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VvTMT2 encodes a putative tonoplast monosaccharide transporter expressed during grape berry (*Vitis vinifera* cv. Sultanine) ripening

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Abstract

Sugars produced by photosynthesis are transported to the berries as sucrose *via* the phloem and imported into the fleshy cells through the plasma membrane and they accumulate in the vacuoles as glucose and fructose during ripening. Therefore, this process requires sugar transporter activity of plasma membrane as well as tonoplast. To examine sugar transport processes in *Vitis vinifera*, a full-length cDNA clone encoding a monosaccharide transporter designated as *VvTMT2* (*Vitis vinifera* Tonoplast Monosaccharide Transporter 2) was isolated by RT-PCR from the ripening stage of *V. vinifera* 'Sultanine' berries (GeneBank accession number: JX233818). *VvTMT2* cDNA contains a 2220-bp ORF encoding a predicted protein of 739 amino acids with a molecular mass of 79.2 kDa. According to the hydropathy pattern, VvTMT2 possesses 11 transmembrane-spanning domains with an extended cytoplasmic loop between transmembrane helices 6 and 7. This loop spans 371 amino acids, which is approximately 4–5 times longer than the corresponding structures in all other known monosaccharide transporters from prokaryotes and eukaryotes. VvTMT2 exhibited high similarities with other tonoplast monosaccharide transporters from different plant species. A search for *cis*-regulatory elements in the *VvTMT2* promoter region showed that this gene is probably regulated by phytohormones, sugars and abiotic stresses. A semiquantitative RT-PCR was conducted to determine the *VvTMT2* expression pattern using mRNAs isolated from various organs such as berries, young and mature leaves, tendrils, roots and pollens. VvTMT2 was highly expressed during the initiation of ripening and over-ripening of berries. However, transcript levels of *VvTMT2* were undetectable in some vegetative organs, suggesting that expression of *VvTMT2* gene is tissue and organ specific. Thus, *VvTMT2* gene may be involved in hexose transport from the cytoplasm to the vacuoles during berry ripening and over-ripening.

Keywords: Monosaccharide transport; sugar sensing; tonoplast; vacuole; *Vitis vinifera*
Abbreviations: MFS- Major Facilitator Superfamily; MST-Monosaccharide transporters; NCBI-National Centre for Biotechnology Information; ORF-Open Reading Frame; RT-PCR-Reverse Transcription-PCR; SUC-Sucrose Transporter; Transporter; TMT-Tonoplast Monosaccharide Transporter; VGT-Vacuolar Glucose

Introduction

Sugars play essential roles in metabolism and regulate many developmental and physiological processes in plants (Koch, 1996; Smeekens, 1998; Sheen et al., 1999). Although some plant species can also translocate sucrose derivatives (Büttner and Sauer, 2000), sucrose, produced by photosynthesis in source organs, represents the main form of reduced carbon transported in the sieve elements of the phloem. In sink organs, unloaded sucrose can be directly imported from the apoplast by sucrose transporters or monosaccharide transporters after cleavage by cell wall-bound invertases (Williams et al., 2000; Smith et al., 2005). Sugars can also be distributed at the subcellular level into different organelles. In addition, sugars can be imported into vacuoles for transient or long-term storage (Voitsekhovskaja et al., 2006). Accumulation of large amounts of sugars in the vacuoles of storage organs in plants such as sugar beet (Doll et al., 1979; Briskin et al., 1985; Getz and Klein, 1995) and sugarcane (Thom et al., 1982) have already been reported. Both facilitated-diffusion and active-transport mechanisms have been described for the transport of monosaccharide and sucrose across the vacuolar membrane (Thom and Komor, 1984; Daie and Wilusz, 1987; Martinoia et al., 1987; Neuhaus, 2007). Monosaccharide transporters (MSTs) are members of the major facilitator superfamily (MFS) and possess 12 predicted transmembrane α-helices with 2 modules of 6 helices connected by a central loop varying in length (Büttner and Sauer, 2000; Lemoine, 2000). MSTs are multigene families and have been isolated from several plant species. For example, the MST gene superfamily contains 53 genes in *Arabidopsis* (Lalonde et al., 2004) and 59 genes in *Vitis vinifera* (Afoufa-Bastien et al., 2010). At least 10 of monosaccharide transporters have been characterized as plasma membrane-localized monosaccharide importers (Lalonde et al., 2004; Büttner, 2007); however little is known about sugar transporters in the tonoplast. The first MST immunolocalized in the tonoplast was cloned from sugar beet and did not show any transport activity in yeast (Chiou and Bush, 1996). Recently, immunolocalization of HvSUT2 to the tonoplast has been shown using a GFP-fusion protein (Endler et al., 2006). In addition, the tonoplast monosaccharide transporter (TMT) subfamily consists of three proteins in *Arabidopsis* (AtTMT1-3) and their orthologs have been found in other plants such as *V. vinifera*, *Oryza sativa*, and *Hordeum vulgare* (Wormit et al., 2006). Recently, TMTs and vacuolar glucose (Glc) transporters have been characterized in *Arabidopsis* and direct or indirect transport

assays support their involvement in an import function as both types of transporters (Wormit et al., 2006; Aluri and Büttner, 2007). Grape berries offer an interesting model for studying monosaccharide transport and storage in sink tissues (Agasse et al., 2009; Afoufa-Bastien et al., 2010). In grapevines, sucrose is the main form of photoassimilate transported to the grape berry and stored in the vacuole of berry pericarp cells, which is the main site for hexose accumulation (Coombe, 1992). The initiation of ripening, also called véraison, is characterized by accumulation of glucose and fructose in the vacuoles of the mesocarp cells (Coombe, 1992; Davies and Robinson, 1996). In the cytoplasm of the mesocarp cells, sucrose and hexoses must be transported into the vacuole via tonoplast transporters.The identification and characterization of genes encoding hexose transporter from *V. vinifera* is important and will help elucidate development processes and ripening of berries. Previous studies have shown that sugar transporters expressed during berry ripening are involved in the phloem or sink-cell loading of sugar. Four sucrose transporters (Davies et al., 1999; Ageorges et al., 2000; Manning et al., 2001) and seven hexose transporters (Fillions et al., 1999; Vignault et al. 2005; Hayes et al., 2007) have been characterized in *V. vinifera* and are expressed during different stages of berry development. The *Vitis* genome contains three genes encoding TMT (Afoufa-Bastien et al., 2010) and to date, identification and characterization of these genes has not been reported for *V. vinifera*. Cloning and expression analysis of a full-length cDNA encoding a putative tonoplast monosaccharide transporter **(**TMT) from *V. vinifera* during berry ripening and analysis of gene expressions in different organs were reported in this study.

Results

Cloning of the full-length V. vinifera TMT2 cDNA

Total RNA extracted from the ripening stage of *V. vinifera* 'Sultanine' berries was first reverse transcribed. The cDNA obtained from the reverse transcription was amplified using specific primers for *VvTMT2* gene. A full-length cDNA sequence with a complete ORF encoding a hexose transporter was obtained and designated as *VvTMT2* on the basis of similarity to the predicted sequence of *VvTMT2*. The gene has been submitted to the GenBank database (accession number: JX233818). The cloned *VvTMT2* gene shared 96% sequence identity with the predicted sequence of *VvTMT2* (GSVIVT01023868001) and contained a 2220-bp ORF. The ORF of the *VvTMT2* gene encodes 739 amino acids with an estimated molecular mass of 79.2 kDa and an isoelectric point of 5.21. The *VvTMT2* gene was located on chromosome 3 and consisted of five exons.

Comparison of VvTMT2 protein and conserved motifs

The predicted amino acid sequence of VvTMT2 is characterized by the presence of an MFS signature between amino acids 7 and 717, a sugar transporter signature I between amino acids 63 and 80 and a sugar transporter signature II between amino acids 105 and 130 (Fig. 1). The deduced amino acid sequence of the *VvTMT2* cDNA includes two phosphorylation sites (casein kinase II and protein kinase C) and several repeats of N-glycosylation and N-myristoylation sites (InterProScan). The predicted amino acid sequence of VvTMT2 was compared with sugar transporters from other species through BLASTP and PSi-BLAST searches. A phylogenetic tree and multiple sequence alignments were constructed using 18 sugar transporter protein sequences from different plants (Fig. 2). VvTMT2 shared high sequence identity with *Ricinus communis* (80%; GenBank accession number, XP_002520608.1), *Arabidopsis thaliana* (73%; GenBank accession number, NP_195256.3) and *Zea mays* (59%; GenBank accession number, NP_001147067.1) while it displayed lower similarity with *O. sativa* (20%; GenBank accession number, BAB19862.1). Plant MSTs, TMTs, sucrose transporters and vacuolar transporters formed four clearly distinct clusters as expected (Fig. 2) and VvTMT2 grouped with TMTs from other plant species. The *VvTMT2* gene was located on chromosome 3 over 5 kb of genomic DNA and consisted of 5 exons and 4 introns (Fig. 3). The hydropathy pattern was analyzed according to Kyte and Doolittle (1982) suggests the presence of 11 transmembrane-spanning domains with a large central hydrophilic fragment between transmembrane domains 6 and 7 which spans approximately 371 amino acids (Fig. 4).

Differential expression of the VvTMT2 gene

To examine the precise tissue- and developmental-specific expression profiles of *VvTMT2* gene, the levels of transcripts in berries at different developmental stages, as well as in other organs were analyzed. The expression profile of the *VvTMT2* gene was examined by semiquantitative RT-PCR using *VvTMT2* gene-specific primers and primers specific to *Vitis* actin gene (*VvActin*) used as control. The *VvTMT2* gene was highly expressed in berries. While transcript level of *VvTMT2* gene was increased from six weeks after fruit set-up to véraison, it was dramatically decreased after véraison (Fig. 5A). Then, its expression was up-regulated again at the over-ripening stage of berries (Fig. 5A). The transcription levels of *VvTMT2* were barely detectable in old leaves while the transcript levels of *VvTMT2* were undetectable in roots, pollen, young and mature leaves and tendrils (Fig. 5B).

Promoter region analysis of the VvTMT2 gene

Putative *cis*-regulatory elements involved in the transcriptional regulation of the *VvTMT2* gene were searched in a 2.2-kb promoter region (Table 1). PLACE analysis performed for the promoter region revealed several *cis*-regulatory elements. These sequences regulate the expression in different plant organs such as leaves, shoots, roots, seeds, flowers and pollen. They were also predicted to be responsive to different phytohormones (ABA, SA, auxin, cytokinin, ethylene and gibberellin), as well as environmental factors (light, O2, cold and dehydration). Several copies of light-response motifs (SORLIP1AT, SORLIP5AT, INRNTPSADB, IBOXCORENT, IBOXCORE, IBOX and GT1CONSENSUS) were identified in the promoter of *VvTMT2* gene (Table 1). Interestingly, four sugar-response motifs (SURE1STPAT21, TATCCAOSAMY, SREATMSD and PYRIMIDINEBOXOSRAMY1A) and two ABA-response motifs (MYCCONSENSUSSAT and DPBFCOREDCDC3) were present in the promoter region of *VvTMT2* gene (Table 1).

Discussion

Sugars play many essential functions in plant cells. Therefore, it is not surprising that plants possess a large number of sugar transporter isoforms exhibiting tightly controlled cell- and tissue-specific expression patterns (Büttner and Sauer, 2000). Despite extensive knowledge of sugar transport across the plasma membrane in higher plants,

Table 1. *Cis*-acting elements identified in the *VvTMT2* promoter. The promoter-region analysis was performed using PLACE. *Cis*-element names, sequence motifs, signaling pathways and copy number are presented for each element.

Cis-element name	Response	Sequence	Number of copies/ *VvTMT2* promoter
ACGTATERD1	Dehydration, etiolation	ACGT	3
ACGTTBOX	Seed	AACGTT	1
AMYBOX1	Amylase, seed	TAACARA	1
ANAERO1CONSENSUS	Anaerobic genes involved in fermentation	AAACAAA	4
ANAERO2CONSENSUS	Anaerobic genes involved in fermentation	AGCAGC	1
ARR1AT	Response regulatory	NGATT	9
BOXCPSAS1	Negative light regulation	CTCCCAC	1
BOXIINTPATPB	Plastid genes	ATAGAA	3
CAATBOX1	Seed	CAAT	10
CACTFTPPCA1	Mesophyll specific expression	YACT	15
CARGATCONSENSUS	Flowering time, low temperature	CC(W)6GG	1
CCAATBOX1	Heat shock element	CCAAT	1
CGACGOSAMY3	Amylase	CGACG	1
CIACADIANLELHC	Circadian, light, leaf, shoot	CAANNNNATC	1
CPBCSPOR	Cytokinin, chlorophyll, chloroplast	TATTAG	3
CTRMCAMV35S	Enhancer	TCTCTCTCT	27
CURECORECR	Copper, oxygen, hypoxic	GTAC	1
DOFCOREZM	Leaf, shoot	AAAG	17
DPBFCOREDCDC3	ABA, embryo, seed	ACACNNG	1
EBOXBNNAPA	Storage protein, seed	CANNTG	9
EECCRCAH1	Low-CO2	GANTTNC	1
GARE1OSREP1	Gibberellin, aleurone, seed	TAACAGA	1
GATABOX	Chlorophyll a/b, light regulation, leaf, shoot	GATA	14
GCCCORE	Pathogene response, ethylene	GCCGCC	1
GT1CONSENSUS	Light regulation, salicylic acid, leaf, shoot	GRWAAW	12
GT1GMSCAM4	Leaf, shoot	GAAAAA	5
GTGANTG10	Pollen	GTGA	8
IBOX	Light-regulated, leaf, shoot	GATAAG	2
IBOXCORE	Light, leaf, shoot	GATAA	8
IBOXCORENT	Light	GATAAGR	1
INRNTPSADB	Light-responsive, initiator, TATA-less promoter,	YTCANTYY	5
LECPLEACS2	Ethylene	TAAAATAT	2
MARTBOX	Scaffold attachment region	TTWTWTTWTT	1
MYB1AT	Dehydration response, ABA, leaf, shoot	WAACCA	1
MYBCOREATCYCB1	Cell cycle, M-phase	AACGG	1
MYBST1	Tumorigenesis, differentiation	GGATA	3
MYCATERD1	Early responsive to dehydration, water	CATGTG	5
MYCCONSENSUSAT	ABA, leaf, seed, cold, dehydration	CANNTG	9
NODCON1GM	Nodulin consensus sequence	AAAGAT	2
NODCON2GM	Nodulin consensus sequence	CTCTT	1
NTBBF1ARROLB	Auxin, tissue specific expression, root, shoot, meristem	ACTTTA	1
OSE1ROOTNODULE	Organ specific, infected cells in root nodules	AAAGAT	2
OSE2ROOTNODULE	Organ specific, infected cells in root nodules	CTCTT	1
P1BS	Phosphate starvation response	GNATATNC	2
PALBOXAPC	Elicitor, light responsiveness	CCGTCC	1
POLASIG1	Poly A	AATAAA	3
POLASIG2	Poly A	AATTAAA	2
POLASIG3	Poly A	AATAAT	5
POLLEN1LELAT52	Pollen specific expression	AGAAA	7
PRECONSCRHSP70A	Plastid response element, chlorophyll	SCGAYNR(N)12HD	2
PYRIMIDINEBOXOSRAMY1A	Sugar repression, GA, embryo, seed	CCTTTT	2
RAV1AAT	Rosette leaves, roots	CAACA	3
ROOTMOTIFTAPOX1	Root	ATATT	5
S1FBOXSORPS1L21	Plastid protein, leaf, down-regulation	ATGGTA	3
S1FSORPL21	Plastid protein, leaf, down-regulation	ATGGTATT	1
SEF3MOTIFGM	Seed	AACCCA	1
SEF4MOTIFGM7S	Storage protein, seed	RTTTTTR	6
SORLIP1AT	Light, phytochrome	GCCAC	1
SORLIP5AT	Light, phytochrome	GAGTGAG	1
SP8BFIBSP8BIB	Amylase, tuberous root	TACTATT	1
SREATMSD	Sugar repressive element, axillary bud outgrowth	TTATCC	1
SURE1STPAT21	Sucrose, root	AATAGAAAA	1
TAAAGSTKST1	Guard cell specific expression	TAAAG	3
TATAPVTRNALEU	Re-initiation	TTTATATA	1
TATCCAOSAMY	Sugar starvation, gibberellin	TATCCA	1
WBOXATNPR1	Salicylic acid, disease resistance	TTGAC	2
WRKY71OS	Pathogen response, gibberellin repression	TGAC	2

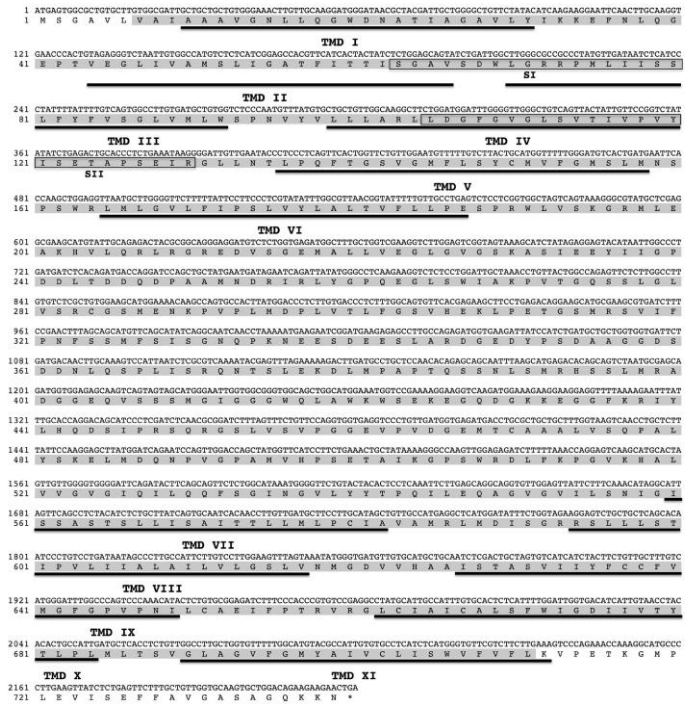

Fig 1. The nucleotide and deduced amino acid sequences of VvTMT2. Sugar transporter protein signatures (SPI and SP II) are indicated by open boxes. The MFS domain located between amino acid residues 7 and 717 is highlighted in gray boxes. The transmembrane domains I, II, III, IV, V, VI, VII, VIII, IX, X, and XI are underlined.

very few tonoplast sugar transporters have been isolated and characterized. Here, we have identified a cDNA encoding a putative tonoplast hexose transporter in grapes, with strong homology to known monosaccharide transporters from other plants. The TMT subfamily in *V. vinifera* contains three genes with a genomic structure that consists of four exons and five introns (Afoufa-Bastien et al., 2010). Besides the high sequence homology found within this subfamily, the genomic structure is highly conserved with respect to the positions and numbers of introns. According to 12x proteome data base screening, *VvTMT2* gene (GSVIVT01023868001) also contains five introns. Thus, TMT-type sugar transporters are not restricted to *V. vinifera*, as homologs have been identified in sugarcane, *Arabidopsis* and pineapple (Casu et al., 2003; Wormit et al., 2006; Antony et al., 2008).VvTMT2 also possesses an extended cytoplasmic loop which is approximately 4–5 times longer than the corresponding structures in all other known monosaccharide transporters from prokaryotes and eukaryotes (Henderson, 1991; Mueckler, 1993). This extended cytoplasmic loop appears to have no significant function in controlling substrate affinity or transport (Schulze et al., 2000); it has a proposed function in sucrose sensing based on the similarity to extended domains of the yeast sensors RGT2 and SNF3 (Kühn, 2003; Lalonde et al., 2004; Sauer 2007); however, this remains to be experimentally proven. *In silico* predicted amino acid sequence of VvTMT2 contains conserved motifs between members of the MFS and the signatures of typical sugar transport proteins. Possible posttranslational modifications are suggested by the presence of eight different putative protein kinase c and putative casein kinase II phosphorylation sites, which may show involvement of phosphorylation in the regulation of VvTMT2 activity, as it was suggested by the OeMST2 and sugar beet sucrose transporters (Roblin et al., 1998; Conde et al., 2007). Additionally, the VvTMT2 protein has several consensus sequences for potential N-myristoylation and N-glycosylation sites (InterProScan). Presence of these putative posttranslational modification sites in the VvTMT2 protein suggest that its sugar transport activity may also be modified by posttranslationally. In *Arabidopsis*, TMT proteins are predicted to function *via* a proton-coupled antiport mechanism that allows the active transport and accumulation of hexoses (glucose and fructose) in the vacuole, particularly in response to stresses—such as cold, drought and salinity—that promote sugar accumulation (Wormit et al., 2006). Therefore, it would be interesting to examine how *VvTMT2* is regulated at the transcriptional and post-translational levels under stress conditions. In source tissues, MSTs are required in the plastid membrane for the export of fixed carbons. MSTs are also required in the tonoplast where vacuolar storage provides the soluble sugars to the cytosol (Linka and Weber, 2010; Wingenter et al., 2010). Our knowledge of the biological functions of TMTs in plants is extremely limited. Members of TMTs as well as members of the VGT subfamilies localize to the tonoplast membrane and transport glucose and fructose into the vacuolar lumen. Recently, AtTMT1 and AtTMT2 have been shown to play functional roles in both cellular carbon balance and whole-plant carbohydrate partitioning (Wormit et al., 2006). Some members of the VGT subfamily have been shown to play a role in carbon allocation during germination and development of *Arabidopsis* (Aluri and Büttner, 2007). The expression of *VvTMT2* differed from tissue to tissue and varied depending on the developmental stage, indicating tissue- and developmental stage-specific expression of *VvTMT2*. Tissue-specific expression of *VvTMT2* has also been reported in grapes on the basis of macroarray expression data (Afoufa-Bastien et al., 2010). Additionally, *VvTMT2* transcript level was higher in the fruit than in leaves. In grape berries, sugar accumulation begins at the onset of ripening (véraison), which is marked by a 10-fold increase in hexose content (Davies and Robinson, 1996). The majority of hexose

is imported into the berry flesh cells during the later phases of berry maturation (post véraison) (Zhang et al., 2006). Interestingly, *VvTMT2* expression was induced during the ripening and over-ripening stages, which coincides with the accumulation of hexoses in berries. Therefore, it is likely that VvTMT2 is involved in the transport of hexose from the cytoplasm to the vacuoles during berry ripening and over-ripening. Previous studies have shown that sugar sensing and control of gene expression are involved in various physiological processes, including fruit development (Smeekens, 2000). In grape berries, *VvHT1* (*Vitis vinifera Hexose Transporter1*) is expressed shortly after véraison, when sugars start to accumulate (Fillion et al., 1999) and *VvHT1* expression is induced by glucose and sucrose (Atanassova et al., 2003). Therefore, we cannot exclude the possibility that *VvTMT2* expression might be also regulated by its own substrate, which is correlated with the accumulation of hexoses. It has recently been reported that overexpression of AtTMT1 led to an increase in phloem transport rates, whereas the *tmt*1-2::tDNA double mutant had a decrease in export rate compared to both wild-type and the overexpressing line (Wingenter et al., 2010). Considering its high expression in berries, it is tempting to suggest that VvTMT2 is probably involved in phloem unloading. Leaf senescence can be induced by low nutrient, especially low nitrogen supply (Ono et al., 1996; Crafts-Brandner et al., 1998). Interestingly, nitrogen deficiency often results in sugar accumulation (Ono et al., 1996; Ono and Watanabe, 1997). Furthermore, experiments on sugar regulated senescence indicate that glucose and fructose accumulate in Arabidopsis leaves until late senescence (Quirino et al., 2001; Pourtau et al., 2004). As hexoses accumulate during leaf senescence, *VvTMT2* might be responsible for hexose accumulation in the vacuoles of old leaves. The *in silico* identification of *cis*-regulatory elements in the promoter of *VvTMT2* suggests that this gene is regulated by different phytohormone and sugar signals and/or abiotic and biotic stresses. The regulation of the expression of sugar transporter genes by sugars was previously described for VvHT1 (Atanassova et al., 2003; Cakir et al., 2003; Conde et al., 2006), OsSUT1 (Matsukura et al., 2000), BvSUT1 (Vaughn et al., 2002; Ransom-Hodgkins et al., 2003) and AtTMTs (Wormit et al., 2006). The presence of sugar-responsive motifs in the promoter of *VvTMT2* also suggests regulation of this gene by sugars. The *in silico* analysis of the *VvTMT2* promoter also showed two ABA-responsive motifs. Although the function of these regulatory elements remains to be tested, this finding is in agreement with the fact that the expression of *VvHT1* gene (grape hexose transporter) is induced by ABA (Cakir et al., 2003). The gene *VvHT5* has also been demonstrated as regulated by ABA (Hayes et al., 2007). Because of *VvTMT2* homologous to known monosaccharide transporters in other organisms, a yeast mutant deficient in hexose transport (EBY.VW4000; Reifenberger E et al., 1997) was used to test the ability of cloned VvTMT2 to regulate hexose transport. The hexose transport activity was unable to detected by heterologous expression of *VvTMT2* in yeast mutants lacking endogenous hexose carriers (data not shown), which is in agreement with the results obtained for sugarcane (Casu et al., 2003) or *Arabidopsis* homologs (Wormit et al., 2006; Aluri and Büttner, 2007). However, the possibility that VvTMT2 may be involved in monosaccharide transport into the vacuole cannot be excluded as it was the case for *Arabidopsis* homologs. For this purpose, it would be interesting to isolate vacuoles of yeast expressing *VvTMT2* and analyze for sugar transport activity.

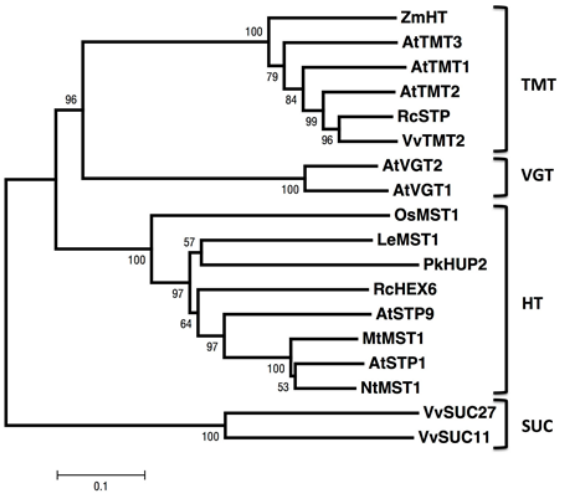

Fig 2. Phylogenetic analysis of VvTMT2 and other MSTs from different plants. The phylogenetic tree was constructed using MUSCLE and phylogenetic analysis was performed with the NJ method by using MEGA4. Bootstrapping was performed with 100 replicates. TMT, tonoplast monosaccharide transporter; VGT, vacuolar glucose transporter; HT, hexose transporter; SUC, sucrose transporter. The GenBank accession numbers for the sequence designations are as follows: AtTMT1, NP_173508.1; AtTMT3, NP_190717.1; AtTMT2, NP_195256.3; AtVGT1, NP_186959.2; AtVGT2, NP_850836.2; AtSTP9, NP_175449.1; AtSTP1, NP_172592.1; VvSUC11, ADP37121.1; VvSUC27, ADP37123.1; RcSTP, XP_002520608.1; RcHEX6, AAA79857.1; NtMST1, CAA47324.1; PkHUP2, CAA47323.1; LeMST1, CAA09419.1; MtMST1, AAB06594.1; OSMST1, BAB19862.1; and ZmHt, NP_001147067.1.

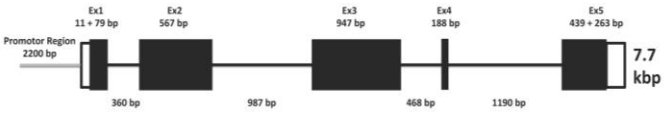

Fig 3. Structure of the *VvTMT2* gene. The promoter region**,** the protein-coding region, the untranslated region, and the introns are represented by gray line, filled rectangles, open rectangles, and straight lines, respectively. The numbers indicate the size of each region in base pairs.

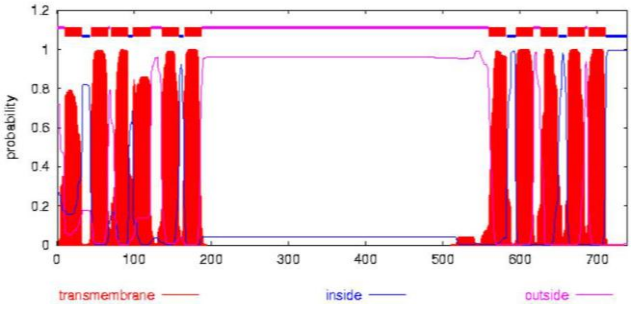

Fig 4. Hydropathy analysis of the deduced amino acid sequence of VvTMT2. VvTMT2 contains eleven putative membrane-spanning domains. The hydropathy plot was generated using the method reported by Kyte and Doolittle (1982). Putative membrane-spanning domains are indicated with thick horizontal bars.

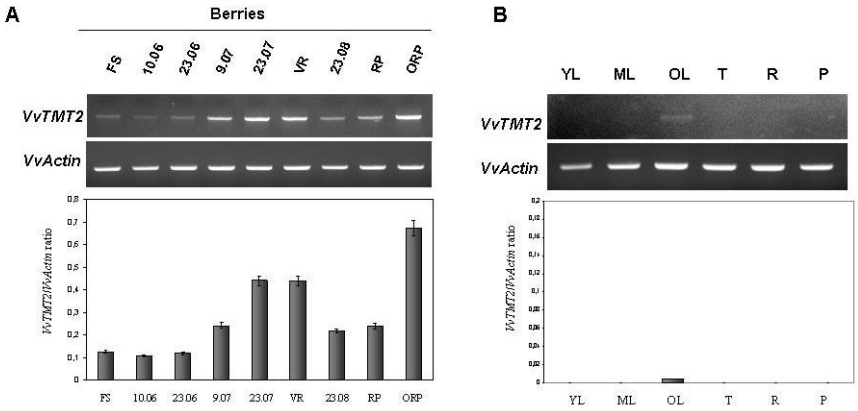

Fig 5. Expression pattern of *VvTMT2* in different organs from *Vitis vinifera*. (A) Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of *VvTMT2* from *Vitis vinifera* 'Sultanine' at different stages of berry development. Total RNA was extracted from berries between May 26 and September 17 at 2-week intervals during the 2008 growing season. May 26: fruit set (FS); August 6: véraison (VR); September 3: ripening (RP); September 17: over-ripening (ORP). The expression of *VvTMT2* was determined by semiquantitative RT-PCR. *VvActin* was amplified as a control to ensure that the equal amounts of templates were added to each RT-PCR reaction. The relative expression was calculated by taking the ratio of the intensity of the *VvTMT2* band over the intensity of the *VvActin* band using the Bio1D software (Vilber Lourmat). The data were analyzed independently in triplicate. The error bars represent standard deviation (SD). (B) Semiquantitative RT-PCR analysis of *VvTMT2* in different organs of *V. vinifera* 'Sultanine'. Total RNA was extracted from leaves, pollen, flowers and tendrils. The expression of *VvTMT2* was determined by semiquantitative RT-PCR.*VvActin* was amplified as a control to ensure that the equal amounts of templates were added to each RT-PCR reaction.The relative expression was calculated by taking the ratio of the intensity of the *VvTMT2* band over the intensity of the *VvActin* band using the Bio1D software (Vilber Lourmat). The data were analyzed independently in triplicate. The error bars represent SD. YL: young leaves; ML: mature leaves; OL: old leaves; P: pollen; R: roots; T: tendrils.

Materials and methods

Plant material

All expression analyses were performed on berries, leaves, roots, tendrils and pollen sampled from an 8-year-old vineyard of *V. vinifera* 'Sultanine' from the Ege University Agricultural Experiment Station (Izmir, Turkey). Berries were harvested at 2-week intervals from fruit in the over-ripe stage in the 2008 growing season (between May 26 and September 17, 2008). Tissues were collected and frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Sequence analyses

A total of 18 full-length coding sequences (CDSs) from different species, including *V. vinifera*, were used for phylogenetic analysis. The protein sequence alignment was generated using the CLUSTALX1.81 program and phylogenetic analyses were performed using protein sequences with maximum likelihood and neighbor joining (NJ) methods. An NJ tree was constructed using MEGA4 (Kumar et al., 2008). A search for *cis*-regulatory elements in promoter sequences was performed using the Plant *Cis*-Acting Regulatory DNA Elements Database (PLACE: http//www.dna.affr.c.po.JP/PLACE/index.html). The molecular weights and isoelectric points of the deduced amino acid sequence were predicted using Compute pI/Mw (http://av.expasy.org). Conserved domains were predicted using InterProScan http://www.ebi.ac.uk/InterProScan/). Identification of eukaryotic signal peptides and prediction of their cleavage sites were performed with the SignalP 3.0 program,available at http://www.cbs.dtu.dk/services/SignalP/ (Nielsen et al., 1997).

Cloning of VvTMT2 cDNA

The *VvTMT2* cDNA was obtained from the berries of *V. vinifera* 'Sultanine' during the ripening stage. First-strand cDNA was synthesized from 2 µg of total RNA in a 20 µl reaction mix using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas) with oligo (dT) primers. The forward primer VvTMT2 F 5′-GCG GCC GCC ACC ATG AGT GGA GCT GTG CTT GTG-3′ and the reverse primer VvTMT2 R 5′- GC GTC GAC TCA GTT CTT CTT CTG TCC AGC-3′, corresponding to the predicted sequence of *VvTMT2* (GSVIVT01023868001), were used to amplify the open reading frame (ORF) of the cDNA. After reverse transcription, the reaction mixture was used in PCR reactions to synthesize the full-length of the cDNA. PCR was performed with an initial denaturation at 94°C for 2 min; followed by 25 cycles of denaturation at 94°C for 45 s, annealing at 64 °C for 30 s and extension at 72°C for 2 min; and then a final extension at 72°C for 10 min. The products were gel purified and subcloned into the pGEM-T Easy cloning vector according to the manufacturer's instructions. Automated sequencing of the *VvTMT2* cDNA clone was performed by the Refgen Center, DNA Sequencing Facility (Ankara, Turkey). The nucleotide sequence of *VvTMT2* was submitted to the GenBank database under the accession number JX233818. Sequence data were analyzed using the DNASTAR software (http://www.dnastar.com). Homology searches in databases were carried out using the BLAST program (http://www.ncbi.nlm.nih.gov/blast).

RNA analysis by semi quantitative RT-PCR

Total RNA was extracted according to the method described by Davies and Robinson (1996) with an additional step of selective precipitation with 2 M LiCl. For semiquantitative RT-PCR, total RNA was treated with RNase-free DNaseI (Fermentas, USA) in order to eliminate genomic DNA and it was purified using the RNeasy purification kit (Qiagen, Germany) according to the RNA clean-up protocol provided by the manufacturer. First-strand cDNA was synthesized from 2 µg of total RNA in a 20 µl reaction mix using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas) with oligo (dT) primers. After reverse

transcription, the reaction mixture was diluted 5 fold and used in PCR reactions. The primer sequences for VvTMT2 and the internal control gene actin (XM_002282480) were as follows: VVTMT2, forward primer VvTMT2F 5′- ATG AGT GGA GCT GTG CTT GTG GCG-3′ and reverse primer VvTMT2R 5′- GAT CCG CGT TGA GAT CGA GGG ATG CT-3′; actin, forward primer VvActinF 5′-GGC ACA TTC TAC AAT GAG CTT CG-3′ and reverse primer VvActinR 5′-AGA GGA CTT CTG GAC AAC GG-3′. PCR reactions were performed in 25-µl volumes containing 1 µl (5-fold dilution) of first-strand cDNA as a template, 0.2 µM of each primer, 0.2 µl of DreamTaq DNA polymerase (5 U/µl) and 2.5 µl of 10× PCR buffer. PCR conditions included an initial denaturation at 94°C for 3 min; 18 cycles of 95°C for 20 s, 62°C for 45 s and 72°C for 2 min; and a final extension at 72°C for 10 min. The optimal number of amplification cyles (between 15 and 35) for each transcript was determined at the exponential phase of amplification. The linear range of detection for each transcript (*VvTMT2* and *VvActin*) was 18 cycles for the leaves, roots, berries, tendrils and pollen. The resulting PCR products were separated on 1% agarose gels. The band intensity was then quantified using the BIO1D software (Vilber Lourmat). Three independent experiments were performed with similar results.

Conclusions

In this study, we isolated *VvTMT2*, the first tonoplast monosaccharide transporter, from *V. vinifera*. The expression of *VvTMT2* was increased in berries during ripening and overripening suggesting its role in hexose accumulation from the cytoplasm to the vacuoles. Since the expression of *VvTMT2* was only detected in old leaves, VvTMT2 might play a role in hexoses accumulation in the vacuoles during leaf senescence. The existence of phosphorylation motifs in the sequences of VvTMT2 suggest the possibility of post-translational regulation of sugar fluxes across the vacuolar membrane. The *in silico* identification of *cis*-regulatory elements in the promoter of *VvTMT2* revealed that this gene may be regulated by different phytohormone and sugar signals and/or abiotic and biotic stresses. Finally, the isolation and the expression pattern of *VvTMT2* in vegetative and reproductive organs may contribute to a better understanding of sugar transport in grapes.

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