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Cloning, Localization, and Expression Analysis of a New Tonoplast Monosaccharide Transporter from *Vitis vinifera* L

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Abstract Tonoplast sugar transporters are important for sugar partitioning, immobilization, and accumulation during fruit development and ripening. Here we report the cloning, localization, and functional analysis of one of these transporters in grape berries (*Vitis vinifera* L.). This clone, named *VvTMT1*, encodes a 742-aa protein with a calculated molecular mass of 80.2 kDa. Predicted membrane topology and phylogenetic analysis suggest that *VvTMT1* belongs to the major facilitator superfamily of membrane carriers. Semiquantitative RT-PCR suggests that *VvTMT1* is a sink-specific transporter, whose expression decreases with berry development. Heterologous expression of *VvTMT1* in yeast can partially restore growth of the *hxt*-null strain in glucose and other monosaccharide media, indicating that VvTMT1 is a functional monosaccharide transporter. Induction of VvTMT1-GFP fusion protein expression in transgenic yeast revealed its tonoplast localization. The subcellular localization of VvTMT1 in plants was shown by immunogold labeling of grape berry mesocarp cells and VvTMT1-GFP transient expression in tobacco epidermis cells. Based on the above analyses of VvTMT1, this is the first report of a functional tonoplast-localized monosaccharide transporter in grapevine.

Keywords Grape berry · Immunogold electron microscopy · Subcellular localization · Tonoplast · Tonoplast monosaccharide transporter · Vacuole · *Vitis vinifera* L

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Introduction

Sugars fulfill many essential functions throughout the higher plant's life cycle. As solutes, sugars are the basic material for a large number of anabolic and catabolic reactions (Wormit and others 2006), and they provide the osmotic driving force for cell enlargement (Stadler and others 1999). Moreover, sugars serve as regulatory signals, activating or repressing different signal-transduction pathways (Lalonde and others 1999) and modulating gene expression (Koch 1996). In grape berries and other fruits, sugar content is one of the most important criteria for commercial quality.

Sucrose is one of the primary forms of photoassimilate transported to berries during grape development. The movement of sugars into berries is a tightly regulated process that requires several essential transport steps across membranes. Sugar transporters, controlling photoassimilate partitioning within cells and among organs, play essential roles in phloem apoplastic loading, transport, and unloading (Riesmeier and others 1993; Gottwald and others 2000). All of the sugar transporter genes reported to date belong to a large gene family, known as the major

facilitator superfamily (MFS). All members contain 12 putative transmembrane α-helices and a central hydrophilic region, with similar structures found in both prokaryotes and eukaryotes (Marger and Saier 1993). Sugar transporters can be identified based on sequence homology, functional similarity, and predicted topology models (Maiden and others 1987; Baldwin and Henderson 1989; Griffith and others 1992). Two main subfamilies of the MSF are the disaccharide (or sucrose) transporters (DSTs) and the monosaccharide (or hexose) transporters (MSTs).

Many sugar transporters have been characterized by widely used heterologous expression systems (Lemoine 2000), and their numbers are rapidly increasing. Various detection techniques, among them *in situ* hybridization, immunohistochemistry, and fluorescence protein labeling, have been used to study temporal and spatial expression patterns of sugar transporters. In grape, three putative sucrose transporter genes—*VvSUC11* (also called as *VvSUT1*), *VvSUC12*, and *VvSUC27*—have been identified. Two of them have been functionally characterized as proton–sucrose symporters by heterologous expression in yeast, and they are all predicted to be located in the plasma membrane (Sauer 2007). Fifty-nine putative hexose transporter genes have been predicted based on protein-motif recognition (Jaillon and others 2007). Six full-length cDNAs (*VvHT1–VvHT6*) coding for MSTs (Fillion and others 1999; Vignault and others 2005; Hayes and others 2007) and a cDNA encoding a putative plastidic hexose transporter (*VvpGLT*) (Terrier and others 2005) have been cloned from various grape cultivars. Sugar-uptake activities of VvHT1, VvHT4, and VvHT5 have been demonstrated by heterologous expression in the *hxt*-null mutant yeast EBY.VW4000 (Wieczorke and others 1999), but attempts to confirm the transport activity of VvHT2 and VvHT6 in yeast have had little success (Agasse and others 2009). The plasma membrane localization of VvHT1, VvHT3, VvHT4, and VvHT5 has been confirmed by immunofluorescence, immunolabeling, and confocal microscopy of C-terminal GFP-fused proteins (Vignault and others 2005; Hayes and others 2007), whereas it is speculated that VvHT2 and VvHT6 are tonoplast-localized hexose transporters (Agasse and others 2009).

Although sugar transporters across plasma membranes have attracted much attention (Williams and others 2000), very little is known about the corresponding transporters in the tonoplast. The vacuole is the main cellular storage pool for long- and short-term storage of primary metabolites, such as monosaccharides, disaccharides, sugar alcohols, amino acids, and so on. The composition of the vacuole and its volume regulation depend on the coordinated activities of transporters and channels localized in the tonoplast (Reisen and others 2005). A putative tonoplast-localized hexose transporter from sugar beet was first reported by immunochemical localization (Chiou and Bush 1996), but its sugar-transporting activity was not described. More recently, the barley carrier protein HvSUT2 and *Arabidopsis* sucrose carrier AtSUT4 have been localized to the tonoplast using proteomic approaches (Endler and others 2006). The first tonoplast MST (AtTMT) was functionally identified in *Arabidopsis*. Transient heterologous expression of AtTMT-GFP fusion proteins of all three isoforms confirmed their vacuolar membrane targeting (Wormit and others 2006). These results are supported by proteome analyses of the vacuolar membranes of *Arabidopsis* mesophyll cells (Carter and others 2004). In addition, vacuolar membrane localization of the putative hexose (AcMST1) and inositol (AcINT1) transporters from pineapple (Antony and others 2008) and the putative sucrose (LjSUT4) transporter from *Lotus japonicus* (Reinders and others 2008) has also been suggested. However, their precise roles and biochemical activities have not been verified.

In grape, berries trap high concentrations of hexose in the vacuoles of the mesocarp cells from véraison to ripening. The aim of the present work was to clone and functionally analyze a putative tonoplast MST from grape berries and further determine its protein localization and expression pattern in grapevine.

Materials and Methods

Plant Material

Grape (*Vitis vinifera* L. cv. Riesling) berries were harvested from the Sino-French Demonstration Farm, Huailai, China, in 2007. Eight to ten randomly selected bunches were sampled and transported to the laboratory at approximately 4°C, then the berries were peeled and seeded, immediately frozen in liquid nitrogen, and stored at −80°C for further analysis.

Grape Berry cDNA Library Construction

Total RNA of grape berries collected 45 days after flowering (stage II) was isolated as described by Davies and Robinson (1996). RNA was then treated with RNase-free DNase I (Takara, Dalian, China). The purity and quality of the RNA were checked by gel electrophoresis and $OD_{260/280}$ ratio, and RNA concentration was calculated. First-strand cDNA was synthesized with a SuperScript RT-PCR kit (Invitrogen, Carlsbad, CA, USA), and used for candidate sugar transporter cDNA cloning. This same procedure was performed on other grapevine tissue samples for semiquantitative RT-PCR.

cDNA Cloning

Sequences of three *Arabidopsis thaliana* vacuolar sugar transporters—*AtTMT1–3* (GenBank accession numbers NM_101937, NM_119696 and NM_115008, respectively)—were subjected to a Blast search against the *Vitis* database of Genoscope (http://www.genoscope.cns.fr/vitis), and three homologous sequences were found. Accordingly, we designed specific primers (Table 1) to amplify their full-length sequences from the aforementioned cDNA library. The PCR products were cloned into the pMD19T simple vector (Takara) for amplification and sequencing, and subsequently named *VvTMT1*, *VvTMT2*, and *VvTMT3,* respectively.

Phylogenetic and Sequence Analyses

Sequence analysis was performed on the BLAST server (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). A phylogenetic tree showing the relationships between VvTMTs and sugar transporters from *A. thaliana* and other organisms was constructed by aligning amino-acid sequences with the program ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). An unrooted tree was constructed and viewed using TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Putative signal peptides were predicted using SignalP 3.0 (Bendtsen and others 2004), and transmembrane helices were predicted with the THMMH Server v. 2.0 (Krogh and others 2001).

Plasmid Construction for Yeast Expression

Standard molecular biology protocols were performed as described by Sambrook and others (1989). *VvTMT1* cDNA was excised from the pMD19T-VvTMT1 construct by *Xho*I/*Sac*II digestion and inserted into the corresponding restriction sites of the pDR195 yeast shuttle vector (Rentsch and others 1995). The pDR195-VvTMT1 construct was confirmed by DNA sequencing.

The open reading frame (ORF) encoding GFP (green fluorescent protein) from *Aequorea victoria* was amplified by PCR from the plasmid pBI221-GFP, using the primer pair GFP-F and GFP-R (Table 1), and cloned into the pMD19T simple vector, yielding the plasmid pMD19-GFP. The *Bam*HI/*Xba*I fragment of pMD19-GFP was subcloned into the yeast expression vector pYES2.0 (Invitrogen), which allows expression of full-length cDNA under control of the inducible yeast *GAL1* promoter, yielding the plasmid pYES2-GFP. The *VvTMT1* coding sequence was amplified using primers VvTMT1-ZF and VvTMT1-BR (Table 1),

Table 1 Primers used for cloning, expression, and RT-PCR assays

Primer pairs	Primer sequence (5′–3′)
VvTMT1F	CCGCTCGAGATGAACGGAGCTGTGCTGGTAGC
VvTMT1R	GATGCCGCGGTCAGTTGTTCTTGGCATCAGTAGC
VvTMT2F	ATGAACGGAGCTGTGCTAGTGG CTATTGCAGCCGC
VvTMT2R	CCGCATTCAGTCATTCTTTGCTGCAGTAACCTGCCT
VvTMT3F	ATGAGTGGAGCTGTGCTTGTGGCGATTGCTGCTGCTGTG
VvTMT3R	GACTAGTCAGTTCTTCTTCTGTCCAGCACTTGCACCAAC
GFP-F	CGGGATCCATGAGTAAAGGAGAAGAAC
GFP-R	GCTCTAGATTATTTGTATAGTTCAT
VvTMT1-ZF	CCAAGCTTATGAACGGAGCTGTGCTGGT
VvTMT1-BR	ATGGGTACCGTTGTTCTTGGCATCAGTAG
VvHT1F	CCGAGCTCATGCCGGCTGTCGGAGGCT
VvHT1R	CGCGGATCCTACATTCTTAACAGGGTAGT
TMT1-FS	GGACATATGGCAAACCAGAGTGTGC
TMT1-RA	AGACTCGAGTGGTTGAACCATTGC
VvTMT1-SF	CGGGGTACCATGAACGGAGCTGTGCTG
VvTMT1-SB	GGCGAGCTCTCAGTTGTTCTTGGCATCA
GFP-S	GCTCTAGAATGAGTAAAGGAGAAG
GFP-A	GGGGTACCTTTGTATAGTTCATCCA
VvTMT1S	ATGCGCTCTCACATTTTGGA TCT
VvTMT1A	AGCAGCCTGCTTTGCACCCA CA
VvHT1S	CACATTCATCATAGCCCAAATCTTC
VvHT1A	CAGGGTAGTTTTCCTTGACCAGTTC
Vv18SF	TGGCCTTCGGGATCGGAGTAA
Vv18SR	ATCCCTGGTCGGCATCGTTTAT

which introduced *Hind*III sites at the N-terminal end and *Kpn*I sites at the C-terminal end of the *VvTMT1* ORF and replaced the stop codon of the original *VvTMT1* ORF. This modified *VvTMT1* sequence was subcloned in-frame to the 5′ end of *GFP*, yielding the plasmid pYES2-VvTMT1-GFP. As a positive control, *VvHT1* cDNA (GenBank accession number AJ001061) was cloned upstream of *GFP* using the primer pair VvHT1F and VvHT1R (Table 1), yielding the plasmid pYES2-VvHT1-GFP. All of the constructs were validated by DNA sequencing.

Transformation into Glucose-transport-deficient Yeast

The pDR195-VvTMT1 plasmid was used to transform *Saccharomyces cerevisiae* strain EBY.VW4000 (Wieczorke and others 1999) by the lithium acetate method (Gietz and others 1992). Transformants were selected on solid yeast nitrogen base (YNB) medium minus uracil and containing 2% maltose. The faster-growing colonies were selected and verified by PCR after incubation for 3 days at 30°C. Positive clones were replica-plated onto the same medium containing 2% glucose or fructose instead of maltose, and the growth phenotype on glucose or fructose medium was assessed after 7 days at 30°C. Cells transformed with the empty pDR195 vector were used as controls.

For in vivo localization of VvTMT1, the EBY.VW4000 yeast strain was transformed with the pYES2-VvTMT1-GFP plasmid, and in parallel, pYES2-VvHT1-GFP and pYES2-GFP were transformed as additional controls. Transformants were selected and verified as described above. Fast-growing colonies were picked and incubated in liquid YNB medium lacking uracil and containing 2% maltose as the carbon source. The transformed yeast cells were washed with physiological saline, induced by the addition of 2% galactose, and incubated at 30°C for 12 h. The cells were observed by fluorescence microscopy (Nikon, Eclipse TE2000-E) at an excitation wavelength of 488 nm.

Complementation Test

For growth complementation, individual positive transformants containing the pDR195-VvTMT1 plasmid were cultured in uracil drop-out medium to an OD_{600} of 1.0, reinoculated in fresh medium to an OD_{600} of 0.1, and grown for 6 h. The exponential-phase cells were harvested, rinsed, and resuspended in distilled water to an OD_{600} of 0.2. Serially diluted (1:10, 1:100, and 1:1000) yeast cell resuspension (10 µl) was spotted on synthetic medium plates containing 2% D-glucose or D-fructose. The growth of yeast colonies on these solid media was recorded after 7 days of incubation at 30°C. Transformants with an empty pDR195 vector were used as negative controls for the growth tests. For yeast growth assay, growth in the same liquid synthetic medium was monitored by measuring OD_{600} in a spectrophotometer.

Preparation of Anti-VvTMT1 Antiserum

A 633-bp DNA fragment was amplified from the plasmid pYES2-VvTMT1-GFP using the primer pair TMT1-FS and TMT1-RA (Table 1). The PCR product was digested with *Nde*I/*Xho*I and inserted into the digested pET31b vector resulting in a translational fusion of the histidine-binding protein. Expression of the fusion protein was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mmol/L and incubation at 30°C for 6 h. The bacteria were lysed on ice by ultrasonication, and the recombinant protein containing the His-tag sequence was purified by Ni-NTA His Bind® Resin (Novagen®, EMD Chemicals) according to the manufacturer's instructions. The purified target protein (500 µg) was injected (subcutaneously and intramuscularly) into two New Zealand White rabbits after being emulsified with an equal volume of Freund's complete adjuvant. Rabbits were given four booster injections at 10-day intervals with antigen in Freund's incomplete adjuvant, and the antiserum was collected 10 days after the last injection. The purified rabbit anti-His-tag-TMT1 IgG was kept for further use.

Localization of VvTMT1 by Immunogold Electron Microscopy

Small (1 mm^3) pieces of mesocarp were cut from the middle part of berries, and tissue was prepared as previously described (Zhang and others 2001). Ultrathin sections (approximately 90–100 nm) were cut with a diamond knife on an OM-U3 (LKB-8800) ultramicrotome. The immunogold reaction was performed on ultrathin sections with 1/50 diluted rabbit anti-VvTMT1 antiserum and 1/50 diluted 15-nm gold particle-labeled goat anti-rabbit IgG (Sigma, St. Louis, MO, USA) as the first and secondary antibody, respectively. Control sections were not subjected to incubation with the primary antibody nor incubated with negative rabbit serum before secondary antibody incubation. At least three replicates of the control experiments were performed for each sample. After immunoreaction, sections were stained with alkaline lead citrate for 15 min. Samples were observed with a JEM-1230 transmission electron microscope (Kyoto, Japan) at 80 kV.

Chimeric Constructs and *Agrobacterium* Transformation

The entire ORF of *VvTMT1* was inserted into the modified binary vector pCAMBIA 1300+ containing the super CaMV35S promoter, using the primer pair VvTMT1-SF

and VvTMT1-SB (Table 1), resulting in the plasmid p1300S-VvTMT1. The ORF of *GFP* was amplified from the plasmid pMD19-GFP by PCR using the primer pair GFP-S and GFP-A (Table 1), which introduced N-terminal *Xba*I and C-terminal *Kpn*I sites. The sequenced *GFP* cDNA fragment was cloned into the p1300S-VvTMT1 vector using the *Xba*I/*Kpn*I sites, leading to the final GFP-VvTMT1 construct under the control of the super CaMV35S promoter and yielding the plant transformation vector p1300s-GFP-VvTMT1. The sequence of the GFP-VvTMT1 construct was determined by DNA sequencing and restriction digestion of plasmids recovered from transformed *Escherichia coli* DH5α. The plasmid p1300s-GFP-VvTMT1 was used to transform *Agrobacterium tumefaciens* strain EHA105. Transgenic *Agrobacterium* was selected on LB-agar plates containing rifampicin (50 μg/ml) and kanamycin (50 μg/ml) at 28°C.

Transient Expression of VvTMT1 in Tobacco

Transient expression of the GFP-VvTMT1 fusion construct in leaf epidermal cells of *Nicotiana tabacum* was performed essentially as described by Batoko and others (2000) with minor modifications. Individual *Agrobacterium* colonies were cultured at 28°C to the stationary phase, washed, and resuspended in infiltration medium containing 50 mM MES (pH 5.6), 0.5% glucose, 2 mM NaH_2PO_4, and 100 μM acetosyringone to an OD_{600m} of 0.5. The bacterial suspension was injected into the abaxial epidermis of leaves of 5–6-week-old tobacco plants using a needleless syringe. Plants were incubated for 2–3 days at 20–25°C under constant light and the leaves were examined 3 days post-inoculation.

RT-PCR for Quantitative Expression Analysis

Semiquantitative RT-PCR analysis was performed using DNase I-treated total RNA extracted from the tissues of interest as described above. PCRs were conducted at the linear phase of the exponential reaction for each gene with the following gene-specific primer pairs: VvTMT1S and VvTMT1A for *VvTMT1* amplification and VvHT1S and VvHT1A for *VvHT1* amplification (Table 1). The reaction conditions were as follows: 95°C for 2 min followed by 30 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 30 s. The PCR products were assayed by agarose-gel electrophoresis. The semiquantitative band density was determined using 1D Image Analysis software (Scientific Imaging Systems, Shanghai, China). The expression of *VvTMT1* and *VvHT1* in each tissue was checked in triplicate, and data were normalized with the constitutively expressed housekeeping gene *18S* rRNA using the primer pair Vv18SF and Vv18SR (Table 1).

Results

Cloning of *VvTMT* cDNA Sequences

Complete sequencing of the grape genome revealed the existence of sugar transporter genes that had not been identified in previous screenings. To identify these unknown vacuolar-transporter-like sequences, we performed a Blast search against the *Vitis* database of Genoscope with the sequences of AtTMT1–3. Three annotations, GSVIVT00019321001, GSVIVT00002919001, and GSVIVT00036283001 on chromosomes 7, 18, and 3, respectively, exhibited the highest homologies with their *Arabidopsis* counterparts (59–72%). The corresponding cDNAs comprising the entire ORFs were amplified from the cDNA library generated from grape berries 45 days after anthesis, subcloned into the pMD19T simple vector to yield pMD19-VvTMT constructs for sequencing, and subsequently named *VvTMT1*, *VvTMT2*, and *VvTMT3*.

VvTMT1 has a 2,229-bp ORF potentially encoding a 742-aa protein, with a calculated molecular mass of 80.2 kDa and an isoelectric point (*pI*) of 5.18. This is similar to the *pI* values of some vacuolar membrane transporters and different from those of many plasma membrane transporters. VvTMT2 and VvTMT3 were predicted to be 740 and 739 aa in length, with calculated molecular masses of 79.4 and 79.2 kDa and *pI* values of 4.97 and 5.15, respectively. Sequence analysis revealed that the three VvTMTs share 66% nucleotide sequence identity in the coding region and 63% identity in their amino acid sequences (Fig. 1). Phylogenetic analysis of predicted VvTMTs and VvHTs distributed them into different clades (Fig. 2), indicating that VvTMTs are members of the MST family and not the DST family. Analysis of the deduced amino acid sequences by TMHMM revealed the presence of 11 membrane-spanning domains with an extraordinarily large central hydrophilic loop between transmembrane domains 6 and 7 (Fig. 3). This topological model is consistent with the structures proposed for the MSTs and DSTs identified to date in microbes, mammals, and plants (Griffith and others 1992; Henderson and others 1992; Büttner and Sauer 2000). The hydrophilic loop of VvTMT1 was about 376 aa long, nearly four to six times larger than the corresponding structures in all other known MSTs from prokaryotes and eukaryotes, but similar to that of AtTMTs.

According to SignalP analysis, all three VvTMT proteins contained a signal peptide and predicted cleavage site between residues 19 and 20. Sugar transporter signatures, including GRR motifs in loops 3 and 8, and in particular motifs corresponding to the PESPR/PETKGR motif after helices 6 and 11, were all present in the predicted amino acid sequences of the VvTMTs. VvTMT1 had three

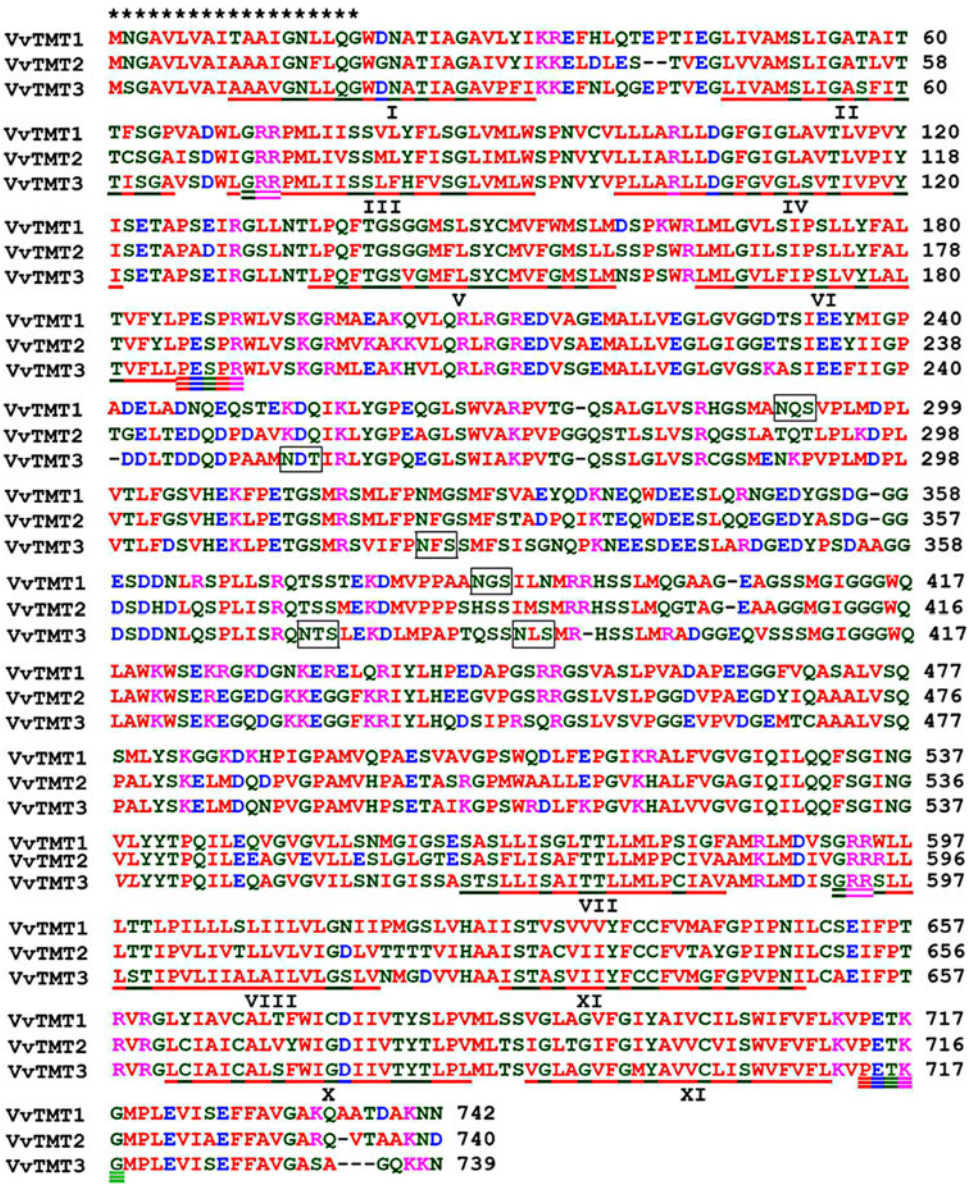

Fig. 1 Amino acid sequence alignment of putative grapevine monosaccharide transporters. VvTMT1, VvTMT2, and VvTMT3, cloned in this study, were aligned with ClustalW by introducing gaps (*dashed line*) to maximize identity. Putative signal peptides are marked with *asterisks* and transmembrane helices are *underlined* with a single line and numbered I–XI. Potential glycosylation sites are *boxed*. The GRR motif in loops III and VIII is indicated by *double underlining*, and the PESPR/PETKGR motif after helices 6 and 11 is *triple underlined*

consensus sequences for potential *N*-glycosylation (asparagine residues 22, 290, and 386). N22 was located within the first putative transmembrane-spanning domain and is therefore not likely to be glycosylated. However, N290 and N386 were located within the putative external loop between transmembrane domains 6 and 7 and thus are potential candidates for *N*-glycosylation. VvTMT3 also contained four consensus sequences for *N*-glycosylation on this external loop (N253, N322, N373, and N389), while VvTMT2 did not. Although specific evidence for glycosylation of plant MSTs is lacking, it is interesting to note that *N*-glycosylation is required for high-affinity glucose transport by a related human glucose transporter, GLUT1 (Asano and others 1991).

Immunolocalization and Expression Pattern of *VvTMT1* in Grape

To assess the physiological functionality of *VvTMT1*, the subcellular localization of its protein was studied by colloidal gold immunoelectron microscopy with VvTMT1-specific antibody, and its gene expression pattern was assayed by semiquantitative RT-PCR. As shown in Fig. 4a, no immunogold particles were detected near the outside surface or inside of the vacuoles of the pulp cells when polyclonal antibody against VvTMT1 was omitted during the immunolabeling or when rabbit preimmune serum was used instead (data not shown). In the ultrathin slices of berry samples from the first, second, and third developmental

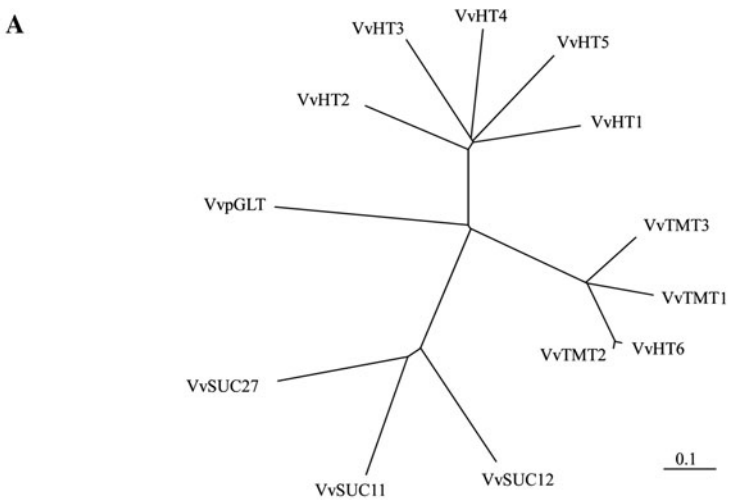

Fig. 2 Analysis of sequence homologies among the characterized or predicted sugar transporters of *Vitis vinifera* L. **a** Phylogenetic tree showing the close relationship between VvTMT1, VvTMT2, and VvTMT3 and their nearest neighbor VvHT6. **b** Similarity/identity matrix of the 13 *V. vinifera* sugar transporters, confirming the relationships demonstrated in **a**. Similarity/identity values for proteins VvTMT1–VvTMT3 are in *black boxes*, values of VvHT6 are in *gray boxes*

B

	% identity													
% similarity		VvHT1	VvHT2	VvHT3	VvHT4	VvHT5	VvHT6	VvpGLT	VvTMT1	VvTMT2	VvTMT3	VvSUC11	VvSUC12	VvSUC27
	VvHT1		53.6	54	55.9	55.7	14.5	22.3	13.9	14.2	13.7	12.6	10.6	12.5
	VvHT2	69.7		49.6	53.8	49.6	16.5	23.1	14.9	16.4	15.4	12.7	12.9	13.5
	VvHT3	68.7	65.8		54.4	53.6	16.3	20.9	14.3	16.2	14.0	11.0	11.1	13.3
	VvHT4	70.6	69.6	71.2		51.9	15.3	20.5	15.5	14.7	16.1	15.7	12.4	15.1
	VvHT5	70.9	66.3	70.3	69.2		15.2	21.7	14.5	13.5	13.1	12.2	11.2	13.6
	VvHT6	26.7	28.5	28.4	28.5	28.2		14.2	73.3	98.7	72.6	10.0	13.5	11.2
	VvpGLT	37.9	11.1	38.1	39.1	38.4	28.1		15.1	14.5	15.9	13.2	13.8	12.8
	VvTMT1	26.6	27.3	28.5	28.0	27.5	84.3	28.5		73.0	71.9	9.7	14.2	8.6
	VvTMT2	26.4	28.0	28.0	27.3	26.6	98.7	28.1	83.9		72.6	9.9	13.9	10.4
	VvTMT3	26.6	28.0	27.4	28.4	27.0	83.3	28.4	82.5	83.2		13.1	12.3	11.3
	VvSUC11	27.9	28.5	27.1	28.9	28.8	21.5	29.2	20.2	20.1	25.4		39.8	49.0
	VvSUC12	24.6	29.0	21.8	25.7	26.9	25.3	26.9	25.8	25.7	25.5	53.1		36.9
	VvSUC27	25.1	27.2	28.0	29.8	28.9	22.2	24.8	20.8	20.3	20.7	62.4	52.9	

stages, immunogold particles were distributed mainly in the vacuole and tonoplast, whereas the cell wall, plasma membrane, and chloroplasts had many less, if any. There was a marked difference in gold particle density in the vacuoles of the three different berry development stages (Fig. 4b, c, d).

To investigate changes in *VvTMT1* expression level, semiquantitative RT-PCR (normalized to *18S* rRNA) was performed with specific primers using RNA isolated from leaves, roots, stems, and berries at different development stages (Fig. 4e). Expression of *VvTMT1* differed among the various organs, with its transcript being most abundant in young fruit and then in young leaves, and showing a weaker expression signal in mature fruits and leaves. Decreasing *VvTMT1* accumulation during berry development and ripening was also revealed by Northern blotting (supplementary material). In contrast, *VvHT1*, used for comparison, exhibited relatively high expression levels in mature leaves, the strongest expression in the shoot stems, and weaker expression in post-véraison berries. This observation was in line with the findings of Hayes and others (2007).

Heterologous Expression of *VvTMT1* in Yeast

To further demonstrate its sugar transporter function, we expressed VvTMT1 in the yeast strain EBY.VW4000. This strain is completely lacking in glucose uptake due to concurrent knockout of endogenous hexose transporters, and can grow only on medium containing maltose as the carbon source. The pDR195-VvTMT1 and pDR195 constructs were used for the transformation. URA^+ transformants were selected on maltose as a permissive carbon source and subsequently replica-plated onto synthetic medium containing 2% glucose or 2% fructose as the sole carbon source. pDR195-VvTMT1-positive transformants showed partially restored growth on glucose and slight growth on fructose, whereas the transformants containing pDR195 were unable to form visible colonies on glucose or fructose media (Fig. 5a).

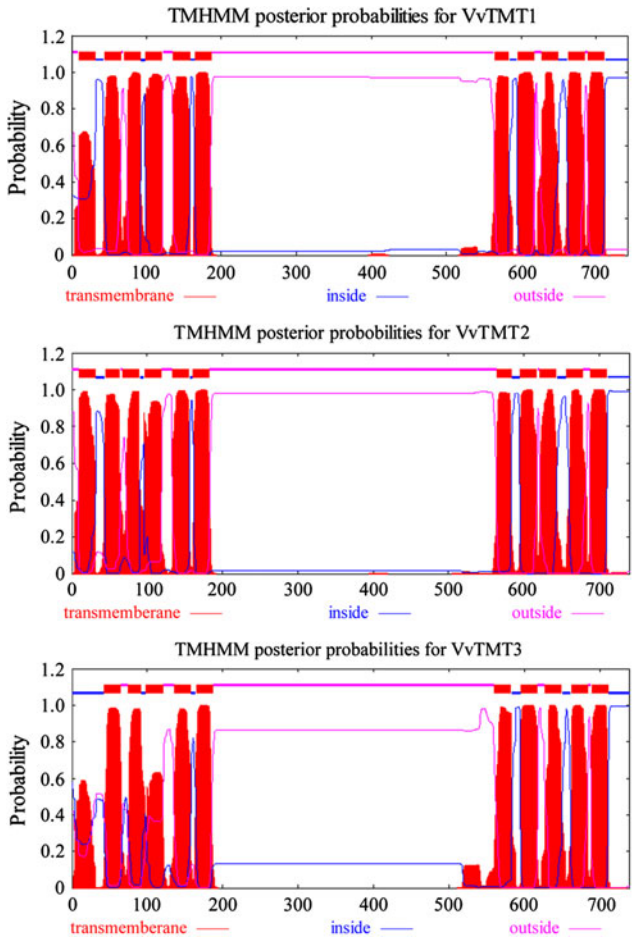

Fig. 3 Hydropathy profiles and membrane-spanning models of VvTMT1–VvTMT3. The calculation was performed using an algorithm published by Krogh and others (2001). *Blocks*, *lower lines*, and *upper lines* at the top of each profile indicate putative transmembrane domains, peptides facing the inside of the membrane, and peptides facing the outside of the membrane, respectively

The putative complementary role of VvTMT1 in transgenic yeast growth was assayed in liquid synthetic media without uracil and with different monosaccharides as the carbon source (Fig. 5b). *VvTMT1* mediated growth of the *hxt*-mutant yeast cells in 2% glucose and 2% fructose, as well as in 2% galactose and xylose. No growth was observed with the transformants containing the empty vector. The growth tests on the various sugar-containing media confirmed that VvTMT1 is a functional MST that plays a complementary role in glucose assimilation, partially restoring yeast cell growth.

To determine its possible subcellular localization, GFP-tagged VvTMT1 was expressed in the mutant *hxt*-null yeast strain. Transformed cells containing pYES2-TMT1-GFP, pYES2-HT1-GFP, and pYES2-GFP were cultured separately in selective media containing maltose, with gene expression under the control of the GAL1 promoter. Distribution of the fusion proteins in the living cells was examined by fluorescence microscopy. A strong green fluorescent signal was observed after galactose induction, whereas the signal in cells without galactose induction was very weak. As shown in Fig. 5C1, the fluorescence pattern of cells expressing pYES2-TMT1-GFP was significantly different from that of cells expressing pYES2-HT1-GFP or pYES2-GFP: in the former, bright fluorescence was concentrated in the internal structures, which resembled vacuoles, suggesting that VvTMT1 might be a tonoplast protein. In contrast, the fluorescence in cells expressing pYES2-HT1-GFP was predominantly localized at the cell periphery (Fig. 5C2), which is consistent with the plasma membrane localization reported by Vignault and others (2005). The fluorescence signal in cells expressing pYES2-GFP was homogeneously distributed in the cytoplasm (Fig. 5C3). Both plants and yeast use vacuole-targeting peptide domains, and some plant vacuole-targeting signal peptides are recognized by yeast (Gal and Raikhel 1994). Our results indicated that VvTMT1 contains a vacuole-targeting signal peptide sequence that is recognized by yeast. VvTMT1 targeting to the tonoplast in transgenic yeast would explain why this MST can only partially restore strain EBY.VW4000's growth on monosaccharide media.

Agrobacterium-mediated Transient Expression of VvTMT1 in Tobacco

To investigate the subcellular localization of VvTMT1 *in planta*, we transiently expressed a *GFP-VvTMT1* fusion construct under the control of the CaMV35S promoter in the epidermal cells of tobacco leaves. Confocal microscopy of transformed tobacco leaves revealed the vacuolar localization of VvTMT1 *in planta*. Figure 6 shows GFP-VvTMT1 fluorescence in the stomatal apparatus of tobacco leaves. No fluorescence was observed in the protoplasm of the guard cells, but clear fluorescence of the fusion protein was found in the tonoplast, labeling the vacuole membrane and not the plasma membrane.

Discussion

Vacuoles are critical in higher plants for their role in cell turgor regulation, maintenance of cellular ion homeostasis, detoxification of heavy metals and xenobiotics, storage of various secondary metabolites, and fruit quality formation (Agasse and others 2009). During berry development and maturation, all of these processes depend on the participation of specific and efficient transport proteins such as the sugar transporters, aquaporins, organic acid transporters, and other MFS proteins (Marger and Saier 1993; Robinson and Davies 2000). To date, however, only a few

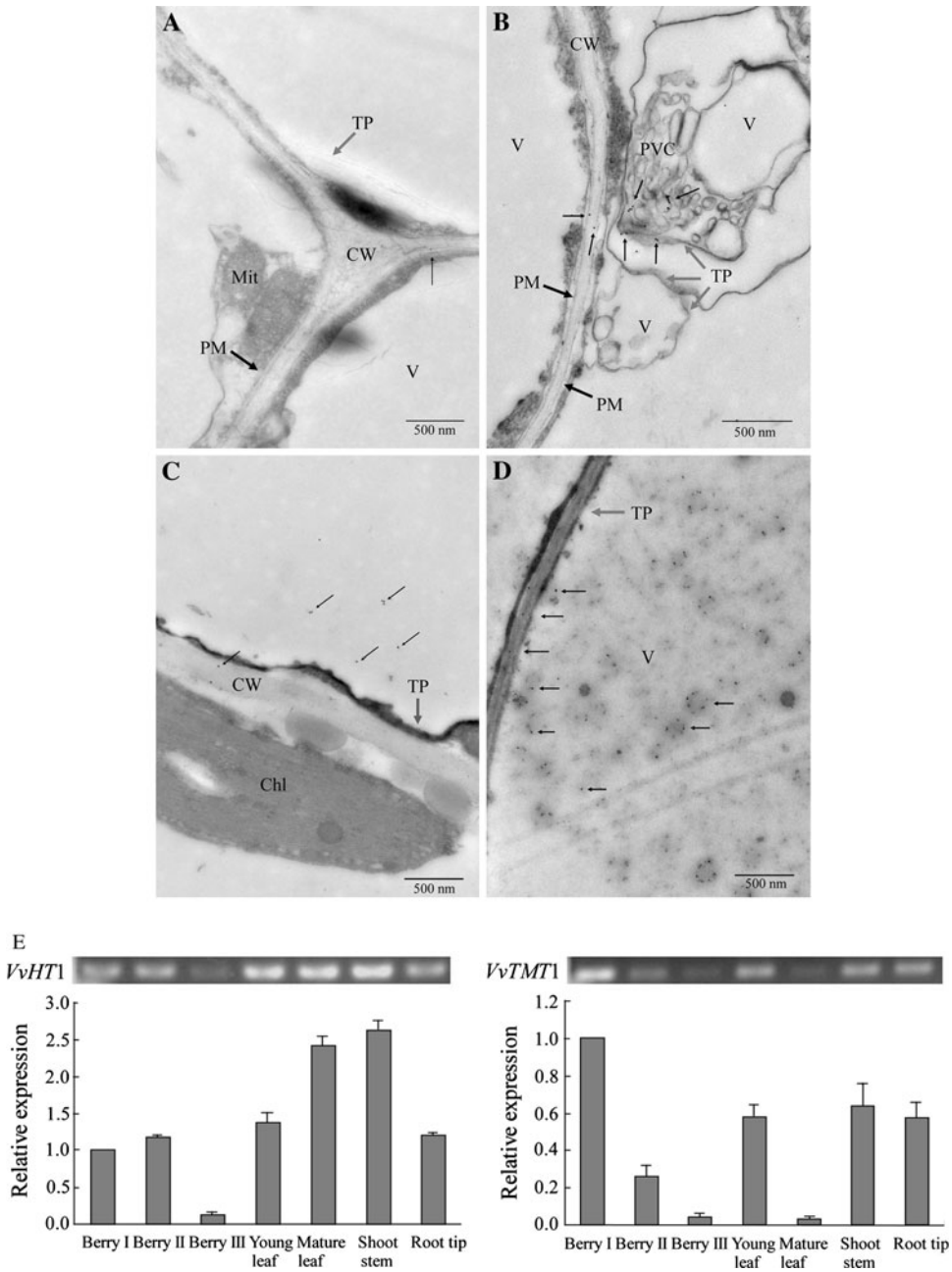

Fig. 4 Expression and immunogold electron microscopy localization of VvTMT1 in grape berry. Samples were taken from stages I, II, and III at 15, 45, and 75 days after flowering, respectively. **a** Control section of stage I berry without incubation with first antibody. **b** Stage I berry, gold particles can be found inside the prevacuolar compartments and vacuole, on the tonoplast, and a few near the plasma membrane are indicated by *arrows*. **c** Stage II berry, gold particles are seen mainly in the vacuole and on the tonoplast, a few on the plasma membrane. **d** Stage III berry, with a relatively high density of gold particles found in the vacuole. *CW* cell wall; *TP* tonoplast membrane; *PM* plasma membrane; *V* vacuole; *PVC* prevacuolar compartments; *Chl* chloroplast; *Mit* mitochondria. *Thin arrows* point to the gold particles, *thick arrows* point to the plasma membrane (*black*) or tonoplast membrane (*gray*). **e** Semiquantitative RT-PCR analysis of *VvTMT1* and *VvHT1* expression using RNA isolated from young leaves, mature leaves, young shoots, root tips, and berries of three development phases. *18S* rRNA was used as a control for constitutive expression and each value is the mean of three replicates. Error bars are standard errors

tonoplast-localized proteins have been functionally characterized.

Recently, proteomic analyses of higher plant vacuoles (Carter and others 2004; Shimaoka and others 2004; Szponarski and others 2004; Endler and others 2006; Jaquinod and others 2007; Schmidt and others 2007) have revealed some new, potentially tonoplast-localized solute transporters. However, proteomic methods might provide false information on the presence of proteins in specific subcellular fractions as a result of contamination from other organelles. Thus, independent proof is required to confirm or disprove their vacuolar membrane localization and physiological function.

Although several MSTs and DSTs have been functionally characterized and localized at the cellular level in grape (Davies and others 1999; Ageorges and others 2000; Manning and others 2001; Vignault and others 2005; Hayes and others 2007), none have been functional vacuolar membrane-localized sugar transporters. Here we describe the functional characterization and subcellular localization of VvTMT1, a new member of the *V. vinifera* MST family.

Searching GenBank with predicted amino acid sequences revealed a high similarity of VvTMT1 with other sugar transporters. A phylogenetic tree based on the deduced amino acid sequences of bacteria and plant MSTs was composed of mainly three distinct clusters: the first was

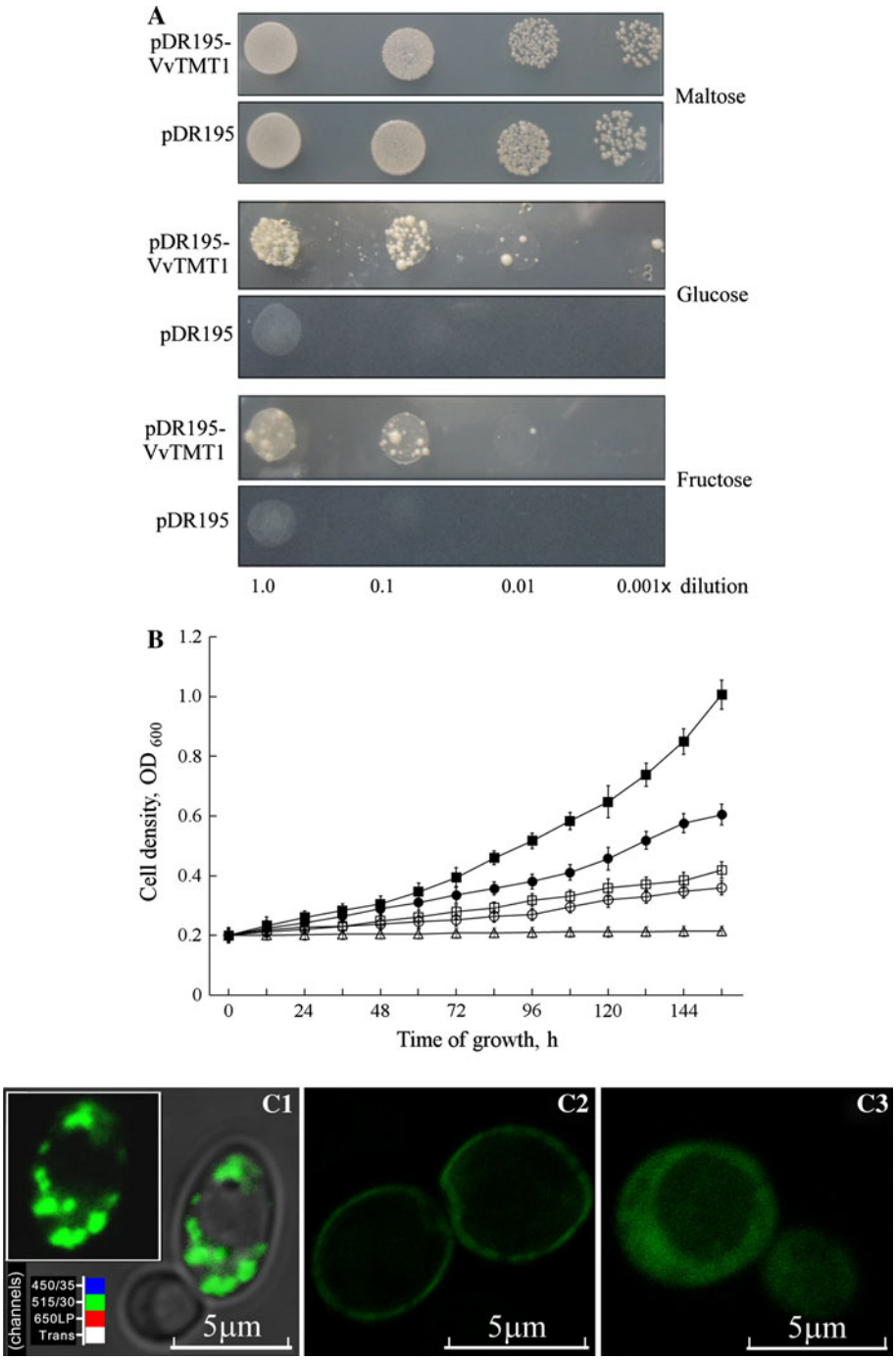

Fig. 5 Functional expression of *VvTMT1* cDNA in the *S. cerevisiae* EBY.VW4000 mutant defective in monosaccharide uptake. **a** Growth phenotype of the mutant complemented by the empty vector (pDR195) and the vector carrying VvTMT1 (pDR195-VvTMT1) on medium containing 2% maltose, glucose, or fructose as the sole carbon source. **b** Growth curves of the *S. cerevisiae hxt* mutant strain expressing the *VvTMT1* gene. Strain EBY.VW4000 containing plasmid pDR195-VvTMT1 was grown at 30°C on selective medium with different sugars. Growth was monitored by measuring the OD_{600} at the indicated times. *Black squares*, 2% glucose; *black circles*, 2% fructose; *white squares*, 2% galactose; *white circles*, 2% xylose; *white triangles*, EBY.VW4000 with the empty vector on 2% glucose. **c** Subcellular localization of VvTMT1-GFP fusion protein in transgenic yeast. *C1* Merged bright- and dark-field confocal microscopy images resemble vacuolar structures. *Inset*: dark-field image of yeast cell expressing the VvTMT1-GFP fusion construct. *C2* Dark-field image of yeast cells expressing the VvHT1-GFP fusion construct; fluorescence signal is strong in the yeast plasma membrane. *C3* Dark-field image of yeast cells expressing GFP alone; no specific localization of the fluorescence, which is evenly distributed in the cell. Scale bar = 5 µm

made up of inositol transporters and bacterial transporters D-XylE and YwtG; the second contained hexose transporters from monocots, including rice, barley, corn, and sorghum; and the third consisted of VvTMT sequences. Within the third cluster, VvTMT3 was most closely related to the *Populus trichocarpa* sugar transporter. No significant diversity was found among the transporters in plants, from monocots to dicots, and VvTMT1 showed slight phylogenetic distance relative to VvTMT2 and VvTMT3 (Fig. 7).

The predicted amino acid sequence of VvTMT1 shared high overall homology with those of previously reported plant MSTs such as *Arabidopsis* AtTMT2 (71.9%), *Zea mays* ZmHT (71.3%), and *Oryza sativa* OsSTP (73.6%). The highest homology was found with the MST of *Populus trichocarpa* (76.6%). Our in silico analyses revealed that VvTMT1 contains amino acid motifs which are conserved among members of the MFS and the amino acid signatures of typical sugar transport proteins. In addition, the

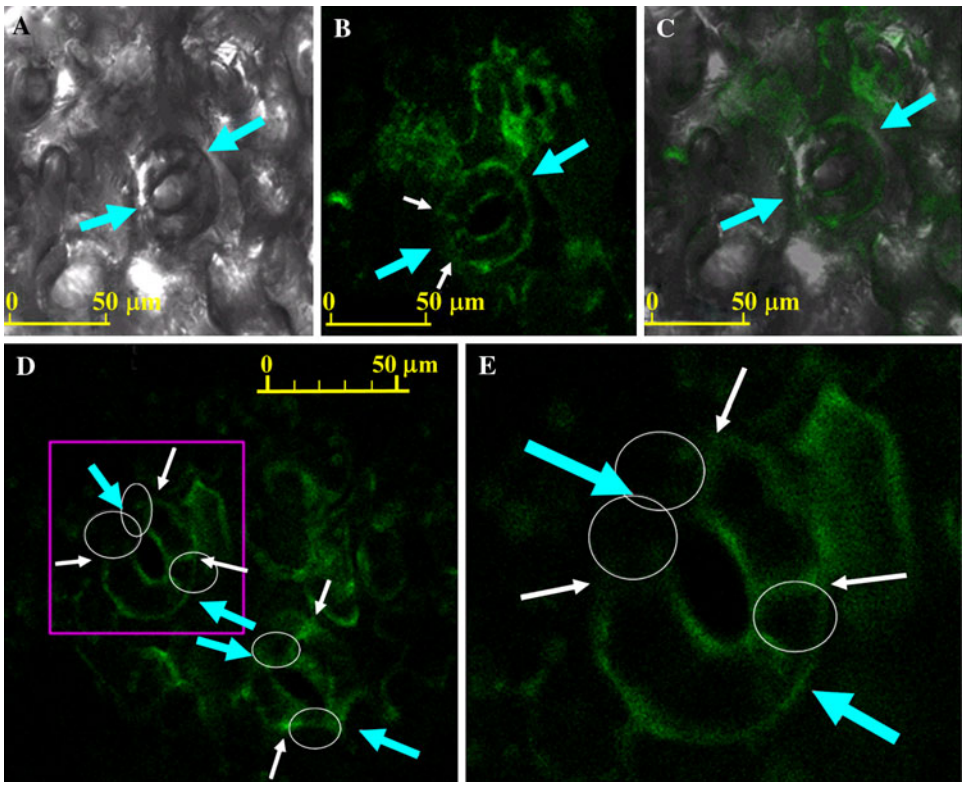

Fig. 6 Subcellular localization of GFP-VvTMT1 in the epidermal cells of tobacco. The CaMV35S-GFP-VvTMT1 construct was used to transform *Nicotiana tabacum* leaves that were subsequently infected with *A. tumefaciens* EHA105. Confocal microscopy was used to visualize membrane localization of the fusion protein. **a** Bright-field image. **b**, **d** Fluorescent images. **c** Merged fluorescent and bright-field images. In the view of the stomatal apparatus (**b**, **d**, **e**), GFP can be seen at the tonoplast of the inner side of the guard cells. A close-up **e** of the boxed section in **d** shows vacuolar membrane localization of the fusion protein. The *thin arrows* point to the labeled vacuole membrane of the inner side of the guard cells, the *thick arrows* point to the junction of two guard cells, and the *circles* indicate the space occupied by the protoplasm, which separates the guard cell tonoplast and plasma membrane. Scale bar = 50 µm

VvTMT1 protein has several putative protein kinase C phosphorylation and *N*-myristoylation sites. These potential post-translational modifications may also affect the protein's sugar transport activity.

Although sequence homologies enable the assignment of putative functions to unknown genes, the actual transport function of VvTMT1 needed to be tested precisely by homologous or heterologous expression. To date, heterologous expression of eukaryotic sugar transporters has been achieved in either *E. coli* (Frommer and Ninnemann 1995) or *Xenopus* oocytes (Reinders and others 2008). Expression in *E. coli* is stable, but very difficult to achieve because of its high susceptibility to external interference, and in oocytes, expression is laborious and unpredictable. *S. cerevisiae* has proven to be a good choice of organism for heterologous expression of plant sugar transporters, and the *hxt*-null yeast strain EBY.VW4000 has been successfully used for MST confirmation (Wieczorke and others 1999; Vignault and others 2005; Conde and others 2007). *VvTMT1* expressed in the *hxt*-null strain showed typical MST properties, and the restoration of yeast growth was similar to that achieved upon complementation with the plasma membrane hexose transporters (Vignault and others 2005; Conde and others 2007), with a relatively slower growth rate and a preference for glucose over other monosaccharides (Fig. 5a, b). Fructose was not significantly transported by VvTMT1, and galactose and xylose were only very poorly transported, at least in the concentration range tested (Fig. 5b).

Yeast growth experiments provide important evidence for the functionality of cloned genes, but it is not direct evidence (Schneider and others 2008). It is possible that recovery of the strain's growth might be due, for example, to mistargeting of the protein to the plasma membrane. We further analyzed the intracellular localization of VvTMT1 in the *hxt*-null yeast mutant strain EBY.VW4000, which carried a construct of the VvTMT1 protein fused to the N-terminus of GFP. As shown in Fig. 5C1, VvTMT1-GFP localized preferentially to the yeast vacuoles, as compared to the plasma membrane labeling by VvHT1-GFP as a positive control (Fig. 5C2). This is in agreement with the results of the previous yeast growth tests.

As heterologous expression in yeast does not necessarily provide information about the intracellular localization of

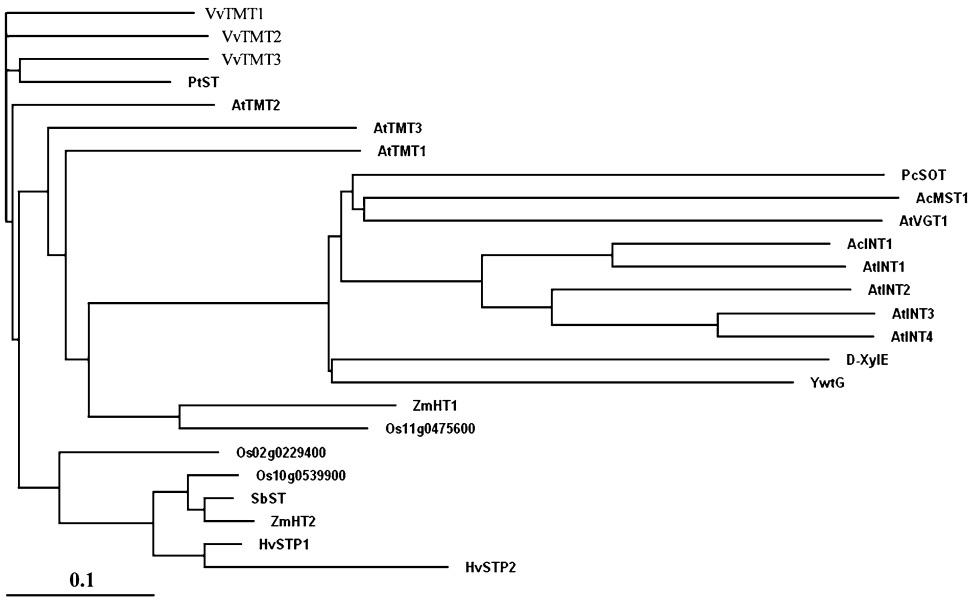

Fig. 7 Unrooted phylogenetic tree showing comparison of monosaccharide transporter sequences from grapevine and other organisms. Multiple sequence alignment was performed with ClustalX. The sequences were retrieved from the NCBI and the rice TIGR database with the following accession numbers: AtTMT1 (At1g20840), AtTMT2 (At4g35300), AtTMT3 (At3g51490), AtVGT1 (At3g03090), AtINT1 (At2g43330), AtINT2 (At1g30220), AtINT3 (At2g35740), AtINT4 (At4g16480), AcMST1 (EF460876), AcINT1 (EF460877), ZmHT1 (ACG25339), ZmHT2 (ACG45075), HvSTP1 (CAD58958), HvSTP2 (CAD58959), Os02g0229400 (NP_001046359), Os10g0539900 (NP_001065182), Os11g0475600 (NP_001067890), PtST (XP_002328276), PcSOT (AAM44082), SbST (XP_002467580), D-XylE (NP_347967), YwtG (YP_080913). Unrooted trees were constructed using the neighbor-joining method and viewed using TreeView software

the protein in plants, we transiently expressed the GFP-VvTMT1 fusion protein in tobacco epidermal cells and followed its localization. Figure 6 showed that GFP-VvTMT1 fluorescence was restricted to the vacuolar membrane within stomatal guard cells, implying that VvTMT1 was not differently targeted in yeast. Immunolabeling (Fig. 4) of grape berry further proved that VvTMT1 is localized in the tonoplast and not the plasma membrane.

Clues about the physiological role of VvTMT1 can be culled from its expression patterns in various organs. According to the semiquantitative RT-PCR results, *VvTMT1* was mostly expressed in sink tissues such as young leaves, root tips, and young berries. A striking feature of *VvTMT1* expression in grape was the relatively high accumulation of transcripts at the early stages of berry development (Fig. 4e). This suggests that VvTMT1 is likely involved in meeting the carbohydrate demands of dividing cells during the cell-division phase, or facilitating the subsequent rapid hexose gain that occurs during the cell-expansion phase. *VvTMT1* expression decreased gradually at the later stages of berry development, indicating that it might not mediate significant amounts of sugar import into pulp cells during grape ripening. Note that the transcript levels presented in Fig. 4e represent average levels for the berry pulp as a whole, and we cannot, therefore, rule out the possibility of localized regions of high expression within specific cell types in the pulp (Fig. 4b–d). *VvTMT1* transcripts were abundant in young leaves and berries, indicating that it could play an important role in sugar utilization, storage, or compartmentalization in these tissues. Taken together, the expression of *VvTMT1* is associated mainly with sink tissues where its encoded protein transports sugars across the tonoplast.

It is worth noting that the expression pattern of *VvTMT1* in berries was similar to that of the grape invertase genes *VvGIN1* and *VvGIN2* (Davies and Robinson 1996), that is, high early in berry development and declining sharply at the start of hexose accumulation. *VvTMT1* was expressed coordinately with *VvGIN*s, indicating that a developmental signal or endogenous and exogenous factors might trigger metabolic pathways that synchronously express these genes. Recent studies have shown that the expression of some sugar transporter genes is regulated by intragenic sequences (Sivitz and others 2007) and controlled by regulatory elements outside the 5′ region (Taylor 1997). Moreover, the *VvTMT1* expression pattern was very different from that of *VvSUC11* and *VvSUC12*, whose upregulated expression is consistent with a role in sucrose import during ripening; on the other hand, it was similar to that of *VvSUC27*, whose downregulated expression is unexplained (Davies and others 1999). The downregulation

of *VvTMT1* in ripening fruit implies that the tonoplast at this stage requires sugar flux in only one direction, and immobilization of the sugar stored in the vacuole limits it to movement back across the tonoplast. The existence of phosphorylation motifs in the *VvTMT1* cDNA sequence suggests post-translational regulation of sugar fluxes across the vacuolar membrane. The possible differential expression of sugar transporters in fruits implies that sugar accumulation in berries is a complicated process regulated by complex mechanisms.

In conclusion, three putative tonoplast monosaccharide transporter genes—*VvTMT*1–3—were cloned from grape berries. The sugar transporter function of VvTMT1 was confirmed by heterologous expression in yeast, which also localized the transporter protein to the tonoplast. Immunogold localization in grape berry cells, and transient expression in tobacco, supported its role as a tonoplast transporter. VvTMT1 is differentially expressed in different sink tissues and organs of grapevine. The detailed roles of VvTMT1 in berry development, sugar accumulation, and its upstream regulation await further investigation.

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