Annals of Botany **87**: 125–129, 2001
doi:10.1006/anbo.2000.1316, available online at http://www.idealibrary.com on IDEAL®

Functional Characterization of Two Ripening-related Sucrose Transporters from Grape Berries

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Received: 18 August 2000 Returned for revision: 21 September 2000 Accepted: 3 October 2000

The ripening of grape (*Vitis vinifera* L.) berries is associated with a large accumulation of glucose and fructose in the vacuoles of the fruit cells. These hexoses are derived from sucrose, which is released from the phloem and may be taken up by parenchyma cells prior to hydrolysis. We have expressed two putative ripening-related sucrose transporters from grape berries, VvSUC11 (synonymous with VvSUT1) and VvSUC12, in an invertase deficient yeast strain to characterize their transport activities. Sucrose was taken up by yeast transformed with either transporter at an optimum pH of $<4{\cdot}5$ and with a Michaelis constant (K_m) of 0·9–1·4 mM. The uptake of sucrose through VvSUC11 and VvSUC12 was inhibited by protonophores and by vanadate. This is consistent with an active uptake mechanism involving proton cotransport, typical of sucrose/H^+ symporters. The transporters from grape berries were functionally similar to Scr1, a sucrose transporter from *Ricinus* cotyledons. It is likely that in grape berries VvSUC11 and VvSUC12 facilitate the loading of sucrose from the apoplast into the parenchyma cells.

Key words: Fruit, grape berries, plasma membrane, sugars, sucrose transporters, *Vitis vinifera*.

INTRODUCTION

In most plants carbohydrates are transported long distances as sucrose. The movement of sucrose from source to sink tissues via the phloem has been the subject of much investigation over recent years (Kühn *et al*., 1999; Williams *et al*., 2000). The movement or loading of sugars into the phloem from source tissues, primarily leaves, has been characterized in greater detail than the process of phloem unloading into sink tissues such as roots, seeds and tubers. The mechanism of sucrose uptake into fruits, which are major sink organs in some plants, is poorly understood.

Grape berries, when ripe, contain high levels of sugars that are important for flavour and fermentation. Sugars also provide the osmotic driving force for cell expansion (Stadler *et al*., 1999) as well as modulating gene expression (Koch, 1996) through signalling mechanisms (Lalonde *et al*., 1999). The movement of sugars into fruits is a tightly regulated process that is coordinated with growth and development. The accumulation of sugars in grape berries begins at the onset of ripening, known as veraison, and is marked by a ten-fold increase in hexose content (Davies and Robinson, 1996). Glucose and fructose, the major soluble sugars in the fruit, are present in approximately equimolar amounts. The cell vacuole is the main site of this massive hexose accumulation and sucrose is the main form of photoassimilate transported to the grape berry (Coombe, 1992).

Biochemical evidence indicates that the conversion of sucrose to hexoses is likely to occur in the vacuole of grape berries and involves the action of two soluble invertases (Davies and Robinson, 1996). Although the genes encoding these invertases exhibit substantially reduced expression after veraison, sufficient invertase activity appears to be present throughout the ripening phase to account for the cleavage of sucrose. Recently, two cDNAs encoding hexose transporters have been cloned from grape berries (Fillion *et al*., 1999). Analysis of their expression in developing fruit indicates they are differentially regulated during ripening. The expression of one of these transporters, Vvht2, parallels the initial rise in hexose accumulation of the berries whereas the expression of the other ripening-related form, Vvht1, is not closely related to the increase in the sugar content of the fruit. The possibility that several hexose transporters are expressed in the fruit implies that regulation of hexose transport is complex.

Regardless of the exact location of sucrose hydrolysis and the mechanism of hexose transport into the vacuole, it is evident that total sugar movement into the fruit depends on the rate of sucrose unloading from the phloem. Molecular evidence for sucrose transporters in fruits has only recently been obtained. A partial cDNA clone from strawberry fruit encodes a putative sucrose transporter whose gene is expressed during ripening (Manning, 1998), in parallel with increases in sucrose, glucose and fructose (Forney and Breen, 1986). The uptake of sucrose into strawberry fruit discs is sensitive to the sulfhydryl inhibitor PCMBS, indicating the presence of a plasma membrane sucrose transporter (Ofosu-Anim *et al*., 1996). More recently, three putative sucrose transporter cDNAs have been identified in grape fruit (Davies *et al*., 1999; Ageorges *et al*., 2000). The

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0305-7364/01/010125+05 $35.00/00

genes encoding two of these transporters, VvSUC11 (also termed VvSUT1) and VvSUC12, had similar expression patterns in the fruit, their mRNAs being up regulated at the commencement of hexose accumulation. Expression of a gene encoding the third sucrose transporter, VvSUC27, was markedly different, its transcript showing reduced expression as berry ripening progressed.

Functional analysis of the biochemical properties of sucrose transporters from fruits such as grape berries is an essential first step in understanding the roles of these proteins in the ripening process. A preliminary study has demonstrated the ability of yeast transformed with VvSUT1 to transport sucrose (Ageorges *et al*., 2000). Here we have extended this observation and compared the functional characteristics of both sucrose transporters that exhibit enhanced expression during the ripening of grape berries (VvSUC11 and VvSUC12) in a heterologous yeast expression system.

MATERIALS AND METHODS

Cloning of VvSUC11 and VvSUC12 into yeast expression vector

The full-length cDNA inserts of *VvSUC11* and *VvSUC12* cloned into pBluescript (Stratagene, Amsterdam) plasmid vector (Davies *et al*., 1999) were excised by NotI and ligated into the NotI site of the yeast shuttle vector NEV-N carrying the URA3 selectable marker (Sauer and Stoltz, 1994). Vectors with inserts in the sense and antisense orientations and recircularized vector lacking an insert were used to transform *E. coli* DH5α cells. Transformants were selected on ampicillin plates and inserts analysed by restriction digest. Insert orientation was confirmed by BigDye terminator sequencing (PE Biosystems, Warrington, UK) using PMA1 (Serrano *et al*., 1986) promoter and terminator primers.

Transformation of yeast

Constructs containing *VvSUC11* and *VvSUC12* and the empty NEV-N vector were transformed into SEY2102, an invertase deficient strain of *Saccharomyces cerevisiae* (Emr *et al*., 1983), by the lithium acetate/polyethylene glycol procedure (Ito *et al*., 1983). URA + transformants were selected on minimal plates (2 % agar, 2 % glucose, 0·67 % yeast nitrogen base without amino acids, 0·5 % ammonium sulfate, 19·2 mg l^{-1} histidine and 19·2 mg l^{-1} leucine). Using DNA prepared by the method of Hoffman and Winston (1987), transformants were verified by PCR with primers specific to vector and insert. Yeast cells transformed with empty (NEV-N) vector and with the vector containing the Scr1 sucrose carrier from *Ricinus communis* named nSC4+ (Weig and Komor, 1996) were controls.

Preparation of yeast cells for sucrose uptake assays

Yeast transformants were grown at 30°C in 500 ml baffle flasks containing 100 ml minimal medium (without agar) in a shaking incubator (Labline Orbit, Jencons Scientific, Leighton Buzzard, UK). Cultures were grown to an OD_{600} between 0·5 and 1·0 and the cells collected by centrifuging at 1000 *g* for 5 min in an MSE Mistral 2000 centrifuge (MSE, Loughborough, UK). Pellets from four replicate flasks were combined and resuspended in 15 ml of 50 mM sodium phosphate buffer pH 5·0. The cells were washed by centrifugation at 1000 *g* for 15 min and the resulting pellet resuspended in 15 ml of phosphate buffer and recentrifuged. The pellet was finally resuspended at an OD_{600} of 2·0 to 2·5 in 50 mM sodium phosphate buffer at the pH used for the uptake assay.

Sucrose uptake assays

Sucrose assays were performed in polypropylene tubes. An equal volume of [U-^{14}C]sucrose (7·4 kBq ml^{-1}; Amersham Life Science, Amersham, UK) in 50 mM sodium phosphate was added to the washed cell suspensions. Samples (1 ml) were incubated for 30 min at 32°C with agitation unless indicated. Cells were collected onto 0·8 μm nitrocellulose by vacuum filtration and washed with 2·5 ml ice cold 50 mM sodium phosphate buffer pH 5·0. Radioactivity bound to the filters was determined in a scintillation counter. Background or non-specific sucrose uptake was determined in yeast cells transformed either with empty vector or with vector containing the sucrose transporter cDNAs in the antisense orientation. Sucrose uptake increased in these cells to a maximum over 5 min then remained constant (see Fig. 1). Thus the uptake of sucrose into transformed yeast was calculated as the radioactivity associated with each sample minus the steady state background radioactivity. Assays were performed in triplicate and all experiments were repeated three times. Experiments were standardized by reference to the data obtained for sucrose uptake into yeast transformed with Scr1. Data are expressed as means ± s.e. of the mean of three experiments unless indicated.

RESULTS

Yeast cells transformed with either the empty NEV-N vector or a vector with an antisense construct of Scr1 exhibited saturable binding of sucrose 5 min after addition of the sugar (Fig. 1). A linear rate of sucrose uptake was maintained for the first 30 min in yeast cells with the sense constructs of Scr1, VvSUC11 and VvSUC12 (Fig. 2). The lack of sugar accumulation above background with antisense VvSUC11 and VvSUC12 (data not shown) confirms that the grape clones are sugar transporters. The uptake of sucrose by all three transporters was pH dependent. In a previous study, the Scr1 transporter exhibited maximum sucrose uptake at pH 5·0 (Weig and Komor, 1996). Here, the rate of sucrose uptake into the Scr1-transformed cells increased considerably between pH 5·0 and 4·5 and this was also the case for the grape transporters (Fig. 3). Source- (leaf) and sink- (root) specific sucrose/H^+ symporters from carrot expressed in yeast exhibited a similar dependence on pH with the rate of uptake tailing off at pH 4·5 (Shakya and Sturm, 1998). The estimated K_m values for sucrose uptake into yeast transformed with VvSUC11 and VvSUC12 were

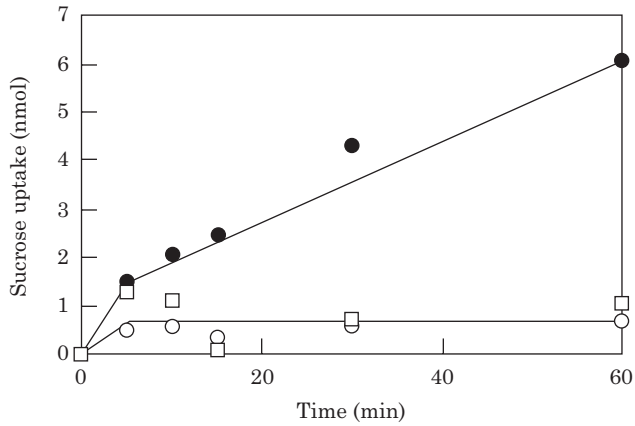

FIG. 1. The time course of sucrose accumulation by *Saccharomyces cerevisiae* SEY2101 transformed with either Scr1 in the sense (●) or antisense (○) orientation, or the empty Nev-N vector (□). Accumulation was assayed from a solution containing 300 μM sucrose at 32°C and pH 5·0. Data are from a characteristic experiment.

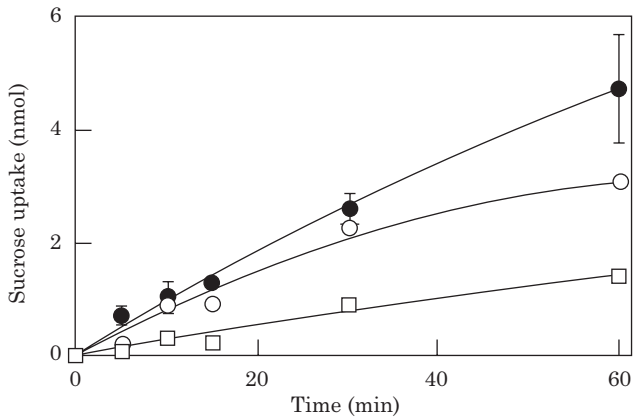

FIG. 2. The time course of sucrose uptake into *Saccharomyces cerevisiae* SEY2101 transformed with either Scr1 (●), VvSUC11 (○) or VvSUC12 (□) in the sense orientations. Uptake was assayed from a solution containing 300 μM sucrose at 32°C and pH 5·0. Uptake was calculated as the total sucrose accumulated by the yeast minus the sucrose bound non-specifically. Data are means from three experiments. For clarity, standard errors are shown for SEY2101 transformed with Scr1 only.

around 1 mM at pH 5·0 (Fig. 4), the value for Scr1 being about half that estimated previously (Weig and Komor, 1996). Similar values have been reported for sucrose transporters from a variety of plant tissues when expressed in yeast (Lemoine, 2000) and for plasma membrane vesicles isolated from leaves (Delrot, 1989). Uptake into yeast transformed with the *Ricinus* and grape transporters was reduced at sucrose concentrations in the external medium above 1·5 mM (Fig. 4).

In the presence of 0·3 mM sucrose, the proton uncouplers carbonyl cyanide-ρ-trifluoromethoxyphenylhydrazone (FCCP) and 2,4-dinitrophenol (2,4-DNP) at 100 μM reduced the rate of sucrose uptake by the three transporters by up to 70 %. Under similar conditions, the uptake of sucrose was reduced 10–35 % by 100 μM vanadate, an inhibitor of P-type H^+-ATPases. These observations

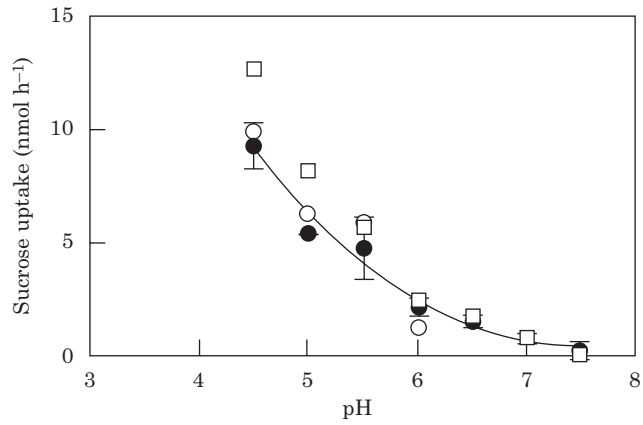

FIG. 3. The pH dependence of sucrose uptake into *Saccharomyces cerevisiae* SEY2101 transformed with either Scr1 (●), VvSUC11 (○) or VvSUC12 (□) in the sense orientations. Uptake was assayed from a solution containing 300 μM sucrose at 32°C and pH 5·0 over 30 min. Uptake was calculated as the total sucrose accumulated by the yeast minus the sucrose bound non-specifically. Data are means from three experiments. For clarity, standard errors are shown for SEY2101 transformed with Scr1 only.

support the hypothesis that sugar uptake mediated by VvSUC11 and VvSUC12 is an energy dependent process mediated by a proton cotransport mechanism. The sulfhydryl-modifying reagent *N*-ethylmaleimide (NEM) at the same concentration had relatively little effect on uptake.

DISCUSSION

It was previously demonstrated that yeast transformed with VvSUT1 could take up sucrose (Ageorges *et al.*, 2000). We have extended this observation by determining the transport characteristics of both VvSUC11 and VvSUC12 in an invertase deficient yeast strain with reference to a previously characterized sucrose transporter, Scr1, from *Ricinus* cotyledon. The data obtained confirm that VvSUC11 and VvSUC12 catalyse the active uptake of sucrose into yeast cells and do not merely facilitate diffusion of the disaccharide. The uptake of sucrose by these transporters is highly dependent on pH and is saturable, distinguishing them from sucrose binding proteins (Overvoorde *et al.*, 1996). The VvSUC11 and VvSUC12 transporters exhibit responses to pH, sucrose and inhibitors (2,4-DNP, FCCP and NEM) that are not readily separable from those of Scr1. The similarity of the transporters from the grape berry, a strong sink tissue that accumulates sugars, and the transporter from *Ricinus* cotyledon, an organ that exports sucrose to other parts of the seedling, is striking. This suggests that they are involved in a similar process (sucrose uptake into cells) although they are present in tissues with different sucrose dynamics. Similarly, the properties of sucrose/H^+ symporters from source and sink tissues of carrot expressed in yeast were functionally indistinguishable (Shakya and Sturm, 1998). The yeast expression system is useful for the preliminary characterization of transporters as shown here, but it does have limitations (Rentsch *et al.*, 1998). If differences in the kinetics of sucrose uptake

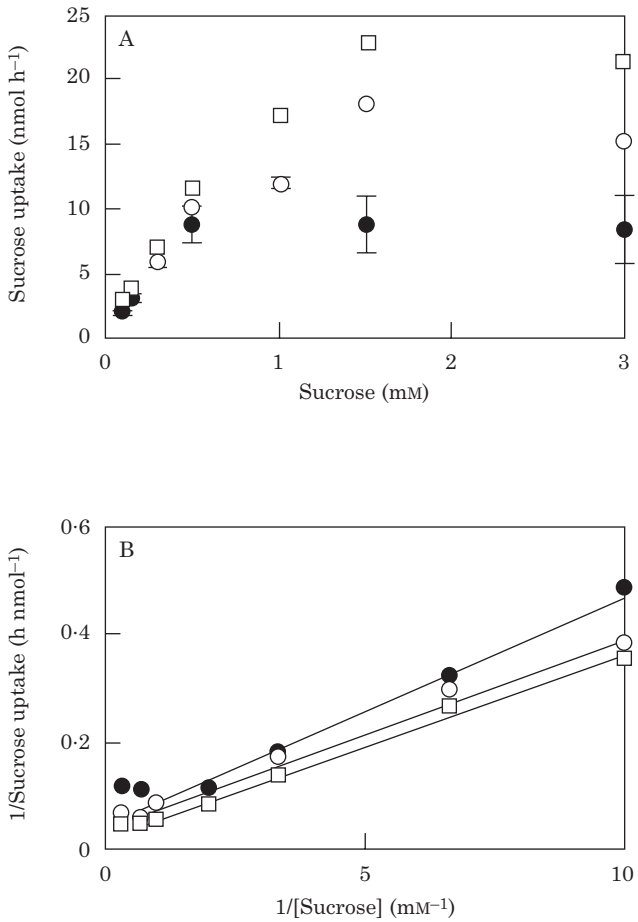

FIG. 4. A, The dependence of sucrose uptake into *Saccharomyces cerevisiae* SEY2101 transformed with either Scr1 (●), VvSUC11 (○) or VvSUC12 (□) in the sense orientations on the sucrose concentration in the assay solution. Initial rates of sucrose uptake were determined over 30 min at 32°C from a solution pH 5·0. Uptake was calculated as the total sucrose accumulated by the yeast minus the sucrose bound non-specifically. Data are means from three experiments. For clarity, standard errors are shown for SEY2101 transformed with Scr1 only. B, Lineweaver-Burke transformation of the data presented in A. Regression lines are shown to data obtained at sucrose concentrations below 1·5 mM. Kinetic parameters were $K_m = 0{\cdot}92$ mM, $V_{max} = 21{\cdot}5$ nmol h^{-1} for Scr1, $K_m = 0{\cdot}88$ mM, $V_{max} = 24{\cdot}8$ nmol h^{-1} for VvSUC11 and $K_m = 1{\cdot}36$ mM, $V_{max} = 40{\cdot}7$ nmol h^{-1} for VvSUC12.

between transporters from source and sink tissues are to be discriminated, techniques with higher resolution may be required. Electrophysiological measurements in *Xenopus* oocytes, for example, enable the relationship between membrane potential and the stoichiometry between protons and sucrose cotransport to be studied directly and the transport mechanism to be determined in detail (Boorer *et al.*, 1996; Lemoine, 2000). Understanding the role of protons in the transport of sugars is particularly relevant to fruits in which high rates of sucrose uptake will be supported by acidification of the apoplast (Almeida and Huber, 1999).

A number of models for phloem unloading have been postulated (Kühn *et al.*, 1999) but there have been few studies on sucrose transporters from sink tissues. The expression patterns of VvSUC11 and VvSUC12 in grape berry (Davies *et al.*, 1999; Ageorges *et al.*, 2000) during veraison suggest that these transporters may be important in the accumulation of sugars for fruit expansion and flavour. Understanding the role of invertases in the cleavage of sucrose and how the monosaccharide transporters (Fillion *et al.*, 1999) partition hexoses in the grape berry will be of key importance in elucidating the mechanism of phloem unloading in this fruit.

ACKNOWLEDGEMENTS

We thank Drs E. Komor and A. Weig from the University of Bayreuth for the yeast strain SEY2102 and the plasmid nSC4+. We also thank Dr A.J. Thompson, HRI, for critically reading this manuscript. This work was supported by the Biotechnology and Biological Sciences Research Council (UK).

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