# Solute transport across plant cell membranes

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## ABSTRACT

The transport of solutes across cell membranes, including organic nutrients, such as sugar, osmolytes, ions or metabolic waste products, is of extreme importance in all-living systems. Up to 14% of the genome of all organisms represents information for transport proteins, which reflects the importance of such process. Transporters are also involved in the transduction of environmental and endogenous signals. Several transport systems have been identified and fully characterised at both molecular and biophysical levels in a wide variety of living organisms, from bacteria to humans, with the bacterial lactose permease (LacY) being a good example of such successful studies. The majority of transporter proteins are very well conserved throughout living systems, and some of them, such as sugar transporters, belong to a large family (SP, Sugar Porter). In higher plants, the photoassimilated carbon is transported from mature leaves throughout the phloem, mainly in the form of sucrose, as in the grapevine, or mannitol, as in the olive tree, to heterotrophic organs such as developing leaves, flowers, fruits and roots, which rely on its supply for their growth and development. Thus, the unlocking of the mechanisms of photoassimilate transport into plant sink tissues, as well as their regulation, has an important basic and applied relevance. Moreover, as most living organisms, plants also face a continuous battle against adverse environmental factors like increasing soil salinity, heat and drought. In this context, solute transport also has a relevant role in plant defence. For instance, the efficient exclusion of $Na^+$ excess from the cytoplasm and vacuolar $Na^+$ accumulation are the most important steps towards the maintenance of ion homeostasis under salt stress. The production, transport and accumulation of compatible solutes like mannitol are also important plant responses to salinity and drought. Like animals, where important diseases such as depression and hypertension are commonly treated with drugs targeted to specific transporters, plants have also benefited from the extensive and ongoing study of membrane transport. The present review provides an overview on the investigation that has been conducted in our laboratory under the scope of this fascinating topic.

## 1. INTRODUCTION

### 1.1. Membrane transport – fundamental aspects

Plants are full of solutes, both inorganic ions and low-molecular-mass organic molecules, and expend considerable amounts of energy and resources in acquiring or synthesising these solutes. Solute transport occurs over a large range of scale, some 10 orders of magnitude, from the order of 10 nm to cross a cell membrane to the order of 100 m to ascend the tallest tree. But the nature of events and driving forces that underlie transport over such huge differences in scale are extremely different for the same solute. A potassium ion carried to the top of a tree in the xylem is dissolved in water, but a potassium ion being transported across a membrane by a carrier is not in solution but instead is bound reversibly to a transporter protein [1].

Transport of solutes such as nutrients, ions or metabolic waste products across cell membranes is of the utmost importance in all-living systems (reviewed by [2]). This is reflected in the observation that up to 14% of the genome of all organisms represents information for transport proteins. Almost all transmembrane transport processes are conducted by integral membrane proteins, sometimes functioning in association with extracytoplasmic receptor proteins and/or cytoplasmic energy-coupling and regulatory proteins. Transport proteins are often referred to as transporters, carriers, permeases, porters or channels. An extensive database and detailed description of the Transport Classification (TC) system, approved by the International Union of Biochemistry and Molecular Biology (IUBMB), is available on http://www.tcdb.org/ comprising all relevant biochemical, biophysical, physiological, genetic, and sequence data presently known. A

transporter mediates either **facilitated diffusion** or **active transport** of solutes and metabolites in or/and out of the cell. Facilitated diffusion, which is the simplest process that a transporter can mediate (in that case also called facilitator), is an equilibrative protein-mediated process that is not coupled to metabolic energy and, therefore, incapable of giving rise to concentration gradients of the transported substrate across the membrane. Active transporters transduce free energy stored in an electrochemical ion gradient (**secondary active transport**) or released by hydrolysis of ATP (**primary active transport**) into a substrate concentration gradient across a membrane. Other primary sources of free energy for primary active transport are redox energy and light. Channels mediate the passage of solutes, usually ions or water [3], in a diffusion-limiting manner from one side of the membrane to the other via a pore formed by specific residues of the constituent protein. Frequently, channels serve as selective ion-conducting pores, many of which open in response to a gating event to move ions down an electrochemical gradient. However, many other hydrophilic and hydrophobic substrates are also transported by these transport systems. Facilitators and ion channels do not transduce energy (reviewed by [2, 4, 5]).

The glucose uptake mechanisms and its kinetics in *Saccharomyces cerevisiae* were studied in detail over the last 30 years, but several conclusions were not easily accepted among different groups. Transport studies in this yeast have produced complex multiphasic uptake kinetics and considerable doubts prevailed on whether the incorporation of glucose was resultant of two different saturable uptake systems or of a single saturable component complemented by a first-order uptake kinetics. However, glucose uptake studies performed in yeast strains mutated in all hexose transporter (HXT) genes known to be responsible for transporter-mediated saturable transport revealed a total absence of sugar transport, including its linear component. This evidence suggests the possibility that several gene products with distinct substrate affinities work simultaneously in *S. cerevisiae* to produce complex multiphasic kinetics in glucose uptake. The molecular identification of transport systems becomes even more complicated considering that high- and low-affinity uptake systems might not necessarily be produced by distinct genes. These studies in *S. cerevisiae* challenged some major dogmas on membrane transport biochemistry and validated the importance of working in microbial model systems where single gene knock-outs and genetic, biochemical and molecular analyses can be easily performed (reviewed by [2]).

The continued study of the mechanisms and regulations of solute transport across plant cell membranes has an important basic and applied relevance. The transport of solutes like sugars, organic acids, minerals, protons and water across the plasma membrane and cytosolic membranes of plant cells, through specific transport systems, has important implications for cell homeostasis and growth, and thus to plant physiology. The same applies to nutrient acquisition from the soil, which is also governed by the general rules of membrane transport [6]. Solute transport across plant cell membranes is the main topic of the investigation in progress in our laboratory and is addressed in the present review.

### 1.2. Sugar transport in plants

The transport of assimilates throughout the phloem provides the materials needed for the build-up of herbaceous plants and trees and has long been recognised as a major determinant in crop yield. Indeed, 80% of the carbon photosynthetically fixed in the leaf is exported through the plant vascular system to the roots, reproductive structures, and storage and developing organs, depending on the supply of sugars for their growth and development. Several improvements in yield potential have resulted more from an increase in the proportion of accumulated carbon in the harvestable organs than from genetic increases in photosynthesis. Thus, it is of the utmost importance to understand the mechanisms and regulations of sugar transport into plant sink tissues ([7] and references therein). In sink organs, sucrose from the phloem can be imported from the apoplast via direct sucrose transporters (DSTs). Alternatively, it can be hydrolysed to glucose and fructose by cell wall-bound invertases and taken up via monosaccharide transporters (MSTs) (Figure 1) (reviewed by [8]). Many MSTs and DSTs have been characterised from a molecular and functional standpoint in herbaceous plants, but much less has been done in sugar transporters from ligneous species such as *Olea europaea* and *Vitis vinifera*.

The first MST cDNA identified in higher plants was *AtSTP1* (*Arabidopsis thaliana* Sugar Transporter Protein 1). It was functionally characterised as a hexose-proton **symporter**, by complementation of hexose transport null-mutant yeasts [9]. Since then, many clones have been identified in various plant species where they belong to multigene families: the *Arabidopsis* genome displays 53 homologous sequences encoding putative MSTs, distributed into 7 distinct clusters ([10], reviewed by [11]). The kinetic properties of the encoded proteins have been studied mainly by heterologous expression in yeasts or *Xenopus* oocytes and all the transporters characterised so far are energy-dependent $H^+$-symporters [12].

Analysis of expression shows that plant MSTs are highly regulated, such as in response to pathogen attack [13] or after wounding, but the mechanisms underlying regulation by sugar levels are not well understood. While in the unicellular green alga *Chlorella* and in *V. vinifera* berries some hexose carrier genes are induced by the substrate genes encoding hexose transporters, in *Chenopodium rubrum* they are constitutively expressed and not regula-

ted by the sugar ([7] and references therein, [14]). Some studies developed in our laboratory have addressed this topic as referred to below.

### 1.3 Two important plant models to study photoassimilate transport: *Vitis vinifera* and *Olea europaea*

Grapes (*Vitis* spp.) are economically the most important fruit species in the world. The development and maturation of grape berries have received considerable scientific interest because of both the uniqueness of such processes to plant biology and the importance of these fruits as a significant component of the human diet and wine industry. For the winemaker, the most important attribute of *V. vinifera* is its capacity to store enormous quantities of sugar, phenolics and other key compounds in the berries. From the plant point of view, the ripe phenotype is the summation of biochemical and physiological changes that occur during fruit development and make the organ edible and desirable to allow the dispersion of the seeds by animal activity. Thus, the understanding of how and when various components accumulate in the berry, including sugars, organic acids, phenolics, ions, etc., is of critical importance to adjust grape growing practices and thus modify wine typology. Molecular and biochemical studies of grape berry development and ripening conducted by several research groups worldwide, including our own (see section 3.2 in this review), have resulted in significant gains in knowledge over the past years (review by [15]).

*O. europaea* is a symbolic species and one of the most important and widespread fruit trees in the Mediterranean basin. Today, olive is one of the most extensively cultivated fruit crops in the world. Over 750 million olive trees are cultivated worldwide, with about 95% being located in the Mediterranean basin. In Portugal, *O. europaea* has a wide distribution, with a cultivated area of 430,000 hectares, predominantly in the centre and southern areas of the country. It represents an important economic and environmental species, making Portugal the eighth major olive producing country in the world, accounting for 280,000 tons of table olives and 40,000 tons of olive oil production per year. Olive oil has a well-balanced composition of fatty acids, with small amounts of palmitate, and it is highly enriched in the moneonic acid oleate. This makes it both fairly stable against auto-oxidation and suitable for human health. Nevertheless, it is the presence of minor components, in particular phenolics, contributing to the oil's high oxidative stability, colour and flavour, that makes olive oil unique among other oils. The biochemistry of the olive tree is also particular. *O. europaea* is one of the few species able to synthesise both polyols (mannitol) and oligosaccharides (raffinose and stachyose) as the final products of the photosynthetic $CO_2$ fixation in the leaf. Further discussion on this topic will be addressed later in this paper (see section 4.1). These carbohydrates, together with sucrose, can be exported from leaves to fruits to fulfil cellular metabolic requirements and act as precursors to oil synthesis. Additionally, developing olives contain active chloroplasts capable of fixing $CO_2$, which contribute to the carbon economy of the fruit (reviewed by [16]).

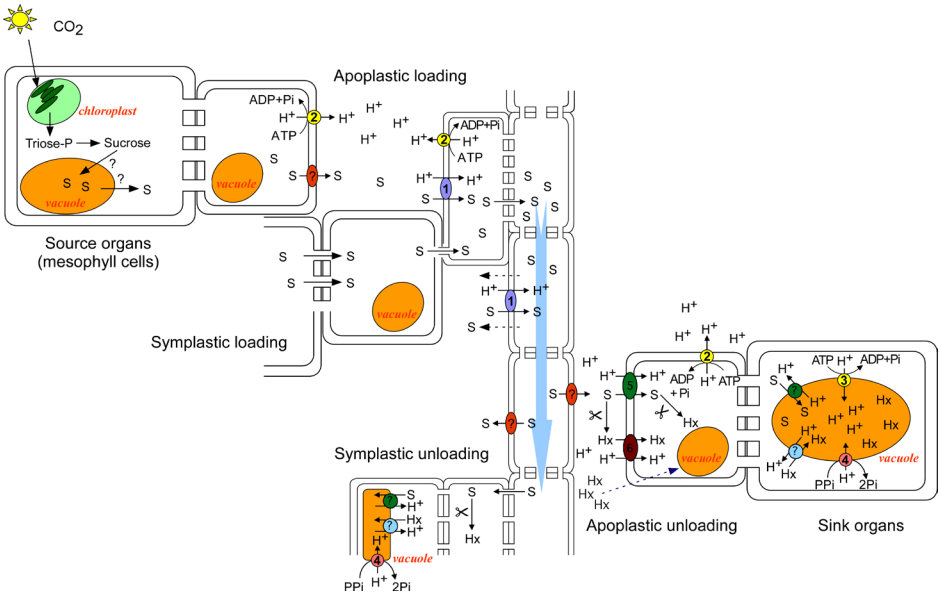


Figure 1: Speculative model for long-distance sugar transport through the phloem in higher plants (adapted from [60]). In the mesophyll sucrose may be loaded into de sieve elements/companion cell complex either through plasmodesmata or via the apoplast. Apoplastic loading requires sucrose export from the mesophyll or the vascular parenchyma and reuptake into the sieve elements/companion cells (SE/CC) complex. Hydrostatic pressure drives phloem sap movement toward sink tissue. Apoplastic phloem unloading implies the existence of a sucrose exporter (yet unidentified) at the sink tissue. Import of sucrose and other solutes into sink tissue may occur through plasmodesmata or sucrose transporters. In addition to plasmodesmal and transporter mediated uptake, cells in the sink take up hexoses after sucrose hydrolysis by an apoplastic invertase. Apoplastic sugars may be incorporated by endocytosis (see figure 10) or through low-affinity transport systems (blue dotted arrow; [7, 44, 51, 52]). The vacuolar transport system may consist of sucrose/$H^+$ antiporters and hexose/$H^+$ antiporters, or uniporters (not shown), but the knowledge about sugar transport mechanisms across the tonoplast is limited. (1) Source sucrose/$H^+$ symporter; (2) plasma membrane $H^+$-ATPase; (3) tonoplast $H^+$-ATPase; (4) pyrophosphatase (V-$H^+$-PPase); (5) sink sucrose/$H^+$ symporter; (6) hexose/$H^+$ symporter; ✂, invertase.

## 2. SOLUTE TRANSPORT ACROSS THE TONOPLAST

### 2.1 The vacuole of plant cells and tonoplast transporters

Vacuoles fulfil highly specialised functions depending on cell type and tissue, and plant developmental stage. They are widely diverse in form, size, content, functional dynamics and play central roles in plant growth, development and stress responses. They have pivotal roles in pH and ion homeostasis, turgor pressure maintenance, protein

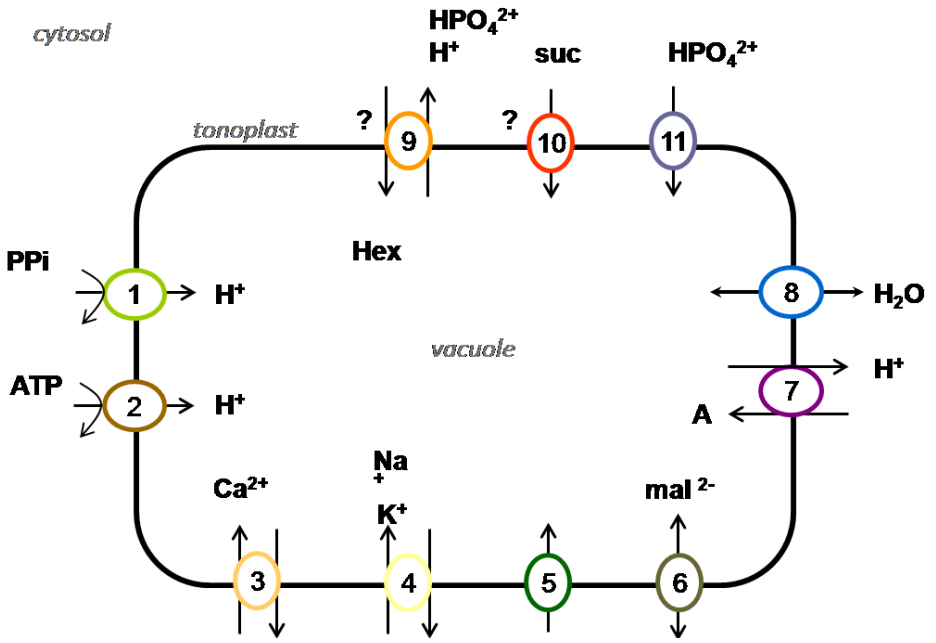


Figure 2: Grape berry vacuolar transport systems identified at molecular level or postulated from transport experiments. 1, V-H⁺-PPase; 2, V-H⁺-ATPase [28, 61]; 3, $Ca^{2+}/H^+$ antiport system [28]; 4, cation/H⁺ antiporter (VvNHX; [62]); 5, malate (mal) transporter (VvtDT; [40]); 6, malate channel (VvALMT9; [40]); 7, MATE transporter implicated in the uptake of acylated anthocyanin (A) [63]; 8, tonoplast intrinsic proteins (TIPs; [64]); 9, monosaccharide transporter; 10, sucrose (suc) transporters; 11, phosphate transporter (N. Fontes and co-workers, unpublished data, 2011). PPi, pyrophosphate. Reproduced with permission from the American Journal of Enology and Viticulture [18].

turnover, sequestration of toxic compounds and pigmentation. For instance, in the grape berry cells, the vacuole is the main reservoir of key solutes such as sugars and phenolics, playing a major role during fruit development and ripening. Berry development is accompanied by modifications in size, composition, colour, texture, flavour, and pathogen susceptibility, mostly due to changes in vacuolar content. The tonoplast membrane controls the passage of inorganic and organic solutes to and from the cytoplasm through a wide range of pumps, carriers, ion channels and receptors (Figure 2) (reviewed by [17-19]).

The electrogenic $H^+$ pumps V-H⁺-ATPase and V-H⁺-PPase are major components of the vacuolar membrane of plant cells. The V-H⁺-ATPase is present in the membranes of different internal acidic organelles in eukaryotic cells and has a complex structure: a peripheral $V_1$ sector which contains three copies of the A- and B-subunits, responsible for the catalytic activity, and the subunits C-H which form a central stalk linking $V_1$ to the hydrophobic membrane-embedded $V_o$ sector. The $V_o$ sector contains the a-subunit and six copies of the c-subunit, which form a proton conducting channel. As in their F-type homologues, where ATP is regenerated by induced conformational changes due to a rotatory mechanism, parts of the V-H⁺-ATPases have been shown to rotate when ATP is supplied, suggesting a very similar enzymatic mechanism for both proton pumps. In contrast to the V-H⁺-ATPase, the V-H⁺-PPase consists of a single polypeptide and exists as a dimer of subunits of 71–80 kDa. It is distributed among most land plants, but only in some algae, protozoa, bacteria and archaea, and it uses PPi as its energy source (reviewed by [17, 18]).

Vacuolar $Na^+/H^+$ antiporters have been investigated as the key to salt tolerance in plants. This topic has been extensively reviewed, including by our group [17]. The antiporter mediates transport of $Na^+$ into the vacuole and its activity was first demonstrated in red beet storage tissue by the group of Eduardo Blumwald (UC Davis, USA). Nass and co-workers [20] identified for the first time in yeast the $Na^+/H^+$ antiporter *Nhx1*. The first plant $Na^+/H^+$ antiporter, *AtNHX1*, was isolated from *Arabidopsis* [21, 22] and its overexpression suppressed some of the salt-sensitive phenotypes of the *nhx1* yeast strain [22]. Since then, several $Na^+/H^+$ antiporter genes have been characterised in plants such as rice, *Atriplex gmelini*, *Beta vulgaris*, *Brassica napus*, cotton, wheat and grapevine. Six AtNHX isoforms were found in *Arabidopsis*, and for five of them $Na^+/H^+$ transport activity has been demonstrated (see the references in [17]). AtNHX1 has nine transmembrane domains, with the hydrophilic C-terminal domain facing the vacuolar lumen and the N-terminus facing the cytosol. Three hydrophobic regions do not appear to span the tonoplast membrane and appear to be membrane-associated instead [23]. More recently, it was proposed that the C-terminal domain faces the cytosol and that the antiporter has 12 transmembrane domains [24].

Besides proton pumps and $Na^+/H^+$ antiporters, whose activity seems pivotal for plant response to salinity, several tonoplast proteins mediating the uptake of sugars, organic acids, water, ions, secondary compounds, etc. have been cloned in plant cells, some of them functionally characterised in heterologous systems. In a recent review we focus on the solute storage function of grape berry vacuoles, as well as on their structure and diversity relatively to the development and ripening of the grape berry [18].

### 2.2. Transport assays in tonoplast vesicles to study plant response to salinity

A significant part (approx. 20%) of the world's cultivated land and nearly half of the irrigated land are affected by salinity, which has become a serious threat to agricultural production (reviewed by [17]). Two stress factors are associated with excessive salinity: an osmotic component resulting from the reduced water availability caused by an increase in osmotic pressure in the soil, and an ionic stress resulting from a solute imbalance, causing changes in the $K^+/$

$Na^+$ ratio and increasing the concentration of $Na^+$ and $Cl^-$ in the cytosol. Sodium toxicity is mainly produced due to the similarity of $Na^+$ and $K^+$ ions to plant transporters and enzymes. Plant cells typically maintain a high $K^+$/ $Na^+$ ratio in the cytosol with relatively high $K^+$, in the order of 100-200 mM, and low $Na^+$, of about 1–10 mM. Over the last decades a lot of research has been done to study the mechanisms of salt homeostasis in plants and to identify genes implicated in salt tolerance. As a result, several plants were already successfully genetically engineered to improve resistance to salt. It is now well established that plasma membrane and tonoplast $Na^+/H^+$ exchangers, which provide the efficient exclusion of $Na^+$ excess from the cytosol to the apoplast and to the vacuole, respectively, are key players in the maintenance of ion homeostasis inside the cell.

In our laboratory, the mechanisms at the tonoplast level involved in the response of *Solanum tuberosum* and *Populus euphratica* to salinity were investigated. While the latter has been used as a plant model to study resistance against salt and osmotic stresses, potato is moderately-salt sensitive and an important food crop in the world. Both studies were conducted in suspension-cultured cells. Although it is recognised that *in vitro* tissue cultures do not accurately simulate normal plant physiological conditions, they provide a convenient experimental system that has already yielded a lot of useful information on several key physiological, biochemical, and molecular processes such as sugar transport, gene expression, as well as plant salt stress tolerance ([25] and references therein).

In *S. tuberosum*, the activity of V-$H^+$-PPase and V-$H^+$-ATPase and the involvement of $Na^+$ compartmentalisation in the vacuole were investigated as a mechanism of salt tolerance [25]. Tonoplast enriched-vesicles were prepared from control and 150 mM NaCl-tolerant calli lines by differential centrifugation, and sucrose gradient and their purity was checked with marker enzymes. To evaluate the proton pumping activities of V-$H^+$-PPase and V-$H^+$-ATPase, the fluorescence quenching of the pH sensitive probe ACMA was measured with a Perkin-Elmer LS-5B fluorometer. The excitation wavelength was set at 415 nm and the emission wavelength was set at 485 nm. After the addition of tonoplast vesicles to the appropriate buffer (pH 7.2) containing ACMA, the reaction was started by the addition of either ATP or PPi (to energise V-$H^+$-PPase and V-$H^+$-ATPase, respectively). Results showed that ATP- and pyrophosphate (PPi)-dependent $H^+$-transport were higher in the salt-tolerant line than in control cells. The polypeptide pattern after SDS-PAGE of tonoplast proteins from control cells differed from that of the NaCl-adapted line. To gain further insights into the regulation mechanisms of both proton pumps in response to salt, tonoplast proteins were probed with polyclonal antibodies raised against the V-$H^+$-PPase and the V-$H^+$-ATPase A subunit from the mung bean. Results confirmed that changes in V-$H^+$-PPase activity are correlated with increased protein amount. However, western blotting analysis revealed that the relative amount of subunit A of the V-$H^+$-ATPase remained constant in NaCl tolerant calli, despite the observed increase in both hydrolytic and $H^+$-pumping activity in the salt-tolerant cell line, suggesting that a mechanism of post-translational regulation is probably involved.

To evaluate the involvement of a $Na^+/H^+$ antiport system on $Na^+$ sequestration into the potato vacuole, the effect of $Na^+$ in the dissipation of a pre-established pH gradient was measured in tonoplast vesicles from control and salt-adapted cell lines. Results showed that the initial rates of proton efflux followed Michaelis–Menten kinetics and the $V_{max}$ of proton dissipation was 2-fold higher in NaCl-tolerant calli when compared to the control. $H^+$-coupled exchange was specific for $Na^+$ and $Li^+$ and not for $K^+$. Authors concluded that the observed increase of the electrochemical proton gradient across the tonoplast, through the up-regulation of both V-$H^+$-ATPase and V-$H^+$-PPase, combined with the stimulation of $Na^+/H^+$ antiport activity in response to salt, strongly suggests that $Na^+$ sequestration into the vacuole contributes to salt tolerance in *S. tuberosum* cell lines [25].

A similar conclusion was attained in *P. euphratica* cell cultures using identical approaches [26]. It was observed that *P. euphratica* suspension-cultured cells are highly tolerant to elevated salinity, being able to grow with up to 150 mM NaCl in the culture medium without substantial modification of the final population size when compared to the control cells in the absence of salt. Remarkably, at a salt concentration of 300 mM, cells were unable to grow but remained highly viable (71% viability) up to 17 days after subculture. This observation led us to assess in more detail their capacity to tolerate salt upon addition of NaCl pulses up to a 1 M concentration. Cells were cultivated in media with 2.5% (w/v) sucrose without salt, aliquots were collected at the mid-exponential growth phase, and 0.5-1 M of NaCl was added. Results showed that suspension cells remained close to 100% viable 24 h after a 1-M-NaCl pulse, although when compared to control cells they displayed a smaller size and a denser cytoplasm. To study viability, fluorescein diacetate (FDA) was used. FDA is permeable to the intact plasma membrane and is converted into a green fluorescent dye, fluorescein, by a function of internal esterases, showing green colour in viable cells under the epifluorescence microscope.

When tonoplast vesicles were purified from cells cultivated in the absence of salt and from salt-stressed cells, it was observed that vacuolar V-$H^+$-PPase seemed to be the primary tonoplast proton pump in *P. euphratica*. However, its activity decreased with exposure to NaCl, in contrast to the sodium-induced increase in the activity of

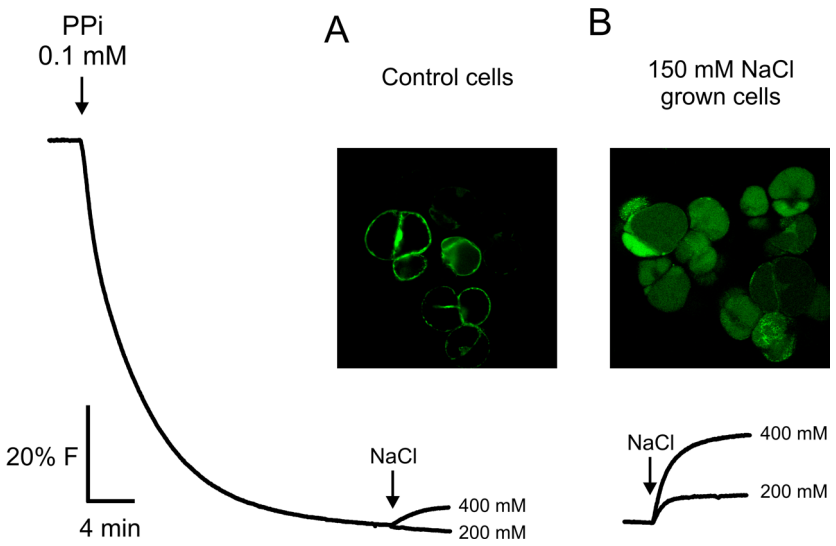


Figure 3: Dissipation of a PPi-dependent $H^+$ gradient upon addition of 200 mM and 400 mM NaCl (final concentrations) to tonoplast vesicles isolated from *P. euphratica* suspension-cultured cells grown in the absence of salt (A) and in the presence of 150 mM NaCl (B). *Inserts*: Confocal imaging of $Na^+$ accumulation in P. euphratica suspension cells stained with Sodium Green. [17, 26].

vacuolar V-$H^+$-ATPase. As referred to above, many studies have shown that vacuolar $Na^+/H^+$ antiport activity is enhanced by salt stress, but there has been very few direct links made between this increased activity and Na+ sequestration into the vacuole. To determine whether $Na^+$ accumulates inside the vacuole in *P. euphratica*, both control and cells subjected to 150-mM-NaCl treatment for 24 h were stained with the fluorescent $Na^+$ probe Sodium Green. Confocal and epifluorescence microscopy observations showed that suspension-cultured cells subjected to a salt pulse accumulated $Na^+$ in the vacuole when compared with control cells. In agreement, the tonoplast $Na^+/H^+$ exchange system was shown to be upregulated by salt and, indirectly, by a salt-mediated increase of V-$H^+$-ATPase activity [26] (Figure 3).

### 2.3. Approaches with intact vacuoles from grape berry cells

During the ripening of grape berries, vacuoles accumulate water, sugars and secondary metabolites. However, in spite of the basic and applied importance of these transport steps, their molecular bases are still not completely understood. In this context, approaches aiming at the isolation and purification of intact vacuoles from grape cells are a prerequisite to understand the physiology of this organelle during grape berry development and ripening [27, 28]. Also, the increasing impact of proteomic studies in plant biology has generated an unexpected interest in the purification of this extremely fragile organelle and led to independent proteomic studies focused on intact vacuoles from *Arabidopsis* [29-31].

The isolation of sufficient amounts of high quality protoplasts is a prerequisite for their use as a source of intact vacuoles. Grape berry protoplasts have been isolated from both grape berry tissues [32, 33] and cultured cells [27, 28] through enzymatic digestion of the cell walls with cellulase Y-C and pectolyase Y-23. Vacuoles from grape cell suspensions are isolated after osmotic lysis of protoplasts at a relatively high temperature followed by a Ficoll step gradient centrifugation. Usually, only 2% of the cytosolic marker glucose-6-phosphatase is recovered in the vacuolar fraction, suggesting that this sample is strongly depleted in protoplasts and cytosolic contaminations. Microscopy observations, flow cytometry analysis and functional studies also supported the purity of the vacuole fraction. Results showed that the tonoplast stained strongly with the fluorescent dye FM1-43 [*N*-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl)pyridinium dibromide] and most vacuoles maintained an internal acidic pH, as assessed by the lipophilic phenazine dye Neutral Red. FM1-43 exhibits weak fluorescence in aqueous medium, but shines brightly when inserted into membranes. Flow cytometry analysis of vacuole samples incubated with the calcium-sensitive fluorescent probe Fluo-4 AM revealed a well-defined subpopulation of intact vacuoles.

Additional studies with the pH sensitive probe ACMA showed that intact vacuoles are able to generate and maintain a pH gradient through the activity of V-$H^+$-ATPase and V-$H^+$-PPase. Also, the addition of $CaCl_2$ to energised intact vacuoles through the activation of V-$H^+$-PPase resulted in an immediate dissipation of the proton gradient, suggesting the involvement of a proton-dependent $Ca^{2+}$ transport system. As shown by epifluorescence microscopy with calcium-sensitive fluorescent probe Fluo-4 AM, intact grape vacuoles accumulate high amounts of calcium (Figure 4). The first plant $Ca^{2+}/H^+$ antiporter identified was named CAX1 [34] and more recently, similar proteins were found in both *Arabidopsis* [35] and rice [36].

Overall, purified intact vacuoles are good experimental models to study the acidification mechanisms of the vacuole lumen and solute uptake. In spite of its importance for crop quality, the mechanisms and regulations of sugar import and storage in the vacuole of grape berry cells are still obscure, although some information is already available in *Arabidopsis* [10, 31, 37]. The same applies to tartaric and malic acid uptake mechanisms. Both acids severely affect grape and wine quality, determining wine pH and providing a freshness sensation as well as microbial stability. In *Arabidopsis*, malate exchange between the vacuole and the cytosol is mediated by a

tonoplast malate transporter (AttDT) and a malate channel (AtALMT9) [38, 39]. In the grape berry, some malate transporter candidates have been identified [40]. Nothing is yet known about tartaric acid transport across the tonoplast. Studies aiming at the elucidation of the transport mechanisms of sugars and organic acids (malic and tartaric) across the tonoplast of grape berry cells are in progress in collaboration with other national (ITQB, Manuela Chaves) and international research groups (The Institute of Vine and Wines Sciences – ISVV, Bordeaux, Serge Delrot).

Furthermore, the cloning and functional characterisation of grape copper transporters, aiming at the identification of key steps involved in the detoxification and tolerance of the grapevine to copper stress are in progress in our laboratory in collaboration with Mohsen Hanana (Tunisia) and Eduardo Blumwald (USA). Preliminary results with intact grape vacuoles show that copper is accumulated in the vacuole through a carrier-mediated active transport system (V. Martins and co-workers, unpublished).

## 3. SUGAR TRANSPORT IN *OLEA EUROPAEA* AND *VITIS VINIFERA* AND FRUIT RIPENING

### 3.1. Sugar transport in olive cells

In *O. europaea* L. sugars are the main soluble components in olive tissues and play important roles, providing energy and acting as precursors for olive oil biosynthesis. Glucose, fructose and galactose are the main sugars found in the olive pulp but appreciable quantities of mannitol are also present [41]. The transport of glucose in *O. europaea* has been studied in our laboratory both in heterotrophic cell suspension and fruit tissues [14, 16, 42].

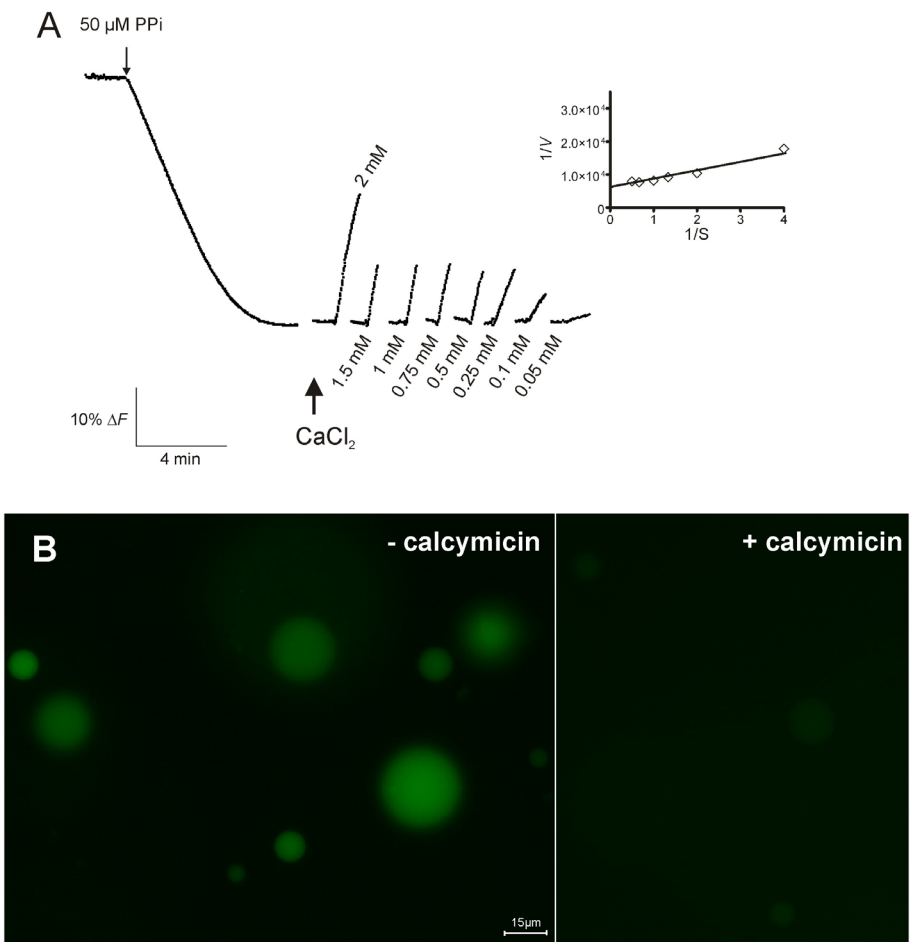


Figure 4: Study of the involvement of $H^+$-dependent $Ca^2$ uptake in intact vacuoles purified from grape protoplasts. Effect of $Ca^{2+}$ on the pre-formed transmembrane proton gradient (A). *Insert*: Lineweaver-burk plot of the initial velocities of fluorescence recovery as function of $Ca^{2+}$ concentration. Intact vacuoles labelled with the calcium fluorescent probe Fluo-4 AM in the absence and in the presence of 300 µM calcymicin, observed under the fluorescence microscope (B). Reproduced from Fontes et al. [28].

In a preliminary experiment, cell suspensions of *O. europaea* var. Galega Vulgar were tested for their capacity to use sucrose, lactose, glucose, galactose, fructose, mannitol and glycerol as sole carbon and energy sources. Growth experiments were performed in mineral medium supplemented with 0.5% of each sugar. Among these substrates, only lactose and glycerol were not able to promote cell growth. Mannitol and glucose behaved as good carbon and energy sources, suggesting that a transport mechanism for these substrates should be present at the plasma membrane of these cells. To estimate the initial uptake rates of D- or L-[$^{14}$C]glucose, a protocol previously used by the group to study solute transport in yeasts [43] was optimised. Briefly, 1 ml of cell suspension was added to 10-ml flasks, under shaking (100 rpm). After 2 min of incubation, at 25ºC, the reaction was started by the addition of 40 µl of an aqueous solution of radiolabelled sugar at the desired specific activity and concentration. Results showed that cell suspensions grown in batch culture with 0.5% (w/v) glucose were able to transport D-[$^{14}$C]glucose according to Michaelis–Menten kinetics (Figure 4) associated with a first-order kinetics. The linear component of total glucose uptake was more evident for D-[$^{14}$C]glucose concentrations ≥ 2 mM. The monosaccharide carrier exhibited high affinity ($K_m$ approx. 50 µM) and was able to transport D-glucose, D-fructose, D-galactose, D-xylose, 2-deoxy-D-glucose and 3-*O*-methyl-D-glucose, but not D-arabinose, D-mannitol or L-glucose [14].

As referred to before, all the MSTs characterised so far are energy-dependent $H^+$-symporters [12]. To study the energetics of the monosaccharide transport system

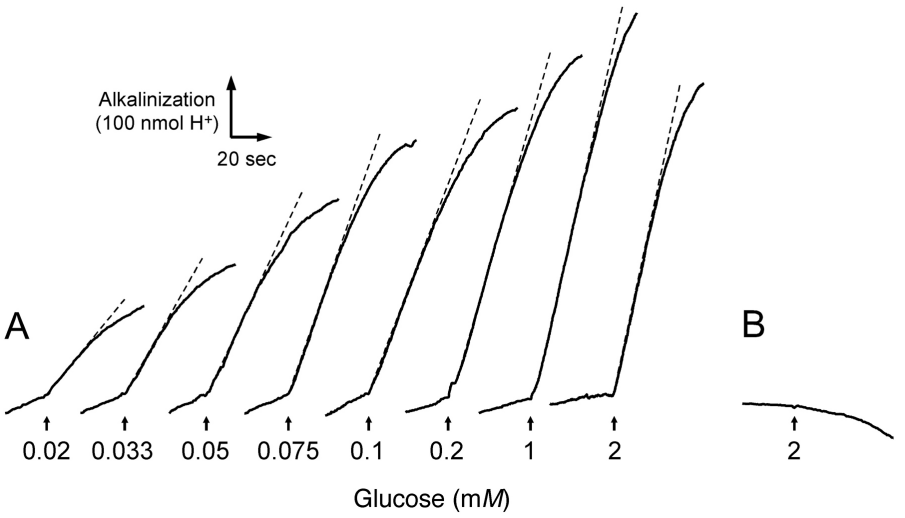


**Figure 5:** Proton movements, at pH 5.0, associated with the addition of glucose to cell suspensions of the yeast strain *Brettanomyces bruxellensis* ISA 1791 cultivated, at 26°C and pH 4.5, in mineral media with 0.1% (A) and 2% glucose (B). Reproduced with permission from the American Journal of Enology and Viticulture [43].

of *O. europaea*, the initial rates of proton uptake upon addition of glucose were measured with a standard pH meter connected to a recorder, as described earlier for studies in yeasts [43]. In the wine spoilage yeast *Brettanomyces bruxellensis* the first evidence for the involvement of an $H^+$-dependent carrier in glucose transport by the high-affinity transport system was the presence of proton movements associated with glucose uptake by 0.1% glucose-grown cells. Initial velocities of proton disappearance upon addition of 0.02 to 2 mM glucose to weakly buffered cell suspensions, at pH 5.0, are shown in Figure 6. Conversely, the addition of glucose to cell suspensions of 2% glucose-grown cells was not associated with proton influx [43]. This approach was successfully used to study proton uptake in plant cells. Results showed that glucose uptake was associated with proton uptake, which also followed Michaelis–Menten kinetics (Figure 5). To study the accumulative capacity of the carrier, a pre-requisite of an active (concentrative) transport system, the non-metabolisable glucose analogue 3-*O*-methyl-Dglucose (3-*O*-MG), was used, since it behaved as a substrate for the monosaccharide carrier. Results showed that the transport of radiolabelled 3-*O*-MG was accumulative (40-fold, at pH 5.0) and the protonophore CCCP (carbonyl cyanide m-chlorophenylhydrazone) strongly inhibited sugar accumulation. The results were consistent with the involvement of a monosaccharide: proton symporter with a stoichiometry of 1:1 [14].

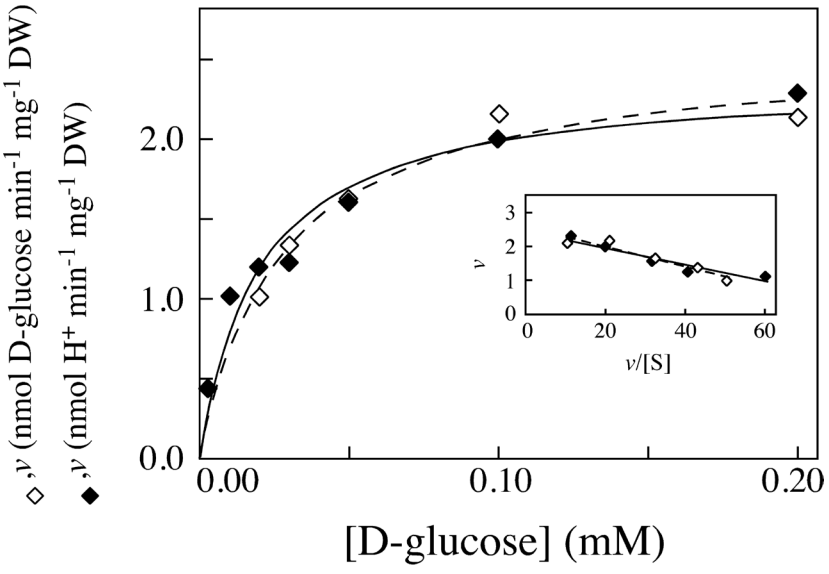


**Figure 6:** Initial uptake rates of protons and D-[$^{14}$C]glucose, at pH 5.0, by suspension cells of *Olea europaea* grown with 0.5% (w/v) glucose. Initial alkalinisation rates of the extracellular medium were measured upon addition of glucose to cell suspensions weakly buffered with 10 mM potassium phosphate. The same cell suspensions were used to measure initial D-[$^{14}$C]glucose uptake. For each case best fitting transport kinetics were determined by the application of GraphPad software. Adapted from Oliveira et al. [14].

Remarkably, when cells were grown with 3% (w/v) glucose, the uptake of D-[$^{14}$C] glucose followed first-order kinetics, and monosaccharide:$H^+$ symporter activity was not detected. At that time we proposed that the first-order kinetics observed in 0.5% and 3% sugar-grown cells was produced exclusively by passive diffusion of the sugar [14]. This conclusion was later disproved [44] as referred to below.

These clear-cut data provided a solid basis to conclude that in *O. europaea* cells sugar levels have a regulatory effect on sugar transport, because the activity for monosaccharide transport was repressed by high sugar concentrations [14]. The well-documented sugar-catabolic repression in yeasts (show in figure 6) has also become evident in plant cells.

More recently, a cDNA encoding a monosaccharide transporter, designated *OeMST2* (*Olea europaea* monosaccharide transporter 2) was cloned using total RNA isolated from olive fruit and degenerate primers corresponding to conserved regions of MSTs from higher plants. 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 hydropathy pattern analysed according to Kyte and Doolittle suggests the presence of 12 transmembrane spanning domains with a large central hydrophilic fragment be-

tween transmembrane domains 6 and 7. This model is consistent with the structure proposed concerning the MSTs and DSTs identified so far in higher plants (Figure 7). Expression of this cDNA in an hxt-null *S. cerevisiae* strain deficient in glucose transport restored its capacity to grow further and to transport glucose. The encoded protein showed high affinity for D-glucose ($K_m$, 25mM) and was also able to recognise D-galactose and the analogues 3-*O*-MG and 2-deoxy-D-glucose, but not D-fructose, D-arabinose, sucrose or D-mannitol. Additional biochemical evidence confirmed that OeMST2 is an $H^+$/monosaccharide transporter that may be negatively regulated by glucose at the transcriptional level. The expression of *OeMST2* was studied during the ripening process of olives. Results showed that transcript levels increased during fruit maturation, suggesting that *OeMST2* is involved in the massive accumulation of monosaccharides in olive fruits during ripening (Figure 8) [42].

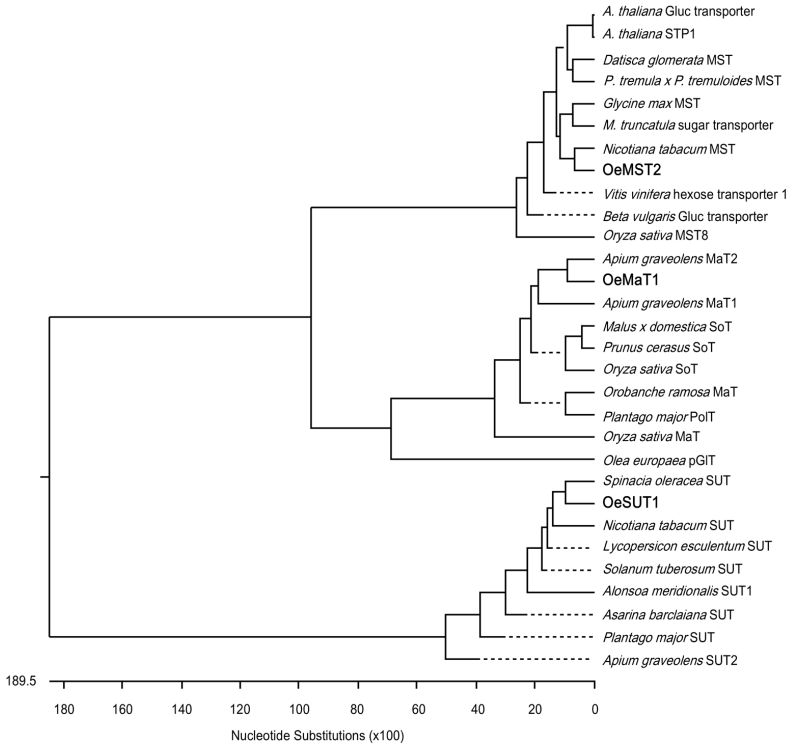


**Figure 7:** Phylogenetic tree showing the comparison between plant monosaccharide, polyol and sucrose transporters.

### 3.2. Sugar transport in grape cells

During ripening of grape berries there is a massive accumulation of soluble sugars and synthesis and accumulation of a wide range of phenolic compounds and aroma precursors. These processes play major roles in the quality of the berries and wine. Sucrose derived from leaf photosynthesis is exported *via* the phloem to the berries. From veraison and throughout ripening the berries accumulate roughly equal amounts of glucose and fructose, reaching over 1M of each hexose. This implies that phloem-transported sucrose is hydrolysed at some step during its transport from the sieve tube to the vacuole of the mesocarp cell. In the grape, 59 putative hexose transporter encoding genes have been identified based on protein motif recognition. Six full length cDNAs encoding for MST and named *VvHT1* to 6 were previously cloned from various grape cultivars such as Pinot Noir, Ugni Blanc, Chardonnay, Cabernet Sauvignon and Syrah. Uptake activities of the plasma membrane hexose transporters VvHT1, VvHT4 and VvHT5 have been demonstrated by heterologous expression in the hxt-null mutant yeast, but attempts to confirm the transport activity of VvHT6 have had little success (reviewed by [11, 45]).

Heterotrophic suspension-cultured cells from grapes (CSB, Cabernet Sauvignon Berry) were used as a model system to study glucose transport and its regulation [7]. Results showed that cells transport D-[$^{14}$C]Glucose according to simple Michaelis-Menten kinetics superimposed on first-order kinetics. Additional biochemical analysis supported that the saturating component is a high-affinity, broad-specificity $H^+$-dependent transport system. It is known that the expression of DSTs and MSTs may be affected by various parameters, including light, water and ion status, wounding, fungal and bacterial attacks, and

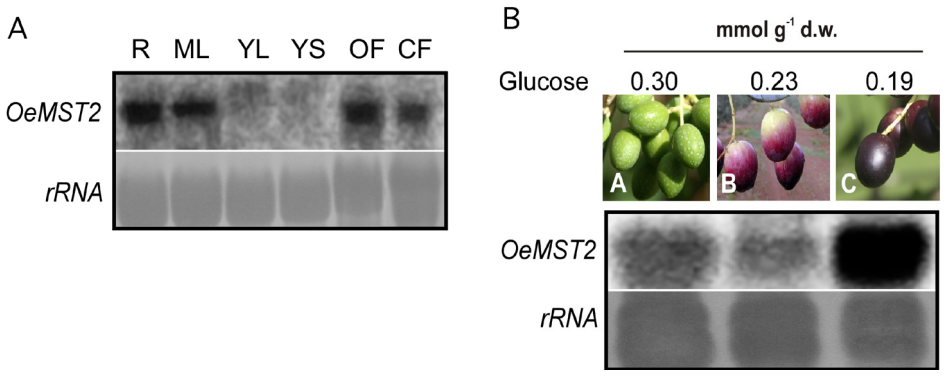


**Figure 8:** Expression of *OeMST2* (*Olea europaea* Monosaccharide Transporter 2) in the different organs and tissues of *O. europaea*. (A) Twenty µg of total RNA from roots (R), mature leaves (ML), young leaves (YL), young stems (YS), open flowers (OF) and closed flowers (CF) were hybridised with a gene specific probe from 3´-untranslated region. (B) Northern blot analysis of *OeMST2* in olive fruit at different maturation stages (A: green; B: cherry; C: black). Glucose concentration determined by Marsilio et al. [41]. Adapted from Conde et al. [42].

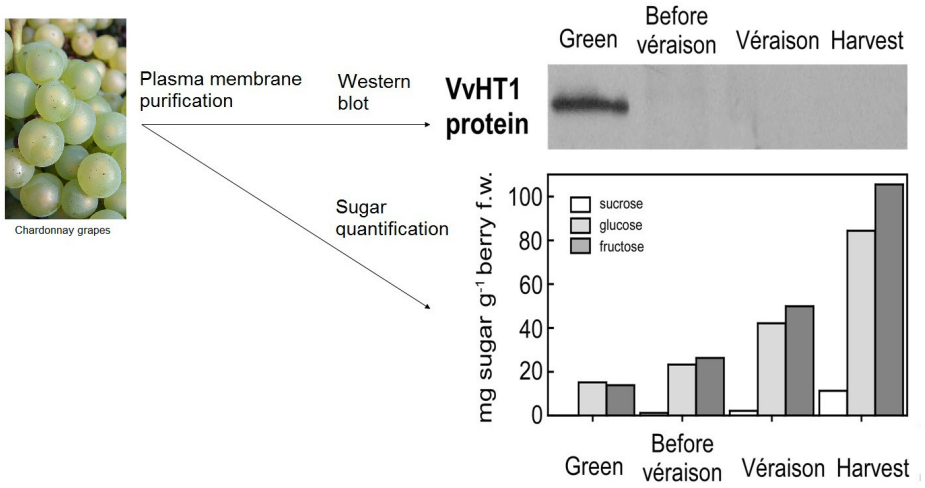


Figure 9: Sugar content and western blot analysis of VvHT1 (*Vitis vinifera* Hexose Transporter 1) levels in plasma membrane of berry flesh cells. Adapted from Conde et al. [7].

hormones, and they are generally not expressed in the same tissues or at the same developmental stages [46]. Also, besides their role as carbon and energy sources, sugars synthesised during the light phase can act as regulatory signals affecting gene expression, allowing the plant to tailor its metabolism in source tissues to face the demands in sinks. Because sugar transporters play such a key role in source-sink interactions, it is likely that their expression and activity are tightly regulated by sugar levels [14, 47]. Observations in CSB cells showed that glucose concentration in the medium tightly regulate the transcription of VvHT1, a monosaccharide transporter previously characterised in the grape berry [48], as well as VvHT1 protein amount and monosaccharide transport activity. All the remaining putative monosaccharide transporters identified so far in grapes were poorly expressed and responded weakly to glucose. Additional evidence supported that this plant sugar transporter may be controlled by its own substrate both at transcriptional and post-transcriptional levels. VvHT1 transporter was shown to be abundant at the green stage of grape berry development when low amounts of sugar are present, which is consistent with the illustrated regulating role of glucose in *VvHT1* expression. It was concluded that VvHT1 contributes to the early steps of glucose and fructose accumulation in the berry flesh cells after the disaccharide had been hydrolysed by apoplastic invertases (Figure 9) [7].

More recently, the physiological/structural status of the soft ripened berry was studied with isolated mesocarp cells from both wine and table grape varieties [19]. Bright-field, fluorescence and confocal microscopy and flow cytometry highlighted the organisation of berry flesh cell, function and viability. FM1-43 staining coupled to confocal microscopy imaging showed the integrity of the plasma membrane and the architecture and complexity of the intracellular membranous system (Figure 10). In this study, the fluorescent non-metabolisable glucose analogue 2-NBDG was used to evaluate the capacity to incorporate sugars of berry-derived protoplasts. Two-deoxy-D-glucose and its corresponding fluorescent probe (2-NBDG) are potent competitive inhibitors of D-glucose uptake [7, 44]. As a result, a brightly fluorescent cytosol with several fluorescent spots was observed 16 h after incubation. Single sections obtained by confocal microscopy showed that the analogue is confined to a particular type of intracellular vesicles, possibly vacuoles or endocytic vesicles (Figure 10). Also, time-lapse imaging after protoplast staining with FM1-43 confirmed a dynamic vesicle trafficking at the plasma membrane level, which may be involved in the endocytosis of apoplastic sugars and other solutes [19]. The confirma-

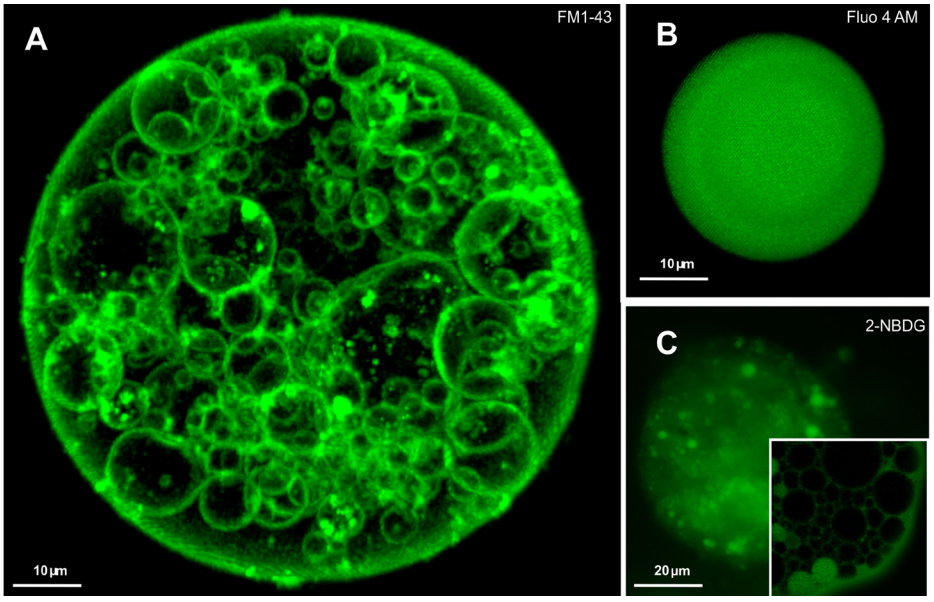


Figure 10: Mesocarp cells from fully ripened berry observed under the confocal microscope. Protoplasts were imaged by confocal laser scanning after being immersed overnight with the styryl dye FM1-43, and a maximum Z projection of 20 sections covering approximately 30 µm is represented (A). Intact vacuoles imaged after staining with the calcium fluorescent probe Fluo-4 AM (B). Plasma membrane integrity assessed under the fluorescence microscope with the fluorescent glucose analogue 2-NBDG (16 h incubation) (C). Insert: single section of protoplast loaded with 2-NBDG observed by confocal microscopy 16 h after incubation. Reproduced with permission from the American Journal of Enology and Viticulture [19].

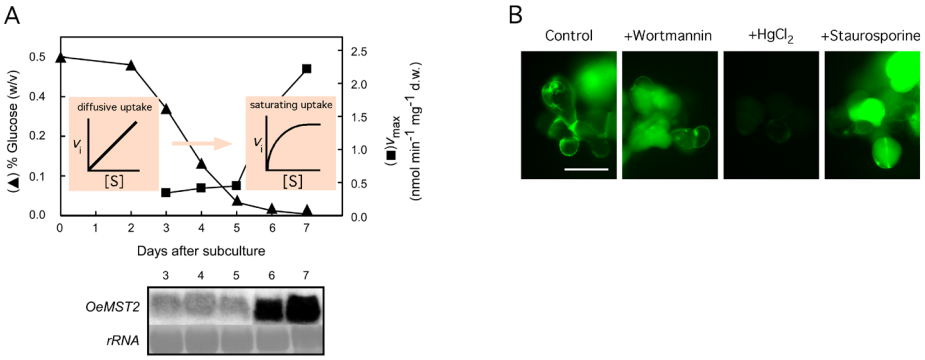


**Figure 11:** Diffusive and saturating glucose transport in suspension-cultured cells of *O. europaea*. (A) In cells collected from the culture medium up to day 5 after subculture (glucose-sufficient cells), uptake is linear with respect to [$^{14}$C]-glucose concentration (up to 100 mM). As glucose is depleted from the culture medium the activity of a saturating monosaccharide transport system becomes apparent together with *OeMST2* transcription. (B) Fluorescent micrograph of glucose-sufficient cells after incubation with 5 mM of the glucose analogue 2-NBDG for 10 min at 25 °C. Bar=100 µm. Adapted from Conde et al. [42, 44].

tion of these observations will have important repercussions on our understanding of the grape berry physiology.

Although several progresses have been achieved in the understanding of sugar unloading and compartmentalisation in the grape berry and in sink tissues in general, this area of research is still open to further and undoubtedly very useful developments.

### 3.3. The intriguing nature of the diffusive sugar transport

In several organisms, solute transport is mediated by the simultaneous operation of saturable and non-saturable (diffusion-like) uptake, but the nature of the diffusive component of sugar transport has been a matter of intense debate by the scientific community. This intriguing topic was extensively reviewed by Conde et al. [2]. Non-saturable (linear) glucose incorporation has been observed in yeasts, *Penicillium ochrochloron* hyphae, animal cells and plants. Transport experiments in plant cells and tissues have shown multiphasic kinetics with two components: a saturable high-affinity, low-capacity component and a linear (non-saturable) low-affinity, high-capacity component.

The nature of the diffusive glucose transport found in *O. europaea* cell cultures [14] was investigated in more detail by Conde et al. [44]. As referred to before, glucose uptake in olive cells is mediated by a glucose-repressible, $H^+$-dependent active saturable transport system that is superimposed on a diffusive component. The latter represents the major uptake mode when high external glucose concentrations are present. Indeed, in cells cultivated with high sugar concentrations, D- and L-[U-$^{14}$C]glucose were shown to be exclusively taken up by a non-saturable system able to sustain both cell growth and metabolism (Figure 11). Low activation energies were estimated from the initial glucose uptake at different temperatures by intact cells and plasma membrane vesicles of 4 and 7 kcal mol$^{-1}$, respectively, similarly to the values described for free diffusion of glucose in water [49] and for the permeation of water through aquaporins [50], but significantly lower than the activation energy for simple diffusion of glucose across phospholipids vesicles, of 12 kcal mol$^{-1}$. In addition, $HgCl_2$ inhibited both the linear component of sugar uptake in sugar sufficient cells and plasma membrane vesicles, suggesting a protein-mediated transport. Diffusive uptake of glucose was also inhibited by propionic acid, suggesting that this putative protein can be regulated by cytosolic pH changes, much like the gating of some aquaporins, and stimulated by the protein kinase inhibitor staurosporine. This set of evidence prompted the conclusion that a channel-like structure whose transport capacity may be regulated by intracellular protonation and phosphorylation/dephosphorylation can account for the diffusional component of glucose. The possible involvement of an aquaporin-like channel may not be discarded, implying a direct role of AQPs in organic nutrition [3].

Following the observations in cultured cells described above, the involvement of a channel-like protein was recently proposed for glucose uptake in *Arabidopsis* root tips where glucose and sucrose accumulation was insensitive to extracellular pH and protonophores [51]. Interestingly, more recently a new class of sugar transporters, named SWEETs, which are low-affinity glucose uniporters, were identified and characterised [52]. The SWEET family contains 17 members in *A. thaliana*. The biochemical properties of *AtSWEET1* are markedly similar to the unidentified transport activity characterised in roots by Chauduri et al. [51]. However, AtSWEET1 expression in roots is low, implicating other AtSWEET paralogues for this function [52].

## 4. TRANSPORT AND METABOLISM OF POLYOLS IN *OLEA EUROPAEA* AND IMPLICATIONS FOR SALT AND DROUGHT STRESS TOLERANCE

### 4.1. *Olea europaea* coordinates mannitol transport and metabolism to cope with salinity and osmotic stress

Polyols are the reduced form of aldoses and ketoses and are present in all living organisms [53]. In several plants, these sugar alcohols are, together with sucrose or raffinose-like sugars, direct products of photosynthesis and function as carbon skeletons and energy sources that are phloem-translocated between source and sink organs. Mannitol is the most widely spread polyol in nature and has been observed in over 100 vascular plant species of several families including the Apiaceae (celery, carrot, parsley), Oleaceae (olive, privet) and Rubiaceae (coffee) [16]. Phlo-

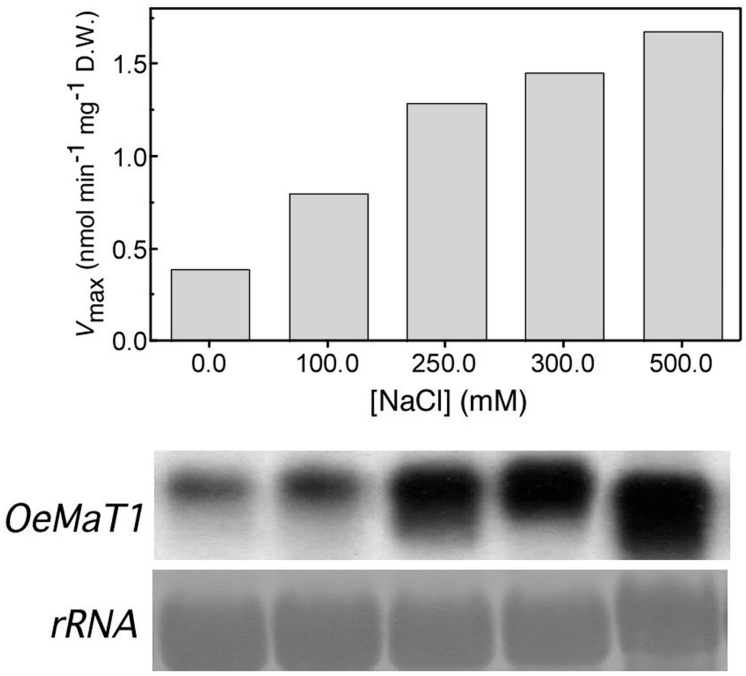


Figure 12: Effect of salt stress on *O. europaea* mannitol transport activity and *OeMaT1* expression. Dependence of the $V_{max}$ of mannitol transport and *OeMaT1* transcription on NaCl concentration in the culture medium. Increasing salt levels substantially enhance mannitol uptake capacity via OeMaT1. Adapted from Conde et al [56].

em transport of mannitol may be more advantageous for plants than sucrose due to its more reduced nature. This results from the initial step of mannitol oxidation catalysed by $NAD^+$-dependent mannitol dehydrogenase (MTD; see below) that generates NADH, thus allowing for more ATP production than in the catabolism of an equal amount of glucose. Furthermore, sugar alcohols, because of their water-like hydroxyl groups, may mimic the structure of water and maintain an artificial sphere of hydration around macromolecules during decreased cellular water potential, functioning as compatible solutes and osmoprotectants, providing tolerance to salinity (reviewed by [54]). Mannitol is a major photosynthetic product and one of the main phloem-translocated carbohydrates in olive [16]. In the olive pulp, significant levels of mannitol are present and together with glucose, fructose and galactose are the most abundant sugars [41].

Mannitol is synthesised in mature leaves from mannose-6-phosphate, by the joint action of a mannose-6-phosphate phosphatase and a NADPH-dependent mannose-6-phosphate reductase. It is then transported through the phloem to heterotrophic sink tissues, where it can be either stored or oxidised to mannose through the action of MTD and utilised as carbon and energy source [reviewed by [16, 54, 55].

The elucidation of the role played by mannitol not only as carbon and energy source for plant growth, but also as a protecting osmolyte against drought, soil salinity and the resultant oxidative stress is of critical importance to allow for the enhancement of the yield potential of *O. europaea* and other plants. Studies conducted in our laboratory have yielded useful information concerning the biochemical and molecular mechanisms involved in polyol uptake and metabolism in olive ([56], Conde et al. 2011, submitted). Transport experiments with radiolabelled mannitol showed that a mannitol:$H^+$ symport system operates in *O. europaea* cells with the following kinetic parameters: $K_m$ = 1.3 mM and a $V_{max}$ = 1.3 nmol min$^{-1}$ mg$^{-1}$ D.W. Competition experiments showed that dulcitol, sorbitol and xylitol competitively inhibited mannitol uptake, whereas glucose and sucrose did not. Reverse transcription-PCR (RT-PCR) performed on mRNA extracted from cultured cells exhibiting high mannitol transport activity allowed for the cloning of a partial *O. europaea* mannitol carrier *OeMaT1*. Remarkably, mannitol transport activity and the expression of *OeMaT1* severely increased simultaneously upon the addition of NaCl (Figure 12). This provides evidence of an up-regulation controlled at the transcriptional level that allows for enhanced mannitol transport capacity under salt stress, whereas, contrarily, salt strongly represses mannitol dehydrogenase activity. This should allow for the intracellular accumulation of mannitol in order to compensate for the decrease of external water activity, providing a tolerance mechanism to salinity in *O. europaea* [56] (Figure 13).

Northern analysis showed that *OeMaT1* transcripts increased throughout maturation of olive fruits, suggesting that an

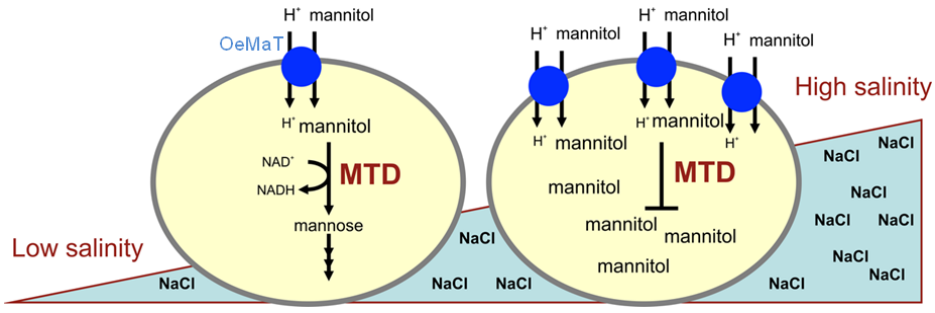


Figure 13: . Regulation and coordination of mannitol transport and metabolism as a mechanism to respond to salt and drought tolerance in *O. europaea*. The significant increase in mannitol transport levels is correlated to a severe inhibition of mannitol oxidation by OeMTD, allowing the intracellular accumulation of mannitol. Adapted from Conde et al [56].

OeMaT is involved in the accumulation of mannitol during ripening of olive. Thus, mannitol transport and compartmentalisation by OeMaT are important to allocate this source of carbon and energy, as well as for salt tolerance and olive ripening. Currently, analytical, biochemical and molecular approaches using leaves and heterotrophic sink tissues from olive are providing further clues about the role of mannitol transport and intracellular oxidation in the overall cell response to salinity and osmotic stress. Protein extraction followed by spectrophotometric enzymatic activity assays allowed for the determination of the kinetic parameters of $NAD^+$-mannitol dehydrogenase (MTD). The corresponding cDNA was cloned and named *OeMTD1* (accession number ABR31791.1). In both mannitol- and sucrose-grown cells *OeMTD1* expression was compared with MTD activity, *OeMaT1* expression and carrier-mediated mannitol uptake (Conde et al. 2011, submitted).

### 4.2. Is mannitol important in *Vitis vinifera*?

The role of mannitol as a carbon and energy source and/or osmoprotectant is yet to be demonstrated in the grapevine. Recent work developed in our laboratory in collaboration with the research group of Manuela Chaves (ITQB, Oeiras) allowed for the cloning of a gene encoding a putative *Vitis vinifera* Mannitol Transporter (*VvMaT1*) and of a gene encoding a putative *V. vinifera* Mannitol Dehydrogenase (*VvMTD1*) from the grape berry mesocarp and from grape cultured cells, respectively (Conde et al. unpublished). Both cDNAs display high similarities to the respective homologues in olive and celery. A preliminary biochemical characterization of VvMTD1 appears to suggest a response of mannitol metabolism under salt and osmotic stress similar to the one in *O. europaea*. Moreover, mannitol accumulation in the grape berry may also serve as an indicator of not only the levels of drought stress that the plant may have been subjected to, but also of the richness in polyphenol and general flavour as well as fruit quality.

Localisation, functional analysis and regulation studies are being undertaken by our lab in plant tissues and cell suspensions to elucidate the coordination between membrane transport of mannitol and its intracellular oxidation on grapevine defence against drought. Progress in this area will be of the utmost interest given the economic value surrounding *V. vinifera*.

## 5. CONCLUSIONS AND FUTURE PERSPECTIVES

The understanding of solute transport in higher plants has significantly increased during the last decades. Both the biological role of genes involved in transport processes and the way they are regulated have been studied in detail by the use of several molecular biology techniques. For instance, the biochemical properties of plant sugar transporters have mostly been elucidated through functional expression in yeast cells and *Xenopus* oocytes. The first plant monosaccharide transporter gene *HUP1* was cloned in 1989 from *Chlorella kessleri* [57] and heterologously expressed in yeast one year later [58]. Similarly, in 1999, the first plant $Na^+/H^+$ antiporter was isolated from *Arabidopsis* and heterologously expressed in yeast to demonstrate its function in response to salt [21, 22]. In the grapevine, the sequencing of the genome and the development of high throughput techniques, such as microarrays, will provide rapid progress in the study of the transport of important solutes for grape berry ripening, such as sugar, organic acids, phenolics, minerals and water.

Some plants have efficiently adapted to survive under salinity, drought, cold or intense heat, being good models to study the complexity of the mechanisms responsible for plant ion homeostasis. The discovery of plasma membrane and tonoplast $Na^+/H^+$ antiporters, and how they are regulated, was a landmark in the study of plant response to salinity. This area of research has been extremely explored and several papers report the successful improvement of plant stress resistance through overexpression of a single gene encoding the $Na^+/H^+$ exchanger. However, the introduction in nature of genetic engineered plants is still controversial. As stated by T. Flowers [59] "transgenic technology will undoubtedly continue to aid the search for the cellular mechanisms that underlie tolerance, but the complexity of the trait is likely to mean that the road to engineering such tolerance into sensitive species will be long" and "experience suggests authors should avoid hyperbole in their titles and summaries, as this does little service to the long-term aim of improving the salt tolerance of crops in the field." Overall, the continued study of the biochemical and molecular mechanisms of solute transport in plants, and how transport is regulated in response to endogenous signals and environmental stress, are of the utmost basic and applied importance. In the context of the ongoing climate changes this knowledge may lead to important improvements of the yield potential through the selection of more adapted varieties and adjustment of growing practices, or through the application of environmentally safe and membrane-transported exogenous compounds, avoiding the introduction in nature of genetically manipulated plants.

## GLOSSARY

**Facilitated diffusion:** The simplest process that a transporter can mediate (in that case also called facilitator), is an equilibrative protein-mediated process that is not coupled to metabolic energy and, therefore, incapable of giving rise to concentration gradients of the transported substrate across the membrane.

**Active transport:** Active transporters transduce free energy stored in an electrochemical ion gradient (**secondary active transport**) or released by hydrolysis of ATP (**primary active transport**) into a substrate concentration gradient across a membrane. Other primary sources of

free energy for primary active transport are redox energy and light.

**Symporter:** Mediates secondary active transport; the transporter protein moves different solutes across the biological membrane in de same direction. For instance, a plant monosaccharide/H+ symporter accumulates glucose inside the cell transducing the free energy released from the dissipation of the $H^+$ gradient. Both substrates transiently bind to the membrane protein before being co-transported.

**Veraison:** An original French term (*véraison*) used in viticulture to denote the onset of the berry ripening (when the berry starts changing in color). Portuguese viticulturists often use the term "fase de pintor".

## ACKNOWLEDGMENTS

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## REFERENCES

1. Yeo A (2007) General Introduction. In *Plant Solute Transport* (Yeo AR & Flowers TJ, eds). Blackwell publishing, UK.

2. Conde A, Diallinas G, Chaumont F, Chaves M & Gerós H (2010) Transporters, channels or simple diffusion? Dogmas, atypical roles and complexity in transport systems. *Int J Biochem Cell Biol* **42**, 857-868.

3. Gomes D, Agasse A, Thiébaud P, Delrot S, Gerós H & Chaumont F (2009) Aquaporins are multifunctional water and solute transporters highly divergent in living organisms. *BBA-Biomembranes* **1788**, 1213-1228.

4. Miller C (2000) Ion channels: doing hard chemistry with hard ions. *Curr Opin Chem Biol* **4**, 148-151.

5. Saier Jr MH (2000) A functional-phylogenetic classification system for transmembrane solute transporters. *Microbiol Mol Biol Rev* **64**, 354-411.

6. Sousa MF, Façanha AR, Tavares RM, Lino-Neto T & Gerós H (2007) Phosphate transport by proteoid roots of Hakea sericea. *Plant Sci* **173**, 550-558.

7. Conde C, Agasse A, Glissant D, Tavares RM, Gerós H & Delrot S (2006) Pathways of glucose regulation of monosaccharide transport in grape cells. *Plant Physiol* **141**, 1563-1577.

8. Williams LE, Lemoine R & Sauer N (2000) Sugar transporters in higher plants - a diversity of roles and complex regulation. *Trends Plant Sci* **5**, 283–290.

9. Sauer N, Friedlander K & Graml-Wicke U (1990) Primary structure, genomic organization and heterologous expression of a glucose transporter from *Arabidopsis thaliana*. *EMBO J* **9**, 3045-3050.

10. Büttner M (2007) The monosaccharide transporter-like gene family in *Arabidopsis*. *FEBS Lett* **581**, 2318-2324.

11. Agasse A, Vignault C, Kappel C, Conde C, Gerós H & Delrot S (2009) Sugar transport and sugar sensing in grape. In *Molecular Biology & Biotechnology of the Grapevine* (Roubelakis-Angelakis KA, ed), pp 105-139. Springer Academic Publishers, Netherlands.

12. Büttner M & Sauer N (2000) Monosaccharide transporters in plants: structure, function and physiology. *Biochim Biophys Acta* **1465**, 263-274.

13. Azevedo H, Conde C, Gerós H & Tavares RT (2006) The Non-host Pathogen *Botrytis cinerea* Enhances Glucose Transport in *Pinus pinaster* Suspension-cultured Cells. *Plant Cell Physiol* **47**, 290-298.

14. Oliveira J, Tavares RM & Gerós H (2002) Utilization and transport of glucose in *Olea europaea* cell suspensions. *Plant Cell Physiol* **43**, 1510-1517.

15. Conde C, Silva P, Fontes N, Dias ACP, Tavares RM, Sousa MJ, Agasse A, Delrot S & Gerós H (2007) Biochemical changes throughout grape berry development and fruit and wine quality. *Food* **1**(1), 1-22.

16. Conde C, Delrot S & Gerós H (2008) Physiological, biochemical and molecular changes occurring during olive development and ripening. *J Plant Physiol* **165**, 1545-1562.

17. Silva P & Gerós H (2009) Regulation by salt of vacuolar $H^+$-ATPase and $H^+$ pyrophosphatase activities and $Na^+/H^+$ exchange. *Plant Signal Behav* **4** (8), 32-40.

18. Fontes N, Gerós H & Delrot S (2011) Grape berry vacuole: a complex and heterogeneous membrane system specialized in the accumulation of solutes. *Am J Enol Vitic* doi: 10.5344/ajev.2011.10125.

19. Fontes N, Côrte-Real M & Gerós H (2011) New observations on the integrity, structure and physiology of flesh cells from fully ripened grape berry. *Am J Enol Vitic* doi: 10.5344/ajev.2011.10126.

20. Nass R, Cunningham KW & Rao R (1997) Intracellular sequestration of sodium by a novel $Na^+/H^+$ exchanger in yeast is enhanced by mutations in the plasma membrane $H^+$-ATPase. *J Biol Chem* **272**, 26145-26152.

21. Apse MP, Aharon GS, Snedden WA & Blumwald E (1999) Salt tolerance conferred by overexpression of a vacuolar $Na^+/H^+$ antiport in Arabidopsis. *Science* **285**, 1256-1258.

22. Gaxiola RA, Rao R, Sherman A, Grisafi P, Alper SL & Fink GR (1999) The *Arabidopsis thaliana* proton transporters, AtNhx1 and Avp1, can function in cation detoxification in yeast. *Proc Natl Acad Sci USA* **96**, 1480-1485.

23. Yamaguchi T, Apse MP, Shi H & Blumwald E (2003) Topological analysis of a plant vacuolar $Na^+/H^+$ antiporter reveals a luminal C terminus that regulates antiporter cation selectivity. *Proc Natl Acad Sci USA* **100** (21), 12510-5.

24. Sato Y & Sakaguchi SM (2005) Topogenic properties of transmembrane segments of *Arabidopsis thaliana* NHX1 reveal a common topology model of the $Na^+/H^+$ exchanger family. *J Biochem* **138**, 425-431.

25. Queirós F, Fontes N, Silva P, Almeida D, Maeshima M, Gerós H & Fidalgo F (2009) Activity of tonoplast proton pumps and $Na^+/H^+$ exchange in potato cell cultures is modulated by salt. *J Exp Bot* **60**, 1363-1374.

26. Silva P, Arnoldo R, Façanha AR, Tavares RM & Gerós H (2010) Role of tonoplast proton pumps and Na+/H+ antiport system in salt tolerance of *Populus euphratica* Oliv. *J Plant Growth Regul* **29**, 23-34.

27. Papadakis AK, Fontes N, Gerós H & Roubelakis-Angelakis KA (2009) Progress in grapevine protoplast technology. In *Molecular Biology & Biotechnology of the Grapevine* (Roubelakis-Angelakis KA, ed), pp 429-460. Springer Academic Publishers, Netherlands.

28. Fontes N, Silva R, Vignault C, Lecourieux F, Gerós H & Delrot S (2010) Purification and functional characterization of protoplasts and intact vacuoles from grape cells. *BMC Res Notes* (http://www.biomedcentral.com/1756-0500/3/19).

29. Carter C, Pan S, Zouhar J, Ávila EL, Girke T & Raikhel N (2004) The vegetative vacuole proteome of Arabidopsis thaliana reveals predicted and unexpected proteins. *Plant Cell* **16**, 3285-3303.

30. Shimaoka T, Ohnishi M, Sazuka T, Mitsuhashi N,

Hara-Nishimura I, Shimazaki KI, Maeshima M, Yokota A, Tomizawa RI & Mimura T (2004) Isolation of intact vacuoles and proteomic analysis of tonoplast from suspension-cultured cells of *Arabidopsis thaliana*. *Plant Cell Physiol* **45**, 672-683.

31. Endler A, Meyer S, Schelbert S, Schneider T, Weschke W, Peters SW, Keller F, Baginsky S, Martinoia E & Schmidt UG (2006) Identification of a vacuolar sucrose transporter in Barley and Arabidopsis mesophyll cells by a tonoplast proteomic approach. *Plant Physiol* **141**, 196-207.

32. Fontes N, Delrot S & Gerós H (2010) A method for the isolation of protoplasts from grape berry mesocarp tissue. *Recent Pat Biotechnol* **4**, 125-129.

33. Fontes N, Gerós H, Anastasia K, Papadakis AK, Delrot S & Roubelakis-Angelakis KA (2010) Isolation and use of protoplasts from grapevine tissues. In *Methodologies and Results in Grapevine Research* (Delrot S, Medrano H, Or E, Bavaresco L & Grando S, eds), pp 277-293. Springer Academic Publishers, Netherlands.

34. Hirschi K, Zhen R, Cunningham KW, Rea PA & Fink GR (1996) CAX1, a $H^+/Ca^{2+}$ antiporter from Arabidopsis. *Proc Natl Acad Sci USA* **93**, 8782-8786.

35. Cai X & Lytton J (2004) The cation/$Ca^{2+}$ exchanger superfamily: Phylogenetic analysis and structural implications. *Mol Biol Evol* **21**, 1692-1703.

36. Kamiya T & Maeshima M (2004) Residues in internal repeats of the rice cation/$H^+$ exchanger are involved in the transport and selection of cations. *J Biol Chem* **279**, 812-819.

37. Aluri S & Büttner M (2007) Identification and functional expression of the Arabidopsis thaliana vacuolar glucose transporter 1 and its role in seed germination and flowering *Proc. Natl. Acad. Sci. USA* **104**, 2537-2542.

38. Emmerlich V, Linka N, Reinhold T, Hurth MA, Traub M, Martinoia E & Neuhaus HE (2003) The plant homolog to the human sodium/dicarboxylic cotransporter is the vacuolar malate carrier *Proc Natl Acad Sci USA* **100**, 11122-11126.

39. Kovermann P, Meyers S, Hörtensteiner S, Picco C, Scholz-Starke J, Ravera S, Lee Y & Martinoia E (2007) The Arabidopsis vacuolar malate channel is a member of the ALMT family *The Plant J* **52**, 1169-1180.

40. Rongala J (2008) Identification and localization of vacuolar organic aid carriers in grapevine berries. Ph.D. thesis, University of Adelaide, Faculty of Science, School of Agriculture, Food and Wine, Waite Campus.

41. Marsilio V, Campestre C, Lanza B & De Angelis M (2001) Sugar and polyol compositions of some European olive fruit varieties (*Olea europaea* L.) suitable for table olive purposes. *Food Chem* **72**, 485–490.

42. Conde C, Agasse A, Silva P, Lemoine R, Delrot S, Tavares RM & Gerós H (2007) OeMST2 encodes a monosaccharide transporter expressed throughout olive fruit maturation. *Plant Cell Physiol* **48**, 1299-1308.

43. Silva P, Cardoso H & Gerós H (2004) Studies on the wine spoilage capacity of *Brettanomyces/Dekkera* spp. *Am J Enol Vitic* **55**, 65-72.

44. Conde C, Silva P, Agasse A, Tavares RM, Delrot S & Gerós H (2007) An Hg-sensitive channel mediates the diffusional component of glucose transport in olive cells. *BBA-Biomembranes* **1768**, 2801-2811.

45. Agasse A, Vignault C, Glissant D, Conde C, Gerós H & Delrot S (2007) Molecular aspects of sugar transport and its regulation during grape berry ripenning. In *Macromolecules and Secondary Metabolites of Grapevine and Wine* (Jeandet P, Clément C & Conreux A, eds), pp. 12-22. Intercept, Lavoisier, Paris, London, New York.

46. Kühn C, Barker L, Burkle L & Frommer WB (1997) Macromolecular trafficking indicated by localization and turnover of sucrose transporters in enucleate sieve elements. *Science* **275**, 1298–1300.

47. Roitsch T (1999) Source-sink regulation by sugar and stress. *Curr Opin Plant Biol* **2**, 198–206.

48. Vignault C, Vachaud M, Cakir B, Glissant D, Dedaldechamp F, Buttner M, Atanassova R, Fleurat-Lessard P, Lemoine R & Delrot S (2005) VvHT1 encodes a monosaccharide transporter expressed in the conducting complex of the grape berry phloem. *J Exp Bot* **56**, 1409–1418.

49. Stein WD (1986) Transport and diffusion across cell membranes. San Diego: Academic Press.

50. Tsukaguchi H, Shayakul C, Berger UV, Mackenzie B, Devidas S, Guggino WB, van Hoek AN & Hediger MA (1998) Molecular characterization of a broad selectivity neutral solute channel. *J Biol Chem* **273**, 24737-24743.

51. Chaudhuri B, Hörmann F, Lalonde S, Brady SM, Orlando DA, Benfey P & Frommer WB (2008) Protonophore- and pH-insensitive glucose and sucrose accumulation detected by FRET nanosensors in Arabidopsis root tips. *Plant J* **56**, 948–962.

52. Chen LQ, Hou BH, Lalonde S, Takanaga H, Hartung ML, Qu XQ, Guo WJ, Kim JG, Underwood W, Chaudhuri B, Chermak D, Antony G, White FF, Somerville SC, Mudgett MB & Frommer WB (2010) Sugar transporters for intercellular exchange and nutrition of pathogens. *Nature* **468** (7323), 527-532.

53. Noiraud N, Maurousset L & Lemoine R (2001) Identification of a mannitol transporter, AgMaT1, in celery phloem. *Plant Cell* **13**, 695-705.

54. Stoop JMH, Williamson JD & Pharr DM (1996) Mannitol metabolism in plants: a method for coping with stress. *Trends Plant Sci* **5**, 139–144.

55. Noiraud N, Maurousset L & Lemoine R (2001) Transport of polyols in higher plants. *Plant Physiol Biochem* **39**, 717–728.

56. Conde C, Silva P, Agasse A, Lemoine R, Delrot S, Tavares R & Gerós H (2007) Utilization and transport of mannitol in *Olea europaea* and implications on salt stress tolerance. *Plant Cell Physiol* **48**, 42-53.

57. Sauer N & Tanner W (1989) The hexose carrier from *Chlorella*. cDNA cloning of a eucaryotic $H^+$-cotransporter. *FEBS Lett* **259**, 43–46.

58. Sauer N, Caspari T, Klebl F & Tanner W (1990) Functional expression of the *Chlorella* hexose transporter in *Schizosaccharomyces pombe*. *Proc Natl Acad Sci USA* **87**, 7949–7952.

59. Flowers TJ (2004) Improving crop salt tolerance. *J Exp Bot* **55**, 307-19.

60. Lalonde S, Boles E, Hellmann H, Barker L, Patrick JW, Frommer WB & Ward JM (1999) The dual function of sugar carriers: Transport and sugar sensing. *The Plant Cell* **11**, 707-726.

61. Terrier N, Deguilloux C, Sauvage FX, Martinoia E & Romieu C (1998) Proton pumps and anion transport in *Vitis vinifera*: The inorganic pyrophosphatase plays a predominant role in the energization of the tonoplast. *Plant Physiol Biochem* **36**, 367-377.

62. Hanana M, Cagnac O, Yamaguchi T, Hamdi S, Ghorbel A & Blumwald E (2007) A grape berry (*Vitis vinifera* L.) cation/proton antiporter is associated with berry ripening. *Plant Cell Physiol* **48**, 804-811.

63. Gomez C, Terrier N, Torregrosa L, Vialet S, Fournier-Level A, Verriès C, Souquet J-M, Mazauric J-P, Klein M, Cheynier V & Ageorges A (2009) Grapevine MATE-type proteins act as vacuolar $H^+$-dependent acylated anthocyanin transporters. *Plant Physiol* **150**, 402-415.

64. Fouquet R, Léon C, Ollat N & Barrieu F (2008) Identification of grapevine aquaporins and expression analysis in developing berries. *Plant Cell Rep* **27**, 1541-1550.