Pathways of Glucose Regulation of Monosaccharide Transport in Grape Cells[1]

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Grape (*Vitis vinifera*) heterotrophic suspension-cultured cells were used as a model system to study glucose (Glc) transport and its regulation. Cells transported D-[^{14}C]Glc according to simple Michaelis-Menten kinetics superimposed on first-order kinetics. The saturating component is a high-affinity, broad-specificity H^+-dependent transport system (K_m = 0.05 mM). Glc concentration in the medium tightly regulated the transcription of *VvHT1* (*Vitis vinifera hexose transporter 1*), a monosaccharide transporter previously characterized in grape berry, as well as VvHT1 protein amount and monosaccharide transport activity. All the remaining putative monosaccharide transporters identified so far in grape were poorly expressed and responded weakly to Glc. *VvHT1* transcription was strongly repressed by Glc and 2-deoxy-D-Glc, but not by 3-*O*-methyl-D-Glc or Glc plus mannoheptulose, indicating the involvement of a hexokinase-dependent repression. 3-*O*-Methyl-D-Glc, which cannot be phosphorylated, and Glc plus mannoheptulose induced a decrease of transport activity caused by the reduction of VvHT1 protein in the plasma membrane without affecting *VvHT1* transcript levels. This demonstrates hexokinase-independent posttranscriptional regulation. High Glc down-regulated *VvHT1* transcription and Glc uptake, whereas low Glc increased those parameters. Present data provide an example showing control of plant sugar transporters by their own substrate both at transcriptional and posttranscriptional levels. VvHT1 protein has an important role in the massive import of monosaccharides into mesocarp cells of young grape berries because it was localized in plasma membranes of the early developing fruit. Protein amount decreased abruptly throughout fruit development as sugar content increases, consistent with the regulating role of Glc on *VvHT1* expression found in suspension-cultured cells.

Phloem transport of assimilates provides the materials needed for the buildup of herbaceous plants and trees and has long been recognized as a major determinant in crop yield. Indeed, 80% of the carbon photosynthetically fixed in the leaf is exported through the plant vascular system to the roots, reproductive structures, and storage and developing organs, depending on the supply of sugars for their growth and development (Chiou and Bush, 1998; Williams et al., 2000). Past improvements in yield potential have resulted more from an increase in the proportion of accumulated carbon in the harvestable organs than from genetic increases in photosynthesis (Gifford et al., 1984). It is therefore important to understand the mechanisms and regulations of sugar transport into sink tissues.

In sink organs, Suc from the phloem can be imported from the apoplast via direct Suc transporters (DSTs). Alternatively, it can be hydrolyzed to Glc and Fru by cell wall-bound invertases and taken up via monosaccharide transporters (MSTs; for review, see Williams et al., 2000). Many MSTs and DSTs have been characterized from a molecular and functional standpoint in herbaceous plants, but much less has been done on the sugar transporters from lignous species. A Glc transporter from grape (*Vitis vinifera*; Fillion et al., 1999; Vignault et al., 2005), a polyol transporter from sour cherry (*Prunus avium*; Gao et al., 2003), and sorbitol transporters from apple (*Malus domestica*) leaves (Watari et al., 2004) assumed to function as proton-sugar transporters were recently cloned and expressed in yeast (*Saccharomyces cerevisiae*).

Expression of DSTs and MSTs may be affected by various parameters, including light, water and ion status, wounding, fungal and bacterial attacks, and hormones, and they are generally not expressed in the same tissues or at the same developmental stages (Kühn et al., 1997). In the model plant Arabidopsis (*Arabidopsis thaliana*), *AtSTP1* is expressed in leaves and other organs, including stems, flowers, and roots (Sauer et al., 1990), suggesting redundant functions,

[1] This work was supported in part by the Pessoa Program (GRICES/EGIDE), the Conférence des Présidents d'Université, the Conselho de Reitores das Universidades Portuguesas, the Fundação para a Ciência e a Tecnologia (research project no. POCI/AGR/56378/2004; grant no. SFRH/BD/10689/2002 to C.C. and grant no. SFRH/PBD/17166/2004 to A.A.), and the Conseil Régional Poitou-Charentes (to D.G.).

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Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.106.080804.

whereas *AtSTP4* is sink specific (Truernit et al., 1996) and *AtSTP2* is expressed in developing pollen (Truernit et al., 1999). The mechanisms of these regulations are still poorly understood (Delrot et al., 2000). Light and diurnal rhythm may control the expression and activity of some transporters either directly as physical signals and/or because they affect the sugar content of the cell. This control can occur at the level of gene expression or it may affect mRNA and protein turnover (Kühn et al., 1997). Expression of *LeSUT1* in tomato (*Lycopersicon esculentum*; Kühn et al., 1997) and of *DcSUT1* in carrot (*Daucus carota*; required for Suc loading into the phloem) is affected by light. On the contrary, *DcSUC2* expression is not diurnally regulated in the storage root (Shakya and Sturm, 1998). Expression of *AtSTP1* is repressed by light and under the control of diurnal rhythm in guard cells of Arabidopsis (Stadler et al., 2003), and *AtSTP4* is regulated in response to environmental factors, such as wounding or pathogen infection (Truernit et al., 1996).

Besides their role as carbon and energy sources, sugars synthesized during the light phase can act as regulatory signals affecting gene expression. The ability to sense altered sugar concentrations is important in the context of resource allocation, allowing the plant to tailor its metabolism in source tissues to face the demands in sinks. Because sugar transporters play such a key role in source-sink interactions, it is likely that their expression and activity are tightly regulated by sugar levels (Roitsch, 1999). However, the mechanisms underlying regulation by sugars in plants are not as well understood as in yeast and there is some discrepancy about the effect of sugars on the control of sugar transporters. The Suc transporter *BvSUT1* is repressed by Suc (Chiou and Bush, 1998; Vaughn et al., 2002; Ransom-Hodgkins et al., 2003), whereas *OsSUT1* is up-regulated by Suc (Matsukura et al., 2000). Down-regulation of monosaccharide transport was also observed in suspension-cultured cells of olive (*Olea europaea*; Oliveira et al., 2002). By contrast, MST genes are constitutively expressed in *Chenopodium rubrum* and not regulated by sugar (Roitsch and Tanner, 1994). The expression of the grape *VvHT1* MST has been reported to be induced by Suc and palatinose in grape cell suspensions (Atanassova et al., 2003). Sorbitol uptake by peach (*Prunus persica*) tree buds is inhibited by Glc via a hexokinase (HXK)-dependent pathway, but the steps (transcription, translation, targeting, and activity) affected by this process were not investigated in detail (Maurel et al., 2004). The effect (induction or repression) of sugars on transporter genes may depend on the concentration of the sensed sugar. This is the case for *VfSUT1*, a Suc transporter expressed in broad bean (*Vicia faba*) cotyledons (Weber et al., 1997) and for yeast hexose transporter gene expression (Rolland et al., 2001). Eventually, sugars may affect sugar transport not only at the transcriptional level, but also by acting on mRNA stability and protein biosynthesis and activity, as documented in yeast (Boles and Hollenberg, 1997).

The mechanisms and regulation of monosaccharide transport have not been characterized so far in grapevine. Most insight has been given into the cloning of grape hexose transporter genes *VvHT. VvHT1* (AJ001061; Fillion et al., 1999) was characterized as a MST by heterologous expression in tobacco (*Nicotiana tabacum*; Leterrier et al., 2003) and yeast (Vignault et al., 2005). In developing berries, *VvHT1* transcript amount is high shortly after fruit set and then decreases until véraison. Although preliminary studies by reverse transcription-PCR reported that *VvHT1* expression slightly increases after véraison in Ugni-Blanc berries (Fillion et al., 1999), detailed microarray analysis suggested that the second peak of expression does not occur in berries from Chardonnay, Shiraz, and Cabernet Sauvignon varieties (Terrier et al., 2005). Expression of the *VvHT1* promoter-reporter gene construct in tobacco cells showed that *VvHT1* expression is enhanced by sugars (Atanassova et al., 2003). A grape abscisic acid, stress, ripening-induced protein was isolated by means of a one-hybrid approach using as a target a small fragment of the *VvHT1* promoter containing two sugar-responsive elements. This abscisic acid, stress, ripening-induced protein may be part of the transcriptional complex mediating the sugar-inducible expression of *VvHT1* (Cakir et al., 2003).

In this article, we report that in grape cell suspensions VvHT1 operates as a high-affinity, broad-specificity monosaccharide-proton cotransport system repressed by Glc. Accordingly, in grape berries, VvHT1 protein was detected only in the early stages of fruit development when sugar is almost absent. High Glc repression of *VvHT1* involves both a HXK-dependent decrease in the *VvHT1* transcript level and a HXK-independent decrease in the VvHT1 protein amount in the plasma membrane.

RESULTS

Growth in Batch Cultures with Suc

In this work, heterotrophic suspension-cultured cells obtained from grape were used as a model system to study monosaccharide transport into sink cells. Cells were cultivated in liquid mineral medium with Suc as the sole carbon and energy source. Extracellular Suc was completely hydrolyzed within 4 d and growth occurred along with Glc and Fru consumption (Fig. 1). The maximal specific growth rate (μ_{max}) was 0.27 d^{-1}. Prior to reaching the maximal population size, sugar deficiency caused a restriction in the specific growth rate and growth arrest occurred after monosaccharide depletion. This led us to characterize in more detail the hexose transport system and its regulation by Glc in this experimental model.

Monosaccharide Transport

Transport studies were performed with grape suspension-cultured cells grown with 2% Suc (initial

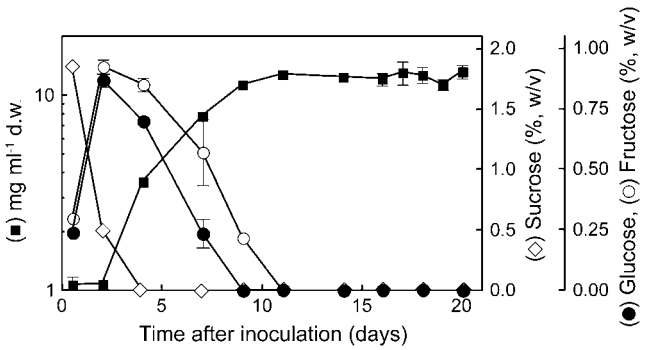

Figure 1. Dry weight and sugar concentration in grape suspension-cultured cells grown with an initial concentration of 2% Suc.

concentration) and collected 10 to 12 d after subculture when the monosaccharides resulting from Suc hydrolysis had declined to residual levels (see Fig. 1). The method used to measure Glc transport in olive suspension-cultured cells (Oliveira et al., 2002) proved to be suitable for grape cells and uptake of D-[^{14}C]Glc was linear up to 180 s (data not shown). Most of the transport experiments were done at an external pH value of 5.0 to impose a transmembrane proton gradient. The initial uptake rates of D-[^{14}C]Glc over a concentration range of 0.02 to 50 mM are shown in Figure 2A. The Eadie-Hofstee plot was biphasic, suggesting the involvement of two transport modes for Glc. Computer-assisted nonlinear regression analysis (GraphPad Prism software) showed Michaelis-Menten kinetics superimposed on first-order kinetics. For the saturable phase of transport, the kinetic parameters were $K_m = 0.05 \pm 0.15$ mM Glc and $V_{max} = 1.45 \pm 0.04$ nmol Glc min^{-1} mg^{-1} dry weight; for the diffusion-like component, $k_d = 0.08$ μL min^{-1} mg^{-1} dry weight. When the uptake of the Glc analog L-[^{14}C]Glc was measured over the same concentration range, only the linear, diffusion-like component was apparent (Fig. 2B). Carrier-mediated transport was calculated as the difference between D- and L-Glc uptake (Fig. 2A, dotted line) and the values for the kinetic parameters did not differ much from those estimated by GraphPad software: $K_m = 0.045$ mM Glc and $V_{max} = 1.39$ nmol Glc min^{-1} mg^{-1} dry weight.

The competitive kinetics of Glc uptake were made in the presence of various unlabeled sugars to test the specificity of the transport system. Figure 3A shows the initial uptake rates of D-[^{14}C]Glc in the presence or absence of 20 mM unlabeled D-Fru. Eadie-Hofstee plots indicated that D-Fru behaves as a competitive inhibitor, although high concentrations had to be used to achieve significant inhibition of D-[^{14}C]Glc uptake (400-fold the K_m value for Glc). When initial uptake rates of 0.02 to 0.5 mM D-[^{14}C]Fru were measured, Michaelis-Menten kinetics were also obtained (Fig. 3B), suggesting carrier-mediated transport. The kinetic parameters were $K_m = 0.5$ mM Fru and $V_{max} = 1.43$ nmol Fru min^{-1} mg^{-1} dry weight. This V_{max} value was similar to that estimated for Glc uptake, suggesting the involvement of the same transport system for both substrates. In addition, a clear inhibition of D-[^{14}C]Fru uptake was obtained with a low concentration (1 mM) of unlabeled Glc.

D-Gal and D-Xyl appear to be transported by the same monosaccharide transport system as Glc and Fru (Fig. 3C). The disaccharide Suc (data not shown), the polyol D-mannitol, and the pentose D-Ara did not affect D-[^{14}C]Glc transport, thus appearing not to be recognized. The effects of the D-Glc analogs 2-deoxy-D-Glc (2-dG), 3-*O*-methyl-D-Glc (3-*O*-MG), and L-Glc on D-[^{14}C]Glc transport are shown in Figure 3D. 2-dG and 3-*O*-MG also competitively inhibited D-[^{14}C]Glc

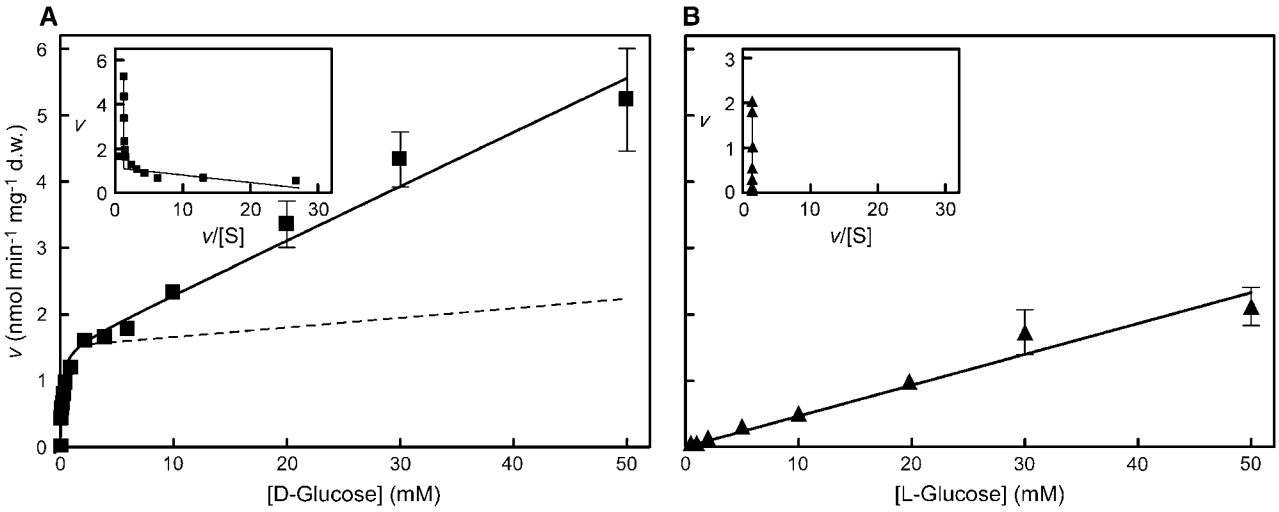

Figure 2. Glc transport by grape suspension-cultured cells cultivated with 2% Suc as in Figure 1. Initial uptake rates of D-[^{14}C]Glc (A) and L-[^{14}C]Glc (B) at pH 5.0 by cells collected at the end of the exponential growth phase when total sugar concentration had fallen to around 0.1%. Dotted line represents the difference between D-[^{14}C]Glc and L-[^{14}C]Glc uptake. Insets, Eadie-Hofstee plots of the initial Glc uptake rates.

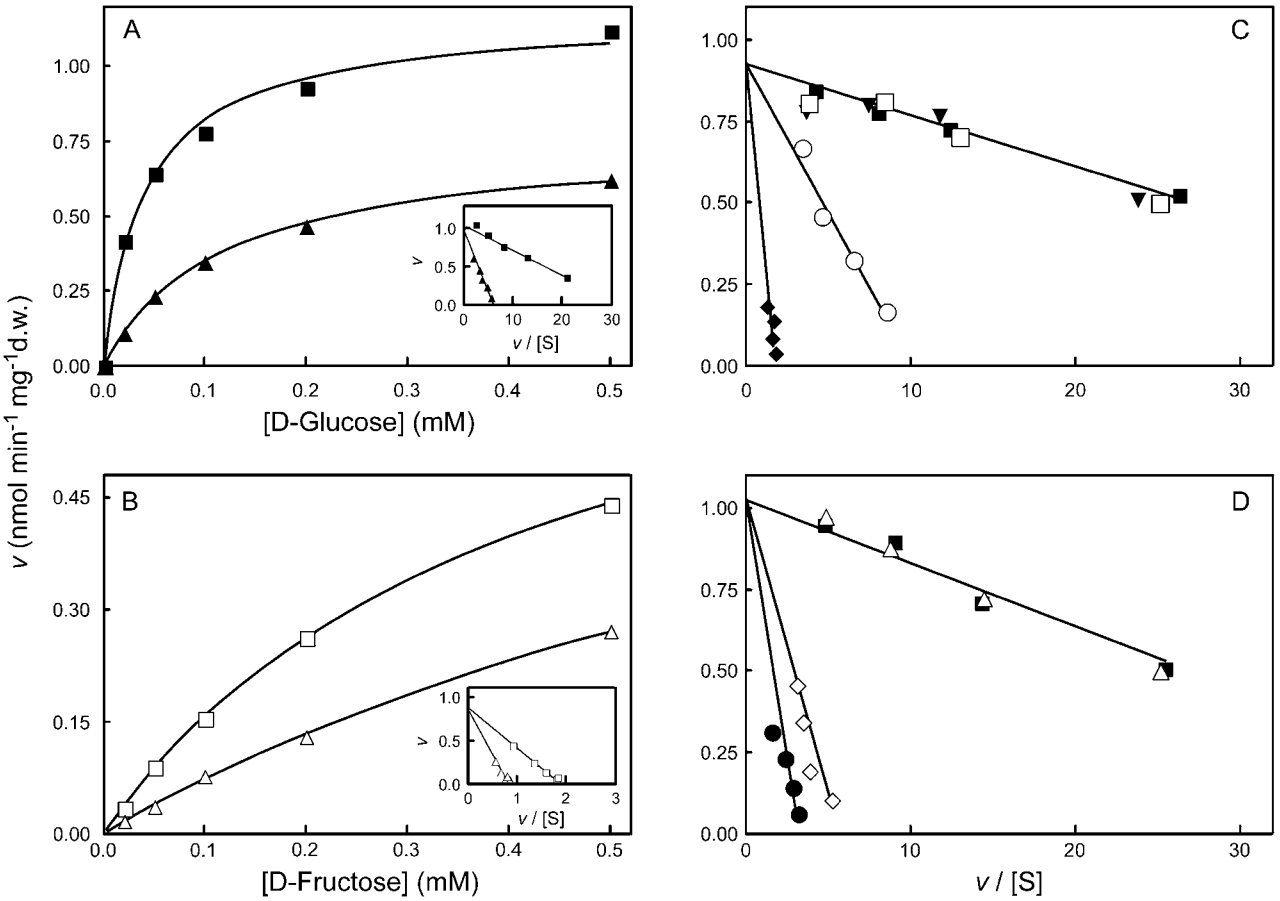

Figure 3. Specificity of the grape monosaccharide transport system. A, Initial uptake rates of D-[^{14}C]Glc in the absence (■) and in the presence (▲) of 20 mM Fru. B, Initial uptake rates of D-[^{14}C]Fru in the absence (□) and in the presence (△) of 1 mM Glc. Insets, Eadie-Hofstee plots of the initial uptake rates of D-[^{14}C]Glc and D-[^{14}C]Fru. C, Eadie-Hofstee plots of the initial uptake rates of D-[^{14}C]Glc in the absence of other sugars (■) and in the presence of 5 mM Xyl (○), 5 mM Gal (♦), 5 mM mannitol (□), and 5 mM Ara (▼), or in the presence (D) of the following Glc analogs: 8 mM L-Glc (△), 0.5 mM 2-dG (◇), and 0.5 mM 3-*O*-MG (●). Transport was measured at pH 5.0 with cells cultivated with 2% Suc, as in Figure 1, and collected at the end of the exponential growth phase when total sugar concentration had fallen to around 0.1%, as in Figure 2.

uptake, meaning that they are also recognized as substrates by the monosaccharide transport system. As expected, L-Glc did not inhibit D-[^{14}C]Glc transport.

Measurements of Glc-induced proton fluxes in grape cell suspensions were made to test directly the hypothesis of a proton-Glc cotransport mechanism. A transient alkalinization of the extracellular medium occurred upon addition of 2 mM D-Glc (final concentration), suggesting that Glc uptake is dependent on the proton gradient (Fig. 4A). The initial velocity of proton uptake was 1.5 nmol H^+ min^{-1} mg^{-1} dry weight, similar to the maximal capacity of the carrier-mediated D-Glc transport. Initial velocities of proton uptake induced by the addition of 2 mM D-Glc decreased by 75% from pH 4.5 to 5.5 (data not shown). The protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) inhibited Glc transport up to 80%, consistent with the hypothesis that a H^+-dependent active transport system was involved (Fig. 4B). Because proton-sugar cotransport mechanisms are associated with a net influx of positive charges into the cells, the effect of dissipating the transmembrane electric potential on Glc uptake was also studied. Addition of the lipophilic and highly permeant cation tetraphenylphosphonium (TPP^+) markedly decreased the initial uptake rates of 0.02 to 0.5 mM D-[^{14}C]Glc (Fig. 4B). The nonmetabolizable Glc analog 3-*O*-MG was used to study the cumulative capacity of the transport system. The analog was concentrated in the intracellular compartment about 25- and 15-fold at pH 5.0 and 7.0, respectively, and CCCP prevented this accumulation (Fig. 4C).

Glc Regulation of MSTs

Several plasma membrane MST homologs (*VvHT*s) and a plastidic Glc transporter (*pGLT*) have been cloned from grape berries. Specific probes were designed in the 3′ noncoding sequences of *VvHT1* (accession no. AJ001061), *VvHT2* (AY663846), *VvHT3* (AY538259), *VvHT4* (AY538260), *VvHT5* (AY538261),

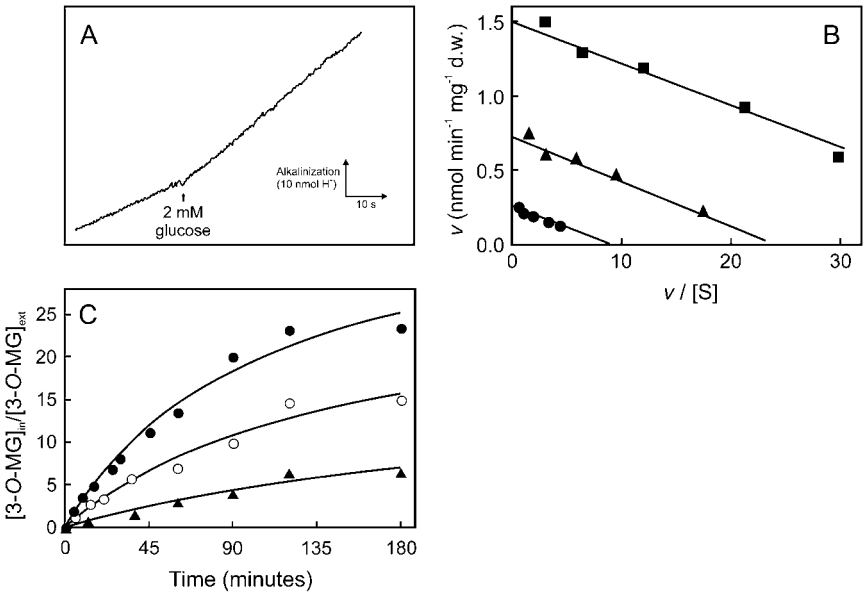

Figure 4. Energetics of the grape monosaccharide transport system. A, Proton uptake, at pH 5.0, associated with the addition of 2 mM Glc to a weakly buffered cell suspension. B, Eadie-Hofstee plots of the initial uptake rates of D-[^{14}C]Glc, at pH 5.0, in the absence (■) or in the presence of 0.05 mM CCCP (●) and 10 mM TPP^+ (▲). C, Accumulation of labeled 3-*O*-MG at pH 7.0 (○) and pH 5.0 in the absence (●) and in the presence (▲) of 0.05 mM CCCP. Initial extracellular concentration of labeled 3-*O*-MG is 0.1 mM. Cells were cultivated with 2% Suc as in Figure 1 and collected at the end of the exponential growth phase when total sugar concentration had fallen to around 0.1% as in Figure 2.

VvHT6 (AY861386), *VvHT7* (AY854146), and *pGLT* (AY608701) and used to test the corresponding transcript amounts. Monosaccharide transport activity and expression patterns of those transporters were studied with cells grown in mineral medium in the presence of 1% Glc (Fig. 5). The most intense signal on RNA blots was observed after hybridization with *VvHT1*, with little expression of *VvHT2*, *VvHT3*, *VvHT4*, *VvHT5*, *VvHT7*, and *pGLT*. The amounts of *VvHT1* transcripts reached a maximal level at day 4 when the $[Glc]_{medium}$ declined below 10 mM, and a high transcription was maintained up to day 6. *VvHT6* transcript amounts constantly decreased after subculture. Transport activity increased abruptly from basal levels at day 4, reaching maximal activity 7 d after subculture. The amount of VvHT1 protein in the plasma membrane was monitored with a polyclonal antibody directed against the C-terminal part of VvHT1 (Vignault et al., 2005). Plasma membranes were purified by a discontinuous Suc gradient from a microsomal fraction prepared from grape cell suspensions collected at various times throughout the culture. The data (Fig. 5A) indicate that the amount of VvHT1 protein present in the plasma membrane began to increase strongly after day 4. The strong expression of *VvHT1* and the correlation of *VvHT1* protein with Glc uptake suggest a major contribution of this transporter in uptake. *VvHT1* transcript levels decreased at day 7 probably due to a too-long Glc starvation period, whereas both transporter protein and uptake activity remain high. Altogether, these results suggest that *VvHT1* expression and monosaccharide transport activity are regulated by Glc levels in the culture medium. To check whether these data may be extended to intact plant tissue, VvHT1 protein amount in plasma membrane of berry mesocarp through maturation was investigated. The amount of the MST decreased abruptly throughout fruit development as sugar content increased, consistent with the repressing role of Glc on *VvHT1* expression (Fig. 5B).

To test this hypothesis more directly, 150 mM Glc was added to cells collected at day 5 when they displayed high transport activity and high levels of *VvHT1* transcripts together with low levels of expression of *VvHT2* to *VvHT7* and p*GLT*. *VvHT1* was by far the most strongly expressed transporter at time 0. Addition of Glc induced a rapid decrease of *VvHT1* transcripts, and transport activity reached basal levels within 12 h (Fig. 6C). In the control cells, *VvHT1* transcripts were maintained at a high level and an increase of Glc transport activity was observed (Fig. 6A). Similarly, after the addition of the same concentration of polyethylene glycol, Glc uptake activity increased and high *VvHT1* transcription was maintained, demonstrating that Glc repression is not due to an osmotic effect (Fig. 6B). Although there was a slight expression of some other *VvHT* homologs in all treatments, they were not repressed by sugar addition and did not prevent the decrease of Glc transport activity induced by Glc. Thus, although it cannot be excluded that some of these transporters contribute to the residual Glc uptake measured after Glc addition, most of the Glc uptake measured in the cells is associated with *VvHT1* expression and is strongly inhibited by Glc.

To test the possible involvement of the HXK-signaling pathway in Glc-mediated *VvHT1* repression, various additions were made to the cells: (1) 150 mM D-Glc in the presence of the HXK inhibitor mannoheptulose (MHL); (2) 3-*O*-MG, a Glc analog that cannot be phosphorylated; and (3) 2-dG, an analog that can be phosphorylated, but is not further metabolized. Blockage of HXK activity in cells treated with 10 mM MHL resulted in derepressed levels of *VvHT1* transcripts (Fig. 6D). Similarly, high levels of *VvHT1* transcripts

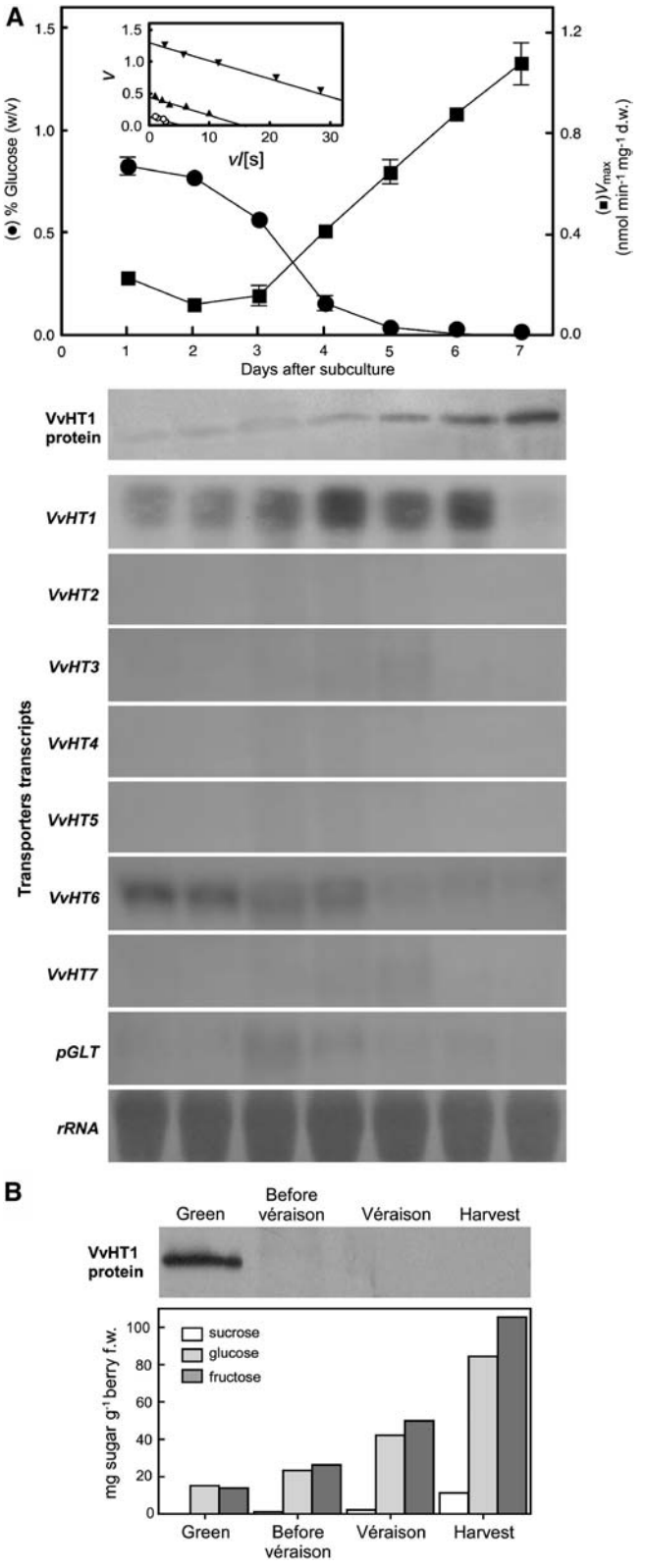

Figure 5. A, Activity of the monosaccharide transport system (V_{max}), western-blot analysis of VvHT1 levels, and northern analysis of *VvHT* and *pGLT* genes in grape suspension-cultured cells along with sugar depletion. Inset, Eadie-Hofstee plots of the initial uptake rates of D-[^{14}C]Glc, at pH 5.0, in cell aliquots harvested from the culture at day 1 (○), day 4 (▲), and day 7 (▼). B, Sugar content and western-blot analysis of VvHT1 levels in the plasma membrane of berry flesh cells at different stages of grape berry development.

were maintained in cells treated with 150 mM 3-*O*-MG (Fig. 6E). However, in both situations, transport activity decreased to basal levels, suggesting that *VvHT1* expression may be regulated posttranscriptionally. The addition of 150 mM 2-dG strongly decreased the amount of *VvHT1* transcripts, as well as transport activity (Fig. 6F). Taken together, these results strongly support the involvement of a HXK-mediated signal responsible for Glc repression of *VvHT1* transcription.

Because *VvHT1* appeared to be transcriptionally repressed upon Glc addition, repressing conditions should be relieved by Glc exhaustion in the medium. To test this assumption, 3-d-old cells grown with 2% Glc were collected when total sugar concentration was >1.5% and transferred to sugar-free medium. D-[^{14}C]Glc uptake and expression of MST genes were followed at selected times after transfer. Glc depletion promoted a 4-fold increase in Glc uptake activity within 30 h (Fig. 7A) associated with a transient induction of *VvHT1* transcription, with the strongest signal being reached 12 h after sugar removal. Transcript levels of *VvHT2*, *VvHT3*, *VvHT6*, *VvHT7*, and *pGLT* were also detected 12 h after sugar starvation, although in much lower amounts than *VvHT1* transcripts (Fig. 7B). The prolonged absence of Glc in the medium resulted in the complete disappearance of all *VvHT* transcripts 24 h after sugar removal. This is in agreement with the absence of *VvHT* transcription at day 7 after subculture (Fig. 5A).

Although high Glc concentrations repress *VvHT1* expression, minimal Glc amounts are required for induction of *VvHT1* transcription. To strengthen the idea that Glc also functions as a positive signal for *VvHT1* induction, Glc was added to Glc-starved cells 30 h after Glc removal according to the conditions of Figure 7A. Figure 7C shows that 12 h after Glc addition, *VvHT1* transcripts increased to levels observed in Glc-repressing conditions (see Fig. 5A up to day 3; Fig. 6C upon Glc addition). In addition, in cells treated with either Glc plus MHL or 3-*O*-MG, a stronger accumulation of *VvHT1* transcripts was observed possibly as a result of HXK impairment in mediating the Glc repression signal, similar to the data of Figure 6, D and E.

Comparing the data of Figure 6C, when Glc addition promoted a decrease of transport activity and *VvHT1* transcription, with those of Figure 6, D and E, when high *VvHT1* message levels were maintained although a drop of transport activity was observed, suggests that posttranscriptional mechanisms can be operating. These mechanisms would lead to the inactivation of transporter proteins or a decrease of protein abundance in the membrane. To test this hypothesis, the amount of VvHT1 protein in the plasma membrane was studied in cells collected 24 h after addition of either Glc, 3-*O*-MG, 2-dG, or Glc plus MHL, as

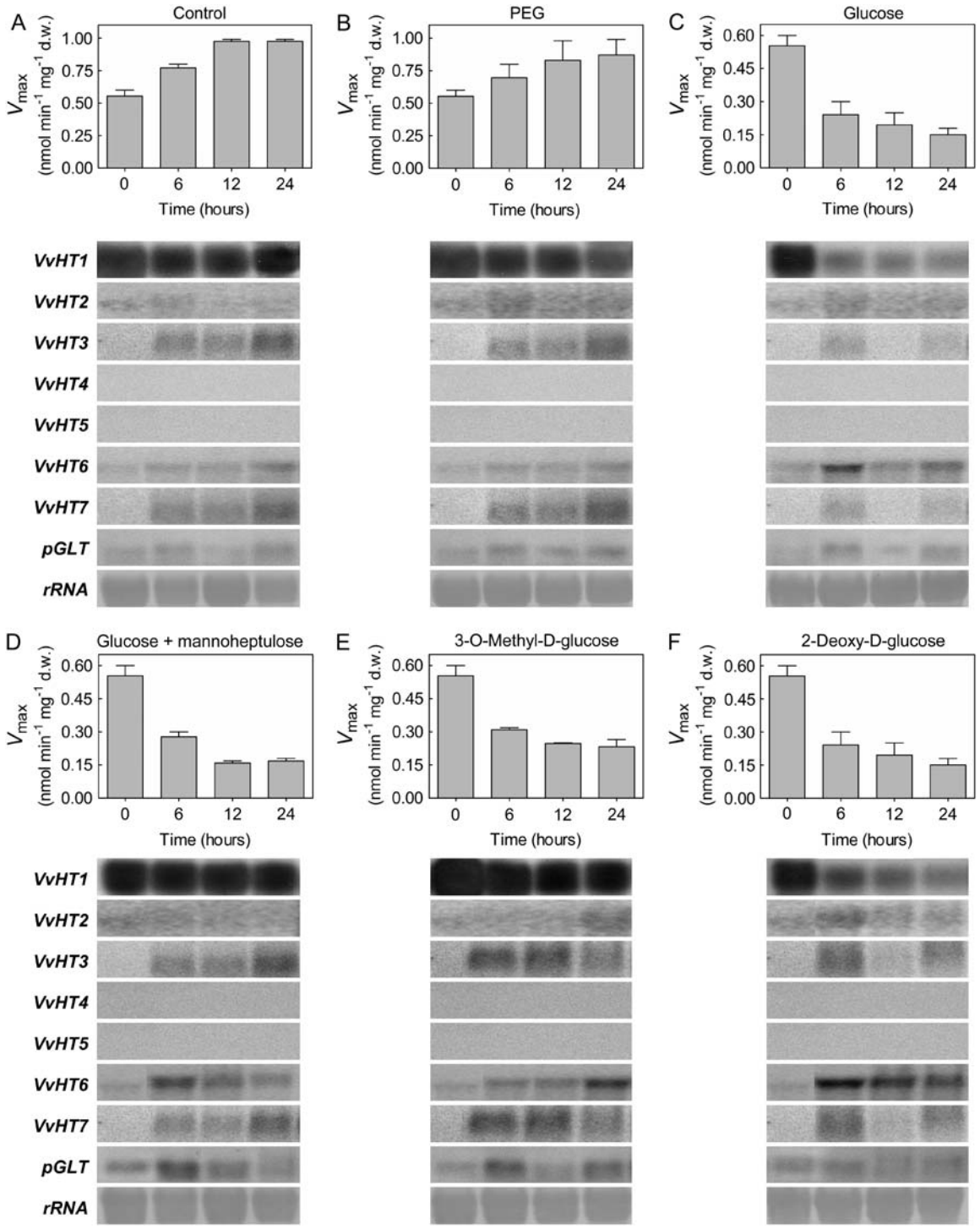

Figure 6. Repression of the grape monosaccharide transport system by different sugars. D-[^{14}C]Glc uptake (V_{max}) and *VvHT* homolog transcripts were measured in cell aliquots at time periods indicated after the addition of 150 mM polyethylene glycol, 150 mM Glc, 150 mM Glc plus 10 mM MHL, 150 mM 3-*O*-MG, and 150 mM 2-dG to cultures at day 5 in the conditions described in Figure 5.

described in Figure 6. The western blot showed that a decrease in transport activity was always correlated with a reduction in the abundance of VvHT1 protein (Fig. 8), even in those cases where the transcript level was not affected.

DISCUSSION

Relatively little is known about the regulation of sugar transport activity in plants. Suc transporters may be transcriptionally repressed by phosphorylation-dependent mechanisms (Vaughn et al., 2002; Ransom-Hodgkins

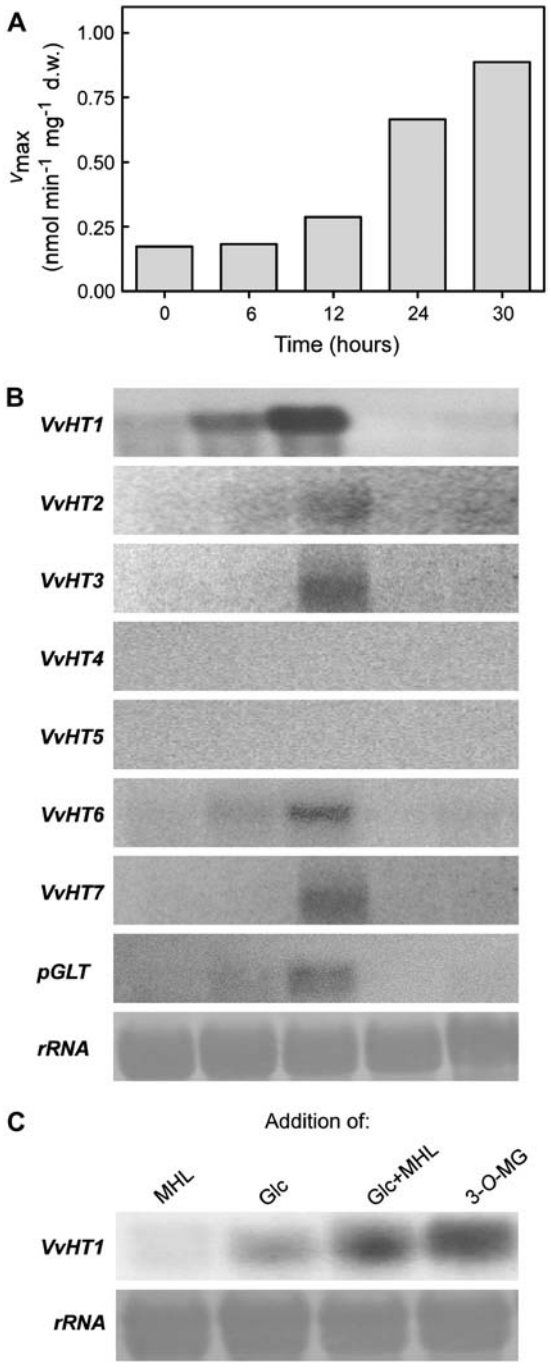

Figure 7. Activity of the monosaccharide transport system (V_{max}; A) and *VvHT* homolog transcription (B) in sugar-starved suspension-cultured cells. Cells were grown with 2% Glc and transferred at day 3 to the same medium without sugar. C, *VvHT1* transcription 12 h after addition of 10 mM MHL, 150 mM Glc, 150 mM Glc plus 10 mM MHL, and 150 mM 3-*O*-MG to cells cultivated during 30 h in the absence of sugar as indicated in A.

et al., 2003) and indirect evidence suggests that Suc transporters may be regulated by phosphorylation (Roblin et al., 1998). The *AtSTP4* MST may be induced by fungal and bacterial elicitors (Truernit et al., 1996; Fotopoulos et al., 2003) and the nonhost pathogen *Botrytis cinerea* enhances Glc transport in *Pinus pinaster* suspension-cultured cells depending on NADPH oxidase and calcium influx, but not mitogen-activated protein kinase (Azevedo et al., 2006). In contrast, the fungal elicitor cryptogein blocks monosaccharide transport in tobacco cells through a calcium-dependent process (Bourque et al., 2002). Although *VvHT1* transcription has been shown to be induced by sugars in grape cell suspensions, the presence in its promoter region of both positive (inducing) and negative (repressing) sugar response cis-elements (Fillion et al., 1999; Atanassova et al., 2003) may suggest that it is under more complex control by sugars. This work provides an extensive description of the hexose transport system operating in grape cell suspensions and investigates in detail the mechanisms and signaling pathways involved in the control of hexose transporters by Glc. A wealth of information is already available on plant sugar transporters in terms of gene structure and expression, whereas much less is known about the regulation of transport activity in relation to gene expression. The problem is that plant organs are often not accessible to such studies. The use of suspension-cultured cells to study sugar uptake and its regulatory mechanisms offers a number of distinct advantages over the intact plant where bulk diffusion, tissue penetration barriers, and cell heterogeneity impair kinetic studies. Additionally, in cell suspensions, the plasma membrane is readily amenable for challenging with exogenous sugars, analogs, and transport inhibitors. Despite the necessary cautions needed to extrapolate the results to a multicellular level, they provide a convenient experimental system that has already yielded a lot of useful information on sugar transport mechanisms and regulation (Roitsch and Tanner, 1994; Ehness and Roitsch, 1997; Oliveira et al., 2002; Cakir et al., 2003), as well as in other key physiological processes, such as cell cycle (Riou-Khamlichi et al., 1999), hormonal signaling (Cakir et al., 2003), and regulation of gene expression (Graham et al., 1994;

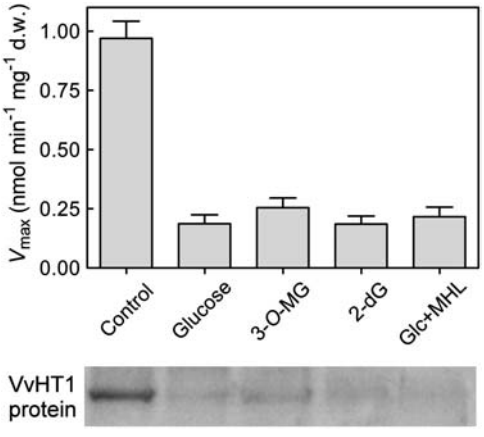

Figure 8. Activity of the monosaccharide transport system (V_{max}) and VvHT1 protein amount in the plasma membrane. Cells were collected 24 h after Glc, 3-*O*-MG, 2-dG, and Glc plus MHL had been added to cell suspensions, according to conditions described in Figure 6.

Cheng et al., 1999). Furthermore, in this work, we demonstrated that when young berries start to accumulate monosaccharides the amounts of VvHT1 protein (Fig. 5B) strongly decrease in accordance with previous results on *VvHT1* transcription (Terrier et al., 2005), indicating that the regulations described in grape cell suspensions are physiologically relevant.

Characterization of the Hexose Transport System in Grape Cell Suspensions

Heterotrophic suspension-cultured cells of grape are able to take up D-Glc by a high-affinity saturable component superimposed on a nonsaturating component (Fig. 2). The saturable component involves a proton-Glc transport mechanism as indicated by the following observations: (1) Glc addition to weakly buffered cell suspensions is associated with a transient alkalinization of the extracellular medium (Fig. 4A); (2) the V_{max} of proton uptake is similar to the V_{max} of carrier-mediated D-Glc uptake, suggesting a stoichiometry of 1 H^+ to 1 Glc, and depends on extracellular pH; (3) the analog 3-*O*-MG is concentrated intracellularly and the accumulation ratio is higher at low pH (Fig. 4B); (4) dissipation of the proton-motive force by CCCP significantly inhibits initial velocities of D-Glc uptake and 3-*O*-MG accumulation (Fig. 4, B and C); and (5) D-Glc transport is sensitive to TPP^+, suggesting that $\Delta\Psi$ is an important component of proton-motive force involved in Glc accumulation. Grape cultured cells are able to accumulate the nonmetabolizable Glc analog 3-*O*-MG up to 25-fold. The Nernst-Plank equation may be used to calculate the transmembrane Glc gradient maintained by a proton-Glc symporter with a stoichiometry of 1 H^+ to 1 Glc. An accumulation factor between 700 and 2,000 would be expected at 25°C and pH_{ext} = 5.0, assuming an intracellular pH of 7.0 and $\Delta\Psi$ of −50 to −120 mV. The accumulation ratio observed is thus somewhat lower than that theoretically derived from this equation. However, given that the cytosolic volume only represents about 10% of the cell volume, the estimated accumulation ratio is probably much closer to the theoretical value.

The high affinity (K_m = 50 μM Glc) measured for the H^+-dependent monosaccharide transport may be important for cell growth in media where the sugar supply rapidly becomes limiting. Similar K_m values were measured for monosaccharide uptake in suspension-cultured cells of tobacco (Verstappen et al., 1991), carrot (Krook et al., 2000), and olive (Oliveira et al., 2002), in guard cell protoplasts of pea (*Pisum sativum*; Ritte et al., 1999), and in yeast expressing MSTs from lower and higher plants (Büttner and Sauer, 2000). The K_m measured here is also in good agreement with the one measured by heterologous expression of *VvHT1* in yeast (Vignault et al., 2005). The monosaccharide transport system of grape exhibited broad specificity given that D-[^{14}C]Glc uptake is competitively inhibited by D-Fru, D-Gal, and D-Xyl, and by the Glc analogs 2-dG and 3-*O*-MG (Fig. 3). D-Glc and D-Fru are both recognized as substrates by the same transport system, although the affinity for D-Glc is much higher than for D-Fru. The lower affinity for D-Fru than for D-Glc (Fig. 3) may explain that D-Glc is consumed before Fru after Suc hydrolysis (Fig. 1). The higher affinity of the monosaccharide transport system for Glc than for Fru seems a general property also found in suspension-cultured cells of bean (*Phaseolus vulgaris*; Botha and O'Kennedy, 1998), carrot (Krook et al., 2000), and olive (Oliveira et al., 2002).

Altogether, these results indicate that the grape H^+-dependent monosaccharide transport system exhibits properties of the MST family members, which transport a range of hexoses and pentoses with K_m values for the preferred substrate around 10 to 100 μM (Büttner and Sauer, 2000). The involvement of high-affinity carrier-mediated transport is adequate for the growth of cells in media containing low sugar levels. However, at extracellular sugar content from 2 to 50 mM, carrier-mediated transport is saturated, contributing only in a small proportion to overall sugar uptake (Fig. 2). Therefore, nonsaturable mechanisms involved in diffusion-like uptake may play important roles in sugar absorption by grape suspension-cultured cells growing at high external sugar levels. Whether the same conclusion may apply to the cells of grape berry in vivo needs further investigation, but may be possible due to the high sugar content of this organ (Coombe, 1992) and in sink tissues in general (Patrick, 1997). Although nonsaturable mechanisms of sugar transport were also reported in other plant cells and tissues (Delrot, 1989; Krook et al., 2000; Oliveira et al., 2002), the underlying mechanisms are still poorly understood. Several mechanisms or a combination of them could account for diffusion-like kinetics: (1) nonspecific permeation of the sugar by free diffusion across the plasma membrane; (2) involvement of carriers with very low affinity; (3) involvement of carriers with Glc channels; and (4) occurrence of a sugar-inducible endocytic process (Etxeberria et al., 2005).

Several lines of evidence indicate that VvHT1 is responsible for the observed monosaccharide/H^+ symporter activity in grape cell suspensions, including (1) its higher expression compared to the other transporters tested; (2) the similar properties (K_m = substrate specificity) described here for grape cell suspensions and for yeast expressing VvHT1 as the sole MST (Vignault et al., 2005); and (3) the parallel between the amount of *VvHT1* transcript and/or protein and the induction (Fig. 5) or repression (Fig. 6) of Glc uptake according to the monosaccharide concentration in the medium. However, the observation that there was a slight expression of some other *VvHT* homologs so far identified in the Vitis genome suggests that *VvHT1* may not be the unique hexose transporter contributing to overall Glc uptake. Some of these transporters, such as *VvHT3*, *VvHT6*, and/or *VvHT7* could contribute to the residual Glc uptake observed in Glc repression conditions, possibly as a result of different regulation mechanisms and/or

kinetic parameters. Indeed, for a given nutrient, multiple transport systems can coexist within a single cell to assure uptake over a broad range of substrate concentrations (Ludewig and Frommer, 2002). In addition, the participation of a yet-unknown Vitis Glc transporter cannot be completely ruled out. Thirteen clusters were recognized in the MST superfamily, with 66 and 22 putative MSTs in the Arabidopsis and rice (*Oryza sativa*) genomes, respectively (Lalonde et al., 2004). The reason for the apparent redundancy is not clear, but fine tuning is probably involved.

Monosaccharide Transport Activity in Grape Cell Suspension Is Induced or Repressed by Glc Depending on Its Concentration in the Medium

Both Suc and Glc are known to play distinct roles in sugar signaling (Lalonde et al., 1999; Wiese et al., 2004). Our data highlight the dual aspect of *VvHT1* expression in response to Glc signals. Indeed, depending on its concentration, Glc may induce or repress *VvHT1* transcription. It induces *VvHT1* in cells experiencing sugar starvation, but it represses *VvHT1* in cells that have been exposed to high sugar levels. Accordingly, we show that monosaccharide uptake is low when the sugar concentration of the medium is high (Figs. 5 and 6) and is induced when this concentration is low. In this regard, higher plant cells resemble the green unicellular algae *Chlorella kessleri* (Komor et al., 1972). This complex regulation may be one of the reasons why contradictory conclusions have been reached on the regulation of sugar transporters by sugars (see introduction). Furthermore, although other transporters are expressed to some extent in these cells, *VvHT1* is the most sensitive to the Glc content of the medium, which led us to investigate the mechanisms of its regulation.

The sharp increase in *VvHT1* expression following transfer to Glc-free medium (Fig. 7B) may result from either a simple derepression of transcription or the cumulative effect of derepression and specific induction of transcription by Glc starvation. RNA-blot data support the first hypothesis because, in the prolonged absence of sugar, *VvHT1* transcripts were virtually absent (Figs. 5A and 7B). Thus, the simple relief of Glc repression is insufficient for constant high-level *VvHT1* expression and a minimal amount of Glc is required for transcriptional activation of the transporter. Indeed, addition of Glc to Glc-starved cells promoted an increase of *VvHT1* transcript levels (Fig. 7C).

Such a dual effect of Glc has also been described for the hexose transporters *HXT2* of yeast (Wendell and Bisson, 1994) and *KHT2* of *Kluyveromyces lactis* (Milkowski et al., 2001). During cotyledon development, fava bean Suc transporter *VfSUT1* expression was also shown to be under dual regulation by sugars (Weber et al., 1997). The dual aspect of *VvHT1* Glc regulation may result from the coexistence of several positive sugar-responsive cis-elements (SURE1 and Suc box 3) and one AMYBOX1 and two AMYBOX2 sugar repression motifs (Fillion et al., 1999; Atanassova et al., 2003).

VvHT1 Expression Is Transcriptionally Repressed by High Glc Concentration via a HXK-Signaling Pathway

Down-regulation of *VvHT1* expression by Glc was further substantiated by the inhibition observed when several monosaccharides were added to cells exhibiting high Glc transport activity and high *VvHT1* transcript levels (Fig. 6). Kinetic analysis of transport activity showed a decrease in V_{max} 6 h after addition of 150 mM Glc, reaching a basal level within 12 h. This sugar concentration, which was also used in previous studies on sugar regulation of Suc transporters (Williams et al., 2000; Vaughn et al., 2002) is lower than that usually employed in studies on sugar sensing (up to 7% Glc, i.e. up to 400 mM [Gibson, 2004]) and may be found in sink tissues (Patrick, 1997). Decreased V_{max} activity is consistent with less VvHT1 protein in the membrane, although other forms of down-regulation, such as protein modification, cannot be ruled out. Decreased levels of symporter protein in the membrane may result either from increased rates of protein turnover and/or from decreased protein synthesis. RNA gel-blot analysis revealed a decrease in *VvHT1* transcript levels, suggesting a drop in transcriptional activity or mRNA stability (Fig. 6). Thus, Glc repression of monosaccharide uptake in grape cell suspensions is at least in part mediated by transcriptional regulation.

Many examples of sugar-induced changes in gene expression have been described (Koch, 2004), but examples of sugar transporter regulation by sugars are rare (Chiou and Bush, 1998; Atanassova et al., 2003; Maurel et al., 2004) and the underlying mechanisms are poorly known. Both HXK-dependent and -independent signaling pathways have been involved in sugar sensing in plants. To investigate the role of HXK in Glc-induced *VvHT1* repression, we studied the effects of Glc analogs and of MHL, a competitive inhibitor of HXK. The results showed that Glc-induced repression of *VvHT1* is reversed by the addition of the HXK inhibitor MHL (Fig. 6). Furthermore, the nonphosphorylable Glc analog 3-*O*-MG did not repress *VvHT1*, whereas 2-dG, which can be phosphorylated and slowly metabolized, decreased the amounts of *VvHT1* transcripts. Although 3-*O*-MG may be phosphorylated by HXK in maize (*Zea mays*; Cortes et al., 2003), the phosphorylation efficiency was 5 orders of magnitude less than for Glc and Man. Our results show that Glc-induced repression of *VvHT1* requires HXK activity, but apparently no further metabolism, which is typical for a HXK-mediated signaling pathway.

The observation that *VvHT1* can be transcribed under conditions in which Glc uptake is weak (i.e. in the presence of 3-*O*-MG) indicates that it may be regulated at a posttranscriptional level. This prompted us to study the amounts of VvHT1 protein under

various experimental conditions. Immunoblot analysis showed that *VvHT1* is also repressed at a posttranscriptional level in response to high Glc concentrations. Indeed, the decrease of Glc transport activity upon addition of Glc, 2-dG, 3-*O*-MG, and Glc plus MHL was accompanied by a decrease of VvHT1 protein in the plasma membrane, regardless the levels of *VvHT1* transcripts, showing that steady-state protein levels and mRNA for *VvHT1* do not always correspond. The same was found under sugar depletion conditions (Fig. 5A, day 7), probably due to a longer half-life of the transporter protein than the corresponding mRNA. A control step affecting either protein translation and/or turnover is most likely involved in the Glc transport reduction induced by 3-*O*-MG and Glc in the presence of MHL. Although the control of a sugar transporter by its own substrate at both transcriptional and posttranscriptional levels has been described in yeast (Wendell and Bisson, 1994; Boles and Hollenberg, 1997), these data illustrate that the same applies in plants. In the case of the sugar beet (*Beta vulgaris*) leaf proton-Suc symporter *BvSUT1*, immunoblot analysis showed that decreased transport activity was caused by a reduction in the abundance of symporter protein (Vaughn et al., 2002) and RNA gel-blot analysis of the leaf symporter revealed that message levels also declined as a result of decreased transcription, demonstrating a clear connection between steady-state mRNA levels and protein amounts. However, other proteins, such as maize invertase (Cheng et al., 1999) and the ATB2/AtbZIP11 transcription factor of Arabidopsis (Rook et al., 1998; Wiese et al., 2004, 2005), are controlled by sugars at both transcriptional and posttranscriptional levels. Altogether, present results suggested that different control steps affecting transcription, mRNA stability, translation, and protein stability or degradation, which do not affect mRNA levels directly, can be operating from the gene to the protein level to coordinate Glc uptake in Vitis cells.

A Model for Glc Regulation of *VvHT1* Expression and Glc Uptake

The data may be summarized by the model detailed in Figure 9. When high Glc is present (left), energy-independent, diffusional uptake is the preferred mode of sugar absorption and is sufficient to sustain cell growth and metabolism. Under these conditions, *VvHT1* expression is maintained at basal levels due to the balance between a positive induction signal generated by the presence of Glc and a repression signal due to high Glc levels sensed by HXK. Additionally, high Glc levels seem to repress Glc transport activity at the protein level, triggering inactivation, mistargeting, and/or proteolysis of VvHT1. This phenomenon is common for rate-limiting proteins, such as transporters, and has been well demonstrated in yeast where it is called carbon inactivation (Busturia and Lagunas, 1985, 1986; Horak and Wolf, 1997). The mechanism by which high Glc levels trigger proteolysis in yeast is poorly known, but Glc appears to induce the synthesis of proteins required for the degradation process. When external Glc decreases to

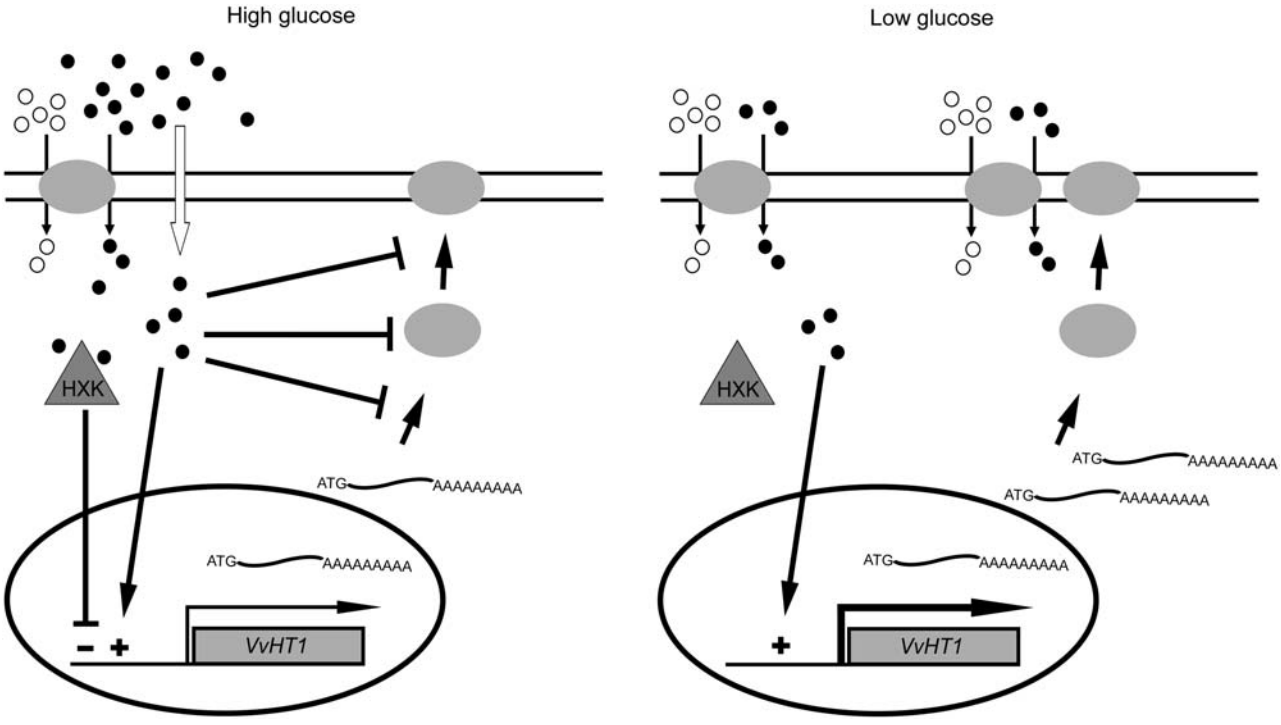

Figure 9. Schematic model of Glc regulation of *VvHT1* expression and Glc transport activity. Glc, ●; H^+, ○.

residual levels (<10 mM Glc, right), the linear transport component no longer sustains Glc transport at a rate sufficient to meet the energy requirements of the cell and the involvement of a concentrative, energy-dependent transport system becomes critical. The absence of the repression signal generated by HXK allows the increase of *VvHT1* transcripts to high levels and, in accordance, the number of high-affinity monosaccharide/H^+ symporters in the plasma membrane increases (increase of V_{max}), ensuring a high-transport capacity at limiting Glc conditions.

In conclusion, these data contributed to the understanding of the mechanisms involved in Glc import into the berry. The regulation of the expression of the MST VvHT1 was investigated at transcriptional, translational, and protein activity levels in a sink model. Also, we reported that the VvHT1 transporter is abundant at the green stage of fruit development, consistent with the illustrated regulating role of Glc on *VvHT1* expression. Although part of the phloem-translocated Suc may be absorbed directly by mesocarp cells through disaccharide transporters (Davies et al., 1999; Ageorges et al., 2000), VvHT1 should contribute to the early steps of Glc and Fru accumulation in the berry flesh cells after the disaccharide had been hydrolyzed by apoplastic invertases. This high-affinity transporter may be involved in the supply of energy for the intense cell division and growth when low apoplastic sugar is available. As ripening proceeds, repression of VvHT1 is most likely associated with monosaccharide accumulation and other sugar transporters should be involved in sugar import into the mesocarp cells.

MATERIALS AND METHODS

Cell Suspension Culture, Growth Conditions, and Treatments

Cell suspensions of grape (*Vitis vinifera*) were maintained in 250-mL flasks on a rotatory shaker at 100 rpm in the dark, at 25°C on mineral medium supplemented with 2% (w/v) Suc or 1% (w/v) Glc, as previously described (Descendit et al., 1996). Cells were subcultured weekly by transferring 10-mL aliquots into 70 mL of fresh medium. Maximal specific growth rates (μ_{max}) were determined from dry-weight measurements. Aliquots (1–5 mL) were filtered through preweighed GF/C filters (Whatman). The samples were washed with deionized water and weighed after 24 h at 80°C. Sugar consumption was monitored by HPLC and the Glc oxidase method (test combination; Boehringer Mannheim).

Estimation of Initial Sugar Uptake Rates

Harvested cells were centrifuged, washed twice with ice-cold culture medium without sugar at pH 5.0, and resuspended in the same medium at a final concentration of 5 mg mL^{-1} dry weight.

To estimate the initial uptake rates of D- or L-[^{14}C]Glc, 1 mL of cell suspension was added to 10-mL flasks under shaking (100 rpm). After 2 min of incubation at 25°C, the reaction was started by the addition of 40 μL of an aqueous solution of radiolabeled sugar at the desired specific activity and concentration. The specific activities were defined according to the final concentration of the sugar in the reaction mixture as follows: 8.33 Bq $nmol^{-1}$ (0.02–0.5 mM Glc), 1.66 Bq $nmol^{-1}$ (1–10 mM Glc), and 0.17 Bq $nmol^{-1}$ (10–50 mM Glc). Sampling times were 0, 60, and 180 s, which ensures linearity of uptake. The reaction was stopped by dilution with 5 mL ice-cold modified Murashige and Skoog medium without sugar and the mixtures were immediately filtered through GF/C filters (Whatman). The filters were washed with 10 mL of the same medium and transferred to vials containing scintillation fluid (OptiPhase HiSafe II; LKB Scintillation Products). Radioactivity was measured in a Packard Tri-Carb 2200 CA liquid scintillation counter (Packard Instruments). The results were corrected for nonspecific binding of labeled sugars to the filters and/or the cells by diluting the cells with 5 mL ice-cold modified Murashige and Skoog medium without sugar before addition of labeled sugar. The values for the nonspecific binding constant of labeled Glc, determined in a range of 0.02 to 50 mM sugar, were 40 ± 3 nL min^{-1} mg^{-1} dry weight (mean ± SD; n = 3). D-[U-^{14}C]Glc (11.28 GBq $mmol^{-1}$) and L-[U-^{14}C]Glc (2.035 GBq $mmol^{-1}$) were obtained from the Radiochemical Centre (Amersham).

Determination of Substrate Specificity

Competition between Glc and other sugars was tested by running competitive uptake kinetics. The concentration range of labeled sugar varied from 0.02 to 0.5 mM and the final concentration of the unlabeled substrate was at least 10-fold higher than the K_m value estimated for the transport system. Initial uptake rates of D-[^{14}C]Fru were estimated as described previously for D- or L-[^{14}C]Glc. The concentration range of labeled Fru varied from 0.02 to 0.5 mM (specific activity = 50 Bq $nmol^{-1}$). Inhibition of Fru transport by 1 mM Glc was assayed as described for inhibition of Glc transport by nonlabeled sugars.

Accumulation Studies

A 10-mL sample of cell suspension was transferred to a 50-mL Erlenmeyer flask under shaking (100 rpm). After 2-min incubation at 25°C, the reaction was started by the addition of an aqueous solution of radiolabeled 3-*O*-MG (specific activity = 50 Bq $nmol^{-1}$) at a final concentration of 0.1 mM. At selected times, 1-mL aliquots were taken from the reaction mixture into 5 mL ice-cold modified Murashige and Skoog medium without sugar and filtered immediately through Whatman GF/C membranes. The filters were washed with 10 mL of the same medium and the radioactivity was counted as indicated above. The intracellular concentrations of 3-*O*-MG were estimated as the ratio between the intracellular and the extracellular 3-*O*-MG concentration, using the intracellular volume obtained as indicated below. 3-*O*-methyl-D-[U-^{14}C]Glc (3.7 MBq $mmol^{-1}$) was obtained from the Radiochemical Centre (Amersham).

Estimation of Initial Rates of Proton Uptake

To estimate the initial rates of proton uptake upon addition of Glc, a standard pH meter (PHM 82 Radiometer A/S) connected to a recorder (Kipp and Zonen) was used as described earlier (Oliveira et al., 2002). The pH electrode was immersed in a water-jacketed chamber with magnetic stirring. A total of 5 mL of grape cell suspension in 10 mM potassium phosphate buffer (about 4 mg mL^{-1} dry weight) were added to the chamber. The pH was adjusted to 5.0 and a baseline was obtained. The desired amount of Glc was added and the subsequent alkalinization curve was monitored. The slope of the initial part of the pH trace was used to calculate the initial rates of proton uptake. Calibration was performed with HCl.

Determination of Intracellular Volume

The methodology used to measure intracellular water volume was a modification of the methods previously described (Rottenberg, 1979; De la Peña et al., 1981) and was based on the quantification of the relative distribution of two radioactive compounds in a cellular suspension: [^{14}C]methoxy inulin, to which biomembranes are impermeable, and [^{3}H]H_2O that equilibrates across biomembranes. After washing with culture medium without sugar, 2 mL of cell suspension were incubated with 5 μL of 9.25 MBq mL^{-1} [^{3}H]H_2O (Amersham; 185 GBq mL^{-1}), and 5 μL of 0.22 mg mL^{-1} [^{14}C]methoxy inulin (New England Nuclear; 192 MBq g^{-1}). The mixture was incubated for 30 min and the cells were pelleted by centrifugation at 4,000*g* for 1 min. The supernatant (100 μL) was added to 5 mL of 1% (w/v) SDS and the same volume of SDS was added to the pellet. After overnight incubation, the mixtures were centrifuged and the radioactivity of 40 μL of each supernatant was measured as described above. Intracellular water volume (V_{int}) was determined according to the expression $V_{int} = V_{sup}\ [(^3H_{pel}/^3H_{sup}) - (^{14}C_{pel}/^{14}C_{sup})]$, where V_{sup} corresponds to the volume of supernatant, $^3H_{pel}$ and $^3H_{sup}$

correspond to the ^{3}H counts in the pellet and in the supernatant, respectively, and $^{14}C_{pel}$ and $^{14}C_{sup}$ correspond to the ^{14}C counts in the pellet and in the supernatant, respectively. A value of 8 ± 3 μL intracellular water mg^{-1} dry weight (mean ± SE, $n = 4$) was obtained.

Calculation of Kinetic Parameters

Data of the initial uptake rates of labeled Glc were analyzed by computer-assisted nonlinear regression analysis (GraphPad Prism software). By this method, the transport kinetics best fitting to the experimental initial uptake rates were determined and estimates for the kinetic parameters were then obtained. Uptake rates are mean values ± SE; n denotes the number of independent experiments.

RNA Gel-Blot Analysis

Total RNAs from grape berry cell suspension samples were isolated by phenol extraction and LiCl 2 M precipitation (adapted from Howell and Hull, 1978). Twenty micrograms of each RNA sample were separated by formaldehyde-agarose gel electrophoresis and transferred onto Hybond N membrane (Amersham Life Science). The 200-bp 3′-specific probes were designed by PCR for each *VvHT* and *pGLT* from sequences available in the National Center for Biotechnology Information International Data Bank and used for RNA-blot analysis. Randomly primed ^{32}P probes (prime-a-gene; Promega) were hybridized and RNA blots were revealed on autoradiographic films and by imaging (Bio-Rad personal molecular imager FX).

Plasma Membrane Isolation

Plasma membrane vesicles were isolated from grape suspension-cultured cells by differential centrifugation and Suc gradient (Nagao et al., 1987; Serrano, 1988). Cells (40–50 g fresh weight) were harvested, centrifuged at 3,000*g* for 1 min, washed twice with distilled water, and suspended in 100 mL of ice-cold buffer containing 250 mM Suc, 20 mM EDTA, pH 8.0, 10 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, and 50 mM Tris-MES, pH 8.0. The mixture was homogenized with an Ultra-Turrax T25 device (IKA WERKE, Janke and Kumkel IKA) for 3 min at 22,000 rpm, on ice, and the homogenate was strained through a layer of cheesecloth and centrifuged at 3,500*g* for 10 min. The supernatant was centrifuged once more at 10,000*g* for 10 min and then at 100,000*g* for 30 min. The pellet was resuspended in 8 mL ice-cold resuspension buffer (15% [v/v] glycerol, 1 mM DTT, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 20 mM Tris-HCl, pH 7.5). The suspension was layered over a 32% and 46% (w/v) discontinuous Suc gradient and centrifuged at 18,000*g* for 3 h in a Beckman SW 28 rotor. In addition to Suc, the gradient solutions contained 10 mM Tris-HCl buffer (pH 7.6), 1 mM EDTA, and 1 mM DTT. The vesicles sedimenting at the 32%-46% Suc interface were collected, diluted with 3 volumes of ice-cold water, and centrifuged at 100,000*g* for 30 min. The pellet was resuspended in the resuspension buffer described above. The vesicles were then frozen under liquid nitrogen and stored at −80°C until use. Protein concentration was determined by the method of Lowry (1951), with bovine serum albumin as the standard. ATPase activity, determined by measuring colorimetrically the release of π (Fiske and Subbarow, 1925), was used to measure the purity of plasma membrane preparations. At pH 6.5, 80% to 90% of the ATPase activity was inhibited by 0.1 mM vanadate.

Western-Blot Analysis

Proteins were separated on 10% SDS-polyacrylamide gels by electrophoresis (Laemmli et al., 1970) and transferred onto nitrocellulose membranes. Immunodetection was performed with VvHT1 antibody as previously described (Zhang et al., 2004; Vignault et al., 2005).

Sugar Quantification in Grape Berries, Plasma Membrane Isolation from Mesocarp Cells, and Immunoblot Detection of VvHT1

Berries from grape cv Chardonnay were collected at the following weeks after flowering: 4 (green stage), 6 (before véraison stage), 8 (véraison stage), and 14 (harvesting). They were deseeded, weighed, immediately frozen in liquid nitrogen, and stored at −80°C. To quantify sugar content, berries were ground in liquid nitrogen, the frozen powder was homogenized in ethanol-water (80% [v/v]), and boiled for 10 min at 80°C to inactivate invertase activity as described by Ageorges et al. (2000). The solution was centrifugated at 15,000*g* for 5 min and sugars in the supernatant were measured by high performance anion-exchange chromatography as described in Ollé et al. (1996). Plasma membrane fractions were purified from the frozen powders of the berries at the different development stages and were used for western-blot analysis of VvHT1 as described above.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AJ001061 (*VvHT1*), AY663846 (*VvHT2*), AY538259 (*VvHT3*), AY538260 (*VvHT4*), AY538261 (*VvHT5*), AY861386 (*VvHT6*), AY854146 (*VvHT7*), and AY608701 (*pGLT*).

ACKNOWLEDGMENTS

We thank Paulo Silva (University of Minho) for expert help in figure design and Magali Vachaud and Cécile Gaillard (University of Poitiers) for helpful advice on VvHT1 immunodetection.

Received March 22, 2006; revised May 30, 2006; accepted May 30, 2006; published June 9, 2006.

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