is that this cDNA encodes a secreted sucrose-hydrolysing activity such as an invertase. These two possibilities can be differentiated in several ways. First, if pS21 encodes a secreted invertase, transformation of yeast with this plasmid should result in growth on sucrose of both the original mutant strain YSH, and its derivatives INV8 or SUSY7 which have been given an internal sucrose-hydrolysing system. In contrast, if the plasmid encodes a sucrose carrier the growth of YSH should be much less vigorous as compared to strains INV8 or SUSY7. The pS21 transformants of SUSY7 grew significantly better than those of YSH (Figure 3) which reduces the likelihood that pS21 encodes a secreted sucrose-cleaving activity. Furthermore no secreted sucrose-hydrolysing activities were detectable in SUSY7-S21 (data not shown). In order to test directly for the ability of SUSY7-S21 to transport sucrose, yeast cells were incubated in medium containing [$^{14}$C]sucrose for a few seconds to minutes, the cells were washed and the radioactivity taken up was measured by liquid scintillation counting. The yeast strain SUSY7-S21 shows a saturable uptake which is significantly higher than the background (Figure 3).

**Table I.** Activation of sucrose transport by preincubation with carbohydrates and amino acids

| Sugar or amino acid (mM) | % activity |
|---|---|
| Control | 100 |
| 10 glucose | 250 |
| 10 fructose | 280 |
| 10 mannose | 230 |
| 10 galactose | 80 |
| 10 glutamine | 90 |

If clone pS21 encodes a sucrose carrier and not a sucrose-hydrolysing activity, short exposure of yeast cells to radioactively labelled sucrose, washing to remove external sucrose, lysis of the cells and subsequent analysis of the products should demonstrate the presence of internalized sucrose. Figure 4 shows the result of a TLC analysis of lysed SUSY7-S21 cells after exposure for 1 min to [$^{14}$C]sucrose, arguing for actual uptake of intact sucrose.

Thus, both the growth characteristics of yeast strains containing pS21 and the biochemical analysis are in agreement with the assumption that pS21 encodes a transport activity for sucrose rather than a sucrose-metabolizing activity.

### *Response of the sucrose carrier activity of pS21 towards various inhibitors is similar to that described for the plant transporter*

The sucrose carrier of several plant species has been described as a sucrose/proton cotransporter, sucrose transport being dependent on the membrane potential and the pH gradient. In agreement with this it was observed that transport of sucrose into the yeast strain SUSY7 containing the plasmid pS21 was stimulated by preincubating the strain in glucose-containing medium before performing the uptake experiments (Figure 3; Table I). The rate of activation by glucose differed between independent experiments but qualitatively the same result was always obtained. In addition to glucose, other monosaccharides such as fructose and mannose also led to a stimulation of sucrose uptake (Table I).

This stimulating effect of various monosaccharides could either be due to a general increase in the energization of the cell or it could be a direct or indirect activation of the transporter itself. Both arsenate and antimycin A which block the ATP generating systems such as glycolysis or respiration lead to transport inhibition. This finding is in agreement with the assumed role of the monosaccharides in serving as energy fuel systems (Table II).

Protonophores such as 2,4-dinitrophenol (2,4-DNP) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) strongly inhibit sucrose transport (Table II). These data support the assumption that the sucrose uptake is mediated by a proton cotransport mechanism. Further support for proton symport comes from the observation that the transport is pH dependent, i.e. maximal in the acidic range, whereas alkalization completely blocks transport (Figure 5).

The sucrose carrier from higher plants is highly sensitive to the SH-modifying agent PCMBS (Giaquinta, 1976; Delrot

**TableII.** Sensitivity of sucrose transport to inhibitors

| Inhibitor | | | % activity |
|---|---|---|---|
| Control | | | 100 |
| 0.5 | μM | CCCP | 65 |
| 2.5 | μM | CCCP | 21 |
| 25 | μM | 2,4-DNP | 61 |
| 100 | μM | 2,4-DNP | 9 |
| 25 | μM | PCMBS | 73 |
| 100 | μM | PCMBS | 21 |
| 1 | mM | NEM | 10 |
| 0.5 | mM | DEPC | 19 |
| 1 | mM | arsenate | 34 |
| 10 | μM | antimycin A | 59 |

Inhibition of sucrose uptake of the yeast strain SUSY7-S21 by different substances, which were added to activated cells 30 s prior to the addition of labelled sucrose.

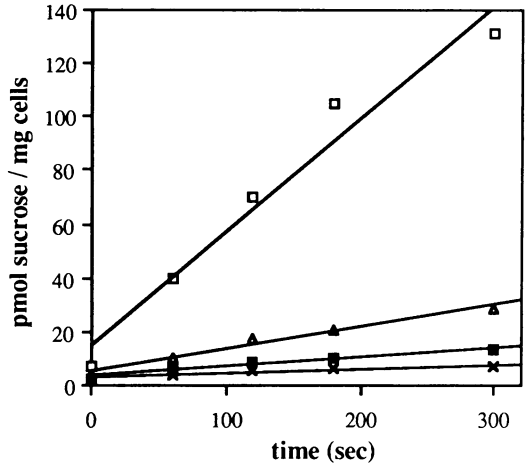


**Fig. 5.** Influence of the pH on the uptake of sucrose by the strain SUSY7-S21. Cells were incubated at different pH values for uptake experiments. (□) pH 4.5; (△) pH 5.5; (■) pH 6.5; (×) pH 7.5.

**Table III.** Specificity of the sucrose carrier

| Inhibitor (mM) | % activity |
|---|---|
| Control | 100 |
| 2 sucrose | 28 |
| 2 maltose | 58 |
| 10 maltose | 37 |
| 2 α-phenyl-glucoside | 7 |
| 2 phloridzin | 16 |
| 2 lactose | 91 |
| 2 raffinose | 110 |
| 10 palatinose | 102 |
| 10 trehalose | 103 |

Inhibition of sucrose uptake of the yeast strain SUSY7-S21 by different sugars, which were added to activated cells 30 s prior to the addition of the labelled sucrose.

*et al.*, 1980) and to diethylpyrocarbonate (DEPC) (Bush, 1989). To link the identity of the cloned transporter to the carrier described in literature, a variety of inhibitors were tested. Sucrose transport in SUSY7-S21 is highly sensitive to PCMBS, NEM and DEPC (Table II; Figure 3). The effect of PCMBS can be partially reversed by the addition of dithiothreitol (DTT) (data not shown).

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AAAAACACACCCAAAAAAAAAACACTACGACTATTTCAAAAAAAACATTGTTACTAGA  60

AATCTTATTATGGCAGGAAGAAATATAAAAAATGGTGAAAATAACAAGATTGCGGGTTCT 120
        M  A  G  R  N  I  K  N  G  E  N  N  K  I  A  G  S

TCTCTTCACTTAGAGAAGAACCCAACAACTCCCCCCGAGGCCGAGGCTACCTTAAAGAAG 180
 S  L  H  L  E  K  N  P  T  T  P  P  E  A  E  A  T  L  K  K
                  ^

CTCGGCCTCGTGGCTTCAGTAGCGGCCGGGGTTCAGTTCGGGTGGGCTTTACAGCTCTCC 240
 L  G  L  V  A  S  V  A  A  G  V  Q  F  G  W  A  L  Q  L  S

CTACTGACCCCGTACGTCCAACTACTGGGCATTCCCCACACTTGGGCCGCCTACATCTGG 300
 L  L  T  P  Y  V  Q  L  L  G  I  P  H  T  W  A  A  Y  I  W

TTGTGCGGCCCAATCTCGGGGATGATTGTCCAGCCATTGGTCGGGTACTATAGTGACCGG 360
 L  C  G  P  I  S  G  M  I  V  Q  P  L  V  G  Y  Y  S  D  R

TGCACCTCCCGCTTCGGCCGACGTCGCCCCTTCATTGCAGCAGGGGCGGCTCTAGTGGCC 420
 C  T  S  R  F  G  R  R  R  P  F  I  A  A  G  A  A  L  V  A

GTAGCGGTGGGGCTAATCGGATTCGCCGCCGATATCGGCGCAGCGTCGGGTGATCCAACG 480
 V  A  V  G  L  I  G  F  A  A  D  I  G  A  A  S  G  D  P  T

GGAAACGTGGCAAAACCCCGGGCCATGGCGGTGTTTGTGGTCGGGTTTTGGATCCTGAC 540
 G  N  V  A  K  P  R  A  I  A  V  F  V  V  G  F  W  I  L  D

GTGGCTAACAACACCCTGCAAGGCCCATGCAGGGCGTTGTTAGCAGACATGGCCGCCGGG 600
 V  A  N  N  T  L  Q  G  P  C  R  A  L  L  A  D  M  A  A  G
                  ^

TCGCAAACCAAAACCCGGTACGCTAACGCCTTCTTCTCCTTCTTCATGGCGTTAGGAAAC 660
 S  Q  T  K  T  R  Y  A  N  A  F  F  S  F  F  M  A  L  G  N

ATCGGAGGGTACGCCGCCGGTTCATACAGCCGCCTCTACACGGTGTTCCCCTTTACCAAA 720
 I  G  G  Y  A  A  G  S  Y  S  R  L  Y  T  V  F  P  F  T  K

ACCGCCGCCTGCGACGTCTACTGCGCCAATCTAAAATCCTGCTTCTTCATCTCCATCACA 780
 T  A  A  C  D  V  Y  C  A  N  L  K  S  C  F  F  I  S  I  T

CTCCTAATCGTCCTCACAATCCTAGCACTTTCCGTCGTAAAAGAGGGTCAAATCACAATC 840
 L  L  I  V  L  T  I  L  A  L  S  V  V  K  E  R  Q  I  T  I

GACGAAATCCAAGAAGAAGAAGACTTAAAAAACAGAAACAATAGCAGCGGTTGTGCAAGA 900
 D  E  I  Q  E  E  E  D  L  K  N  R  N  N  S  S  G  C  A  R
                              ^

CTACCGTTCTTCGGACAATTAATAGGCGCTCTCAAAGATCTACCAAAACCAATGCTAATC 960
 L  P  F  F  G  Q  L  I  G  A  L  K  D  L  P  K  P  M  L  I

CTATTACTAGTAACAGCCCTAAATTGGATCGCATGGTTTCCATTCTTGTTGTTCGATACT 1020
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GATTGGATGGGTAAAGAAGTGTACGGTGGTACAGTCGGAGAAGGTAAATTGTACGACCAA 1080
 D  W  M  G  K  E  V  Y  G  G  T  V  G  E  G  K  L  Y  D  Q

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 G  V  H  A  G  A  L  G  L  M  I  N  S  V  V  L  G  V  M  S

TTGAGTATTGAAGGTTTGGCTCGTATGGTAGGCGGTGCTAAAAGGTTATGGGAATTGTC 1200
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AATATTATCTTGCTGTTTGTTTAGCTATGACGGTGTTAGTTACTAAGTCCGCCGAACAC 1260
 N  I  I  L  A  V  C  L  A  M  T  V  L  V  T  K  S  A  E  H

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CTAGGAGTTCTCAACCTCGCCATCGTTGTACCCCAGATGTTTGTGTCGGTAACAAGTGGG 1500
 L  G  V  L  N  L  A  I  V  V  P  Q  M  F  V  S  V  T  S  G

CCATGGGATGCAATGTTTGGTGGAGGAAATTTGCCAGCATTCGTGGTGGGAGCTGTAGCA 1560
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GCAACAGCCAGTGCAGTTCTTTCATTTACATTGTTGCCTTCTCCACCCCCTGAAGCTAAA 1620
 A  T  A  S  A  V  L  S  F  T  L  L  P  S  P  P  P  E  A  K

ATTGGTGGGTCCATGGGTGGTCATTAAGAAATTTAATACTACTCCGTACAATTTAAACCC 1680
 I  G  G  S  M  G  G  H  *

AAATTAAAAATGAAAATGAAAATTTTTAACCCATGTTCGTTACGTTGTAATTAGAGAGAA 1740

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CTCTGCTTTTTTTTTTTTTTTTTAACGCGACGTGTTTTTGAGATAAGGAAGGGCTAGATC 1860

GAGGATGGGGGAATTGGCAAGAAATTGCTCGGGTATAAATATTTATCCCTCTTTGTAATT 1920

TTCAGTAACATTTAATAGCCAGAAATCAAAAAGTCAAGAAAAATGAAA
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**Fig. 6.** DNA and amino acid sequence of pS21. The translation stop is marked by an asterisk, putative membrane spanning regions are underlined and the potential N-glycosylation sites are indicated by double-underlining and ^.

### *Specificity of the pS21 encoded transport protein*

The affinity of the pS21 encoded carrier protein for various sugars was analysed. The $K_m$ value of the carrier for sucrose is ~1.5 mM, which is in good agreement with data published for various plant systems (data not shown). The uptake system is highly specific as a 10-fold excess of di- and trisaccharides such as palatinose, raffinose, trehalose and

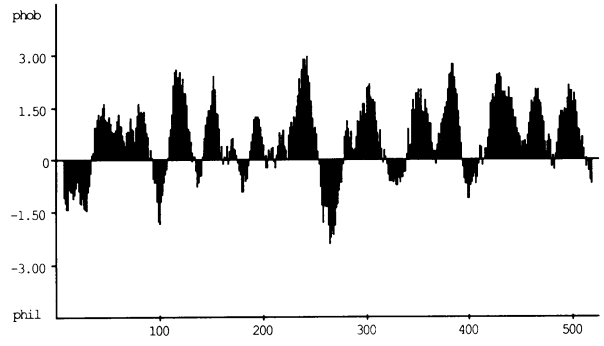


**Fig. 7.** Hydropathy plot calculated from the amino acid sequence of the sucrose carrier as deduced from the nucleotide sequence of the cDNA pS21. The calculation was performed according to Kyte and Doolittle (1982) with a window of 15 amino acids. Hydrophobic regions are given a positive hydropathy index.

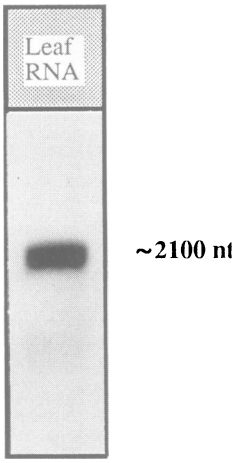


**Fig. 8.** RNA blot analysis of spinach leaf RNA. Hybridization of the cDNA pS21 to total RNA (50 $\mu$g) isolated from leaves of spinach shows the presence of a single RNA species with a size of ~2100 nucleotides.

lactose do not compete, whereas maltose clearly competes (Table III). Transport studies using [$^{14}$C]maltose demonstrate that maltose is also transported by the system, the $K_m$ for maltose was determined to be ~5 mM. This result corroborates uptake studies into leaf tissues (Maynard and Lucas, 1982). The importance of the hydrophobic surface of sucrose (Hitz *et al.*, 1986) for the transport is supported by the finding that $\alpha$-phenylglucoside and phloridzin strongly inhibit sucrose uptake (Table III). As shown above, neither hexoses nor glutamine, one of the major phloem sap constituents, compete.

### *DNA sequence and expression*

As a next step the DNA sequence of clone pS21 was determined. The pS21 cDNA encodes a predicted peptide of 525 amino acids (Figure 6; 55 kDa). The hydropathy plot of the predicted protein reveals the presence of 12 hydrophobic segments of at least 20 amino acids presenting an average hydropathy index of >1.3 (Kyte and Doolittle, 1982) and thus fits the requirements for integral membrane proteins with a 6-loop-6 structure (Figures 6 and 7). It is therefore similar to other amino acid and sugar transporters from pro- and eukaryotes (e.g. Botfield *et al.*, 1992). The N terminus is, as in the case of the yeast amino acid permeases,

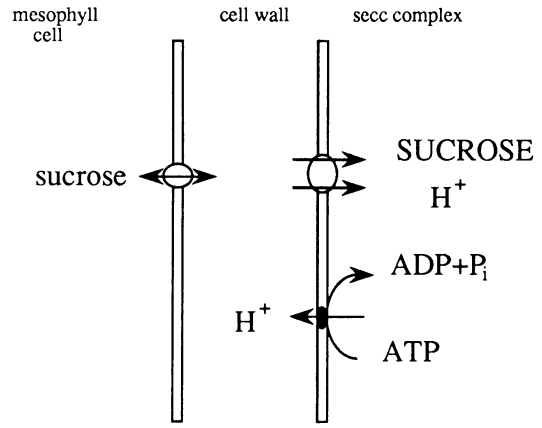


**Fig. 9.** Hypothetical model for active loading of phloem with sucrose. Two membranes separate the mesophyll from the SECC complex. The postulated proton symport must be located at the plasma membrane of the SECC and is energized by a proton gradient generated by the proton ATPase. A second carrier for sucrose must be present at the plasma membrane that allows the sucrose synthesized in the mesophyll to enter the phloem. This carrier could represent a facilitated diffusion mechanism or a sucrose–proton antiport.

hydrophilic (Jauniaux and Grenson, 1990) and hence does not present features of a cleavable signal sequence. No strong sequence homologies were found to other disaccharide carriers such as lactose or maltose permeases from *E.coli* and yeast, or any other proteins in the NBRF protein sequence data bank. Taken together these data suggest that the sucrose permease from spinach belongs to the class of single transport proteins, which mediate both substrate recognition and translocation.

Southern blot analysis of *Eco*RI and *Bam*HI digested genomic DNA from spinach probed with the 1.95 kb insert of pS21 shows only one single band (data not shown). Northern blot analysis of total RNA from mature spinach leaves indicates a single 2100 nucleotide transcript that hybridizes with pS21 cDNA (Figure 8). Thus it is most likely that the clone pS21 represents a full-length clone of this RNA, the small differences being probably due to the missing poly(A) tail and some sequences in the 5′-untranslated leader.

## Discussion

Growth and development of a plant are controlled by the availability of the products of the photoassimilation process. Distribution of metabolites throughout the plant occurs mainly via the sieve elements. Analysis of the composition of the phloem sap of spinach (Riens *et al.*, 1991) showed that ~83% of its organic contents are represented by sucrose whereas the other components (mainly amino acids such as glutamic and aspartic acid) represent 17%. The vast majority of the energy fixed in photosynthetically active leaves is transported throughout the plant in the form of sucrose. The control of the loading and unloading processes of the systems used for long-distance transport, i.e. the phloem, is most likely controlled by membrane-associated steps. Some evidence suggests that loading (and maybe also unloading) of minor components of the phloem sap, e.g. amino acids, occurs via mass flow, thus assigning a central role to the distribution of metabolites within the plant to sucrose as the major constituent of the phloem sap. Therefore understanding, and at a later step, modulating the loading and/or

unloading of sucrose might represent a key step in analysing/modulating the distribution of metabolites via the phloem system. Active loading of the phloem with sucrose is postulated to occur by a proton sucrose symporter from the apoplastic compartment into the phloem (Figure 9). The active transport against a concentration gradient is energized by a plasma membrane-bound $H^+$-ATPase (Humphreys, 1988). A second sucrose transport system acting either as a proton antiporter or facilitating diffusion must be present at the mesophyll plasma membrane to allow the sucrose to enter the apoplast. The data described give strong evidence that clone pS21 isolated from a cDNA library of poly(A)$^+$ RNA from spinach leaves encodes a proton sucrose symporter.

This interpretation is based on the following results. (i) Expression of this carrier in *S.cerevisiae* enables yeast to import sucrose as evidenced by direct uptake measurements. This import is not directly coupled to a metabolization of the sucrose as shown by the reisolation of intact sucrose from the yeast cells. (ii) Expression of clone pS21 allows the yeast strains INV8 and SUSY7 (which both express a cytosolic sucrose-hydrolysing activity) to grow more efficiently on sucrose as the sole carbon source as compared to yeast strains devoid of a sucrose-metabolizing activity, i.e. YSH. This observation, combined with the fact that no invertase activity could be detected, excludes the possibility that pS21 encodes a secreted sucrose-hydrolysing activity. The residual growth of the YSH strain expressing pS21 (but lacking the sucrose-metabolizing activity) on sucrose could be explained by either assuming that sucrose imported by the pS21 encoded protein is either hydrolysed non-enzymatically or that endogenous enzymes display some low sucrose-cleaving activity.

The interpretation that clone pS21 is the main sucrose carrier characterized by vesicle studies in different plant species (cf. Introduction) is based on the following facts. (i) The activity of the carrier is pH dependent which is in agreement with its description as a sucrose proton cotransporter. (ii) The activity is dependent on the pH gradient and the membrane potential as shown by the inhibition of transport by protonophores and inhibition of ATP-generating systems. (iii) The sucrose carrier is inhibited by thiol-modifying agents such as PCMBS which again is in agreement with the description of the endogenous sucrose carrier from plants. (iv) The $K_m$ value of the carrier for sucrose is estimated to be $\sim 1.5$ mM which is in a similar range to that found for sugarbeet plasma membrane vesicles. (v) The specificity towards other derivatives of sucrose reflects the data described for the sucrose carrier in plants.

One interesting result which has been controversial before, is the ability of the sucrose carrier to transport maltose.

Finally, the primary structure of the protein fits well with the characteristics required for a membrane protein. Taken together the most likely interpretation of these data is that pS21 does indeed encode the sucrose carrier from higher plants.

Earlier attempts to identify the sucrose carrier have resulted in the molecular identification of a sucrose-binding protein (Ripp *et al.*, 1988) which however turned out to be clearly different from the carrier. The molecular mass of this protein has been estimated to be $\sim 62$ kDa. By differential labelling experiments a protein with an apparent molecular mass of 42 kda present in plasma membrane fractions of sugarbeet has been identified as a possible sucrose carrier. Antibodies raised against the 42 kDa fraction of plasma membrane proteins were able to inhibit the sucrose uptake systems in isolated membrane vesicles, thus strongly suggesting that the 42 kDa fraction does indeed contain the sucrose carrier protein (Delrot *et al.*, 1991). The molecular mass of the pS21 encoded protein described here is 55 kDa. This is well above the 42 kDa protein identified by Delrot *et al.* (1991), however the apparent molecular mass of membrane proteins as estimated from SDS gels is often well below their actual molecular mass. Further investigations will be necessary to investigate the possible homology of the described proteins.

Functional complementation in yeast was used in the work described here to identify a sucrose carrier from higher plants. To this end a yeast strain was constructed which was unable to grow on sucrose due to the lack of an external invertase but could metabolize sucrose once it was internalized. Two activities were introduced into yeast to allow metabolization of imported sucrose, a sucrose synthase activity from potato and a cytosolic invertase activity. Although in the work described here the aim was to identify a sucrose carrier protein it is obvious that a similar approach can be used to identify other carriers with different substrate specificities as well as secreted sucrose-hydrolysing activities such as invertases. Thus, the approach demonstrated here, i.e. the tailoring of yeast metabolism to identify a specific gene allows a much broader use of yeast for the identification of genes as compared to the use of classical yeast mutants.

A further advantage is that the functional expression of plant membrane transporters in yeast allows rapid analysis of structure–function relationships in the heterologous system.

The availability of such carrier genes together with the possibility of creating transgenic plants where the expression of these carriers are either down-regulated by antisense RNA expression or modulated by their ectopic expression, will undoubtedly result in new insights into the role these carriers play in photoassimilate allocation and partitioning in higher plants.

## Materials and methods

### *Bacteria and yeast strains*

The following strains were used in this study. *E.coli: DH5α supE44 hsdR17 recA1 endA1 gyrA96 thi- 1 relA1. S.cerevisiae:* S288C (*αSUC2 mal mel gal2 CUP1*) Yeast Genetic Stock Center, UC Berkeley. YSH: (YSH 2.64–1A, Gozalbo and Hohmann, 1990) (*suc2::URA3 mal0 leu2 trp1*) was a gift from S.Hohmann, University of Leuven, Belgium. INV8 and SUSY7 are derivatives of YSH-expressing cytosolic invertase and sucrose synthase respectively. YSH-112A1NE: YSH transformed with YEP112A1NE for control experiments. SUSY7-112A1NE: SUSY7 transformed with YEP112A1NE for control experiments. SUSY7 S21: SUSY7 derivative containing the spinach cDNA pS21 encoding a sucrose carrier cDNA. Yeast strains were grown in synthetic dextrose minimal medium (SD medium) supplemented with either glucose (2%) or sucrose (2%).

### *Recombinant DNA techniques*

Oligonucleotides were synthesized on an ABI 380A DNA synthesizer.

| | |
|---|---|
| INV3 | GAGCTGCAGATGGCAAACGAAACTAGCGATAGACCTT TGGTCACA |
| INV4 | GAGACTAGTTTATAACCTCTATTTTACTTCCCTTACTT GGAA |
| SUSY1 | GAGAGAGGATCCTGCAATGGCTGAACGTGTTTTGACTC GTG |

SUSY2 GAGAGAGGATCCTTCATTCACTCAGCAGCCAATGGAA CAGCT
NE1 GTCTAGAGAATTCCCGGGCGGCCGCG
NE2 GATCCGCGGCCGCCCGGGAATTCTCTAGACTGCA

*Yeast expression vectors (YIP128A1/128A2 and YEP112A1NE).* The plasmids YIplac 128 and YEplac 112 (Gietz und Sugino, 1988) were restricted with *Pst*I and *Eco*RI, blunted with T4 polymerase and relegated to remove most of the multiple cloning site. To construct YIP128A1 and YEP112A1, the plasmids were digested with *Sph*I and the 728 bp fragment from pVT100U containing the ADH1 expression cassette was inserted (Vernet *et al.*, 1987). For YIP128A2, the *Sph*I fragment from pVT102U with the polylinker in the opposite orientation was used.

To insert a *Not*I site into the polylinker, YEP112A1 was digested with *Pst*I/*Xba*I and the annealed synthetic oligonucleotides NE1/NE2 were ligated into it. The new polylinker contains the Adh1 promoter and restriction sites for *Pst*I, *Eco*RI, *Sma*I, *Not*I, *Xba*I and *Bam*HI.

To construct 128A1-INV, polymerase chain reaction (PCR) using the oligonucleotides INV3 and INV4 was applied to amplify the truncated invertase gene from PI-3-INV (von Schaewen *et al.*, 1990). The PCR product was digested with *Pst*I and *Spe*I and cloned into the *Pst*I/*Xba*I sites of YIP128A1. The construct was integrated into the genome of YSH via the *Eco*RV site in the *LEU2* gene to yield INV8.

To construct 128A2-SUSY, the sucrose synthase gene was amplified by PCR using the oligonucleotides SUSY1 and SUSY2 from the λ clone of potato (Salanoubat and Belliard, 1987). The PCR product was digested with *Bam*HI and cloned into the *Bam*HI site of YIP128A2. The correct orientation of the insert was demonstrated by *Sac*I digestion. The construct was integrated into the genome of YSH via the *Cla*I site in the *LEU2* gene to yield SUSY7.

*Construction of the spinach cDNA library, yeast transformation and selection.* The source leaf cDNA library in λ ZAPII was a gift from U.Sonnewald (Sonnewald *et al.*, 1992). Phages (1 500 000) were plated, washed from the plates and DNA was prepared on CsCl−sarcosyl gradients (Buckley and Goding, 1988). The inserts were excised with *Not*I and separated on a 1% agarose gel. The size range from <1.5 kb and >1.5 kb was excised and eluted. Inserts were cloned into the yeast expression plasmid YEP112A1NE, previously digested with *Not*I and dephosphorylated. Approximately 90.000 transformants of the <1.5 kb fraction and ~40.000 of the >1.5 kb fraction were obtained. Transformed *E.coli* cells were washed from the plates and DNA was isolated. Fourteen batches of competent INV8 and SUSY7 cells were transformed with 1 μg of the cDNA library (Dohmen *et al.*, 1991). Yeast cells were plated on SD medium containing 2% glucose and $5-10 \times 10^4$ transformants were obtained per μg of DNA. Transformants were washed from the plates in 10 ml SD medium and 10 μl of the transformed cells were transferred to solid SD medium supplemented with 0.5% sucrose. Faster growing colonies were picked after incubation for 4 days at 30°C.

Chemicals were obtained from Sigma. DNA sequence analysis was performed using *Exo*III deletions and synthetic oligonucleotides in conjunction with T7 polymerase (Pharmacia). All other methods were done according to Sambrock *et al.* (1989). Computer analysis was performed with the UWGCG package (Devreux *et al.*, 1987).

### Physiological measurements

*Determination of sugars and enzyme activities.* To test the invertase and sucrose synthase activity in yeast, 3 ml overnight cultures of the respective yeast strains were harvested, washed in yeast lysis buffer (50 mM HEPES pH 7.5, 10% glycerol, 1 mM DTT, 1 mM EDTA, 2 mM PMSF), resuspended in 100 μl of the same buffer and after addition of an equal volume of glass beads (0.5 mm diameter) the suspension was vortexed five times for 15 s, centrifuged and the supernatant was analysed. The activity tests were performed as described by Stitt *et al.* (1989).

*Uptake of radiolabelled sugars.* Cells were grown to the logarithmic phase, washed and resuspended to 1−5% w/v in SD. [$^{14}$C]Sucrose (Amersham, Braunschweig) uptake was determined essentially as described by Cirillo (1989). One milligramme of cells was incubated for 0, 60, 120, 180 and 300 s in a solution containing 0.5 μCi [$^{14}$C]sucrose (3 mCi/mmol final concentration), the reaction was stopped by transfer to 8 ml ice-cold water and filtration on glass-fibre filters (Whatman). Cells were washed three times in 4 ml ice-cold water and the radioactivity taken up by the cells was determined by liquid scintillation counting (Beckman). Due to trace contaminations of glucose in the sucrose, the product had to be purified by preparative TLC (Schleicher & Schüll F1500 in 87% acetone). Assays were generally performed in 0.2 mM sucrose at room temperature. For activation, cells were preincubated for 5 min in 10 mM glucose. For inhibition and competition studies the reagents were added 30 s prior to addition of sucrose (dissolved to 1% w/v of final concentration used in the assay in appropriate solvents). The solvents alone showed no influence on sucrose uptake. MES buffer at a final concentration of 25 mM was used to determine the transport activity at different pH values.

*Detection of sugars after uptake.* Cells were incubated for 2 min in 1 μCi[$^{14}$C]sucrose, collected as described above and sugars were extracted in 300 μl 80% ethanol at 70°C for 60 min. The cleared supernatant (10 μl) was separated by TLC on silica plates (Schleicher & Schüll F1500) in 87% acetone. Chromatograms were autoradiographed on X-ray film for 7 days.

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## References

Anderson,J.A., Huprikar,S.S., Kochian,L.V., Lucas,W.J. and Gaber,R.F. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 3736−3740.
Botfield,M.C., Naguchi,K., Tsuchiya,T. and Wilson,T. (1992) *J. Biol. Chem.*, **267**, 1818−1822.
Buckley,M.F. and Goding,J.W. (1988) *Anal. Biochem.*, **175**, 281−283.
Buckhout,T.J. (1989) *Planta*, **178**, 393−399.
Bush,D. (1989) *Plant Physiol.*, **89**, 1318−1323.
Bush,D. (1990) *Plant Physiol.*, **93**, 1590−1596.
Cirillo,V.P. (1989) *Methods Enzymol.*, **174**, 617−622.
Delrot,S. (1981) *Plant Physiol.*, **68**, 706−711.
Delrot,S., Despequel,J.D. and Bonnemain,J.L. (1980) *Planta*, **149**, 144−148.
Delrot,S., Gallet,O., Li,Z.S., Gaillard,C. and Laloi,M. (1991) In Bonnemain,J.L., Delrot,S., Lucas,W.J. and Dainty,J. (eds), *Recent Advances in Phloem Transport and Assimilate Compartmentation*. Ouest Editions, Nantes, pp. 154−166.
Devreux,J., Haeberli,P. and Smithies,O. (1987) *Nucleic Acids Res.*, **12**, 387−395.
Dohmen,R.J., Strasser,A.W.M., Höner,C.B. and Hollenberg,C.P. (1991) *Yeast*, **7**, 691−692.
Flügge,U.I., Fischer,K., Gross,A., Sebald,W., Lottspeich,F. and Eckerskorn,C. (1989) *EMBO J.*, **8**, 39−46.
Giaquinta,W.T. (1976) *Plant Physiol.*, **57**, 872−875.
Giaquinta,W.T. (1977) *Nature*, **267**, 369−370.
Giaquinta,W.T. (1983) *Annu. Rev. Plant Physiol.*, **34**, 347−387.
Gietz,R.D. and Sugino,A. (1988) *Gene*, **74**, 527−534.
Gozalbo,D. and Hohmann,S. (1990) *Curr. Genet.*, **17**, 77−79.
Heineke,D., Sonnewald,U., Büssis,G., Günter,K., Leidreiter,K., Wilke,I., Raschke,K., Willmitzer,L. and Heldt,H. (1992) *Plant Physiol.*, in press.
Hitz,W., Card,P.J. and Ripp,K.G. (1986) *J. Biol. Chem.*, **261**, 11986−11991.
Ho,L.C. (1988) *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **39**, 355−378.
Humphreys,T.E. (1988) In Baker,D.A. and Hall,J.L. (eds), Solute Transport in Plant Cells and Tissues. Longman, New York, pp. 305−345.
Jauniaux,J.C. and Grenson,M. (1990) *Eur. J. Biochem.*, **190**, 39−44.
Khan,N.A., Zimmermann,F.K. and Eaton,N.R. (1973) *Mol. Gen. Genet.*, **123**, 43−50.
Kyte,J. and Doolittle,R.F. (1982) *J. Mol. Biol.*, **157**, 105−132.
Lemoine,R. and Delrot,S. (1989) *FEBS Lett.*, **249**, 129−133.
Lemoine,R., Delrot,S., Gallet,O. and Larsson,C. (1989) *Biochem. Biophys. Acta.*, **978**, 65−71.
Li,Z., Gallet,O., Gaillard,C., Lemoine,R. and Delrot,S. (1991) *FEBS Lett.*, **286**, 117−120.
Maynard,J.W. and Lucas,W.J. (1982) *Plant Physiol.*, **70**, 1436−1443.
Minet,M., DuFour,M.-E. and Lacroute,F. (1992) *Plant J.*, **2**, 417−422.
Riens,B., Lohaus,G., Heinicke,D. and Heldt,H.W. (1991) *Plant Physiol.*, **97**, 227−233.
Ripp,K.G., Viitanen,P.V., Hitz,W.D. and Franceschi,V.R. (1988) *Plant Physiol.*, **88**, 1435−1445.

Robards,A.W. and Lucas,W.J. (1990) *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **41**, 369–419.
Salanoubat,M. and Belliard,G. (1987) *Gene*, **60**, 47–56.
Sambrock,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning, A Laboratory Manual*. Cold Spring Harbour Laboratory Press, Cold Spring Harbor.
Santos,E., Rodriguez,L., Elorza,M.V. and Sentandreu,R. (1982) *Arch. Biochem. Biophys.*, **216**, 652–660.
Sauer,N., Friedländer,K. and Gräml-Wicke,U. (1990) *EMBO J.*, **9**, 3045–3050.
Sentenac,H., Bonneaud,N., Minet,M., Lacroute,F., Salmon,J.-M., Gaymard,F. and Grignon,C. (1992) *Science*, **256**, 663–665.
Sonnewald,U., Brauer,M., von Schaewen,A., Stitt,M. and Willmitzer,L. (1991) *Plant J.*, **1**, 95–106.
Sonnewald,U., Quick,W.P., MacRae,E., Krause,H.P. and Stitt,M. (1992) *Planta*, in press.
Stitt,M., McC. Lilley,R., Gerhardt,R. and Heldt,H.W. (1989) *Methods Enzymol.*, **174**, 518–552.
Thorne,J.H. (1985) *Annu. Rev. Plant Physiol.*, **36**, 317–343.
Turgeon,R. (1989) *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **40**, 119–138.
Vernet,T., Dignard,D. and Thomas,D.V. (1987) *Gene*, **52**, 225–233.
von Schaewen,A., Stitt,M., Schmidt,R., Sonnewald,U. and Willmitzer,L. (1990) *EMBO J.*, **9**, 3033–3044.
Williams,L.E., Nelson,S.J. and Hall,J.L. (1992) *Planta*, **186**, 541–550.
Wright,K. and Oparka,K.J. (1989) *Planta*, **177**, 237–244.

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