RESEARCH PAPER

Isolation, functional characterization, and expression analysis of grapevine (Vitis vinifera L.) hexose transporters: differential roles in sink and source tissues*

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Abstract

Three hexose transporters (VvHT3, VvHT4, and VvHT5) were cloned from Vitis vinifera L. and functionally characterized in the hexose transport-impaired Saccharomyces cerevisiae mutant EBY.VW4000. Both VvHT4 and VvHT5 facilitated glucose uptake, with $K_m$ of 137 μM and 89 μM, respectively. VvHT3 was not functional in the yeast system but a VvHT3:GFP (green fluorescent protein) fusion protein was targeted to the plasma membrane in plant cells. In young ‘sink’ leaves, transcript levels of all five VvHTs and a cell wall invertase (VvcwINV) were low. In mature leaves, there were increased levels of VvHT1, VvHT3, VvHT5, and VvcwINV transcripts, suggesting that mature leaves may have an increased capacity for apoplastic sucrose hydrolysis and hexose retrieval. In grape berries, VvHT1, VvHT2, and VvHT3 transcript levels were found to be significantly higher than those of VvHT4 and VvHT5. VvHT1 was most highly expressed early in berry development but decreased during the period of rapid sugar accumulation, while VvHT2 and VvHT3 expression remained high during this accumulation phase. VvcwINV expression occurred throughout berry development but peaked just prior to veraison. It is clear that the machinery to transport the hexose molecules produced through the cleavage of sucrose, by cell wall invertase, is present in the berry. This agrees with the suggestion that hexose accumulation to high levels during the ripening phase occurs through an apoplastic pathway. Interestingly, there is no direct relationship between VvHT gene expression and hexose accumulation, which suggests either that transcription is not the main determinant of transport activity or that other transport pathways are also active.

Key words: Grape, hexose transporter, sink, Vitis.

Introduction

Grapevines are an important horticultural crop in many countries where they are cultivated to produce fruit predominantly for wine making but also for juice, and dried and fresh fruit. The sugar content of grapes is of commercial importance in winemaking, not only because its fermentation by yeast produces alcohol, but because it augments the flavour profile of the final wine product. In the warm irrigated regions, berry sugar accumulation is rapid. The requirement for high levels of flavour metabolites has led to fruit being left on the vines for longer to attain these levels. One consequence of this is that wine made from such grapes has an increased alcohol content, which is of concern to the industry. In cooler viticultural regions, fruit may not achieve sufficiently high levels of sugars. In some countries, the fermentation of such grapes may be assisted by the addition of sugar.

Grape berry development can be divided into three stages. During the first phase, imported carbohydrate is utilized for several processes including seed development, cell proliferation and expansion, and the synthesis of organic acids (Coombe, 1992). This is followed by a lag phase...
which lasts approximately 2 weeks, during which there is no increase in berry size. The end of the lag phase is signalled by the inception of berry ripening or ‘veraison’, at which point berries begin to soften and, in the case of red varieties, begin to accumulate anthocyanins (Coombe, 1992). Another major process that occurs during berry ripening is the rapid importation of carbohydrate that accumulates in the vacuoles of berry pericarp cells, which expand significantly in size. At ripeness, berries may contain between 20% and 25% (v/v) hexoses (Lott and Barrett, 1967).

In most plants, sucrose is the major form of carbohydrate loaded into the phloem at photosynthetic source leaves and distributed to heterotrophic sinks such as fruit. Most woody plants, such as grapevine, are thought to be symplastic phloem ‘loaders’ due to the presence of plasmodesmata connecting mesophyll cells with phloem-associated cells (Gamalei, 1989). However, other plant species lack such connectivity and are considered apoplastic loaders (Riesmeier et al., 1994; Bürk et al., 1998). Once sucrose enters the phloem, its passage is driven by hydrostatic pressure and concentration differences between source and sink organs (Münch, 1930). Phloem unloading may also occur within the symplasm, again mediated by plasmodesmatal connections between the phloem termini and adjacent cells, or, alternatively, unloading may occur via efflux into the apoplast and subsequent carrier-mediated uptake by sink cells expressing appropriate membrane-localized transporters. Enzymatic hydrolysis of sucrose by invertase and sucrose synthase within sink organs regulates the rate of unloading via both routes and provides a direct connection between supply and demand (Patrick et al., 2001).

Previous studies have determined that genes potentially involved in phloem unloading or sink cell loading of sugars are expressed during berry ripening. Three sucrose transporters (Davies et al., 1999) and two hexose transporters (HTs) (Fillion et al., 1999) are expressed during various stages of berry development, while vacuolar invertase (vINV) appears to be the predominant source of sucrolytic activity in berries (Ruffner et al., 1990; Davies and Robinson, 1996). To describe further molecular components that contribute to sugar accumulation in grape berries, three new genes encoding HTs have been isolated from grapevine, their function analysed in yeast, and their expression patterns compared, during berry and leaf development, with that of other members of the grapevine HT and the invertase families. The results are discussed in terms of the physiological roles these proteins may play in carbohydrate partitioning in grapevine.

Materials and methods

Berry ripening and leaf development sampling series

Cabernet Sauvignon berries were sampled from the Slate Creek vineyard (Willunga, South Australia, latitude 35° 15′ south, longitude 138° 33′ east) during the 2003–2004 growing season. Flowering was defined as the date on which 50% of flowers within an individual bunch had undergone capfall. All subsequent samplings were conducted at 2 week intervals after this date. Sugar concentration of a subset (12 berries) of sampled berries was estimated by the level of total soluble solids (°Brix) determined with a refractometer (Reichert, Vienna).

Leaves were sampled from potted Cabernet Sauvignon vines grown in temperature-controlled glasshouses. Canes from two independent vines were sampled, with node 1 designated as the first expanding leaf below the growing tip. Leaves at nodes 1–4 were <4 cm long measured along the central vein and represent expanding sink leaves. Leaves at nodes 6 and 7 were almost fully expanded leaves, approximately 8 cm measured along the central vein, while leaves at nodes 9 and 10 were fully expanded source leaves, approximately 10 cm in length.

Grapevine RNA extraction and cDNA synthesis

Total RNA was isolated from grape tissues using the sodium perchlorate method of Rezaian and Krake (1987) with the modifications of Davies and Robinson (1996). Before reverse transcription, 100 μg of total RNA was further purified and DNase treated using an RNeasy Mini Kit (Qiagen) and an RNase-Free DNase Set (Qiagen) according to the manufacturer’s instructions. DNase-treated RNA (2 μg) was reverse transcribed employing the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) using the oligo(dT)20 primer according to the manufacturer’s instructions. Before use in RT-PCR experiments, cDNA reactions were diluted 10-fold to 200 μl with 10 mM TRIS-HCl, pH 7.6.

Cloning of grapevine hexose transporter (VvHT) cDNAs

Sequences of all primers used for cloning, sequencing, and expression analysis of VvHT genes are provided in Supplementary Table S1 available at JXB online. Degenerate primers DEGfwd and DEGrev were designed to conserved amino acid domains (Fig. 1.) using the program CODEHOP (Rose et al., 1998). Partial-length VvHT cDNAs were initially amplified from total RNA extracted from Chardonnay grape berries and leaves using BioTaq Red DNA polymerase (Bioline) according to the manufacturer’s instructions, with degenerate primers DEGfwd and DEGrev using the following cycling conditions: 95 °C, 3 min, followed by 38 cycles of 95 °C for 30 s; 54 °C for 30 s; 72 °C for 1 min. Amplification products were separated by agarose gel electrophoresis, extracted from gel slices using a QIAquick Gel Extraction Kit (Qiagen), and cloned using the pGEM T-Easy Vector System I (Promega).

Full-length VvHT cDNA sequences were obtained using RACE (rapid amplification of cDNA ends) PCR techniques (Frohman et al., 1988). For amplification of 3′ cDNA ends, target cDNA was amplified using 25 ng of a target-specific forward primer and 25 ng of an oligo(dT) adaptor primer, using grapevine Cabernet Sauvignon leaf cDNA as template. For amplification of 5′ cDNA ends, first-strand cDNA was synthesized using 25 ng of a target-specific reverse primer and 1 μg of DNase-treated total RNA. The cDNA was then purified (QIAquick PCR purification kit, Qiagen) and treated with 1 μl of terminal deoxynucleotide transferase (Invitrogen) in the buffer supplied and supplemented with 200 μM dCTP (Invitrogen). Target molecules were amplified from 2 μl of C-tailed cDNA using 25 ng of an oligo(dG)14 forward primer and 25 ng of a target-specific reverse primer. Amplification products were cloned into pGEM T-Easy and sequenced. All target-specific primers were designed to produce approximately 100 nucleotide overlaps with the partial cDNA fragments isolated by degenerate PCR to facilitate unambiguous alignment.
Functional characterization of VvHTs in yeast

Full-length cDNAs encompassing the open reading frames (ORFs) of VvHT1 (Fillion et al., 1999), VvHT3, VvHT4, and VvHT5 were amplified from Cabernet Sauvignon leaf cDNA with HiFi Taq High Fidelity DNA polymerase (Invitrogen) according to the manufacturer’s instructions using forward (F) and reverse (R) primers (see Supplementary Table S1 at JXB online) incorporating the predicted initiation and termination codons. Amplified products were sequenced, cloned into pGEM T-Easy (Promega), and subcloned into the yeast expression vector p426Met25 (Mumberg et al., 1994) which contains the URA3 gene for uracil prototrophy and the Met25 promoter for constitutive expression of the introduced transgene.

Functional testing was carried out in the yeast strain EBY.VW4000 (Wieczorke et al., 1999), which is deficient in glucose transport due to concurrent knock-out of endogenous transporters. EBY.VW4000 was transformed with p426Met25-VvHT1, p426Met25-VvHT3, p426Met25-VvHT4, and p426Met25-VvHTS, and p426Met25 vector alone, using the lithium acetate method of Gietz et al. (1992). After selection for uracil prototrophy, individual transformants were cultured in uracil drop-out media until an OD600 of 1.0–1.2 was reached. Cells were pelleted by centrifugation, washed twice with 25 mM NaHPO4/NaH2PO4 (pH 5.0), and resuspended in this buffer at a final concentration of 10 OD600 units per ml.

For each glucose uptake measurement, one OD600 unit (100 μl) of cell suspension was incubated at 30 °C with shaking for 2 min prior to addition of 100 μl of D-[U-14C]glucose (specific activity 185 kBq μmol⁻¹; Amersham Biosciences) at the specified concentration in 25 mM NaHPO4/NaH2PO4 (pH 5.0) and incubated for 30 min. Duplicate samples of 100 μl were removed from the reaction medium, pipetted directly into 10 ml of cold water, filtered onto GF/C filters (Whatman, #1822021), followed by two washes with 15 ml of cold water. Filters were placed into liquid scintillant and incorporation of radioactivity determined using a Beckman LS3801 liquid scintillation counter. For competitor and inhibitor studies, D-[U-14C]glucose concentration was 100 μM, competing sugars were at 500 μM, and cyanide m-chlorophenylhydrazone (CCCP) was at 50 μM. Transport activity was determined per g FW of yeast cells.

Cellular targeting of VvHT:GFP fusion proteins

The pART7 shuttle vector (Gleave, 1992) was modified to produce pART7-C’gfp which contains the full-length GFP (green fluorescent protein) ORF (lacking the start codon) downstream of the multiple cloning site (T Franks, unpublished results) and used transiently to express VvHT3:GFP and VvHT4:GFP fusion proteins.
in onion epidermal strips. Full-length VvHT3 and VvHT4 cDNAs were amplified using gene-specific primers with HiFi Taq High Fidelity DNA polymerase (Invitrogen) and cloned into pART7-C-gfp to generate C-terminal fusions with GFP, pART7-ATG-GFP (T Franks, unpublished results) which expresses untargeted GFP was used as a control. The localization of GFP fusion proteins was determined by bombardment of GFP constructs into onion epidermal strips and subsequent visualization using confocal microscopy, essentially as described by Selth et al. (2005). The internal surface of onion (Allium cepa) epidermal peel was placed facing up on 1.2% (w/v) water–agar plates and bombarded with each construct. In a 1.5 ml microcentrifuge tube, 400 µg of gold particles in 100 µl ethanol were vortexed for 2 min, centrifuged, washed twice with sterile water, and resuspended in 25 µl of 40% glycerol. While gently vortexing, 1.6 µg of DNA construct. 10 µl of cold 0.1 M spermidine, and 25 µl of 2.5 M CaCl2 were added drop-wise and this mixture incubated on ice for 10 min. The gold particles now coated with precipitated DNA were spun down, washed with 70% ethanol, resuspended in 24 µl of cold absolute ethanol, and 6 µl aliquots were placed onto four sterile filter holders. Onion strips on water–agar were placed inside the gun chamber, covered with a sterile mesh, and bombarded with a pressure of 650 kPa after evacuating the chamber to 90 kPa. After bombardment, tissue was stored in the dark for 48 h and GFP fluorescence visualized using a Bio-Rad Radiance 2100 Confocal Laser Scanning Microscope System (Hanson Institute Detmold Family Trust Cell Imaging Centre, Institute for Medical and Veterinary Science, Adelaide, Australia). The excitation wavelength used for GFP detection was 488 nm.

Quantitative analysis of gene expression by real-time PCR analysis

Expression analysis was carried out by real-time PCR analysis using a SYBR green method on a Rotor-Gene 3000 thermal cycler (Corbett Research, Mortlake, Australia). Each 15 µl PCR contained 330 nM of each primer, 3 µl of diluted cDNA, and 1×ABsolute™ QPCR SYBR® Green ROX Mix (Integrated Sciences). The thermal cycling conditions used were 95 °C for 15 min followed by 40 cycles of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s, followed by a melt cycle of 1 °C increment per min from 65 °C to 96 °C. All primer pairs (see Supplementary Table S1 at JXB online) amplified a single product of the expected size and sequence, which was confirmed by melt-curve analysis, agarose gel electrophoresis, and DNA sequencing. After testing the suitability of antigen (TC45156), ubiquitin (TC38636), and β-tubulin (TC39826) for use as reference genes, actin was selected for normalization of the leaf developmental series, while ubiquitin expression was used as the reference for the grape berry developmental series. The expression of each target gene was calculated relative to the expression of the reference gene in each cDNA tested using Rotor-Gene 6.0 software (Corbett Research, Mortlake, Australia) to calculate Ct values, to reference gene in each cDNA tested using Rotor-Gene 6.0 software of each target gene was calculated relative to the expression of the reference for the grape berry developmental series. The expression as reference genes, actin was selected for normalization of the leaf (TC45156), ubiquitin (TC38636), and predicted peptides of 508, 526, and 536 amino acids, respectively, which were amplified from Cabernet Sauvignon leaf cDNA. Sequencing of clones generated from the PCR product revealed the presence of three distinct partial-length cDNAs with high levels of homology to previously reported plant monosaccharide transporters. RACE PCR in both the 5′ and 3′ directions was successfully used to isolate the complete ORF of these partial transcripts, designated VvHT3 (AY538259), VvHT4 (AY538260), and VvHT5 (AY538261) in recognition of the previously reported grapevine HTs, VvHT1 and VvHT2 (Fillion et al., 1999). Full-length cDNAs were also amplified and cloned from Chardonnay berry skin cDNA and found to be identical to the Cabernet Sauvignon clones. These ORFs encode predicted peptides of 508, 526, and 536 amino acids, respectively, which share approximately 60% amino acid identity with the predicted peptides of VvHT1 and VvHT2 (Fig. 1).

Analysis of the secondary structures of the five VvHT translation products predicts each of these peptides to form 12 transmembrane helices with a cytoplasm-exposed loop between transmembrane helices 6 and 7 of approximately 60 amino acids (Fig. 1). This secondary structure is common to all plant HTs isolated to date and is a typical feature of members of the major facilitator superfamily of uniporter, symporter, and antiporter proteins (Böttner and Sauer, 2000). According to SignalP analysis (Bendtsen et al., 2004), VvHT3 and VvHT4 contain N-terminal sorting signals (Fig. 1), whilst VvHT5, and the previously reported VvHT1 and VvHT2, do not. However, there is significant homology between all VvHTs around the predicted signal peptide cleavage site (residues 32 and 33) of VvHT4. Other functionally important residues identified by mutation analysis of ChHUP1 from Chlorella kessleri (Will et al., 1994) which affect substrate affinity or transport rate, such as Q175, Q295, and V430, are also conserved in the VvHT peptides (Fig. 1).
Phylogenetic analysis of predicted VvHT peptides and HTs isolated from other plant species distributes the grape peptides into different clades, indicating that the different members of the grapevine HT gene family share more homology with sequences from other plant species than with each other (Fig. 2). Indeed, VvHT3, VvHT4, and VvHT5 share 80–84% amino acid identity with homologous sequences from Arabidopsis (AtSTP7, CAB80698), castor bean (RcHex6, AAA79857), and tomato (LeHT2, AJ132224), respectively, compared with only 50–60% amino acid identity to each other (data not shown).

### Functional characterization of VvHTs in yeast

To demonstrate functionality of VvHT3, VvHT4, and VvHT5, each was expressed in the hexose transport-impaired Saccharomyces cerevisiae mutant EBY.VW4000 (Wieczorke et al., 1999). This strain has a very low rate of hexose uptake due to concurrent knockout of 20 endogenous transporter genes, and has been used in recent studies functionally to characterize plant monosaccharide transporters (Scholz-Starke et al., 2003) including VvHT1 (Vignault et al., 2005).

Growth-based complementation assays indicated that VvHT3, VvHT4, and VvHT5 did not mediate sufficient rates of glucose uptake to support yeast growth on glucose media (data not shown). Therefore, it was decided to attempt to measure direct rates of glucose uptake into yeast facilitated by the introduced VvHT proteins using d-[U-14C]glucose. VvHT1 was also included in this uptake assay for comparison, together with EBY.VW4000 containing empty vector which was used as a measure of the basal rate of glucose uptake by this strain. The yeast strain EBY.VW4000 expressing VvHT1, VvHT4, or VvHT5 accumulated labelled glucose at significantly higher rates than the empty vector control over the entire uptake period, indicating that they were functional glucose transporters. VvHT1 was found to facilitate the highest rate of glucose transport under these assay conditions, estimated to be 9.4 μmol min⁻¹ g FW⁻¹ (Fig. 3A), whereas VvHT4 and VvHT5 facilitated lower uptake rates at 2.8 μmol min⁻¹ g FW⁻¹ and 0.12 μmol min⁻¹ g FW⁻¹, respectively. In contrast, the rate of glucose uptake into yeast cells expressing VvHT3 was not significantly different from that in the cells containing the vector-only control plasmid.

Glucose transport mediated by VvHT1, VvHT4, and VvHT5 displayed typical Michaelis–Menten saturation kinetics (Fig. 3B–D). Of the three hexose transporters analysed, VvHT1 had the highest affinity for glucose, with an estimated $K_m$ for glucose of 67 μM (±12.2), and the highest $V_{max}$ at 12.7±0.8 μmol min⁻¹ g FW⁻¹. This is in close agreement with the report of Vignault et al. (2005) where the $K_m$ of VvHT1 was estimated at 70 μM. VvHT4 and VvHT5 displayed lower affinities for glucose, with $K_m$s of 137±25.5 μM and 89±12.1 μM, respectively, and $V_{max}$ values of 4.5±0.36 μmol min⁻¹ g FW⁻¹ and 0.15±0.012 μmol min⁻¹ g FW⁻¹, respectively. Phylegenetic analysis (Fig. 2) grouped VvHT4 with AtSTP3, which is a low affinity hexose transporter from Arabidopsis (Büttner et al., 2000). However, the estimated $K_m$ for glucose of AtSTP3 was 2 mM, approximately 15-fold higher than the $K_m$ of VvHT4 and far higher than the $K_m$ range of 15–80 μM estimated for other plant HTs (Büttner et al., 2000; Büttner and Sauer, 2000). Additionally, the $K_m$ of VvHT5 was almost double the $K_m$ estimated for its close homologue LeHT2 (45 μM; Gear et al., 2000).

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**Fig. 2.** Phylogenetic analysis of predicted protein sequences of monosaccharide transporter-like nucleotide sequences from grapevine and other plant species. Grapevine sequences are shown in bold. Multiple sequence alignment was made with ClustalX and phylogenetic analysis done by the parsimony method with Protpars (Felsenstein, 1989) using CHKUPl as the outgroup. Bootstrap analysis was performed with ClustalX, and values shown at internal nodes indicate the occurrence of these nodes in 1000 replicates. Database accession numbers of the sequences used are: VvHT6 (AA547312), NtpGLT (AA647546), VvGLT (AAU07980), VvHT2 (AY663846), AtSTP3 (AJ012399), RcHex6 (AAS79857), AtSTP4 (AB025631), PhPMT1 (AF061106), AtSTP1 (AJ001664), AtSTP9 (AJ001662), NIMST1 (X66856), Mst1 (U38351), VISTP1 (Z93775), VvHT1 (CAA70777), AtSTP1 (AC007759), AtSTP7 (CAB80698), LeHT2 (AJ132224), AtSTP6 (AJ001659), AtSTP13 (CAC60074), and CHKUPl (X55349).
To test if VvHT1, VvHT4, and VvHT5 may potentially transport other sugars, the rate of D-[U-14C]glucose transport was measured in the presence of fructose, galactose, mannose, and sucrose supplied at 5-fold higher concentrations in an uptake competition assay. Glucose uptake into EBY.VW4000 expressing VvHT1 was inhibited by 61% by galactose and 45% by mannose (Fig. 4), suggesting that VvHT1 may also transport these monosaccharides, whereas fructose and sucrose did not significantly inhibit glucose uptake. Vignault et al. (2005) also observed glucose uptake inhibition by galactose and mannose in competition assays; however, VvHT1 only marginally increased the rate of radiolabelled mannose uptake relative to untransformed yeast cells. This suggests that mannose may bind to the active site in VvHT1, but may not be efficiently transported across membranes by this protein. In contrast, glucose transport mediated by VvHT4 was not significantly inhibited by the competing sugars, while transport by VvHT5 was inhibited 27% in the presence of fructose but not by galactose or mannose (Fig. 4).

Addition of the proton ionophore CCCP strongly inhibited transport activity in all yeast lines tested, suggesting that an electrochemical gradient across the plasma membrane is essential for VvHT1-, VvHT4-, and VvHT5-mediated glucose uptake (Fig. 4). Consistent with this observation, glucose uptake facilitated by VvHT1, VvHT4, and VvHT5 was also found to be sensitive to the pH of the external medium, with uptake rates significantly higher at pH 5.0 than at pH 7.0 (data not shown).

**Subcellular localization of VvHT3 protein in planta**

The demonstrated transport activity of VvHT4 and VvHT5 in the yeast expression system confirms that these transporters are targeted to the plasma membrane in yeast.
However, the apparent inability of VvHT3 to mediate glucose uptake in yeast raised the question as to the subcellular targeting of this protein. In order to examine this in more detail, the VvHT3 ORF was cloned upstream of, and in-frame with, the GFP reporter gene to produce a VvHT3:GFP fusion protein, under control of the cauliflower mosaic virus (CaMV) 35S promoter. A GFP fusion with the transport-active VvHT4 protein (VvHT4:GFP) was also constructed to serve as a positive control for plasma membrane targeting. These constructs, together with a construct containing native, non-targeted GFP, were bombarded into onion epidermal cells and the cellular localization of the fusion proteins determined by confocal microscopy. Non-targeted GFP was found to be distributed in both the cytoplasm and nucleus of bombarded epidermal onion cells (Fig. 5A). In contrast, both VvHT4:GFP (Fig. 5B) and VvHT3:GFP (Fig. 5C, D) were localized exclusively to the periphery of bombarded cells. Careful analysis of serial optical sections through a number of VvHT3:GFP-expressing cells clearly showed that fluorescence was only ever associated with the cell periphery and was not observed around the inner edge (tonoplast side) of the nucleus in these cells (cf. Fig. 5C, 5D). This indicates that VvHT3 is not associated with the tonoplast membrane and suggests that it is targeted to the plasma membrane in planta.

**Differential expression of VvHTs and cell wall invertase during grapevine leaf development**

Grapevine leaves initiate as carbohydrate sinks and develop into source leaves as the rate of photosynthesis rises above requirements for respiration and growth. Although environmental factors such as light and temperature will influence the timing of the transition of individual leaves from sink to source status, there is evidence to suggest that this normally occurs somewhere between node positions 4 and 6, as shown by the rapid increase in sucrose concentrations in leaves after this position (Ruffner et al., 1990). This ontogenetic transition provided an opportunity to examine the potential role of each of the VvHT proteins in sink/source development in grapevine leaves.

The quantitative expression of each VvHT gene was examined in leaves sampled from three positions along Cabernet Sauvignon shoots: (i) young sink leaves (<4 cm length) at nodes 1–4; (ii) almost fully expanded leaves at nodes 6–7 (~8 cm length); and (iii) fully expanded source leaves at nodes 9–10 (~10 cm length). Figure 6 shows that VvHT1 and VvHT3 were the most highly expressed HTs in leaves at all developmental stages measured, and expression of both transporters increased strongly during leaf development. Similarly, VvHT5 was also more highly expressed in mature leaves than in young leaves, but transcript levels were significantly lower than those of VvHT1 and VvHT3. In contrast, the level of expression of VvHT2 and VvHT4 was low in all leaf samples analysed. Interestingly, VvHT2 transcript levels, which were low relative to VvHT1 and VvHT3, were maximal in young sink leaves, but declined sharply during the transition to source leaf status, and were barely detectable in mature source leaves.

Invertase enzymes hydrolyse sucrose to the hexose monomers glucose and fructose, and support phloem unloading at sink organs by maintaining a sucrose gradient between the end of the phloem path and the site of unloading (Patrick et al., 2001). Acid invertase isoforms are localized in the apoplast (cwINV) and vacuole (vINV), while neutral/alkaline forms are found within the cytoplasm (nINV, for neutral invertase). Cell wall invertase is of particular importance when considering the physiological roles of HTs because cwINV activity generates apoplastic hexoses, the substrate of HTs, which may be transported into heterotrophic cells via HT activity. An apoplastic cell wall invertase (GenBank accession no. AY538262) which is strongly up-regulated during powdery mildew infection was cloned from Cabernet Sauvignon (MA Hayes and IB Dry, unpublished results). VvcwINV expression was also found to increase strongly during leaf development and was more highly expressed in mature leaves (Fig. 6). This expression
throughout berry development, dominant sucrolytic activity present in grape berries was down-regulated post-veraison (Fig. 7B). Like VvHT4, VvHT3 transcript levels were high in young berries and declined during the lag phase around veraison, but VvHT3 increased again during the phase of sugar storage. While VvHT4 expression showed little change throughout berry development, VvHT5 transcript increased 3-fold in the final sample collected at 14 WPF even though the overall relative transcript level was very low.

Previous studies have reported that vINV activity is the dominant sucrolytic activity present in grape berries (Ruffner et al., 1990), and two vINV genes, VvG1N1 and VvG1N2, have been shown to be expressed predominantly in pre-veraison fruit (Davies and Robinson, 1996). CWINV activity was measured and observed to be low relative to vINV activity (Ruffner et al., 1990). Zhang et al. (2006) recently reported a steady increase in CWINV protein levels and enzyme activity during the pre-veraison period in a hybrid grape cultivar. At veraison, the cwINV protein and enzyme activity levels increased quite sharply. However, their report did not contain details of changes in CWINV transcript levels during berry development. The present results show that VcvwINV transcript levels increased progressively up to veraison (Fig. 7B). VcvwINV transcript levels declined around 10 WPF, and subsequently increased in the final stages of berry ripening.

Discussion

The grape hexose transporter gene family

In this study, the cloning and functional characterization of three new cDNAs encoding putative hexose transporter proteins from grapevine are reported. Phylogenetic analysis indicates that these cDNAs share most sequence identity with HTs from other plant species and have hence been designated VvHT3, VvHT4, and VvHT5 (Fig. 2). This suggests that the evolutionary ancestors of plants already contained several HT genes, theoretically allowing differential regulation and cell-specific monosaccharide uptake, as observed in higher plants (Büttner and Sauer, 2000).

In addition to five VvHT genes examined in this study, three other sequences that encode putative HTs have been reported from grapevine, but questions remain regarding the function of these genes. GenBank accession no. AB608701, which was cloned from ripening Chardonnay berries, is postulated to be a plastid hexose transporter (S Delrot, personal communication) based on the high degree of homology (77% amino acid identity) it shares with pGlcT from tobacco which encodes a plastidic HT (Weber et al., 2000). A second grape sequence, AB61386, cloned from Chardonnay berries (which is also identical to DQ017393 cloned from Shiraz berries), has been postulated as another member of the VvHT gene family (Vignault et al., 2005) and has been designated VvHT6. However, this cDNA encodes a predicted peptide of 740 amino acids, which is approximately 200 amino acids longer than all of the other VvHT family members (Fig. 1) and all other functional HTs previously characterized from plants (Büttner and Sauer, 2000). The putative VvHT6 protein shares significant amino acid homology with typical plant HTs on either side of an approximately 200 amino acid insertion in the cytoplasmic loop between predicted transmembrane helices 6 and 7 (data not shown) and is most closely related to uncharacterized sequences from Arabidopsis thaliana (AAM19835) and rice (XP_464773). However, the actual transport function of this putative HT is yet to be demonstrated. Finally, Conde et al. (2006) recently reported a putative grapevine HT from Chardonnay berries which they designated VvHT7. However, analysis of the published nucleic acid sequence (AY854146) reveals it to be 99% identical (over 1717 nucleotides) to VvHT3, suggesting it is an allele of the
Thus, it appears that there may be a family of at least seven putative hexose transporters encoded by the *V. vinifera* genome compared with 14 in *A. thaliana* (Buttner and Sauer, 2000) and at least seven in tomato (Dibley et al., 2005). However, of those 21 putative HTs reported in *Arabidopsis* and tomato, to date only eight have been functionally demonstrated to transport monosaccharides: AtSTP1 (Sauer et al., 1990), AtSTP2 (Truernit et al., 1999), AtSTP3 (Buttner et al., 2000), AtSTP4 (Truernit et al., 1996), AtSTP6 (Scholz-Starke et al., 2003), AtSTP9 (Schneidereit et al., 2003), AtSTP11 (Schneidereit et al., 2005), and LeHT2 (Gear et al., 2000). It is therefore critical to validate the hexose transport capacity of these new, putative grapevine transporter proteins before function can be assigned.

To test functionality, VvHT3, VvHT4, and VvHT5 were expressed in the hexose transport-impaired yeast strain EBY.VW4000 (Wieczorke et al., 1999). Initial attempts to demonstrate function by complementation were unsuccessful, indicating that these transporters did not mediate sufficient glucose uptake to support yeast growth (data not shown). However, using a more sensitive assay based on the uptake of radiolabelled glucose, yeast expressing VvHT4 and VvHT5 were found to accumulate glucose at faster rates than yeast transformed with the empty vector, demonstrating them to be functional glucose transporters. Glucose transport facilitated by VvHT4, VvHT5, and VvHT1 was sensitive to external pH (data not shown) and the proton ionophore CCCP (Fig. 4), indicating that these transporters are probably energized by the plasma membrane electrochemical gradient and therefore are likely to be H⁺/glucose symporters. Although only tested directly for CkHUP from the green algae *Chlorella kessleri* (Caspari et al., 1996), all plant HTs are thought to be H⁺-symporters that are driven by the plasma membrane potential or the proton gradient (Buttner and Sauer, 2000).

No evidence could be found to demonstrate that VvHT3 facilitated glucose transport in yeast. Thus, despite the presence of all of the HT-associated hallmarks in the predicted VvHT3 translation product, this protein is not active in yeast. Improper protein folding or a low rate of translation or transcription may have impeded the functional activity of VvHT3 in yeast, but these possibilities were not investigated. Heterologous expression systems are unpredictable, and failure to express the protein in a functional state is commonplace and the underlying causes of this are obscure (Dreyer et al., 1999; Toyofuku et al., 2000; N Sauer, personal communication). Alternatively, VvHT3 may not have been correctly targeted to the plasma membrane in yeast strain EBY.VW4000. Although targeting of VvHT3 in EBY.VW4000 was not further analysed, we were able to demonstrate that VvHT3 is localized, like the functionally active VvHT4 protein, to the plasma membrane of onion epidermal cells, using C-terminal GFP fusion proteins (Fig. 5). One further possibility is that VvHT3 might facilitate transport of a hexose substrate other than glucose, although there is currently no precedent for this as all functionally defined plant HTs transport glucose to some degree (Buttner and Sauer, 2000).

Hexose transporter genes and cell wall invertase are co-ordinately expressed during sink/source development in grape leaves

Carbohydrate produced by autotrophic cell types is loaded into the phloem, usually as sucrose, for distribution to sink organs and other heterotrophic cell types that are reliant on sucrose importation to support growth and normal
metabolic function. Studies on Arabidopsis (Schneider-Stein et al., 2005), tomato (Gear et al., 2000), and grapevine (Fillion et al., 1999) have reported that HTs are most highly expressed in sink organs such as flowers and fruit where they are thought to facilitate the acquisition of sugars unloaded into the apoplast.

The present results indicate that the expression of three members of the grapevine HT gene family VvHT1, VvHT3, and VvHT5 increased sharply as leaves developed (Fig. 6), suggesting that grapevine leaves have increased capacity for apoplastic hexose retrieval after the developmental transition from sink to source. One possible role for VvHT1, VvHT3, and VvHT5 in mature source leaves may be the retrieval of monosaccharides leaked into the apoplast from the conducting complex to allow redistribution along the phloem path. Büttner et al. (2000) originally proposed such a role for AtSPT3, which is also highly expressed in mature Arabidopsis leaves. This is also consistent with the observation that VvHT1 transcript is localized to phloem-associated cells in grape leaves (Vignault et al., 2005). A role for HTs in source leaves is further supported by Arabidopsis microarray data (Genevestigator-Gene Atlas; Zimmermann et al., 2004), which show that the HT genes AtSTP1 (At1g11260), AtSTP3 (At5g61520), AtSTP4 (At3g19930), and AtSTP13 (At5g26340) are more highly expressed in adult leaves than in juvenile leaves.

Quantitative RT-PCR analysis of VvcwINV showed that expression of this gene is co-ordinately regulated with VvHT1, VvHT3, and VvHT5 (Fig. 6), and supports the idea that HTs play a significant role in source leaves. Interestingly, VvHT5 and VvcwINV are also co-ordinately induced by biotic and abiotic stress stimuli (MA Hayes and IB Dry, unpublished results), indicating that both developmental and exogenous cues can trigger signalling pathways that synchronize expression of these genes. Co-ordinate expression of HTs and cwINV genes has previously been observed in Arabidopsis (Fotopoulos et al., 2003) and in Chenopodium rubrum (Ehnh and Roitsch, 1997). Although cwINV genes have traditionally been associated with sink organs and are essential for carbohydrate allocation to symplastically isolated tissues such as pollen (Roitsch et al., 2003) and some seeds (Patrick and Offler, 2001), analysis of Arabidopsis microarray data (Genevestigator-Gene Atlas; Zimmermann et al., 2004) indicates that two Arabidopsis cwINV genes, AtcwINV1 (At3g13790) and AtFruct5 (At1g55120), are also more highly expressed in mature source leaves than in sink leaves. This suggests an additional role for cwINV in source leaves.

Notably, expression levels of VvHT1-5 and VvcwINV are relatively low in sink leaves, consistent with the idea that phloem unloading may occur within the symplasm in leaves at this developmental stage. In young sink leaves, vacuolar invertase expression and activity is high before declining as leaves reach maturity (Ruffner et al., 1990; Davies and Robinson, 1996). High vINV activity may support symplastic phloem unloading because it would help maintain sucrose concentration differences between the end of the phloem path and the cytoplasm of terminal sink cells (Patrick, 1997). Plasmodesmata of young sink leaves are of an open form with large aperture, thought to be capable of massive solute fluxes and therefore able to function as the sole nutrient supply pathway to rapidly expanding tissues (Oparka et al., 1999; Roberts and Oparka, 2003).

**The role of hexose transporters and invertases in sugar accumulation in grape berries**

Phloem unloading can occur within the symplast and be mediated by plasmodesmata, or it may involve an apoplastic step where sugars are released into the apoplast before further transport. In fruits, seeds, and other storage organs that accumulate high concentrations of sugars, apoplastic release is commonly observed and may be a physiological necessity to mediate sustained unloading (Patrick et al., 2001). Indeed, by looking at the movement of symplastic tracer molecules, Zhang et al. (2006) recently confirmed that phloem unloading in grape berries shifts from a symplastic pathway prior to veraison, to an apoplastic pathway during ripening. Based on the reduction of symplastic permeability (Zhang et al., 2006) and the increase in apoplastic solutes (Bondada et al., 2005), both of which occur at veraison, it seems likely that sucrose unloading into the apoplast is the dominant path of phloem unloading in post-veraison berries.

Quantitative RT-PCR analysis did not demonstrate any strict correlation between the expression of the five VvHTs examined in this study and the post-veraison period of rapid sugar import in ripening Cabernet Sauvignon berries (Fig. 7). VvHT1 was found to be most highly expressed in pre-veraison berries and declined during the period of sugar accumulation. This observation is in agreement with the recent findings of Conde et al. (2006), who showed that VvHT1 transcript and protein levels were repressed by glucose in grape cell cultures and suggest that VvHT1 may not mediate significant amounts of sugar import into pulp cells after veraison, but may be involved in the supply of energy for cell division and growth when low levels of apoplastic hexoses are available (Conde et al., 2006).

Based on relative transcript levels alone, VvHT2 and VvHT3 are the most likely candidates to facilitate the transport and accumulation of sugar within pulp cells of ripening Cabernet Sauvignon berries (Fig. 7). However, as the functionality of VvHT3 could not be demonstrated in the yeast heterologous system (Fig. 3) and VvHT2 remains to be functionally characterized, the in planta role of these genes is still in question. Interestingly, although we were able to demonstrate clearly that both VvHT4 and
VvHT5 function as hexose transporters in yeast (Fig. 3), the very low level of expression of these genes in Cabernet Sauvignon berries, relative to VvHT2 and VvHT3, suggests that these transporters may not contribute greatly to sugar import during ripening. However, it must be remembered that the transcript levels presented in Fig. 7 only represent the average levels for the berry pulp as a whole, and this does rule out the possibility of localized regions of high expression within specific cell types within the pulp. Thus, until further information is available on the cellular localization patterns of these HT transcripts, their potential role in sugar importation in developing berries cannot be ruled out.

Cell wall invertase regulates phloem unloading in some sink organs (Patrick and Offler, 2001; Roitsch et al., 2003) and produces hexose substrates that may be acquired by HTs. VvcwINV transcript was detected throughout berry development, but was highest just prior to the period of rapid sugar accumulation that occurs after veraison (Fig. 7). The predicted presence of elevated levels of cwINV enzyme after veraison would be consistent with a role in the cleavage of sucrose unloaded from the phloem into the apoplastic space. Biochemical studies have estimated that cwINV enzyme activity represents only 4% of the total invertase activity present in berries, with the majority due to soluble vINV activity (Ruffner et al., 1990). However, even relatively small amounts of cwINV activity may play an import regulatory role in carbohydrate unloading if it is localized specifically around sites of phloem unloading. Consistent with this idea, Famiani et al. (2000) used a cwINV-specific antibody to examine pre-veraison grape berry sections, and found cwINV epitopes in vasculature-associated cells around developing seeds, and in cells in the area of the dorsal bundle network that underlies the berry epidermal cell layers.

Molecular evidence presented in the current study demonstrates that both HT and cwINV genes are expressed during berry development. This is consistent with the idea that sucrose unloaded into the apoplastic space from the phloem is cleaved into hexoses by cwINV which are subsequently moved into berry pulp cells by VvHT-mediated hexose uptake. However, the lack of a clear correlation between the expression profiles of any of the five HTs tested and the period of rapid sugar accumulation in ripening berries (Fig. 7) remains puzzling. One possible explanation is that the importation of sugars into ripening grape berries is not regulated at the gene transcription level, but instead is strongly influenced by post-transcriptional regulation or protein turnover at the plasma membrane (Kühn et al., 1997). Additionally, the sucrose transporters VvSUC11 and VvSUC12 are induced in co-ordination with increased berry sugar concentrations (Davies et al., 1999), suggesting that direct sucrose uptake by berry pulp cells is another plausible pathway for sugar accumulation in ripening grape berries. However, neither VvSUC11 nor VvSUC12 has been localized at the cellular level. Sucrose transporters isolated from other plants are localized to phloem cells where they are proposed to contribute to sucrose loading into the phloem, unloading into the apoplast, or re-absorption along the phloem path (Stadler et al., 1995; Barth et al., 2003; Carpaneto et al., 2005), while others are expressed in sink cells and are thought to mediate sucrose acquisition (Shakya and Sturm, 1998; Meyer et al., 2004).

Supplementary material
Supplementary material is available at JXB online.
Table S1. Oligonucleotide primers used in this study

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