**OeMST2 Encodes a Monosaccharide Transporter Expressed throughout Olive Fruit Maturation**

Carlos Conde 1, Alice Agasse 1, Paulo Silva 1, Rémi Lemoine 2, Serge Delrot 3, Rui Tavares 1 and Hernâni Gerôs 1,*

1 Departamento de Biologia, Universidade do Minho, Campus de Gualtar 4710-057 Braga, Portugal
2 UMR CNRS 6161, Transport des Assimilats, Laboratoire de Physiologie, Biochimie et Biologie Moléculaires Végétales, Bâtiment Botanique, UFR Sciences, 40 Avenue du Recteur Pineau, 86022 Poitiers Cédex, France
3 Institute of Vine and Wine Sciences (ISVV), University Victor Segalen Bordeaux II, Unité Mixte de Recherches Ecophysiology and Grape Functional Genomics, INRA, 71 Avenue Edouard Bourlaux, 33885, BP 81 Villenave d’Ornon, France

In olive fruits, sugars are the main soluble components providing energy and acting as precursors for olive oil biosynthesis. Large quantities of glucose, fructose, and galactose are often found in olive pulp. To analyze sugar transport processes in *Olea europaea*, a cDNA encoding a monosaccharide transporter, designated OeMST2 (*Olea europaea* monosaccharide transporter 2) was cloned. An open reading frame of 1,569 bp codes for a protein of 523 amino acids and a calculated molecular weight of 57.6 kDa. The protein is homologous to other sugar transporters identified so far in higher plants. Expression of this cDNA in an hxt-null *Saccharomyces cerevisiae* strain deficient in glucose transport restored its capacity to grow on and to transport glucose. The encoded protein showed high affinity for α-glucose (Km, 25 μM), and was also able to recognize α-galactose and the analogs 3-O-methyl-α-glucose and 2-deoxy-α-glucose, but not α-fructose, α-arabinose, sucrose or α-mannitol. Maximal transport activity was high at acidic pH (5.0), and the initial α-[14C]glucose uptake rates were strongly inhibited by the protonophore carbonyl cyanide m-chlorophenylhydrazone, confirming that OeMST2 is a H+/monosaccharide transporter. The expression of OeMST2 was studied during the ripening process. Transcript levels increased during fruit maturation, suggesting that OeMST2 takes part in the massive accumulation of monosaccharides in olive fruits. Monosaccharide:H+ transport system activity and OeMST2 expression were negatively regulated by glucose in suspension-cultured cells. Glucose-mediated OeMST2 repression was impaired by mannoheptulose, suggesting the involvement of a hexokinase-dependent signaling pathway.

Keywords: Glucose — Monosaccharide transporter — *Olea europaea* — Sugar sensing.

Abbreviations: CCCP, carbonyl cyanide m-chlorophenylhydrazone; 2-dG, 2-deoxy-α-glucose; DST, disaccharide transporter; HK, hexokinase; MFS, major facilitator superfamily; 3-O-MG, 3-O-methyl-α-glucose; MST, monosaccharide transporter; RACE, rapid amplification of cDNA ends; PEG, polyethylene glycol; RT–PCR, reverse transcription–PCR; TPP+, tetraphenylphosphonium.

The nucleotide sequence reported in this paper has been submitted to NCBI under accession number DQ087177.

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**Introduction**

*Olea europaea* L. is an evergreen moderately salt-tolerant tree (Therios and Misopolinos 1988; Rugini and Fedeli 1990), traditionally cultivated in the Mediterranean basin, showing a preference for the coast, where olives and olive oil play an important nutritional role. The unadulterated oil that results from crushing of olive fruits is a predominant component of the widely known ‘Mediterranean diet’ to which increasing attention is being paid in nutrition studies. Indeed, worldwide olive oil consumption has multiplied 6-fold over the past 30 years, as a result of the growing knowledge of its protective properties against cardiovascular diseases and cancer. Olive fruit maturation occurs during summer, and harvest takes place between October (green in color but growth completed, cherry stage) and February (fully ripe, black stage). Olive oil is usually extracted from olives harvested in November (Rotondi et al. 2004). High concentrations of sugar are a common phenomenon occurring during the ripening of most fruits. In olive, besides providing energy, sugars act as precursors for olive oil biosynthesis. Glucose, fructose and galactose are the main sugars found in olive pulp, but significant amounts of mannitol, a sugar-alcohol which plays an important role in salt stress resistance in olive, are also present (Marsilio et al. 2001, Conde et al. 2007a).

Higher plants represent complex mosaics of phototrophic and heterotrophic cells and tissues. Reduced carbon is produced through photosynthesis in the mesophyll of mature leaves. They are specialized producers and exporters of sugars and are known as ‘source’ tissues.
Assimilated carbon is transported throughout the phloem, mainly in the form of sucrose, to heterotrophic organs such as developing leaves, flowers, fruits and roots which rely on its supply for their growth and development. These are considered as 'sink tissues' and are specialized importers and consumers of sugar. Thus, phloem transport of assimilates provides the materials needed for the build-up of herbaceous plants and trees, and has long been recognized as a major determinant in crop yield. It is therefore important to understand the mechanisms and regulations of sugar transport into sink tissues.

Phloem unloading may take place either symplasmically via plasmodesmata or apoplastically across the plasma membrane. In the latter case, sucrose may be taken up by sink cells either intact via sink-specific sucrose/H\(^+\) transporters (disaccharide transporters; DSTs) or, after extracellular hydrolysis by cell wall invertases, to glucose and fructose, via monosaccharide/H\(^+\) transporters (MSTs) (Williams et al. 2000). Although both symplasmic and apoplastic unloading may participate in providing sugars to the fruit, the apoplastic pathway, involving transporter proteins, may prevail at the latest stages of fruit maturation (Ruan and Patrick 1995, Zhang et al. 2006).

The current understanding of sugar transport biochemistry in higher plants has significantly increased during the past decade on account of the successful cloning of several genes and cDNA clones encoding sucrose and monosaccharide transport proteins isolated from several plant species. The biochemical properties of plant sugar transporters have mostly been elucidated through functional expression in yeast cells and Xenopus oocytes (reviewed by Büttner and Sauer 2000). All transporters characterized so far are plasma membrane carriers functioning as energy-dependent H\(^+\) symporters. MSTs are members of the major facilitator superfamily (MFS) and are characterized by the presence of 12 transmembrane-spanning domains arranged in a (6+6)-a-helical configuration separated by a large cytoplasmic loop. MSTs are found as multigene families. The tissue and cellular expression patterns of these genes indicate their specific and sometimes unique physiological roles. Some only play a nutritional role and supply sugars to cells for their growth and development, whereas others are also involved in the generation of osmotic gradients required to produce mass flow or movement. Plant cells appear to possess sugars transporters tailored to meet their specific requirements at particular stages of development. Various levels of control regulate these sugar transporters during plant development, sugar status and when the normal environment is disturbed (Delrot et al. 2000, Williams et al. 2000).

As hexoses represent the main soluble components found in olive pulp, understanding of the key steps involved in long-distance transport, and particularly the partitioning and accumulation of monosaccharides in olive fruits may significantly impact the improvement of olive growth and productivity. Here we report the cloning and functional characterization of a full-length cDNA clone of an MST (OeMST2) from O. europaea, expressed throughout the plant. Furthermore, the expression of OeMST2 during ripening was studied, as well as the regulation of its activity by sugar in suspension-cultured cells.

**Results**

**Cloning of a monosaccharide transporter gene**

Hexose uptake in olive fruit is facilitated by the direct movement of sugars from the apoplasma into the flesh cells of the olive fruit. MSTs are postulated to be involved in this process. To identify potential cDNAs encoding MSTs, reverse transcription–PCR (RT–PCR) was performed using total RNA isolated from olive fruit and degenerate primers corresponding to conserved regions of MSTs from higher plants. An amplified cDNA fragment of 953 bp was obtained, cloned and sequenced. The comparison of the deduced amino acid sequence of this PCR product with the corresponding fragments of plant MSTs revealed high similarity at the protein level. Specific primers were designed to perform 5'- and 3'-RACE (rapid amplification of cDNA ends) in order to obtain the full-length cDNA sequence. This approach resulted in the isolation of an 1,816 bp sequence subsequently named OeMST2 (Olea europaea monosaccharide transporter 2, accession No. DQ087177).

OeMST2 has a 1,569 bp open reading frame potentially encoding a protein of 523 amino acid residues with a predicted molecular mass of 57.6 kDa and a pI of 8.76. The hydropathy pattern analyzed according to Kyte and Doolittle suggests the presence of 12 transmembrane-spanning domains with a large central hydrophilic fragment between transmembrane domains 6 and 7. This model is consistent with the structure proposed concerning the MSTs and DSTs identified so far. The predicted amino acid sequence was aligned with several plant sugar transporter-like sequences present in GenBank. The phylogenetic tree constructed from a Clustal alignment of the 30 sugar transporter homologs showed that OeMST2 is most closely related to the tobacco transporter NiMST1. Plant MSTs, sucrose transporters and polyol transporters form, as expected, three clearly distinct clusters (Fig. 1).

**Characterization of OeMST2 in Saccharomyces cerevisiae**

OeMST2 cDNA was inserted in the pDR195 yeast shuttle vector, and the pDR195-OeMST2 construction was checked by PCR and sequencing. The recombinant plasmid containing OeMST2 was used to transform
**S. cerevisiae** strain EBY.VW4000. This strain is unable to grow on hexoses as it lacks the ability to transport these sugars, but it is able to use maltose as a carbon and energy source. Complementation by pDR195-OeMST2 restored yeast growth on 0.1 mM glucose (Fig. 2A). Furthermore, yeast cells expressing OeMST2 were able to transport D-[14C] glucose at a much higher rate than control cells transformed with the empty vector, where glucose uptake was virtually undetected (Fig. 2B,C).

Initial uptake rates of 0.02–0.5 mM D-[14C] glucose by yeast cells complemented with OeMST2 followed Michaelis–Menten kinetics, indicating carrier-mediated transport by OeMST2 (Fig. 2C). A computer-assisted non-linear regression analysis (GraphPad Prism, version 4.0) yielded the following kinetic parameters: $K_m = 25 \pm 2 \mu M$ glucose and $V_{max} = 7.6 \pm 0.3 \text{nmol glucose min}^{-1} \text{mg}^{-1} \text{DW}$, characterizing OeMST2 as a high-affinity glucose transporter. D-[14C] Fructose uptake occurred at a very low rate in the concentration range studied (Fig. 2C) and, accordingly, D-fructose did not inhibit D-[14C] glucose uptake (Fig. 3). However, it cannot be excluded that high fructose concentrations may inhibit glucose uptake, as previously shown in suspension-cultured cells (Oliveira et al. 2002). Also, L-[14C] glucose uptake was residual (Fig. 2C) and L-glucose did not inhibit D-[14C] glucose transport (Fig. 3). These results suggest that both D-fructose and the analog L-glucose are not recognized by OeMST2.

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**Fig. 1** Phylogenetic tree showing the comparison between plant monosaccharide, polyol and sucrose transporters. The amino acid sequences were aligned and compared using DNASTAR-MegAlign software (Genetics Computer Group, Madison, WI, USA). Sequences were obtained through the National Center for Biotechnology Information via the BLAST site (Altschul et al. 1997).
Besides d-fructose and l-glucose, in order to determine in more detail the substrate specificity of OeMST2, the uptake of 0.5 mM D-[14C]glucose was studied in the presence of other putative competitors (Fig. 3A). Sucrose and d-mannitol did not significantly affect glucose transport, suggesting that disaccharides and polyols are not transported by OeMST2. The slight inhibition promoted by sucrose may be due to partial hydrolysis into glucose, mediated by invertase. d-Arabinose was not recognized by OeMST2. In contrast, d-galactose, and the glucose analogs 2-deoxy-d-glucose (2-dG) and 3-O-methyl-d-glucose (3-O-MG), severely inhibited D-[14C]glucose uptake. When the effect of all the sugars tested above was measured over 0.02–0.5 mM D-[14C]glucose uptake (Fig. 3B), it was observed that d-galactose, 2-dG and 3-O-MG behave as competitive inhibitors, suggesting that they are substrates of OeMST2; however, it cannot be excluded that they may merely compete for the carrier binding site.

To study the energetics of OeMST2, D-[14C]glucose uptake was measured at different external pH values. $V_{\max}$ of OeMST2 was higher at pH 5 and decreased about 50% from pH 5 to 7.5 (Fig. 4A), suggesting that glucose uptake is dependent on the proton gradient. In addition, the uptake of 0.5 mM [14C]glucose (saturating concentration), at pH 5.0, was strongly inhibited (80.7 ± 0.13%; $n = 3$) by 50 μM of the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP), indicating that OeMST2 functions as an H+-dependent active transport system. When the effect of the protonophore was tested over the uptake of 0.02–0.5 mM D-[14C]glucose, a typical non-competitive inhibition pattern was observed (Fig. 4B). Since proton–sugar co-transport mechanisms are associated with a net influx of positive charges into the cells, the effect of dissipating the transmembrane electric potential on glucose uptake was also studied. The addition of the lipophilic and highly permeant cation tetraphenylphosphonium (TPP$^+$) also decreased the initial uptake rates of 0.5 mM D-[14C]glucose (40.8 ± 1.2%; $n = 3$) and promoted a non-competitive inhibition pattern, as shown in Fig. 4B. Taken together, these results are in accordance with what is described for most types of plant MSTs operating as symporters mediating the proton-driven transport of a wide range of hexoses and pentoses across the plasma membrane.

Expression of OeMST2 in different organs and tissues

To investigate the expression pattern of OeMST2 in different parts of the plant, total RNAs were extracted from leaves, flowers, roots and stems; and during different development stages of olive fruits. RNA blot analysis was performed using a gene-specific probe corresponding to the 3′-untranslated region of OeMST2. Expression of OeMST2 differed among various organs, with the OeMST2 transcript being more abundant in roots and mature leaves (Fig. 5A). Weaker signals were obtained from the RNA extracted from young leaves and young stems. OeMST2 expression was also high in flowers. Interestingly, a higher transcript amount was found in open flowers when compared with buds.

As referred to in the Introduction, substantial amounts of monosaccharides are present in olive fruit pulp, reaching 1.4 mg g DW$^{-1}$ (Marsilio et al. 2001). To study the involvement of OeMST2 in monosaccharide unloading during olive fruit maturation, RNAs were isolated from olive fruits at the green, cherry and black stages, and the expression of OeMST2 was studied. Fig. 5B shows that, although detectable throughout fruit development, OeMST2 transcript levels have strongly accumulated in the green stage, and decreased during the cherry stage,
Fig. 3  Specificity of monosaccharide uptake mediated by OeMST2. (A) Effect of several mono- and disaccharides, mannitol and glucose analogs on the initial uptake rates of 0.5 mM d-[14C]glucose. Error bars represent the SD from the mean, n = 3. (B) Uptake of 0.02–0.5 mM d-[14C]glucose (filled squares) challenged by an excess (2 mM) of unlabeled sugar: sucrose (open diamonds), α-mannitol (open inverted triangles), α-fructose (filled circles), α-arabinose (open triangles), α-galactose (open squares), 2-deoxy-α-glucose (filled inverted triangles), 3-O-methyl-α-glucose (filled diamonds) and l-glucose (open circles). Insert: Eadie–Hofstee plot of the initial uptake rates of d-[14C]glucose.

Fig. 4  Energetics of glucose uptake mediated by OeMST2. (A) pH dependence of d-[14C]glucose transport. (B) Eadie–Hofstee plots of the initial uptake rates of d-[14C]glucose in the absence (filled circles) and in the presence of 0.03 mM CCCP (filled triangles) and 10 mM TPP⁺ (filled squares). Error bars represent the SD from the mean, n = 3.

Fig. 5  Expression pattern of OeMST2 in the different organs and tissues of O. europaea. (A) A 20 μg aliquot of total RNA from roots (R), mature leaves (ML), young leaves (YL), young stems (YS), open flowers (OF) and closed flowers (CF) was hybridized with an OeMST2-specific probe from the 3’-untranslated region. (B) RNA blot analysis of OeMST2 in olive fruit at different maturation stages. aDetermined by Marsilio et al. (2001). Methylene blue staining shows the quality and quantity of total RNA loaded in each well.
reaching their maximal value during the late stage of ripening (black stage).

**Sugar regulation of OeMST2 expression**

Since sugar transporters play a key role in source–sink interactions, it is likely that their expression and activity are tightly regulated by the surrounding sugar levels (Roitsch 1999). Plant organs are often not accessible to study the mechanisms and regulation of sugar uptake, because bulk diffusion, tissue penetration barriers and cell heterogeneity impair kinetic studies. Therefore, to understand how OeMST2 is regulated by sugar, monosaccharide transport activity and expression patterns of OeMST2 were studied in O. europaea suspension-cultured cells in mineral medium with glucose as carbon and energy source. Although it is recognized that suspension-cultured cells may not be close to normal physiological conditions of sink tissues, they provide a convenient experimental system that has already yielded a lot of useful information on sugar transport mechanisms and regulation because plasma membrane is easily accessible to exogenous sugars, sugar analogs and transport inhibitors (Roitsch and Tanner 1994, Ehness and Roitsch 1997, Oliveira et al. 2002, Cakir et al. 2003, Conde et al. 2006, Conde et al. 2007a).

To test the regulatory effect of glucose on OeMST2 expression, suspension-cultured cells were cultivated with 0.5% (w/v) glucose, and OeMST2 transcript levels were studied by RNA blot with cell aliquots harvested at various time periods. The typical behavior of the cells in culture is depicted in Fig. 6A, confirming earlier data of Oliveira et al. (2002). Although detectable since day 3, OeMST2 mRNA increased abruptly after day 5, when the glucose concentration of the medium declined below 0.025% (w/v), the highest value being reached at day 7, when glucose was completely exhausted from the culture medium. In the same cell samples, measurements of the initial uptake rates of D-[14C]glucose revealed that the activity of the H⁺-dependent monosaccharide transport system started to increase from basal levels at day 5, the maximum transport activity being detected at day 7. This transient sequential phenomenon reflects a close relationship between OeMST2 transcript and glucose transport activity, suggesting that glucose-mediated regulation of carrier expression is mainly controlled at the transcriptional step, although post-transcriptional regulation cannot be ruled out.

To determine if the pattern of OeMST2 expression presented above is the result of general glucose repression, 150 mM glucose was added to cells collected at day 7, displaying high H⁺-dependent monosaccharide transport activity and high levels of OeMST2 mRNA. OeMST2 transcripts completely disappeared 24 h after the glucose pulse, and glucose transport activity measured in the same cell samples dropped to basal levels. The addition of polyethylene glycol (PEG), at an osmotic pressure equivalent to 150 mM glucose, did not inhibit glucose uptake activity, demonstrating that sugar repression is not due to an osmotic effect (not shown), in accordance with previous glucose uptake studies in grape cells (Conde et al. 2006). As previously observed (Oliveira et al. 2002), the addition of mannitol, which is not transported by OeMST2,
also repressed the activity of the monosaccharide transport system, probably because this polyol represents an important carbon and energy source in \textit{O. europaea} (Conde et al. 2007a). In contrast, sucrose did not repress the monosaccharide transport system. Several studies have described sugar-mediated changes in gene expression, and recent research provided convincing evidence that hexoses and hexose kinases (HXKs) play an important role in this pathway for some plant MSTs (Conde et al. 2006). To test the possible involvement of this signaling pathway, several additions were made to the cells: (i) 150 mM \(\alpha\)-glucose in the presence of the HXK inhibitor mannoheptulose (MHL); (ii) 150 mM 3-O-MG, a glucose analog which cannot be phosphorylated; and (iii) 150 mM 2-dG, an analog that can be phosphorylated, but is not further metabolized. The results showed (Fig. 6B) that treatment of cells with \(\alpha\)-glucose plus MHL or 3-O-MG alone resulted in derepressed levels of \textit{OeMST2} transcripts. However, in both situations, \(H^+\)-dependent monosaccharide transport activity decreased to basal levels, suggesting that \textit{OeMST2} expression may be regulated post-transcriptionally. The addition of 2-dG strongly decreased both the amount of \textit{OeMST2} transcripts and glucose transport activity. Taken together, these data suggest that the glucose repression pathway described herein is mediated by HXK as the sugar sensor.

**Discussion**

This work describes the cloning of a full-length cDNA sequence of an MST from \textit{O. europaea} and the characterization of the corresponding gene product by heterologous expression in the yeast \textit{S. cerevisiae}. \textit{OeMST2} was isolated from RNA extracted from olive fruits, an organ that accumulates high concentrations of glucose, fructose and galactose. The predicted amino acid sequence shares a high overall homology with other MST sequences identified in plants, the highest homology being found in \textit{Nicotiana tabacum} MST1. The in silico predicted peptide sequence contains amino acid motifs conserved between members of the MFS and the amino acid signatures of typical sugar transport proteins. Possible post-translational modifications are suggested by the presence of four putative protein kinase C phosphorylation sites and four putative casein kinase II phosphorylation sites. This may suggest a possible involvement of phosphorylation in the regulation of \textit{OeMST2} activity as suggested by the sugar beet sucrose transporter (Roblin et al. 1998). Additionally, \textit{OeMST2} protein has several consensus sequences for potential N-glycosylation and some putative N-myristoylation sites (Expasy tools, ScanProsite). These potential post-translational modifications of the protein may also affect its sugar transport activity.

Heterologous expression of \textit{OeMST2} in an \textit{hxt-null} strain of \textit{S. cerevisiae} confirmed its function as a high-affinity, broad specificity, monosaccharide: \(H^+\) symporter and hence a member of the MST family that comprises sugar carriers transporting a wide range of hexoses and pentoses, with \(K_m\) values for the preferred substrates between 10 and 100 \(\mu\)M (reviewed by Büttner and Sauer 2000). Clues about the physiological role of \textit{OeMST2} may be inferred from the expression pattern in various organs and during fruit development. \textit{OeMST2} transcripts are abundant in roots, flowers, mature leaves and mature fruit. Since flowers are one of the most active sink organs, \textit{OeMST2} might be important in supplying substrates for the active metabolism that occurs in flowers, particularly in the open state. The Arabidopsis monosaccharide transporters \textit{AtSTP6} and \textit{AtSTP11}, for instance, are specifically expressed in flowers and may play a role in the supply of monosaccharides to growing pollen tubes (Scholz-Starke et al. 2003, Schneiderreit et al. 2005). Also in maize, some hexose transporters show preferential expression in late stages of pollen maturation (Datta et al. 2002). A high expression of \textit{OeMST2} in the roots of olive trees, another typical sink organ, may be essential to the import of sugar from the apoplast. \textit{OeMST2} expression was also detected in other sink tissues such as young stems and young leaves, although at a much lower level. In contrast to roots and flowers, which are permanent sinks, young leaves are considered to be sink organs while mature leaves are considered as source organs. The results of RNA blots showed that \textit{OeMST2} is highly expressed in mature leaves (Fig. 5) where it may function as a mechanism for retrieval of carbohydrate leaked into the leaf apoplasm. Indeed, HPLC analysis showed that glucose together with mannitol represent the main soluble sugars in olive leaves (Cataldi et al. 2000). A striking feature of \textit{OeMST2} expression in olive trees is the relatively high accumulation of transcripts that occurs during olive fruit maturation, reaching its maximal level in fully mature black olives, suggesting that monosaccharide transport and compartmentation by \textit{OeMST2} are important to allocate this source of carbon and energy. A differential expression of the mannitol carrier \textit{OeMaT1} was also recently reported during olive fruit maturation (Conde et al. 2007a). Taken together, these results support the involvement of an apoplastic step in the unloading of both sugars and polyols in \textit{O. europaea}, and that both mannitol and sugar carriers play an important role during olive fruit ripening.

Although recent advances have provided a detailed understanding of several key steps in assimilate partitioning, there is little knowledge of the regulatory pathways that control resource allocation. The ability to sense altered sugar concentrations is important in the context of resource allocation, allowing the plant to tailor its
metabolism in source tissues to face the demands in sink tissues. Given the crucial role of MSTs in source–sink interactions it is not surprising that their expression and activity are tightly regulated by sugar levels. Evidence is provided here to show that in *O. europaea* cells, alterations in glucose levels have a pronounced effect on *OeMST2* expression and on proton-coupled glucose transport activity (Fig. 6A). *OeMST2* transcription is negatively regulated by high glucose levels. Accordingly, H⁺-dependent monosaccharide transporter activity is absent when there is a high sugar concentration in the medium. When external glucose decreases to residual levels, a sharp increase in *OeMST2* expression and consequently in monosaccharide:H⁺ transport system activity is observed (Fig. 6A). Down-regulation of *OeMST2* expression by glucose was further supported by the observation that the addition of 150 mM glucose to cells exhibiting high *OeMST2* mRNA levels and a high H⁺-dependent monosaccharide transport activity promoted a decline of *OeMST2* transcripts to undetected levels within 24 h. Accordingly, the activity of the monosaccharide transport system dropped to residual values.

The mechanisms involved in glucose uptake by glucose-sufficient cells of *O. europaea* have been recently studied in detail. In a high sugar concentration, the saturable component is absent and glucose is exclusively absorbed by a non-saturable mechanism able to sustain both cell growth and metabolism. The data demonstrate that the low-affinity, high-capacity, diffusional component of glucose uptake occurs through an Hg-sensitive channel whose transport capacity may be regulated by intracellular protonation and phosphorylation/dephosphorylation (Conde et al. 2007b).

Several studies have demonstrated the role of hexoses as the active signal molecule in sugar sensing in higher plants, and convincing evidence has been presented that substrate flux through HXK is a key step in the sugar-sensing transduction pathway. However, HXK-independent signaling has also been proposed for the regulation of gene expression directly by the disaccharides sucrose or trehalose mediated by a possible plasma membrane sensor (reviewed by Rolland et al. 2006). In the results reported here, a glucose-dependent signaling pathway is described that regulates the expression level of *OeMST2* in an HXK-dependent manner similar to what was recently found in *Vitis vinifera* for *VvHT1* expression (Conde et al. 2006), a monosaccharide transporter characterized in grape berry (Vignault et al. 2005). The way plant cells respond and adapt to shifts in environmental conditions such as the abundance of glucose involves different levels of regulation. In olive cells, the clear parallel between *OeMST2* transcripts and *VvHT1* expression of the monosaccharide:H⁺ symporter system suggests that it is mediated, at least in part, by a drop in transcriptional activity or mRNA stability. However, in the presence of α-glucose plus mannoheptulose, or 3-O-MG alone, *OeMST2* is transcribed but glucose uptake is low (Fig. 6B), indicating that it may also be regulated at a post-transcriptional level. Recently, in grape cells, immunoblot analysis with an anti-VvHT1 antibody showed that steady-state protein levels and *VvHT1* message levels do not always correspond, supporting that *VvHT1* is also repressed at a post-transcriptional level in response to high glucose concentrations (Conde et al. 2006). We propose, therefore, that the observed glucose repression of *OeMST2* expression probably operates via a HXK-generated signal that impairs *OeMST2* transcription and by means of HXK-independent post-transcriptional regulation. However, the possibility that *OeMST2* may not be the sole hexose transporter contributing to overall saturable glucose uptake by suspension-cultured cells cannot be completely discarded at the present time. Nevertheless, the idea that *OeMST2* is the major contributor to monosaccharide:H⁺ symporter activity in olive suspension-cultured cells is supported by the fact that cell suspensions and yeast expressing *OeMST2* as the sole MST displayed similar kinetic properties (*Kₘ* and substrate specificity). Furthermore, there was a close parallelism between the amount of *OeMST2* transcripts and glucose uptake during monosaccharide depletion from the culture medium.

After the identification of the H⁺-hexose co-transporter from *Chlorella kessleri*, HUP1 (Sauer and Tanner 1989), and subsequent demonstration of HUP1 as a glucose transporter by heterologous expression in yeast (Sauer et al. 1990), major progress has been achieved on plant sugar transporters in terms of gene identification and functional analysis. Such advances were strongly enhanced by the heterologous expression approach that nowadays is an important tool to explore the structure–function relationship of the transporter proteins through mutational analysis and some aspects of post-transcriptional regulation. However, much less is known about the regulation of transport activity in relation to gene expression in planta. In fact, the limitations that arise from the investigation of plant transporters within a heterologous expression system (transcriptional and translational processes) can only be circumvented by homologous expression of the cloned transporters in suspension-cultured cells and plant organs (reviewed by Dreyer et al. 1999). This allows physiological analysis in a cell environment closely related to that of the native cell of the cloned transporter.

The isolation of *OeMST2* cDNA from *O. europaea*, the functional characterization of the encoded protein as a monosaccharide:H⁺ transporter by heterologous expression in yeast, the expression pattern of *OeMST2*
in vegetative and reproductive organs of olive plants and, finally, the regulation of OeMST2 in suspension-cultured cells, possibly mediated by a HXK-dependent signaling pathway, contribute to a better understanding of sugar transport in *O. europaea* and more generally in fleshy fruits.

**Materials and Methods**

**Plant material, cell suspensions and growth conditions**

Cell suspensions of *O. europaea* L. var. Galega Vulgar were maintained in 250 ml flasks on a rotatory shaker at 100 r.p.m., in the dark, at 25 °C on modified Murashige and Skoog (MS) medium (Murashige and Skoog 1962), supplemented with 0.5% (w/v) glucose. Cells were subcultured weekly by transferring 10 ml aliquots into 70 ml of fresh medium. Growth was monitored by determination of dry weight for estimation of the maximum specific growth rate (μ<sub>max</sub>). Aliquots of 1–5 ml were filtered through pre-weighed GF/C filters (Whatman, Clifton, NJ, USA). The samples were washed with deionized water and weighed after 24 h at 80 °C. Sugar consumption was monitored by HPLC.

**Transport tests in suspension-cultured cells**

The harvested cells were centrifuged, washed twice with ice-cold culture medium without sugar at pH 4.5, and resuspended in the same medium at a final concentration of 5 mg DW ml<sup>−1</sup>. To estimate the initial uptake rates of d-[U-14C]glucose, 1 ml of cell suspension was added to 10 ml flasks, under shaking (100 r.p.m.). After 2 min of incubation, at 25 °C, the reaction was started with the addition of 40 μl of an aqueous solution of 0.02-0.5 mM radiolabeled sugar (specific activity: 500 d.p.m. mmol<sup>−1</sup>). The reaction was stopped by dilution with 5 ml of ice-cold modified MS medium without sugar, and the mixtures were immediately filtered through GF/C filters (Whatman, Clifton, NJ, USA). The filters were washed with 10 ml of the same medium and transferred to vials containing scintillation fluid (OptiPhase HiSafe II; LKB Scintillation Products). The radioactivity was measured in a Packard Tri-Carb 2200 CA liquid scintillation counter (Packard Instruments Co., Inc., Rockville, MD, USA).

**Cloning of OeMST2 cDNA**

To isolate the OeMST2 clone, degenerate primers were designed from conserved regions of plant hexose transporter cDNA sequences obtained from GenBank. The sequences of the primers were as follows: forward primer, 5'-TGY CHT CCG GWT TGW TIA CIT CHA TG-3'; reverse primer, 5'-GGI CCC CAI GAC CAI GCR AA-3'. RT-PCR was performed on total RNA extracted from olive fruits harvested at the black stage of fruit maturation. The amplified 953 bp fragment was cloned into the pGEM-T easy vector (Promega, Madison, WI, USA) according to the manufacturer's instructions and sequenced.

To obtain the 5' and 3' ends of the final cDNA, RACE-PCR was conducted on the cDNA previously described, using a Clontech SMART RACE kit. The primers were designed according to the partial OeMST2 sequence isolated: OeMST2 5'RACE primer, 5'-TGC TGC GCT GTG TGC AAG CTT TGC G-3'; and OeMST2 3'RACE primer, 5'-ATC CTC CCA TCC GGA TGA ATT-3'.

The total cDNA sequence of OeMST2 was obtained by PCR, through the combination of the information contained in the 5' and 3'RACE products. The forward primer (defined on the 5'RACE product) and the reverse primer (defined on the 3'RACE product) were 5'-ACT TAT AAT CAA AAT GCC CG-3' and 5'-ACC ATT GCC ATT CTA CAA TTC AA-3', respectively. The PCR product was cloned into the pGEM-T easy vector (Promega, Madison, WI, USA) according to the manufacturer's instructions, sequenced and subsequently named OeMST2.

**Heterologous expression of OeMST2 in *S. cerevisiae**

OeMST2 cDNA was excised from the pGEM-T easy vector by a NotI digestion. The pDR195 vector was linearized by a NotI digestion and the extremities generated were dephosphorylated using the SAP enzyme to prevent religation following the manufacturer's recommendations (Fermentas, Life Science). The OeMST2 cDNA fragment was inserted in the corresponding restriction sites of the pDR195 yeast shuttle vector and the construct checked by sequencing. The pDR195-OeMST2 construct and the pDR195 plasmid were used to transform the EBY.VW4000 *S. cerevisiae* strain by a PEG-based method (Dohmen et al. 1991). The EBY.VW4000 strain is completely deficient in glucose uptake due to multiple mutations on the hexose transporters (Wieczorke et al. 1999), but it can grow on a maltose medium. The growth phenotype of the transformants was tested on Yeast Nitrogen Base (YNB) medium depleted of URA and containing glucose or maltose.

**Transport tests in *S. cerevisiae***

*S. cerevisiae* strain EBY.VW4000, carrying either pDR195-OeMST2 or empty pDR195, was grown in YNB maltose medium at 30 °C to an OD<sub>600</sub> of approximately 0.8. Cells were harvested by centrifugation, washed twice with ice-cold distilled water, and suspended in distilled water at a final concentration of about 40 mg DW ml<sup>−1</sup>. To estimate initial uptake rates of radiolabeled sugar, 10 μl of cell suspension was mixed with 30 μl of 50 mM potassium phosphate buffer at pH 5.0 in 10 ml conical tubes. After 2 min of incubation at 26 °C in a water bath, the reaction was started by the addition of 10 μl of an aqueous solution of the radiolabeled sugar with 8,000 d.p.m. mmol<sup>−1</sup> (5-[14C]glucose and L-[14C]glucose) or 16,000 d.p.m. mmol<sup>−1</sup> (d-[14C]fructose) at the desired concentration. Potential sugar competitors or metabolic inhibitors were added to the reaction mixture 10 s prior to the addition of radiolabeled glucose. The reaction was stopped by dilution with 5 ml of ice-cold water, and the mixtures were immediately filtered through GF/C filters (Whatman, Clifton, NJ, USA). The filters were washed with 10 ml of the same medium and transferred to vials containing scintillation fluid, and the radioactivity was measured as indicated above. l-[14C]Glucose (55 mCi mmol<sup>−1</sup>) and d-[14C]fructose (316 mCi mmol<sup>−1</sup>) were obtained from Amersham Biosciences (Little Chalfont, UK).

**RNA gel blot analysis**

Total RNAs from olive suspension-cultured cells were isolated by phenol extraction and 2 M LiCl precipitation (adapted from Howell and Hull 1978). Total RNAs from olive leaves, stems, roots, flowers and olive fruits, harvested at green, cherry and black stages of ripening, were isolated using the hot borate method adapted from Wan and Wilkins (1994). This method produced high yields of total RNA. Total RNAs isolated from leaves, stems, roots, flowers and olive fruits, harvested at green, cherry and black stages of ripening, were isolated using the hot borate method adapted from Wan and Wilkins (1994). This method produced high yields of total RNA. The RNA sample was separated by formaldehyde-agarose gel electrophoresis and transferred onto Hybond N membrane (Amersham Life Science). RNA blot analysis was conducted using a gene-specific probe.
[32P]OMST2 cDNA probe obtained from the 3'-untranslated region and radioactive signals detected by imaging (Bio Rad Personal Molecular Imager FX, Bio Rad Laboratories, Hercules, CA, USA).

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References


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