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A Soybean Sucrose Binding Protein Independently Mediates Nonsaturable Sucrose Uptake in Yeast

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Heterologous expression of a cDNA encoding a 62-kD soybean sucrose binding protein in yeast demonstrates that this protein, independent of other plant proteins, mediates sucrose uptake across the plasma membrane. Sucrose binding protein-mediated sucrose uptake is nonsaturable up to 30 mM sucrose, is specific for sucrose, and is relatively insensitive to treatment with sulfhydryl-modifying reagents. Alteration of the external pH or pretreatment of the yeast cells with protonophores did not significantly affect the rate of ^{14}C-sucrose uptake. This demonstrates that sucrose binding protein-mediated sucrose uptake is not dependent on H^+ movement and delineates it from other plant sucrose transporters. Physiological characterization of sucrose uptake into higher plant cells has shown the presence of both saturable and nonsaturable uptake components. The nonsaturable mechanism is relatively insensitive to external pH, pretreatment with protonophores, and treatment with sulfhydryl-modifying reagents. Sucrose binding protein-mediated sucrose uptake in yeast mimics this physiologically described, but mechanistically undefined, nonsaturable sucrose uptake mechanism in higher plants. Functional characterization of the sucrose binding protein thus defines both a novel component of sucrose uptake and provides important insight into this nonsaturable sucrose uptake mechanism, which has remained enigmatic since its physiological description.

INTRODUCTION

Elucidating the mechanisms regulating the translocation of carbon assimilated during photosynthesis is central to understanding plant growth and development. In many plant species, sucrose is synthesized in the cytosol of photosynthetically active leaves and is loaded into the phloem against a concentration gradient for long-distance transport (Giaquinta, 1983). The movement of carbohydrates in the phloem is dependent on the osmotic gradient maintained by the loading of sucrose in source leaves and the unloading of sucrose in sink tissues that utilize the imported carbon for structural or energetic needs.

Classic biochemical and physiological experiments demonstrate that multiple modes of sucrose uptake operate in concert to mediate the overall distribution of sucrose in plants (Thorne, 1980, 1982; Lichtner and Spanswick, 1981). Using protoplasts from soybean cotyledons, Lin et al. (1984) established that sucrose uptake results from the simultaneous operation of at least two distinct mechanisms—one that is coupled to H^+ flux and is saturable, and another that is independent of H^+ movement and is nonsaturable. Maynard and Lucas (1982a, 1982b) have demonstrated that simple diffusion does not account for this linear component; they estimated that ~43% of sucrose uptake into leaves is mediated by this linear component in the presence of 25 mM sucrose. To understand fully the regulation of sucrose movement and carbon partitioning in higher plants, it is first necessary to identify the proteinaceous components mediating both the saturable and nonsaturable uptake mechanisms.

Heterologous expression of plant cDNAs in an engineered yeast strain resulted in the identification and partial characterization of several sucrose–H^+ symport proteins that belong to a superfamily of proteins containing 12 transmembrane domains (Riesmeier et al., 1992, 1993, 1994; Bush, 1993; Sauer and Stolz, 1994). Sucrose uptake mediated by these sucrose–H^+ symporters (termed SUT or SUC) is saturable, dependent on proton motive force, and sensitive to the sulfhydryl modifying reagents *p*-chloromercuribenzenesulfonic acid (PCMBS) and *N*-ethylmaleimide (NEM). Thus, uptake mediated by the SUT and SUC proteins appears to mediate sucrose uptake in a saturable H^+-dependent manner.

Photoaffinity labeling of membranes isolated from the soybean cotyledon with a photolyzable sucrose analog identified a 62-kD sucrose binding protein (Ripp et al., 1988). Characterization of the cDNA encoding this protein and analysis of the deduced amino acid sequence indicate that this sucrose binding protein is not similar to other membrane transport proteins (Grimes et al., 1992). Overvoorde and Grimes (1994) demonstrated that this hydrophilic sucrose binding protein is bound tightly to the external surface of the plasma membrane with the only putative transmembrane domain present at the N terminus, suggesting that it may behave as either a peripheral or type II membrane protein (i.e., N terminus$_{in}$, one

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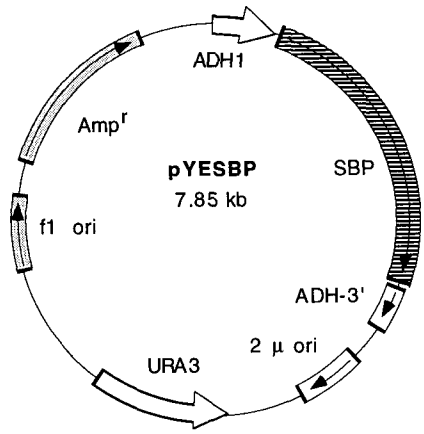

Figure 1. Structure of the Yeast Shuttle Vector Containing the Chimeric Sucrose Binding Protein Gene.

The soybean sucrose binding protein cDNA was subcloned into the yeast shuttle vector pMK195, and its expression was regulated by the yeast *ADH1* promoter and terminated by the 3′ region of the *ADH* gene. The 5′ to 3′ direction of the DNA sequences is indicated by the arrows. The hatched area represents the sucrose binding protein (SBP) open reading frame. The stippled area indicates bacterial-derived sequences. Amp^r, ampicillin resistance; f1 ori, origin of replication; 2 μ ori, yeast origin of replication.

membrane-spanning domain, the bulk of the protein exposed to the extracellular environment, and the C terminus$_{out}$; Singer, 1990).

Despite the lack of similarity between the sucrose binding protein and other known membrane transport proteins, several characteristics of this protein link it to the process of sucrose uptake. The sucrose binding protein binds sucrose specifically, and the photolabeling of this protein is substrate reversible (Ripp et al., 1988). Grimes et al. (1992) demonstrated that the temporal pattern of sucrose binding protein mRNA expression and protein accumulation is strongly correlated with the rate of sucrose uptake into developing soybean cotyledons. Furthermore, the protein is associated with the plasma membrane of several cell types actively engaged in sucrose transport, including the mesophyll cells of young sink leaves, the companion cells of mature soybean phloem, and the cells of developing cotyledons (Grimes et al., 1992; Overvoorde and Grimes, 1994).

It has been impossible to assign a function to the sucrose binding protein due to its unusual topology for a membrane carrier and its lack of similarity with other membrane transport proteins. The association of the sucrose binding protein with the plasma membrane of cells actively engaged in sucrose transport is a compelling argument that this protein has a role in sucrose uptake. These correlative data, however, do not constitute definitive proof of a functional role in the process of sucrose uptake. To test whether the sucrose binding protein is capable of mediating sucrose uptake independent of other plant plasma membrane proteins, it was expressed in the engineered yeast strain *susy7/ura3*. *susy7/ura3* contains a copy of the potato sucrose synthase (SuSy) cDNA and lacks endogenous sucrose transport and invertase activity (Riesmeier et al., 1992). As a result, *susy7/ura3* yeast are unable to grow on a medium containing sucrose as the sole carbon source unless a protein that mediates sucrose uptake across the plasma membrane is expressed ectopically in these yeast.

The results presented here establish the function of the sucrose binding protein by demonstrating that it independently mediates sucrose uptake. In addition, the kinetics of sucrose uptake mediated by the sucrose binding protein in yeast tightly mimic the previously described linear component of sucrose uptake in plants. These results not only define a role for this sucrose binding protein in sucrose uptake but also identify an important component of sucrose transport and provide insight into a novel type of membrane protein involved in the multifaceted process of sucrose transport regulating plant growth and development.

RESULTS

Construction of a Chimeric Gene for the Expression of the Sucrose Binding Protein in the Yeast *susy7/ura3*

A chimeric gene consisting of the yeast alcohol dehydrogenase 1 (*ADH1*) promoter, the sucrose binding protein cDNA, and the *ADH1* polyadenylation signal was constructed in pMK195 to generate the plasmid pYESBP (Figure 1). Table 1 defines the yeast strains used in this study. The yeast strain *susy7* has a spinach sucrose synthase cDNA stably integrated into its genome to mediate the intracellular hydrolysis of sucrose (Riesmeier et al., 1992). However, this yeast strain lacks the ability to transport sucrose and contains a disrupted secreted invertase (*SUC2*) gene and consequently is unable to grow on a medium containing sucrose as the sole carbon source (Riesmeier et al., 1992). To generate a host strain that would

Table 1. Yeast Strains Used in This Study

Strain	Genotype	Source
YSH 2.64-5A	*Matα suc2::URA3 malO leu2-3/112 trp1 ura3-52*	Gozalbo and Hohmann (1990)
susy7/ura3	*Matα suc2::URA3 leu2::SPS malO trp1 ura3*	Riesmeier et al. (1992)
OG1	*Matα suc2::URA3 leu2*::SPS *malO trp1 ura3* + pMK195 (2 μ URA3)	This study
OG2	*Matα suc2::URA3 leu2*::SPS *malO trp1 ura3* + pYESBP (2 μ SBP URA3)	This study
SM1058	*Matα ura3-52 leu2-3, 112 his4 trp1 can1*	Berkower and Michaelis (1991)

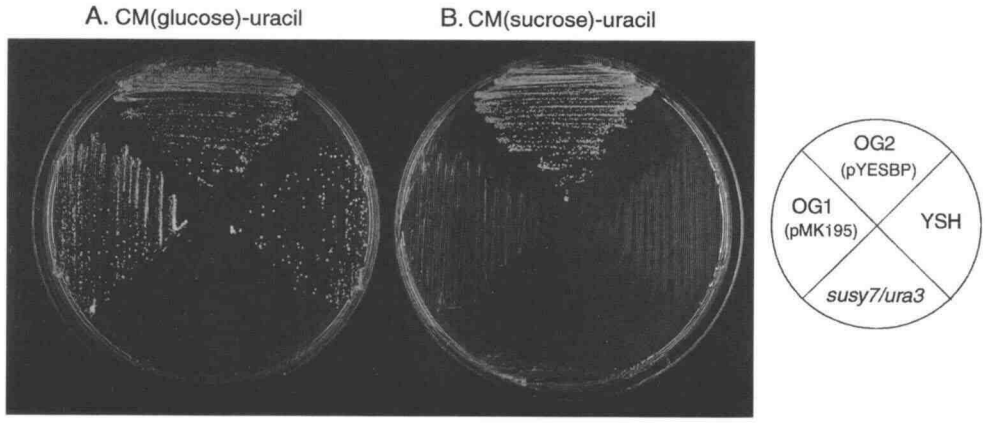

Figure 2. Expression of the Sucrose Binding Protein Restores the Ability of *susy7/ura3* Yeast to Grow on a Medium Containing Sucrose as the Sole Carbon Source.

(A) YSH, *susy7/ura3*, OG1 (*susy7/ura3* containing the pMK195 vector alone), and OG2 (*susy7/ura3* containing the pYESBP plasmid) yeast strains were grown on a complete medium lacking uracil and containing 2% glucose (CM(glucose)-uracil). The presence of the pYESBP plasmid in OG2 cells confers uracil prototrophy to the *susy7/ura3* cells.

(B) YSH, *susy7/ura3*, OG1, and OG2 yeast strains were grown on a complete medium lacking uracil and containing 2% sucrose (CM(sucrose)-uracil) as the sole carbon source. The expression of the sucrose binding protein confers the ability of the OG2 yeast to grow under these conditions.

allow for selection of yeast transformed with the sucrose binding protein expression vector (pYESBP), the *susy7* strain used in this study was selected for uracil auxotrophy. This was done by growing the *susy7* yeast on medium containing 5′-fluoroorotic acid (5′-FOA), which is converted to a toxic product, 5-fluorouracil, by the *URA3* gene product. The *URA3* gene encodes orotidine-5′-phosphate decarboxylase, a key enzyme required for the biosynthesis of uracil. Hence, only yeast containing a disruption in the *URA3* gene are able to grow on a medium containing 5′-FOA. The resulting strain, *susy7/ura3*, was unable to grow on a medium containing glucose and lacking uracil (Figure 2A). Introducing the pMK195 vector or the pYESBP plasmid, containing an intact *URA3* gene, into *susy7/ura3* to generate the yeast strains OG1 and OG2, respectively, restored the ability of the yeast to grow on medium lacking uracil (Figure 2A).

Expression of the Sucrose Binding Protein Confers the Ability of *susy7/ura3* Yeast to Grow by Mediating Sucrose Uptake across the Plasma Membrane

To determine whether the sucrose binding protein was able to restore the ability of the *susy7/ura3* to grow on a medium containing sucrose as the sole carbon source, OG2 yeast cells were plated on a medium containing 2% sucrose and lacking uracil. Figure 2B shows that the expression of the sucrose binding protein in *susy7/ura3* restored the ability of this strain to grow on a medium containing sucrose as a sole carbon source. Although the medium may have contained a small percentage (i.e., <0.01%) of hexoses, the fact that OG1 yeast showed no growth on this medium indicates that this level of hexoses could not sustain the observed levels of growth seen with the OG2 yeast.

Figure 3 shows that the sucrose binding protein is synthesized in the OG2 yeast cell, as indicated by the positive staining of a single polypeptide with the sucrose binding protein antiserum. In untransformed *susy7/ura3* or in *susy7/ura3* containing the

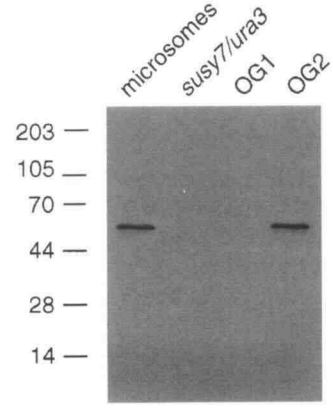

Figure 3. Transformation of *susy7/ura3* Cells with pYESBP Results in Expression of the Sucrose Binding Protein That Is Recognized by Affinity-Purified Antiserum.

Five micrograms of purified soybean microsomal proteins (microsomes) and 10 μg of total protein extract from overnight cultures of *susy7/ura3*, OG1, and OG2 yeast strains were separated by SDS-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose and probed with affinity-purified sucrose binding protein antiserum. The position of the protein size markers (in kilodaltons) is indicated at left.

vector pMK195 alone (OG1), no proteins cross-reacting with the sucrose binding protein antiserum were detected (Figure 3).

The ability of OG2 yeast to grow on a medium containing sucrose as the sole carbon source raises two important questions related to the mechanism of sucrose binding protein–mediated sucrose uptake: (1) Is sucrose hydrolyzed into hexoses before uptake? and (2) If intact sucrose is taken up, is it chemically modified, for example, phosphorylated or methylated, during uptake? To address the first question, ethanol-extractable sugars present in OG2 cells after incubation in a medium containing ^{14}C-sucrose were separated by paper chromatography. Figure 4 shows that extracts of OG2 cells contain a single peak of radioactivity that comigrates with the ^{14}C-sucrose standard (compare circles and squares), suggesting that sucrose is not cleaved into hexoses before being taken up. To confirm that the sucrose binding protein does not encode a cryptic sucrose-hydrolyzing activity, total protein extracts from OG2 and SM1058 cells grown in a medium containing sucrose as the sole carbon source were assayed for invertase activity. SM1058 cells, which possess an intact *SUC2* gene that encodes a secreted invertase, showed high levels of sucrose hydrolyzing activity, whereas the OG2 cells contained no detectable sucrose-hydrolyzing activity (data not shown).

To show that the radiolabel accumulated by the OG2 cells was indeed sucrose, the ethanol-extractable sugars were treated with invertase and then separated using paper chromatography. Figure 4 shows that the ^{14}C-sucrose accumulated by OG2 cells could be cleaved into hexoses by invertase, demonstrating that it remains unmodified during uptake into OG2 cells. Furthermore, if sucrose were chemically modified during uptake into the OG2 cells, it would not crystalize with pure sucrose. To test whether such modification was occurring, the ability of the ^{14}C-sucrose taken up by the OG2 cells to cocrystallize with pure sucrose was tested using three independent ethanol-extractable samples from OG2 cells. Specific activities of 135.0 ± 35 Bq per μmol and 136.7 ± 32 Bq per μmol were obtained after the first and second crystallizations, respectively. These results demonstrate that the ^{14}C-sucrose was recrystallized to a constant specific activity and shows that the sucrose binding protein mediates the uptake of intact, chemically unmodified sucrose into OG2 cells.

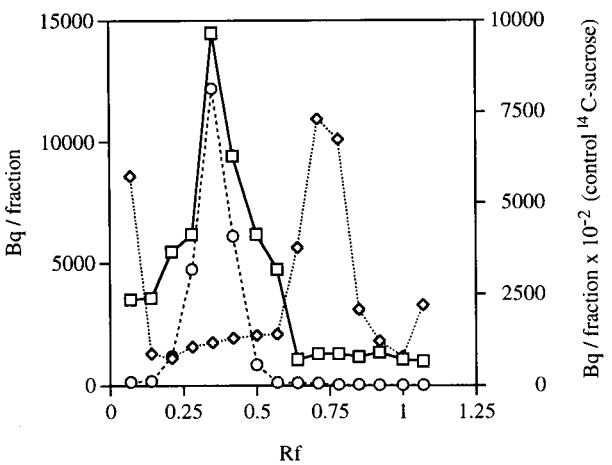

Figure 4. Sucrose Binding Protein Mediates the Uptake of Intact ^{14}C-Sucrose.

After incubating OG2 yeast cells in the presence of ^{14}C-sucrose for 10 min, the extracellular sugars were removed by extensive washing, and the ethanol-soluble sugars were extracted. These sugars were either treated with 40 units of invertase for 2 hr at 55°C (diamonds) or left untreated (squares) and then separated by paper chromatography. After separation, the chromatographic strips were cut into 0.75-cm sections, and the amount of radioactivity present in each fraction was determined. The mobility of pure ^{14}C-sucrose (circles) is shown as a standard for intact sucrose. Rf, radiofrequency.

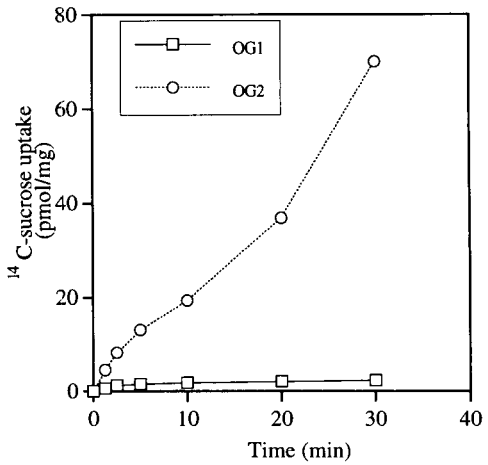

Figure 5. ^{14}C-Sucrose Uptake Mediated by the Sucrose Binding Protein Is Linear over Time.

OG1 and OG2 cells were grown overnight, harvested, and incubated in a solution containing ^{14}C-sucrose. The amount of radioactivity accumulated by the OG1 and OG2 cells at the indicated times was determined and used to calculate the amount of ^{14}C-sucrose uptake. The data shown are the mean of three replicates. The standard error in all cases was smaller than the size of the symbols used.

Time Course and Linear Uptake of Sucrose by OG2 Yeast Are Similar to the Linear Component of Sucrose Uptake in Plants

The uptake of sucrose in plants has at least two components (Sovinick et al., 1974; Thorne, 1980, 1982; Lichtner and Spanswick, 1981; Maynard and Lucas, 1982a, 1982b; Lin et al., 1984; Schmitt et al., 1984), raising the possibility that the sucrose binding protein mediates sucrose uptake by using a distinct mechanism. The kinetics and biochemical characteristics of sucrose binding protein–mediated sucrose uptake were assayed by using the established protocol of Cirillo (1989). Basically, yeast cells were incubated in solutions containing

^{14}C-sucrose for 0 to 30 min and washed extensively to remove extracellular sucrose; the amount of radiolabeled sucrose accumulated was determined by liquid scintillation counting.

Figure 5 shows that the OG2 yeast strain accumulated sucrose in a linear manner over a 30-min time course. In contrast, the OG1 yeast strain did not accumulate significant amounts of radiolabeled sucrose even after 30 min (Figure 5). Because the OG1 yeast did not accumulate radiolabeled sucrose, it is unlikely that the linear accumulation of sucrose seen with the OG2 strain is due to simple diffusion of sucrose across the plasma membrane. Figure 6 shows that the uptake of sucrose remains linear over a broad range of external sucrose concentrations (0.1 to 30 mM) and does not saturate within the range of concentrations tested. Both of these results are consistent with the kinetic characteristics described for the linear component of sucrose uptake in plants (Thorne, 1980, 1982; Lichtner and Spanswick, 1981; Maynard and Lucas, 1982a, 1982b; Lin et al., 1984; Schmitt et al., 1984).

Response of the OG2 Yeast to pH, Protonophores, Protein-Modifying Reagents, and Inhibitors

Previous reports characterizing the linear component of sucrose uptake in plants have demonstrated that it is relatively insensitive to pH, protonophores, and the protein-modifying reagents PCMBS and NEM (Maynard and Lucas, 1982b; Thorne, 1982; Lin et al., 1984; Schmitt et al., 1984). Because uptake mediated by the sucrose binding protein appeared to share similarities with the linear component of uptake, the effects of various treatments on sucrose uptake by the OG2 cells were examined.

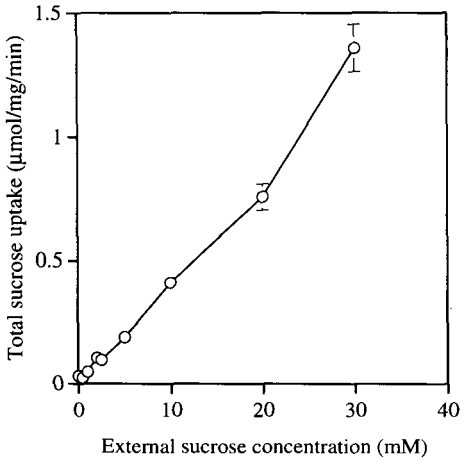

Figure 6. ^{14}C-Sucrose Uptake Mediated by the Sucrose Binding Protein Is Linear and Nonsaturable.

OG2 cells were grown overnight, harvested, and incubated in a solution containing the indicated final concentrations of unlabeled sucrose and ^{14}C-sucrose. The amount of radioactivity accumulated by the OG2 cells after 5 min was determined and used to calculate the total amount of sucrose uptake. The data shown are the mean of three replicates. The standard error is indicated where the value exceeded the size of symbols used.

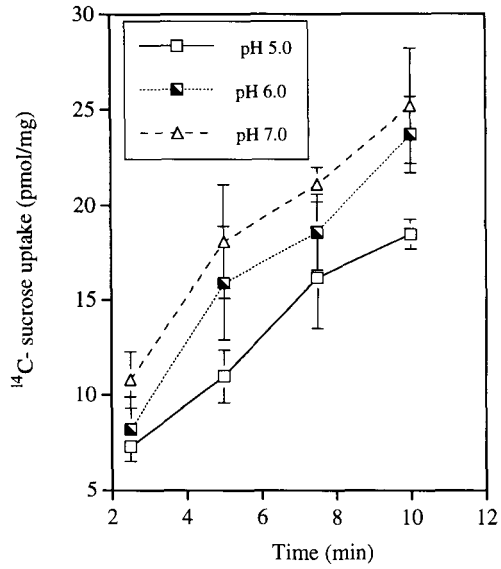

Figure 7. ^{14}C-Sucrose Uptake Mediated by the Sucrose Binding Protein Is Insensitive to pH.

OG2 cells were grown overnight, harvested, and incubated in solutions containing 25 mM Mes adjusted to the indicated pH values and ^{14}C-sucrose. The amount of radioactivity accumulated by the OG2 cells after 2.5, 5, 7.5, and 10 min was determined and used to calculate the amount of ^{14}C-sucrose uptake. The data shown are the mean of three replicates. The standard error is indicated where the value exceeded the size of symbols used.

Several observations indicate that the sucrose uptake facilitated by the sucrose binding protein is not mediated by a H^+–sucrose symport mechanism. Figure 7 shows that the uptake of sucrose by the OG2 strain of yeast is relatively independent of pH. Although the amount of sucrose accumulated increased somewhat with increasing pH, the magnitude of the difference was marginal. In addition, Table 2 shows that protonophores, such as 2,4-dinitrophenol (2,4-DNP) or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), have only a negligible effect on sucrose transport. Finally, Table 3 indicates that preincubation of the OG2 yeast strain in glucose has no stimulatory effect on the rate of sucrose uptake, as has been reported previously for yeast-expressing plant sucrose–H^+ symport transport proteins (Riesmeier et al., 1992, 1993; Sauer and Stolz, 1994).

Unlike the saturable component of sucrose uptake, the linear component of sucrose uptake in higher plants is relatively insensitive to the sulfhydryl-modifying reagent PCMBS and to NEM (Maynard and Lucas, 1982b; Lin et al., 1984; Schmitt et al., 1984). To determine whether sucrose binding protein–mediated sucrose uptake was sensitive to sulfhydryl-modifications, the OG2 strain of yeast was pretreated with these

Table 2. Treatment of the Yeast Strain OG2 Expressing the Sucrose Binding Protein with Protonophores Has Negligible Effect on Sucrose Transport

Treatment[a]	Sucrose (pmol/mg)	% of Control
Control	10.3 ± 2	100
0.5 μM CCCP	10.0 ± 3	97
2.5 μm CCCP	9.3 ± 4	90
25 μM 2,4-DNP	8.2 ± 5	79
100 μM 2,4-DNP	8.4 ± 5	82

[a] Ionophores were added to cells 30 sec before the addition of labeled sucrose solution. Uptake was allowed to proceed for 2.5 min, and the amount of radiolabeled sucrose transported was determined. Values are the mean ± SE of three replicates.

reagents and the effect on sucrose uptake assessed. Table 4 shows that the rate of sucrose uptake by the OG2 strain of yeast was inhibited by ~20% after treatment with 25 μM PCMBS or 1 mM NEM; this is similar to the observed inhibition of the linear component in plants (Lin et al., 1984).

By using a broad range of carbohydrates, the specificity of uptake mediated by the sucrose binding protein was examined. Maltose competitively inhibits both the linear and saturable components of sucrose uptake (Maynard and Lucas, 1982a; Schmitt et al., 1984). Table 3 demonstrates that only sucrose or a 10-fold molar excess of maltose significantly inhibited the uptake of sucrose by the OG2 strain of yeast. Glucose, mannose, and palatinose had no effect on the amount of sucrose accumulated by the OG2 yeast strain (Table 3). Pretreatment with 10 mM trehalose resulted in an ~25% inhibition of sucrose transport, but the physiological significance of this result is unclear at present. These observations are consistent with the previously reported substrate specificity of the sucrose binding protein (Hitz et al., 1986; Ripp et al., 1988) and further support the hypothesis that the sucrose binding protein mediates sucrose uptake in a specific manner.

DISCUSSION

The goal of the experiments described here was to determine whether the sucrose binding protein is capable of mediating sucrose uptake across the plasma membrane independently of other plant proteins. These studies were predicated on the conundrum presented by our earlier data implying a role for this protein in sucrose uptake, based on its spatial and temporal association with cells actively engaged in sucrose transport and on data showing that this sucrose binding protein lacks similarity to other membrane transport proteins (Ripp et al., 1988; Grimes et al., 1992). In fact, due to the hydrophilic nature of the protein and the absence of sequence similarity to other membrane transport proteins, Grimes et al. (1992) suggested that the sucrose binding protein is involved in sucrose transport but is not performing this function independently. Heterologous expression of the soybean sucrose binding protein cDNA in yeast, however, clearly demonstrates that this protein mediates sucrose uptake across the plasma membrane. Therefore, these results establish a functional role for the sucrose binding protein in sucrose uptake and demonstrate that sucrose binding protein–mediated sucrose uptake occurs in the absence of other plant proteins.

Sucrose Binding Protein-Mediated Sucrose Uptake Is Nonsaturable and Mechanistically Distinct from Other Plant Sucrose Transport Activities

Physiological characterization of sucrose transport in higher plants indicates that sucrose uptake is composed of at least three mechanisms with unique biochemical features (Sovinick et al., 1974; Thorne, 1980, 1982; Lichtner and Spanswick, 1981; Maynard and Lucas, 1982a, 1982b; Lin et al., 1984; Schmitt et al., 1984). The first defined mechanism consists of a saturable, H^+-mediated uptake mechanism with a K_m for sucrose between 0.4 and 1.5 mM. This saturable uptake mechanism has a sharp pH optimum at 5.5, is inhibited by treatment with protonophores, and is sensitive to treatment with sulfhydryl-modifying reagents (Maynard and Lucas, 1982a, 1982b; Lin et al., 1984). The second component is diffusion and is an unmediated event. The third defined mechanism consists of a nonsaturable, H^+-independent uptake mechanism. This nonsaturable uptake mechanism is relatively insensitive to external pH or disruption of the H^+ gradient with protonophores and is relatively insensitive to treatment with sulfhydryl-modifying reagents (Sovinick et al., 1974; Thorne, 1980, 1982; Lichtner and Spanswick, 1981; Maynard and Lucas, 1982a, 1982b; Lin et al., 1984; Schmitt et al., 1984).

The ectopic expression of plant cDNAs in *Saccharomyces cerevisiae* has facilitated the identification and functional characterization of several transport proteins, including ion pumps

Table 3. Substrate Specificity of Sucrose Transport in the Yeast Strain OG2 Expressing the Sucrose Binding Protein

Competitor[a]	Sucrose (pmol/mg)	% of Control
Control	11.2 ± 0.8	100
2 mM sucrose	3.0 ± 0.3	26
10 mM glucose	13.0 ± 1.4	115
2 mM maltose	10.5 ± 2.3	94
10 mM maltose	12.0 ± 1.5	57
10 mM trehelose	8.6 ± 1.7	76
10 mM mannose	6.3 ± 1.3	107
10 mM palatinose	10.3 ± 1.1	92

[a] Different sugars were added to cells 30 sec before the addition of labeled sucrose solution. Uptake was allowed to proceed for 2.5 min, and the amount of radiolabeled sucrose transported was determined. Values are the mean ± SE of three replicates.

Table 4. Effect of Treating the Yeast Strain OG2 Expressing the Sucrose Binding Protein with Protein-Modifying Reagents

Treatment[a]	Sucrose (pmol/mg)	% of Control
Control	14.6 ± 0.5	100
25 μM PCMBS	11.9 ± 2.5	82
100 μM PCMBS	14.7 ± 1.9	101
1 mM NEM	11.4 ± 0.3	79

[a] The reagents were added to cells 30 sec before the addition of labeled sucrose solution. Uptake was allowed to proceed for 2.5 min, and the amount of radiolabeled sucrose transported was determined. Values are the mean ± SE of three replicates.

and channels, ATP binding cassette proteins, and amino acid transporters (Bush, 1993; Frommer and Ninnemann, 1995). In addition, functional complementation studies in yeast have resulted in the identification and characterization of several plant sucrose–H^+ symporters (Riesmeier et al., 1992, 1993; Sauer and Stolz, 1994). The transporter genes from spinach, potato, tomato, tobacco, and Arabidopsis encode highly hydrophobic proteins that belong to a large superfamily of metabolite transporters, consisting of two sets of six transmembrane domains separated by a large cytoplasmic loop (Henderson and Maiden, 1990; Frommer and Sonnewald, 1995). Based on the biochemical characteristics of sucrose transport kinetics in yeast, all of these plant sucrose–H^+ symporters mediate sucrose uptake in a saturable, H^+-dependent manner (Riesmeier et al., 1992, 1993; Sauer and Stolz, 1994).

In contrast to the biochemical characterization of this saturable uptake mechanism, no protein(s) has been identified that mediates the nonsaturable uptake of sucrose. The kinetics of sucrose uptake mediated by heterologous expression of the sucrose binding protein in yeast exhibit several features implicative of this nonsaturable, H^+-independent mechanism in higher plants. Perhaps most importantly, the uptake of sucrose mediated by the sucrose binding protein is linear over both time and a broad range (up to 30 mM) of external sucrose concentrations. In addition, sucrose binding protein–mediated sucrose uptake is relatively insensitive to external pH or pretreatment with the protonophores CCCP or 2,4-DNP. Finally, sucrose uptake mediated by the sucrose binding protein is relatively insensitive (inhibited by ~20%) to pretreatment with the sulfhydryl-modifying reagents PCMBS or NEM. These two sulfhydryl-modifying reagents strongly inhibit the saturable uptake mechanism but inhibit the nonsaturable uptake mechanism by only ~20% (Maynard and Lucas, 1982b; Riesmeier et al., 1992). Thus, the biochemical characteristics of sucrose uptake into OG2 cells indicate that the sucrose binding protein mediates nonsaturable, H^+-independent movement of sucrose into yeast.

It remains a formal possibility, however, that the expression of the sucrose binding protein in yeast might activate or stimulate an endogenous, inactive sucrose uptake mechanism. Kühn and Frommer (1995) have shown that the expression of other proteins, including certain kinases, apparently is capable of inducing a sucrose uptake capacity in the *susy7/ura3* yeast strain. This explanation seems unlikely, given the spatial association of the sucrose binding protein with cells involved in active sucrose uptake, the fact that the sucrose binding protein is a plasma membrane protein, and the specific labeling of this protein by a sucrose analog that inhibits sucrose uptake (Ripp et al., 1988; Grimes et al., 1992; Overvoorde and Grimes, 1994).

In summary, this research has elucidated the biochemical function of the sucrose binding protein in sucrose uptake. Sucrose binding protein–mediated sucrose uptake in yeast tightly mimics the kinetic features of a physiologically defined, nonsaturable, H^+-independent uptake mechanism in higher plants. Biochemical characterization of the sucrose binding protein has therefore provided important insight into the proteinaceous components involved in this kinetically distinct, nonsaturable, H^+-independent uptake mechanism. Additional experiments are necessary to determine the mechanism of sucrose binding protein–mediated sucrose uptake and whether the sucrose binding protein mediates the nonsaturable uptake of sucrose in plants. Although numerous transport systems exist in other organisms (Robillard and Lolkema, 1988; Higgins, 1992; Bisson et al., 1993), the sucrose binding protein shares no sequence similarity with these other transport proteins. This observation, coupled with the biochemical characteristics of sucrose binding protein–mediated sucrose uptake and the membrane topology of this protein, suggests that the sucrose binding protein represents a novel mechanism of membrane transport.

METHODS

Yeast Strains, Plasmid Construction, and Transformation into *Saccharomyces cerevisiae*

Yeast YSH 2.64-1A (Gozalbo and Hohmann, 1990) was used to engineer the yeast strain *susy7* (Riesmeier et al., 1992). *susy7/ura3* was selected by growing *susy7* on CM(Glu) containing 2% glucose (Sherman, 1991) and 5′-fluoroorotic acid (5′-FOA; Boeke et al., 1984). OG1 is *susy7/ura3* transformed with pMK195. OG2 is *susy7/ura3* transformed with pYESBP. SM1058 was a gift from S. Michaelis (Johns Hopkins University School of Medicine, Baltimore, MD). Yeast strains were grown at 30°C on complete minimal or uracil dropout media containing 2% glucose (CM[Glu]) or 2% sucrose (CM[Suc]) (Ausubel et al., 1994).

The multiple cloning site following the yeast alcohol dehydrogenase 1 (*ADH1*) promoter in vector YEP112A1 (Riesmeier et al., 1992) was modified by digesting with PstI and BamHI, annealing the oligonucleotides XE1 (5′-GAATTCTAGATCTCGAGCGGCCGCG-3′) and XE2 (5′-GATCCGCGGCCGCTCGAGATCTAGAATTCTGCA-3′), and ligating to generate the plasmid YEP112A1XE. The new multiple cloning site contained the unique restriction sites PvuII, PstI, EcoRI, XbaI, BglII, XhoI, NotI, and BamHI downstream of the *ADH1* promoter. To generate the episomal yeast expression vector pMK195, the vector YEplac195 (Gietz and Sugino, 1988) was cleaved with PstI and EcoRI to remove most of the pUC19 polylinker, blunt-ended with the Klenow

fragment of DNA polymerase I, and religated. The *ADH1* expression cassette from YEP112A1XE was isolated as a 0.7-kb SphI fragment and ligated into the corresponding site of the modified YEplac195 vector to produce pMK195. To construct pYESBP, the sucrose binding protein cDNA was excised from pSBP (Grimes et al., 1992) by digestion with XbaI and SalI and ligated into the XbaI and XhoI sites of pMK195. The construct was confirmed by restriction digests.

susy7/ura3 yeast were transformed with 1 μg of pMK195 or pYESBP plasmid DNA, using a small-scale, LiOAc-based procedure adapted from Gietz et al. (1992). Transformed yeast were plated on solidified CM(Glu)-Ura medium (Ausubel et al., 1994), and 4 to 7 × 10^4 transformants per microgram of DNA were obtained. To characterize the putative transformants, DNA was isolated (Hoffman and Winston, 1987) and used to transform *Escherichia coli* (XL-1 Blue; Stratagene) by electroporation. Plasmid DNA from transformed *E. coli* colonies growing on a solid Luria broth medium containing 50 mg/L ampicillin was isolated (Sambrook et al., 1989) and used to confirm the structure of the re-isolated yeast shuttle vector by restriction digest or polymerase chain reaction, using sucrose binding protein specific primers.

Identification of Sucrose Binding Protein in OG2 Yeast

OG1, OG2, or *susy7/ura3* yeast were grown overnight at 30°C (225 rpm) in YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) to an OD_{600} of 1.2. After harvesting the cells (2.8 mL) by centrifugation (10,000*g* for 10 sec at room temperature), the cells were suspended in 40 μL of sterile water (4°C). The cell suspension was added to 40 μL of preheated (100°C) Laemmli (Laemmli, 1970) sample buffer containing 300 mg of acid-washed glass beads. This mixture was boiled for 2 min, mixed by vortexing for 30 sec, and boiled again. After repeating the boiling and vortexing cycle five times, the slurry was clarified by centrifugation at 10,000*g* for 1 min at room temperature. The supernatant was collected, and the centrifugation was repeated. The final supernatant was collected and loaded on a 10% polyacrylamide gel. After SDS-PAGE, the proteins were either stained with Coomassie Brilliant Blue R 250 to confirm equal loading of protein or electroblotted to nitrocellulose (Towbin et al., 1979). Proteins transferred to nitrocellulose were probed with affinity-purified antibodies to the sucrose binding protein, according to Grimes et al. (1992). Color development was performed using chemiluminescence (ECL; Amersham, Arlington Heights, IL), according to the manufacturer's instructions.

Determination of ^{14}C-Sucrose Uptake

For transport assays, OG1 and OG2 cells grown to an OD_{600} of 0.5 to 1.3 in YPD were harvested by centrifugation, washed twice with 25 mM Mes-KOH buffer, pH 5.5, weighed, and resuspended in the same buffer to yield solutions containing 10 to 30 mg/mL of yeast cells. The uptake assays were started upon the addition of cells to an equal volume of solution containing 25 mM Mes-KOH, pH 5.5, 0.5 to 2.5 μCi of ^{14}C-sucrose, and unlabeled sucrose at twice the desired final concentration. At predetermined time points, aliquots of the uptake solution and cells were collected, and uptake was quenched by transfer to 5 mL of ice-cold water. The cells were collected by filtration through glass fiber filters (GF/A; Whatman, Hillsboro, OR) and washed five times with 5 mL of ice-cold water; the radioactivity taken up by the cells was determined by liquid scintillation counting. All uptake assays, except those used to determine the concentration dependence of transport, were performed in a final concentration of 1 mM sucrose at room temperature. For determining the pH dependence of uptake, the cells were harvested in the same fashion but were washed and suspended in solutions containing 25 mM Mes-KOH at the pH values indicated. All uptake assays were performed at least three times.

For inhibition and competition studies, stock solutions were made by dissolving *p*-chloromercuribenzenesulfonic acid (PCMBS; Aldrich, Milwaukee, WI) in dimethyl sulfoxide, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and 2,4-dinitrophenol (2,4-DNP) in 100% ethanol, and *N*-ethylmaleimide (NEM) and the other sugars in 25 mM Mes, pH 5.5. The stocks were diluted to the concentrations indicated in the tables, with a solution composed of 25 mM Mes, pH 5.5, and 0.5 μCi ^{14}C-sucrose. Uptake assays were started by adding an equal volume of washed cells as described above. Uptake was allowed to proceed for 2.5 min and was terminated by the addition of 5 mL of ice-cold water. Cells were collected and washed, and the amount of radioactivity incorporated was determined by liquid scintillation counting as described above. Control uptake assays containing solvents alone had no effect on sucrose uptake. Invertase staining was performed as previously described by Riesmeier et al. (1992).

Chromatographic Analysis and Crystallization of Radiolabeled Sugars

After harvesting and washing in 25 mM Mes, pH 5.5, the OG2 cells were incubated for 5 or 10 min in 1 μCi of ^{14}C-sucrose, collected on GF/A filters, and washed five times with 5 mL of ice-cold water. The filter was placed in a microfuge tube, and the cells were broken by three cycles of freezing in liquid nitrogen and heating at 70°C; 250 μL of 80% ethanol was added, and the soluble sugars were extracted for 1 hr at 70°C. The extract obtained was centrifuged for 10 min at 10,000*g*, and the supernatant, which contained >85% of the total radioactivity retained by the cells, was dried in a speed vacuum.

For paper chromatography, the dried pellet was resuspended in 10 μL of water, applied to strips (2 × 20 cm) of Whatman No. 3 paper, and separated using *n*-butanol–ethanol–water (52:33:15) as the solvent. The strips were dried and cut into 0.75-cm pieces, and the amount of radioactivity was determined by liquid scintillation counting. To test whether the radiolabeled sugars could be hydrolyzed into hexoses by invertase, the dried sugar pellet was suspended to a final volume of 30 μL in a mixture containing 25 mM Mes, pH 5.5, and 40 units of invertase (Sigma). After incubation at 55°C for 2 hr, the sample was separated by paper chromatography, using the same system. The results presented are representative of at least two independent experiments.

Crystallization was performed by resuspending the dried pellet in 8 μL of water and adding this to 50 μL of hot water containing 120 mg of sucrose. After crystallization for 24 hr at room temperature, the supernatant was aspirated, the crystals dried, and the weight of crystallized sucrose determined. This pellet was then resuspended in hot water to yield a solution containing the same concentration of sucrose as above. One-tenth of the sample was used to determine the amount of ^{14}C-sucrose crystallized; the remainder of this suspension was allowed to recrystallize for 24 hr. The final crystals were dried and weighed, and the amount of ^{14}C-sucrose recrystallized was determined by liquid scintillation counting. The results presented are the mean of three independent experiments.

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