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Biochemical and molecular mechanisms of salt stress tolerance in *Populus euphratica* and *Olea europaea*

Tese de Doutoramento em Ciências
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The reasonable man adapts himself to the world, the unreasonable one persists in trying to adapt the world to himself. Therefore all progress depends on the unreasonable man.

George Bernard Shaw

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The road goes ever on and on…

Abstract

The current work focused in the research subject of membrane transport and plant - environment interactions and two plant models were the target of the studies: *Olea europaea* and *Populus euphratica*. Olive tree is an emblematic species and one of the most important fruit crops in the Mediterranean basin. The halophytic and salt and drought stress tolearant plant *P. euphratica*, which occurs naturally in semiarid areas, has recently been used as a model to study plant defense mechanisms against salt stress. In both plant species we aimed to contribute to the elucidation of the biochemical mechanisms involved in salt response, in particular those involving transmembrane transport steps of photoassimilates and tonoplast transport of protons and salt. The mechanism on how sodium is accumulated in the vacuole in response to salt in *P. euphratica*, and how salt stress may affect the generation and maintenance of a transmembrane proton gradient across the tonoplast were investigated. Biochemical data corroborated the involvement of Na^+/H^+ exchange activity in cell suspensions at the tonoplast level, whose activity increased 6-fold in NaCl-treated cells. Accordingly, confocal and epifluorescence microscopy analyses with the Na^+-sensitive probe *Sodium Green* showed that suspension-cultured cells subjected to a salt pulse accumulated Na^+ in the vacuole. In tonoplast vesicles the V-H^+-PPase activity decreased with exposure to NaCl, in contrast to the observed sodium-induced increase in the activity of vacuolar H^+-ATPase. The increase of both the transmembrane H^+ gradient - generated by tonoplast proton pumps - and the Na^+/H^+ antiport activity in response to salt strongly suggested that Na^+ accumulation into the vacuole contributes to salt tolerance in *P. euphratica*, in line with the confocal microscopy observations. In *O. europaea*, key biochemical and molecular steps involved in the partitioning of sugars and polyols, and how polyols may enhance salt and drought stress resistance were addressed at the protein activity and gene expression levels. Polyols are the reduced form of aldoses and ketoses, present in several species. In *O. europaea* leaves, mannitol was found to be the main soluble carbohydrate, followed by the monosaccharide glucose. Fructose was not detected, probably because it acted as precursor for mannitol biosynthesis. Transport experiments with [^{14}C]mannitol showed that a polyol:H^+ symport system operates in *O. europaea* heterotrophic cultured cells (K_m = 1.3 mM).

Subsequent work led to the cloning of a cDNA sequence of a mannitol carrier which was named *OeMaT1* (*O. europaea* mannitol transporter *1*). In parallel experiments, salt strongly repressed mannitol dehydrogenase activity, the first enzyme responsible for intracellular mannitol oxidation, and down-regulated *OeMTD1* (*O. europaea* mannitol dehydrogenase *1*) transcripts. This should allow for the intracellular accumulation of mannitol in order to compensate for the decrease of external water activity, thus conferring a response mechanism to salinity in *O. europaea.* Subsequent studies on the molecular mechanisms of glucose utilization by olive cells led to the cloning and functional characterization of the monosaccharide transporter *OeMST2*. Heterologous expression of this gene in *Saccharomyces cerevisiae* deficient in glucose transport restored its capacity to grow and to transport glucose. Transcript levels of *OeMST2* increased during fruit maturation, confirming that OeMST2 catalyzes the membrane transport process of hexoses during sugar unloading in the fruits. In addition to this saturable energy dependent transport systems, in a variety of cell types, including plant cells, sugars, polyols and other solutes may be incorporated according to a diffusion-like kinetics, in spite of the real nature of this transport mechanism having been elusive. The measurement of [^{14}C]glucose transport by cells and membrane vesicles in the presence of specific inhibitors, the measurement of activation energies of glucose uptake, among other biochemical approaches, led us to demonstrate that the low-affinity, high-capacity, diffusion-like glucose uptake in olive cells occurs through a channel-like structure whose transport capacity may be regulated by intracellular protonation and phosphorylation/dephosphorylation. The recent publication in Nature reporting the identification and functional characterization of a new class of sugar transporters, named SWEET, which are postulated to be involved in phloem transport and plant nectar production, further strengthened the involvement of low-affinity sugar facilitators in plants.

Resumo

O presente trabalho focou-se no tema do transporte transmembranar de solutos em plantas, em particular no estudo dos mecanismos de transporte envolvidos na interação das plantas com o ambiente. As espécies modelo *Olea europaea* e *Populus euphratica* foram os alvos destes estudos ao nível bioquímico e molecular. A oliveira é uma espécie emblemática desde tempos ancestrais e uma das árvores de fruto mais importantes na bacia Mediterrânica. *P. euphratica* é uma espécie arbórea resistente ao sal e à secura, presente naturalmente em zonas semiáridas, e tem sido usada recentemente como uma planta-modelo para o estudo dos mecanismos de resistência de plantas ao stress salino. No presente trabalho tentámos contribuir para a elucidação dos mecanismos bioquímicos envolvidos na resposta de ambas as plantas à elevada salinidade, mais concretamente aqueles que envolvem o transporte transmembranar de fotoassimilados e a compartimentação no vacúolo de protões e NaCl. Em particular, foram investigados os mecanismos de compartimentação de sódio no vacúolo de *P. euphratica* em resposta à elevada salinidade, bem como o efeito do stresse salino na geração e manutenção de um gradiente transmembranar de protões através do tonoplasto. Os resultados mostraram o envolvimento de um sistema de antiporte do tipo Na^+/H^+ ao nível do tonoplasto de culturas celulares de *P. euphratica*, cuja actividade aumentou significativamente em células tratadas com NaCl. Estudos de microscopia confocal e de fluorescência com a sonda fluorescente *Sodium Green* mostraram que o NaCl se acumula no vacúolo quando as células são expostas a um pulso de sal. Em vesículas de tonoplasto purificadas de células expostas ao sal observou-se uma diminuição da actividade da bomba de protões vacuolar V-H^+-PPase, contrastando com o aumento da actividade da V-H^+-ATPase. No seu conjunto, os resultados sugerem que o aumento do gradiente transmembranar de H^+ gerado pelas bombas de protões do tonoplasto, bem como o aumento da atividade do sistema de antiporte Na^+/H^+, contribuem para a acumulação de sódio no vacúolo em *P. euphratica* em resposta ao sal. Estas observações corroboram os resultados de microscopia confocal que mostraram a compartimentação de sódio no vacúolo. Em *O. europaea* foram estudados ao nível bioquímico e molecular transportadores membranares e enzimas envolvidos no metabolismo de açúcares e de polióis, no sentido de clarificar

o papel dos fotoassimilados nos mecanismos de resposta à salinidade e à secura. Os polióis são formas reduzidas de aldoses e cetoses, presentes em mais de 100 espécies de plantas. Os resultados mostraram que o poliol manitol consiste no principal hidrato de carbono solúvel em folhas de *O. europaea*, seguido do monossacarídeo glucose. A frutose não foi detectada, provavelmente por ser utilizada como precursor biossintético de manitol. Experiências com [^{14}C]manitol demonstraram o envolvimento de um sistema de transporte do tipo simporte poliol:H^+ em culturas celulares heterotróficas de *O. europaea* (K_m = 1.3 mM). Em paralelo, foi clonada a sequência de cDNA de um transportador de manitol, denominado *OeMaT1* (*O. europaea* manitol transporter *1*). A adição de um pulso de sal a culturas celulares reprimiu a actividade da manitol desidrogenase, enzima responsável pelo primeiro passo de oxidação intracelular do manitol, e inibiu a transcrição do gene *OeMTD1* (*O. europaea* mannitol dehydrogenase *1*). Este mecanismo de regulação do transporte e metabolismo intracelular deve contribuir para a acumulação intracelular de manitol de modo a compensar a diminuição da actividade da água no espaço extracelular, constituindo um mecanismo de resposta à salinidade em *O. europaea*. Estudos subsequentes sobre os mecanismos moleculares de utilização de glucose em culturas celulares de oliveira permitiram a clonagem e caracterização funcional do transportador de monossacarídeos *OeMST2*. A expressão heteróloga deste gene numa estirpe mutante de *Saccharomyces cerevisiae* deficiente no transporte de glucose restaurou a sua capacidade de crescer em e de transportar glucose. Ao nível da planta foi observado que os níveis de transcritos do gene *OeMST2* aumentam durante a maturação da azeitona, sugerindo que o OeMST2 está envolvido no descarregamento de açúcares do floema para o fruto. Diversos estudos desenvolvidos numa ampla variedade de modelos celulares têm mostrado que os açúcares, polióis e outros solutos podem ser incorporados de acordo com uma cinética de primeira ordem (do tipo difusional), para além dos mecanismos saturáveis, dependentes de energia, como os descritos neste trabalho para os transportadores de polóis e monossacarídeos. Contudo, a natureza bioquímica e molecular destes mecanismos não saturáveis permanece ainda pouco esclarecida. No presente trabalho desenvolvemos experiências de transporte com substratos radioativos em células e vesículas de membrana plasmática na presença de inibidores específicos, no sentido de procurar compreender a natureza das cinéticas de primeira ordem observadas em culturas celulares de oliveira. No seu conjunto, os

resultados sugeriram o envolvimento de proteínas do tipo canal cuja capacidade de transporte pode ser regulada por protonação intracelular e fosforilação/desfosforilação. A publicação na prestigiada revista Nature sobre a recente identificação e caracterização funcional de uma nova classe de transportadores de açúcares denominada SWEET mostrou que permeases de baixa afinidade podem estar envolvidas no carregamento e descarregamento do floema.

Table of Contents

Abbreviations and Acronyms

°C	Degrees Celsius
2-NBDG	2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl amino]-2-deoxy-D-glucose
3-*O*-MG	3-*O*-methyl-D-glucose
$A_{###}$	Absorbance at wavelength ### nm
aa	Amino acid
ABA	Abscisic acid
ACMA	9-amino-6-chloro-2-methoxyacridine
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BAP	6-benzylaminopurine
bp	Base pair
BSA	Bovine serum albumine
BTP	Bis-tris propane (1,3-bis(tris(hydroxymethyl)methylamino)propane)
cal	Calorie
CAM	Crassulacean acid metabolism
CCCP	Carbonyl cyanide *m*-chlorophenylhydrazone
cDNA	Complementary DNA
Ci	Curie
cv.	Cultivar
CW-INV	Cell wall-bound invertase
ΔΨ	Transmembrane electric potential difference
Δ*G*	Change in Gibbs free energy
D.W.	Dry weight
Da	Dalton (unified atomic mass unit)
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dpm	Disintegrations per minute
DST	Disaccharide transporter family
DTT	Dithiothreitol
E_a	Activation energy
EDTA	Ethylenediaminetetraacetic acid
EST	Expressed sequence tag
F.W.	Fresh weight
FDA	Fluorescein diacetate
FM1-43	N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl)pyridinium dibromide
g	Gram
GC-TOF-MS	Gas chromatography-time of flight-mass spectroscopy
GFP	Green fluorescent protein
h	Hour
ha	Hectare

H_2DCFH-DA	2',7'-dichlorodihydrofluorescin diacetate
HPLC	High performance liquid chromatography
IC_{50}	Half maximal inhibitory concentration
K_m	Michaelis-Menten constant (substrate concentration required to reach half the maximum velocity of the enzymatic reaction)
L	Litre
M	Molar
MFS	Major facilitator superfamily
min	Minute
mol	Mole
MOPS	3-(N-morpholino)propanesulfonic acid
M_r	Relative molecular weight
mRNA	Messenger RNA
MS	Murashige and Skoog
MST	Monosaccharide transporter family
NAA	1-naphthaleneacetic acid
NAD^+	Nicotinamide adenine dinucleotide, oxidized
NADH	Nicotinamide adenine dinucleotide, reduced
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
NGS	Next generation sequencing
$OD_{###}$	Optical density at wavelength ### nm
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PI	Propidium iodide
P_i	Inorganic phosphate
PI3K	Phosphatidylinositol 3-kinase
PMSF	Phenylmethylsulfonyl fluoride
PP_i	Pyrophosphate
PVPP	Polyvinylpolypyrrolidone
RACE-PCR	Rapid amplification of cDNA ends-PCR
RFO	Raffinose family oligosaccharides
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rotations per minute
RT-PCR	Reverse transcriptase-PCR
s	Second
SAP	Shrimp alkaline phosphatase
SD	Standard deviation
SE	Standard error
SE-CCC	Sieve element-companion cell complex
SNP	Single nucleotide polymorphism
SP	Sugar Porter family

TCA	Trichloroacetic acid
TPP^+	Tetraphenylphosphonium cation
Tris	Tris(hydroxymethyl)aminomethane
URA	Uracil
v	Volume
V_{max}	Maximum velocity (rate) of the enzymatic reaction
W	Watts
w	Weight
x*g*	Relative centrifugal force (to Earth's gravitational acceleration)
XTT	Sodium 3'-(1-[phenylamino-carbonyl]-3,4-tetrazolium)-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate
YNB	Yeast nitrogen base

Nucleic acid notation

A	Adenosine
C	Cytosine
G	Guanine
T	Thymine
U	Uracyl
N	A, C, G or T
R	A or G
Y	C or T
M	A or C
S	C or G
W	A or T
K	G or T
V	A, C or G
D	A, G or T
H	A, C or T
B	C, G or T

Amino acid notation

A	Ala	Alanine	**M**	Met	Methionine
C	Cys	Cysteine	**N**	Asn	Asparagine
D	Asp	Aspartic acid	**P**	Pro	Proline
E	Glu	Glutamic acid	**Q**	Gln	Glutamine
F	Phe	Phenylalanine	**R**	Arg	Arginine
G	Gly	Glycine	**S**	Ser	Serine
H	His	Histidine	**T**	Thr	Threonine
I	Ile	Isoleucine	**V**	Val	Valine
K	Lys	Lysine	**W**	Trp	Tryptophan
L	Leu	Leucine	**Y**	Tyr	Tyrosine

List of Publications

Papers in International Journals with Peer-Review

Conde A, **Silva P**, Agasse A, Conde C, Gerós H. 2011. Mannitol transport and mannitol dehydrogenase activities are coordinated in *Olea europaea* under salt and osmotic stresses. *Plant and Cell Physiology* 52(10): 1766-1775.

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Silva P, Conde C, Agasse A, Lemoine R, Delrot S, Tavares RM, Gerós H. Role of mannitol as osmoprotectant in *Olea europaea*. 9th Congress of the Iberian Cytometry Society, 18-21 May 2005, Alfândega, Porto, Portugal (Oral presentation).

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Chapter 1

Introduction:
Solute transporters, plant photoassimilate partitioning and response to salt stress

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1.1. Plant solute transport: a general overview

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 processes. 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, Kaback 2005) being a good example of such successful studies (reviewed by Conde *et al.* 2010). A recent effort to compile all available membrane transport proteins and develop a classification system resulted in the Transporter Classification (TC) system, similar to the Enzyme Commission (EC) system for enzyme classification and approved by the IUBMB (International Union of Biochemistry and Molecular Biology), but incorporating structural, functional and phylogenetic information. This data is compiled on a searchable database, the Transporter Classification Database (http://www.tcdb.org/), which currently incorporates information on over 600 families of membrane transporters, including their description, TC numbers, protein sequence and relevant bibliographic references (Saier *et al.* 2009).

A transporter mediates either facilitated diffusion or active transport of solutes and metabolites in and/or out of the cell. Facilitated diffusion, which is the simplest process that a transporter can mediate (in this case also called facilitator or uniporter), is an equilibrative protein-mediated process that is not coupled to metabolic energy and, therefore, incapable of giving rise to concentration gradients of the substrate across the membrane. Active transporters transduce free energy (ΔG) 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, generally ions, but also water, 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, hydrophobic and neutral substrates are also transported by these transport systems. Facilitators and ion channels do not transduce energy (reviewed by Conde *et al.* 2010).

The study in progress of the biochemical and molecular mechanisms of solute transport across plant cell membranes is very important from a fundamental and applied standpoint. Solutes like sugars, organic acids, minerals, protons and water cross the plasma and intracellular membranes of plant cells through specific transport systems with important implications for cell homeostasis and growth, and, ultimately, to plant productivity. The same is true for the acquisition of nutrients from the soil that is also dependent on membrane transport and regulation processes (Conde *et al.* 2006, Sousa *et al.* 2007).

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, *S*ugar *P*orter). 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 (reviewed by Conde *et al.* 2011a).

Plants are generally well adapted to a wide range of environmental conditions. Even though they have notably prospered in our planet, stressful conditions such as salinity, drought and cold or heat, which are increasingly being observed worldwide in the context of the ongoing climate changes, limit their growth and productivity. Behind the remarkable ability of plants to cope with these stresses and still thrive, sophisticated and efficient mechanisms to re-establish and maintain ion and cellular homeostasis are involved (reviewed by Conde *et al.* 2011a). In this context, solute transport also has a relevant role in plant defence. For instance, the efficient exclusion of excess Na^+ from the cytosol and vacuolar Na^+ accumulation are the most important steps towards the maintenance of ion homeostasis under salt stress (see Chapter 2). The production, transport and accumulation of compatible solutes such as mannitol are also important

plant responses to salinity and drought (see Chapter 3). 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 (reviewed by Conde *et al.* 2011b). Over the last decade, our research group has been focusing on this fascinating topic of research, with special emphasis on important crops, including *Olea europaea*, *Vitis vinifera*, and *Solanum tuberosum*, as well as on the salt-tolerant model tree *Populus euphratica*.

1.2. Activities of vacuolar H^+-ATPase, H^+-pyrophosphatase and Na^+/H^+ exchange in response to salt stress

Approximately 20% of the world's cultivated land and nearly half of irrigated land are affected by salinity, which has become a serious threat to agricultural production, limiting plant growth and productivity worldwide (Rengasamy 2006, Sahi *et al.* 2006). Excessive salinity imposes two stress factors on plants: an osmotic component that results 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 (reviewed by Blumwald *et al.* 2000). Sodium toxicity is caused mainly by the similarity of the Na^+ and K^+ ions to plant transporters and enzymes. Plant cells typically maintain a high K^+/Na^+ ratio in their cytosol with relatively high K^+, in the order of 100 - 200 mM, and low Na^+, of about 1 - 10 mM (Higinbotham 1973).

Several efforts have been undertaken to enhance the salt tolerance of economically important plants by traditional plant breeding as well as by biotechnological approaches (reviewed by Flowers 2004, Karrenberg *et al.* 2006). Traditional breeding programs trying to improve abiotic stress tolerance have had some success, but are limited by the multigenic nature of the trait. The model plant *Arabidopsis thaliana* also proved to be extremely important for assessing functions for individual stress-associated genes due to the availability of knock-out mutants and its amenability for genetic manipulation (reviewed by Bartels and Sunkar 2005). The *in vitro* culture approach has been proved effective in the selection of salt-tolerant cell lines and subsequent regeneration of whole

plants with improved salt tolerance, such as alfalfa (Winicov 1991), rice (Winicov 1996, Miki *et al.* 2001) and potato (Ochatt *et al.* 1999).

Osmolytes like proline, glycine-betaine, trehalose, and sugar alcohols such as mannitol and sorbitol that are abundantly produced and accumulated in salt-treated cells represent a critical component of salt-stress responses. These compounds are expected to work through lowering the osmotic potential of cells or by protecting various cellular structures and proteins during stress (Sahi *et al.* 2006). The addition of NaCl to suspension-cultured cells of *O. europaea* enhanced the capacity of the polyol:H^+ symport system and the amount of *OeMaT1* (*O. europaea* mannitol transporter *1*) transcripts, whereas it strongly repressed mannitol dehydrogenase activity (*OeMTD1*, *O. europaea* mannitol *dehydrogenase 1*), providing intracellular accumulation of mannitol (Conde *et al.* 2007b, 2011c). Similarly, in celery cell suspensions treated with 300 mM NaCl, *Mtd* transcripts decreased in parallel with MTD activity (Williamson *et al.* 1995), while whole plants subject to salt stress displayed mannitol accumulation together with a drastic decrease in mannitol oxidation (Stoop and Pharr 1994, Everard *et al.* 1994). In addition, leaf cells of *Plantago major* displayed an increase in sorbitol content in response to treatment with salt (Pommerrenig *et al.* 2007).

Therefore, the improvement of salt tolerance in plants could be achieved by the increased production of osmolytes or stress proteins that protect or reduce damage caused by salt stress (Zhu 2001). Accordingly, when *Nicotiana tabacum*, *Populus tomentosa* and other plants were genetically engineered to synthesize mannitol through introduction of an *Escherichia coli* mannitol-1-phosphate dehydrogenase (*mtlD*), which catalyzes the biosynthesis of mannitol from fructose, it resulted in more salt-tolerant plants (Tarczynski *et al.* 1993, Hu *et al.* 2005). Additionally, *mtlD* gene transfer and expression in *Arabidopsis* enhanced seed germination under salinity conditions (Thomas *et al.* 1995). Moreover, a relationship between antioxidant defence system and salt tolerance was demonstrated in cotton and sunflower *calli* lines grown under NaCl (Gossett *et al.* 1996, Davenport *et al.* 2003). Gueta-Dahan *et al.* (1997) have also reported that salt tolerance acquisition in a citrus cell line was related with improved resistance to oxidative stress. Concordantly, the exogenous application of mannitol was shown to protect wheat plants from the harmful effects of salt-induced

oxidative stress by enhancing the activity of antioxidant enzymes (Seckin *et al.* 2009).

The ability to compartmentalise salt into the vacuoles is an important step towards the maintenance of ion homeostasis inside the cell. The first plant tonoplast Na^+/H^+ antiporter, *AtNHX1*, was isolated from *Arabidopsis* (Apse *et al.* 1999, Gaxiola *et al.* 1999) and several studies have shown that the exposure to salt up-regulates Na^+/H^+ antiport activity, suggesting a role of the exchanger in salt tolerance. The activity of this secondary transport system is driven by the proton-motive force generated by the vacuolar membrane H^+-ATPase and H^+-pyrophosphatase (V-H^+-ATPase and V-H^+-PPase, respectively; reviewed by Maeshima 2001, Martinoia *et al.* 2007) that also respond to salt levels through transcriptional and post-transcriptional regulation mechanisms. The direct stimulation of the vacuolar Na^+/H^+ antiport system may be coordinated with the increased activity of the vacuolar H^+ pumps, which provide the driving force for the operation of this cation exchanger. Thus, the overexpression of the H^+-pyrophosphatase *Avp1* was reported to confer salt tolerance in *Arabidopsis* transgenic plants (Gaxiola *et al.* 2001). In the present section, the role of the tonoplast Na^+/H^+ exchanger and proton pumps V-H^+-ATPase and pyrophosphatase on plant response to high salinity are dissected, in relation with their regulation by Na^+ and signalling pathways involved on salt sensing.

1.2.1. Two proton pumps energize the vacuolar membrane

The vacuoles of plant cells are widely diverse in form, size, content, functional dynamics and play central roles in plant growth, development, and stress responses (Paris *et al.* 1996, Martinoia *et al.* 2007). They have recognized functions in protein turnover, pH and ion homeostasis, turgor pressure maintenance, sequestration of toxic compounds, and pigmentation. The central vacuole, which can occupy more than 80% of the total plant cell volume, is separated from the surrounding cytosol by the tonoplast membrane, which controls the passage of inorganic and organic solutes to and from the cytoplasm through a wide range of pumps, carriers, ion channels and receptors (Shimaoka *et al.* 2004, Neuhaus 2007), but these proteins are generally less well known than the corresponding plasma membrane proteins. Proteomic methodologies

can provide important insights into the potential functions of these proteins (Maeshima 2001, Carter *et al.* 2004).

The electrogenic proton pumps V-H^+-ATPase and V-H^+-PPase are major components of the vacuolar membrane of plant cells (Maeshima 2001, Martinoia *et al.* 2007). With the notable exception of lemon, where the H^+-PPase can be ruled out as the primary proton pump (Müller *et al.* 1996), all plant species from which vacuolar membranes were studied exhibit V-H^+-PPase activity in addition to V-H^+-ATPase activity. The V-H^+-ATPase is universally present in the membranes of different internal acidic organelles in eukaryotic cells and has an intricate 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 through H, which form a central stalk linking the 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 forms 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 (reviewed by Martinoia *et al.* 2007). In contrast with the V-H^+-ATPase, the V-H^+-PPase consists of a single polypeptide and exists as a dimmer of subunits of 71-80 kDa. It is distributed among most land plants, but it exists only in some algae, protozoa, bacteria, and archaebacteria, and uses PP_i as its energy source (reviewed by Maeshima 2000).

In several plant models, the V-H^+-PPase seems to be able to generate and maintain across the vacuolar membrane a higher pH gradient than the V-H^+-ATPase, at PP_i concentrations in the micromolar range (Shiratake *et al.* 1997, Nakanishi and Maeshima 1998, Terrier *et al.* 2001, Queirós *et al.* 2009). Generally, V-H^+-PPase activity is high in young tissues, whereas V-H^+-ATPase activity is relatively constant during growth and maturation (Martinoia *et al.* 2007). In pear fruit, the ratio of V-H^+-PPase activity to V-H^+-ATPase activity indicated that the V-H^+-PPase is a major H^+ pump of the vacuolar membranes of young fruit, and that the contribution of the V-H^+-ATPase increases with fruit development, and finally, the V-H^+-ATPase becomes the major H^+ pump during the later stages of fruit development (Shiratake *et al.* 1997). In growing tissues and exponentially growing cultured cells, a large amount of PP_i is produced

as a by-product of several metabolic processes, such as DNA and RNA synthesis, sucrose and cellulose synthesis, and more PP_i is available to be used as a source of energy for active transport of protons into the vacuoles (Martinoia *et al.* 2007). Other studies have shown that the activity of the vacuolar V-H^+-PPase may allow the plant cell to conserve the free energy of PP_i in a transmembrane pH gradient driving the synthesis of ATP (Façanha and de Meis 1998).

1.2.2. Regulation of V-H^+-PPase and V-H^+-ATPase activity by salt

The regulation of both V-H^+-ATPase and V-H^+-PPase activity by salt is well reported in the literature; however, to date, no clear correlative pattern has been found for activation or deactivation of both proton pumps in response to salinity. Evidence for a decreased activity of V-H^+-PPase with exposure to NaCl has been described several times (Nakamura *et al.* 1992, Bremberger and Lüttge 1992, Rockel *et al.* 1994, Wang *et al.* 2000, Otoch *et al.* 2001, Silva *et al.* 2010), but it has been shown that the activity of V-H^+-PPase increases in several plants grown within saline environments (Colombo and Cerana 1993, Zingarelli *et al.* 1994, Ballesteros *et al.* 1996, Parks *et al.* 2002, Queirós *et al.* 2009). In a salt-adapted cell line of *S. tuberosum*, the activity of the V-H^+-PPase increased about three-fold over cells cultivated in the absence of salt (Queirós *et al.* 2009). In the halophyte *Suaeda salsa*, only in the case of 100 mM NaCl treatment was V-H^+-PPase activity markedly increased over the entire duration of the experiment, all other treatments only led to a small transient increase of V-H^+-PPase activity or to a decrease of activity compared to controls; thus, under salt stress and osmotic stress conditions in *S. salsa*, V-H^+-PPase activity seems to be less important physiologically than V-H^+-ATPase activity (Wang *et al.* 2001). As discussed by these authors, NaCl responses of the V-H^+-PPase depend on plant species and type of treatment and cannot be generalized.

In some plants, a clear correlation between the activity of V-H^+-PPase and protein amount has been detected, suggesting that increased or decreased protein levels may be at least partly responsible for the stimulation and repression of V-H^+-PPase activity, respectively. This is the case of *S. tuberosum*, where immunoblot analysis showed

that increased amounts of V-H$^+$-PPase protein are present in the tonoplast of NaCl-tolerant *calli*. A control step enhancing transcription or protein translation rates and/or diminishing the turnover of the protein is most likely involved in the *S. tuberosum* cells in response to salt (Queirós *et al.* 2009). Similarly, an increased accumulation of the 68 kDa V-H$^+$-PPase catalytic subunit in vacuolar membrane vesicles isolated from *Salicornia bigelovii* grown in 200 mM NaCl was observed (Parks *et al.* 2002). In tonoplast vesicles from wheat (*Triticum aestivum*) roots exposed to severe NaCl stress (200 mM) for 3 days the strong reduction in V-H$^+$-PPase substrate hydrolysis activity correlated with lower amounts of V-H$^+$-PPase protein (Wang *et al.* 2000). However, the decreased proton transport and hydrolytic activities of the V-H$^+$-PPase in 3-day-old seedlings of *Vigna unguiculata* treated with 100 mM NaCl did not show any correlation with V-H$^+$-PPase protein levels, suggesting that regulation of the activity was due to a partial enzyme inactivation (Otoch *et al.* 2001). There is evidence that transcripts encoding the V-H$^+$-PPase are regulated by salt stress in maize and bean plants (Marivet *et al.* 1994). The physiological significance and the regulation of the gene expression of the V-H$^+$-PPase has been reviewed by Maeshima (2000).

Although a reduced activity of the V-H$^+$-PPase has been observed in some plants in response to salt, it is well documented that increased salt accumulation in the vacuole is likely the result, at least in part, of an increased driving force for Na^+/H^+ exchange provided by and V-H$^+$-PPase or V-H$^+$-ATPase activity, or both. Thus, the overexpression of the vacuolar H$^+$-PPase AVP1 in *A. thaliana* resulted in plants exhibiting higher salt tolerance, which was probably a consequence of an increased proton gradient across the tonoplast (Gaxiola *et al.* 2001).

A general sodium-induced increase in V-H$^+$-ATPase activity in plant response to salt has been reported (Matsumoto and Chung 1988, Reuveni *et al.* 1990, Nakamura *et al.* 1992, Bremberger and Lüttge 1992, Zingarelli *et al.* 1994, Barkla *et al.* 1995, Ballesteros *et al.* 1996, Ayala *et al.* 1996, Ballesteros *et al.* 1997, Vera-Estrella *et al.* 1999, Otoch *et al.* 2001, Wang *et al.* 2001, Ma *et al.* 2002, Qiu *et al.* 2007, Queirós *et al.* 2009, Silva *et al.* 2010). In contrast, the activity of the V-H$^+$-ATPase in *Daucus carrota* was unaffected by salt treatment (Colombo and Cerana 1993) and was even repressed in wheat roots under severe NaCl stress (Wang *et al.* 2000).

In the halophyte *S. salsa*, the main strategy of salt tolerance seems to be an up-regulation of V-H^+-ATPase (Wang *et al.* 2001). The hydrolytic and H^+ pumping activity of the V-H^+-ATPase in tonoplast vesicles derived from leaves increased two-fold in salt-treated leaves (200 mM NaCl) compared with control leaves (Qiu *et al.* 2007). In *Mesembryanthemum crystallinum*, where the tonoplast ATPase seems to be the main enzyme responsible for the energization of malate accumulation in this CAM plant (Bremberger and Lüttge 1992), both V-H^+-ATPase H^+-transport and ATP hydrolytic activity were two-fold higher in vesicles isolated from leaves of plants treated with 200 mM NaCl, when compared with the activity measured in control plants (Barkla *et al.* 1995). In *P. euphratica,* studies showed that cell suspensions respond to salt stress by increasing both the V-H^+-ATPase hydrolytic (Ma *et al.* 2002, Silva *et al.* 2010) and H^+ pumping activities (Ma *et al.* 2002). V-H^+-ATPase proton pumping was also stimulated in NaCl-adapted cells of tobacco (Reuveni *et al.* 1990), in salt-stressed roots of barley (Matsumoto and Chung 1988), mung bean (Nakamura *et al.* 1992) and sunflower (Ballesteros *et al.* 1996), in cowpea seedlings subjected to NaCl (Otoch *et al.* 2001), as well as in *S. tuberosum calli* adapted to 150 mM NaCl (Queirós *et al.* 2009).

Several reports have shown that the activity of V-H^+-ATPase varies in parallel with protein amount. This is the case in cowpea seedlings subjected to NaCl treatment, when western blot analysis of A- and B-subunits of the V-H^+-ATPase revealed that the protein content of the two subunits increased in parallel with the increase of proton transport and hydrolytic activities (Otoch *et al.* 2001). Also, in plants of *M. crystallinum* L., two subunits of the V-H^+-ATPase with M_r of about 27 and 31 kDa showed particularly high intensities only in the CAM state, induced by salt treatment or aging, when the total ATP hydrolytic activity of the tonoplast ATPase was higher. Therefore, the increase in ATPase activity was accompanied by *de novo* synthesis of tonoplast proteins (Bremberger and Lüttge 1992). In *S. salsa*, the up-regulation of V-H^+-ATPase activity is not accomplished by structural changes of the enzyme, but by an increase in protein amount (Wang *et al.* 2001).

Other studies have shown that, in some plants, salt-mediated increase of V-H^+-ATPase activity is not mediated by the increase in protein expression, as in the halophytes *M. crystallinum* (Vera-Estrella *et al.* 1999) and *S. bigelovii* (Ayala *et al.*

1996, Parks *et al.* 2002). In tobacco (Reuveni *et al.* 1990), the relative H^+ transport capacity per unit of 69 kDa subunit of the tonoplast ATPase of vesicles isolated from NaCl-adapted cells was four-fold greater than that observed for vesicles from non-adapted cells. Such correlation between enzyme activity and protein content was also found for the tonoplast V-H^+-ATPase in potato cell lines, when western blotting analysis revealed that the relative amount of A subunit 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 (Queirós *et al.* 2009). Therefore, since the amount of the subunit A is likely to represent the protein level of V-H^+-ATPase, and post-translational modifications such as phosphorylation and/or dephosphorylation, the assembly of other subunits or the action of regulatory proteins might be involved. Phosphorylation and dephosphorylation of proteins is a common example of a post-translational modification that has the potential to alter protein activity (Gaxiola *et al.* 2007). It was shown that V-H^+-ATPases are potential targets of WNK kinases and their associated signaling pathways (Hong-Hermesdorf *et al.* 2006). Recently, the Ser/Thr kinase SOS2 (see below) was implicated in the regulation of V-H^+-ATPase activity in *Arabidopsis*, coordinating changes in ion transport during salt stress (Batelli *et al.* 2007). Proteolysis has also been show to regulate the V-H^+-ATPase. In wheat, the proteolysis of subunit A of V-H^+-ATPase was related to the observed decrease in activity of the proton pump in response to salt stress (Wang *et al.* 2000).

The ability to respond to salinity stress with changes in the gene expression of the vacuolar ATPase might be a prerequisite and a characteristic of salt tolerance in plants (reviewed by Dietz *et al.* 2001 and Kluge *et al.* 2003). It has been shown that the transcript levels of some subunits are up-regulated in response to salt stress. In fully expanded leaves of *M. crystallinum*, there was an increase in the transcript levels of subunit c mRNA but not of subunit A or B, 8 h after salt treatment (Löw *et al.* 1996), which correlates well with the observed increase in activity of the V-H^+-ATPase in vesicles from leaf mesophyll tissue from plants treated with salt (Barkla *et al.* 1995), whereas in roots and young leaves, mRNA levels for all the three subunits increased about two-fold compared to control plants. The expression of vacuolar H^+-ATPase genes does not always involve a fixed stoichiometry of mRNAs for the different subunits, and the mRNA level for subunit c is particularly sensitive to developmental and environmental

changes (Löw *et al.* 1996). Also, the emerging knowledge on subunit isogenes in some species, including *Arabidopsis*, illustrates another level of complexity, the regulation of isogene expression and function of subunit isoforms (Kluge *et al.* 2003).

Moreover, other factors may account for the regulation of tonoplast transport proteins, such as changes in lipid-protein interactions, since alterations in membrane lipid composition and structure have been associated with salt stress (Wu *et al.* 2005, Salama *et al.* 2007), and ATPase activity could be regulated by changes in the membrane lipids (Yu *et al.* 1999, Zhao and Qin 2005).

1.2.3. Regulation of Na^+/H^+ antiport activity by salt

Vacuolar Na^+/H^+ antiporters have been investigated as the key to salt tolerance in plants (Blumwald *et al.* 2000). The antiporter mediates transport of Na^+ into the vacuole. In 1985, Blumwald and Poole demonstrated the activity of the antiporter in tonoplast vesicles from red beet storage tissue (*Beta vulgaris*, Blumwald and Poole 1985) and in 1991, Barkla and Blumwald identified a 170 kDa protein associated with the vacuolar Na^+/H^+ antiport of *B. vulgaris* (Barkla and Blumwald 1991). In yeast, the Na^+/H^+ antiporter *Nhx1*, which contributes to cellular Na^+ homeostasis, was identified by Nass and co-workers (Nass *et al.* 1997). The exchanger was localized to the late endosome/prevacuolar compartment and it was proposed that it may be involved in Na^+ transport, water movement and vesicle volume regulation (Nass and Rao 1998), as well as in osmotolerance following sudden exposure to hyperosmotic media (Nass and Rao 1999). The first plant Na^+/H^+ antiporter, *AtNHX1*, was isolated from *Arabidopsis* by functional genetic complementation of a yeast mutant defective for endosomal Na^+/H^+ activity (Apse *et al.* 1999, Gaxiola *et al.* 1999), and its overexpression suppressed some of the salt-sensitive phenotypes of the *nhx1* yeast strain (Gaxiola *et al.* 1999). Since then, several Na^+/H^+ antiporter genes have been characterized in plants such as rice (*Oryza sativa*, Fukuda *et al.* 1999, Fukuda *et al.* 2004), *Atriplex gmelini* (Hamada *et al.* 2001), *B. vulgaris* (Xia *et al.* 2002), *Brassica napus* (Wang *et al.* 2003), cotton (*Gossypium hirsutum*, Wu *et al.* 2004), wheat (*Triticum aestivum*, Wang *et al.* 2002, Brini *et al.* 2005, Yu *et al.* 2007) and grapevine (*V. vinifera*, Hanana *et al.* 2007). Six

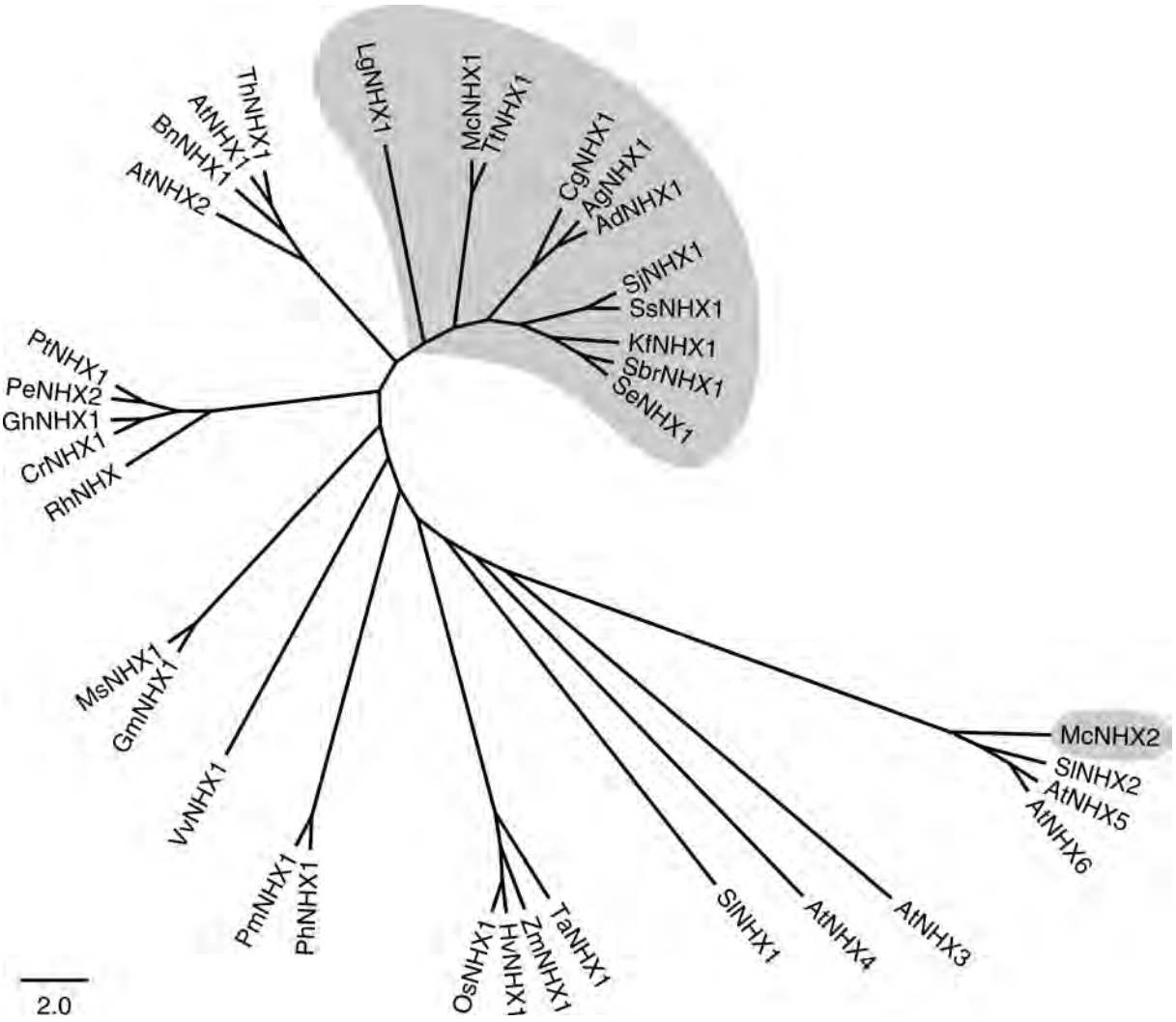

Figure 1.1. Phylogenetic tree of Na^+/H^+ antiporters. Sequence analysis was performed online using Mobyle (http://mobyle.pasteur.fr/). A multiple sequence alignment of several antiporter protein sequences was generated using ClustalW and the neighbour-joining method was used to calculate evolutionary distances. The unrooted phylogenetic tree was constructed using the FigTree software package (FigTree 1.2.2, http://tree.bio.ed.ac.uk/software/figtree/). Antiporter sequences from the following species were used in the construction of the tree: *Atriplex dimorphostegia* (*AdNHX1*, AY211397), *Atriplex gmelini* (*AgNHX1*, AB038492), *Arabidopsis thaliana* (*AtNHX1*, NM_122597; *AtNHX2*, NM_111375; *AtNHX3*, NM_124929; *AtNHX4*, NM_111512; *AtNHX5*, NM_104315; *AtNHX6*, NM_106609), *Brassica napus* (*BnNHX1*, AY189676), *Chenopodium glaucum* (*CgNHX1*, AY371319), *Citrus reticulata* (*CrNHX1*, AY607026), *Gossypium hirsutum* (*GhNHX1*,AF515632), *Glycine max* (*GmNHX1*, AY972078), *Hordeum vulgare* (*HvNHX1*, AB089197), *Kalidium foliatum* (*KfNHX1*, AY825250), *Limonium gmelinii* (*LgNHX1*, EU780457), *Mesembryanthemum crystallinum* (*McNHX1*, AM746985; *McNHX2*, AM748092), *Medicago sativa* (*MsNHX1*, AY456096), *Oryza sativa* (*OsNHX1*, AB021878), *Populus euphratica* (*PeNHX2*, EU382999), *Petunia hybrida* (*PhNHX1*, AB051817), *Plantago maritima* (*PmNHX1*, EU233808), *Populus tomentosa* (*PtNHX1*, AY660749), *Rosa hybrida* (*RhNHX1*, AB199912), *Salicornia brachiata* (*SbNHX1*, EU448383), *Salicornia europaea* (*SeNHX1*, AY131235), *Suaeda japonica* (*SjNHX1*, AB198178), *Solanum lycopersicum* (*SlNHX1*, AJ306630; *SlNHX2*, AJ306631), *Suaeda salsa* (*SsNHX1*, AY261806), *Tetragonia tetragonioides* (*TtNHX1*, AF527625), *Thellungiella halophila* (*ThNHX1*, FJ713100), *Triticum aestivum* (*TaNHX1*, AY461512), *Vitis vinifera* (*VvNHX1*, AY634283), *Zea mays* (*ZmNHX1*, NM_001111751). The shaded area represents halophytic species.

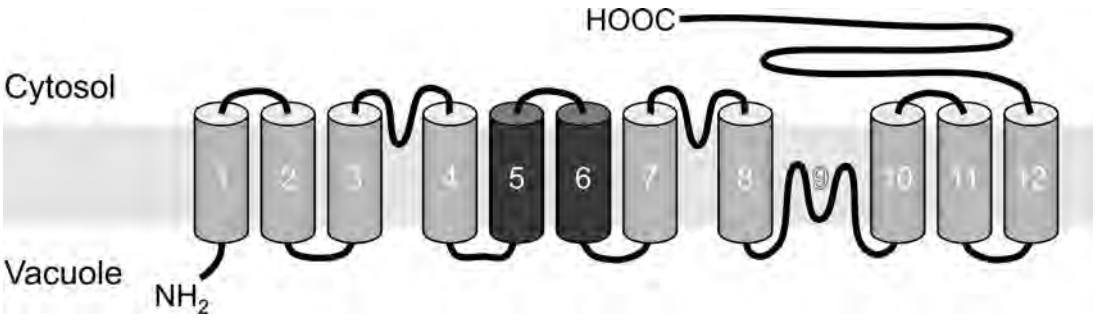

Figure 1.2. Topological model of the *Arabidopsis* Na^+/H^+ exchanger AtNHX1 (accession number NM_122597), showing 12 transmembrane domains, and with a hydrophobic, luminal N-terminal and a hydrophilic, cytosolic C-terminal. The model was constructed and adapted according to the work of Sato and co-workers (Sato *et al.* 2005). The darker transmembrane domains represent the predicted active site (Yamaguchi *et al.* 2003).

AtNHX isoforms were found in *Arabidopsis*, and for five of them Na^+/H^+ transport activity has been demonstrated (Yokoi *et al.* 2002, Aharon *et al.* 2003) (Figure 1.1). *AtNHX1* and *AtNHX2* are the most highly expressed members of this family, and corresponding transcripts are widely distributed, while *AtNHX3* and *AtNHX4* transcripts are almost exclusively present in flowers and roots. Yamaguchi and co-workers reported that AtNHX1 comprises nine transmembrane domains, with the hydrophilic C-terminal domain facing the vacuolar lumen and the N-terminal domain facing the cytosol. Three hydrophobic regions do not appear to span the tonoplast membrane, yet appear to be membrane associated (Yamaguchi *et al.* 2003). However, Sato and Sakaguchi (2005) place the C-terminal domain in the cytoplasm and confirm a structural analogy between AtNHX1 and the human NHE1, with both antiporters having 12 transmembrane domains and AtNHX1 lacking an N-terminal signal peptide (Figure 1.2). These results agree well with the structure proposed for VvNHX1 (Hanana *et al.* 2009).

Chloride channels have already been identified and cloned in plants (Plant *et al.* 1994, Lurin *et al.* 1996) and, in yeasts, mutants lacking the gene *GEF1* encoding a chloride channel are more susceptible to cation toxicity (Gaxiola *et al.* 1998). More recently, two tonoplast Cl^- transporter genes from rice, *OsClC1* and *OsClC2*, were identified and functionally characterized in yeast (Nakamura *et al.* 2004). The level of expression of *OsClC1*, but not of *OsClC2*, was increased by treatment with NaCl. In *P. euphratica,* an enhanced ability of the V-H^+-PPase to create a H^+ gradient in the presence of Cl^- was demonstrated (Silva *et al.* 2010). In fact, results by Chen and co-workers showed that, in salt-stressed *P. euphratica*, young root cortical cells

accumulated Cl^- in the vacuoles when compared with control plants (Chen *et al.* 2002), and in suspension-cultured cells subjected to 200 mM NaCl, a higher amount of Cl^- was found in the vacuole than in the cytoplasm and cell wall (Gu *et al.* 2004). This may be due to an adaptation of salt-tolerant plants to NaCl stress, where a greater permeability of the tonoplast vesicles to Cl^- can allow it to accumulate in the vacuole down its electrical gradient, dissipating an inside-positive membrane potential and thus stimulating the formation of a higher ΔpH through V-H^+-ATPase and V-H^+-PPase activity (Bennet and Spanswick 1983), which can be used in the detoxification of sodium and other cations and in an osmotic pressure increase by means of sodium accumulation in the vacuole (Gaxiola *et al.* 1999). Thus, it appears that this transporter protein could be the physiological counterpart to NHX for the accumulation of Cl^-. As discussed by Martinoia *et al.* (2007), it is not still clear if it works as a channel, as suggested by Nakamura and co-workers (2004), or as a Cl^-/H^+ antiporter.

Contrary to the notion that multiple traits introduced by breeding into crop plants are needed to obtain salt-tolerant plants, the overexpression of the vacuolar Na^+/H^+ antiport has shown to improve salinity tolerance in several plants. The first evidence showed that the overexpression of *AtNHX1* in *Arabidopsis* plants promoted sustained growth and development in soil watered with up to 200 mM NaCl (Apse *et al.* 1999), although recent evidence report that transgenic *Arabidopsis* do not show a significantly improved salt tolerance above that of control plants (Yang *et al.* 2009). In addition, transgenic tomato plants overexpressing *AtNHX1* were able to grow, flower, and produce fruit in the presence of 200 mM NaCl, and sodium accumulated in leaves but not in the fruit (Zhang and Blumwald 2001). Also, transgenic *B. napus* plants, overexpressing the same gene from *Arabidopsis*, were able to grow, flower, and produce seeds in the presence of 200 mM NaCl (Zhang *et al.* 2001), and transgenic tobacco plants overexpressing *GhNHX1* from cotton exhibited higher salt tolerance than wild-type plants (Wu *et al.* 2004). The overexpression of the Na^+/H^+ antiporter gene *OsNHX1* improved the salt tolerance of transgenic rice cells and plants (Fukuda *et al.* 2004).

The role of tonoplast Na^+/H^+ antiporter in plant salt tolerance has been reinforced by several evidences showing that exposure to salt promotes the increase of Na^+/H^+ antiport activity (Garbarino and DuPont 1988, Staal *et al.* 1991, Barkla *et al.* 1995,

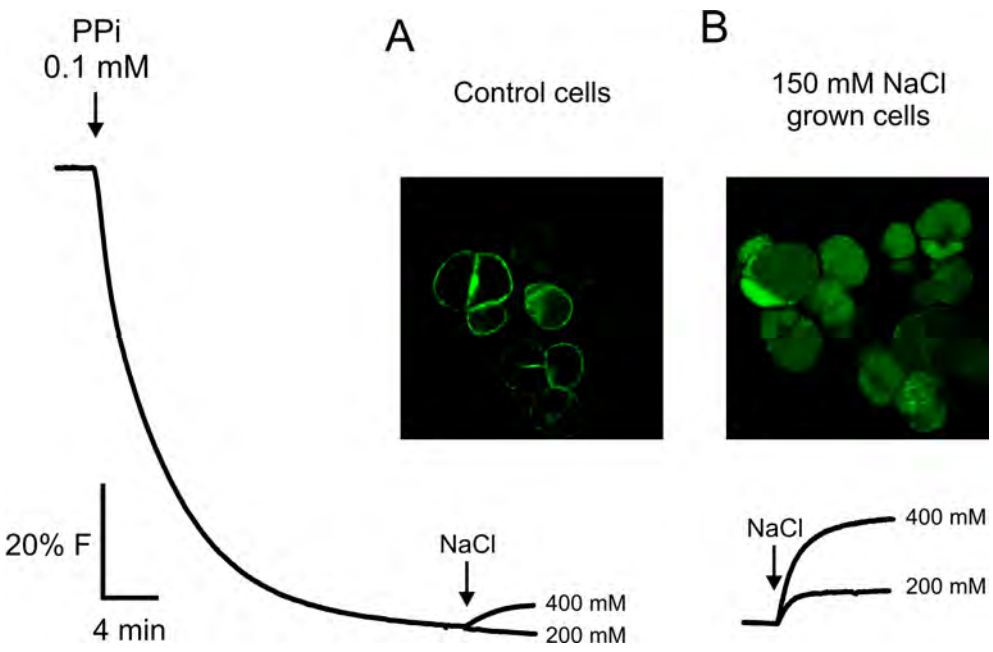

Figure 1.3. Dissipation of a PP_i-dependent H^+ gradient upon addition of 200 mM and 400 mM NaCl (final concentrations) to tonoplast vesicles isolated from *P. euphratica* suspension-cultured cells grown in the absence of salt (A) and in the presence of 150 mM NaCl (B). *Inserts:* Confocal imaging of Na^+ accumulation in *P. euphratica* suspension cells stained with Sodium Green. Adapted from Silva *et al.* (2010).

Ballesteros *et al.* 1997, Parks *et al.* 2002, Queirós *et al.* 2009, Silva *et al.* 2010) (Figure 1.3). Some reports show up-regulation of *NHX* genes (Fukuda *et al.* 1999, Gaxiola *et al.* 1999, Hamada *et al.* 2001, Xia *et al.* 2002, Shi and Zhu 2002, Wang *et al.* 2003, Fukuda *et al.* 2004, Wu *et al.* 2004, Brini *et al.* 2005, Qiu *et al.* 2007, Yu *et al.* 2007), increased protein abundance (Hamada *et al.* 2001, Xia *et al.* 2002, Wang *et al.* 2003, Qiu *et al.* 2007) or regulation at protein activity level (Garbarino and Dupont 1989, Parks *et al.* 2002). Garbarino and DuPont (Garbarino and DuPont 1988, Garbarino and DuPont 1989) have shown that the inducible Na^+/H^+ antiporter activity observed in tonoplast from barley roots grown in the presence of NaCl was due to activation of an existing protein rather than to *de novo* protein synthesis, since the rapid induction was observed in the presence of inhibitors of protein synthesis. As shown below, there can be coordination of activity between the exchangers on the tonoplast and plasma membranes (Qiu *et al.* 2004), and the C-terminal domain of AtNHX1, which may face the vacuolar lumen (Yamaguchi *et al.* 2003), may have a key role in the regulation of the protein activity by binding calmodulin (Yamaguchi *et al.* 2005). Moreover, in *A. gmelini* (Hamada *et al.* 2001), *B. vulgaris* (Xia *et al.* 2002), *B. napus* (Wang *et al.* 2003) and *S. salsa* (Qiu *et al.* 2007), up-regulation of the tonoplast Na^+/H^+ antiport activity is

due to the increase of both transcription and translation. A crosstalk between osmotic stress and vacuole accumulation of Na^+ has been demonstrated in *Arabidopsis*, where osmotic stress activates the synthesis of abscisic acid (ABA), which up-regulates the transcription of *AtNHX1* (Shi and Zhu 2002). Overall, higher-than-normal levels of *NHX* transcripts, protein, and vacuolar Na^+/H^+ antiport activity have been reported in several plants in response to salt, supporting the key role of the Na^+/H^+ exchanger in plant salinity tolerance.

1.2.4. Na^+ sensing

To survive and develop normally, plants must constantly perceive changes in their environment and respond properly through a variety of molecular mechanisms. One of the most important abiotic stresses for crop productivity concerns plant dehydration. Plants suffer from dehydration under high salinity and drought, as well as low-temperature conditions, all of which cause hyperosmotic stress, characterized by a decreased turgor pressure and water loss. Dehydration triggers the biosynthesis of the abscisic acid (ABA) hormone, and it has been known for a long time that a significant set of genes, induced by drought, salt, and cold stresses are also activated by ABA (Boudsocp and Laurière 2005). The mechanisms involved in the sensing of osmotic and salt stress in plants remain poorly understood, and the majority of the available information comes from studies in microorganisms. In yeast, hyperosmotic stress is sensed by two types of osmosensors, SLN1 and SHO1, which feed into the HOG (*h*igh-*o*smolarity *g*lycerol) MAPK (*m*itogen-*a*ctivated *p*rotein *k*inase) pathway (Bartels and Sunkar 2005). In *Arabidopsis*, the SLN1 homologue ATHK1 (*A. thaliana h*istidine *k*inase *1*) functions as an osmosensor and transmits the stress signal to a downstream MAPK cascade. The introduction of the *ATHK1* cDNA into a yeast double mutant, which lacks SLN1 and SHO1, suppressed lethality in high salinity media and activated the HOG1 MAPK pathway (Urao *et al.* 1999). Also, the activity of the plant histidine kinase Cre1 (*c*ytokinin *re*sponse *1*) is regulated by changes in turgor pressure, in a manner identical to that of Sln1, being a probable candidate for sensing osmotic stress in plants (Reiser *et al.* 2003). The gene *NtC7* from tobacco codes for a receptor-like protein functioning in osmotic adjustment, whose membrane location was confirmed in

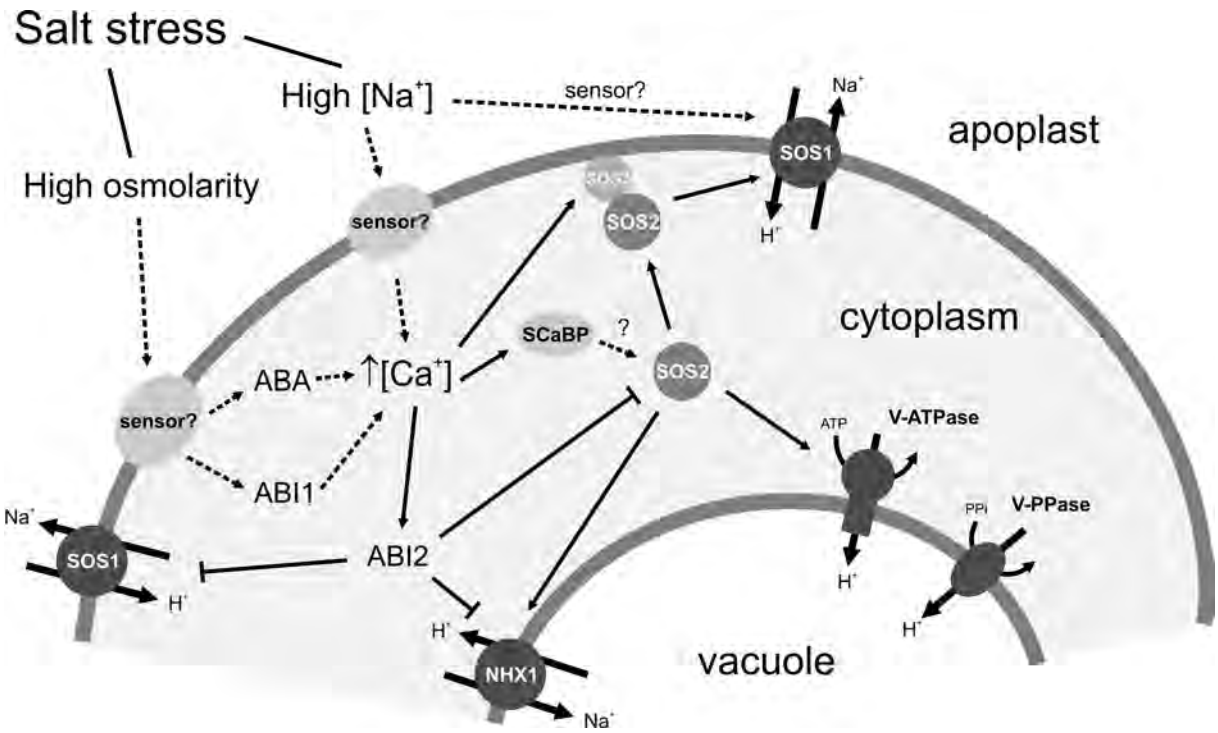

Figure 1.4. Signalling pathways responsible for sodium extrusion in *Arabidopsis* under salt stress. Excess Na^+ and high osmolarity are separately perceived by yet unidentified sensors at the plasma membrane level, which then induce an increase in cytosolic Ca^{2+} concentration. This increase is then sensed by SOS3 which activates SOS2. The activated SOS3-SOS2 protein complex phosphorylates SOS1, the plasma membrane Na^+/H^+ antiporter, resulting in the efflux of Na^+ ions (Zhu 2003). SOS2 has also been shown to regulate NHX1 antiport activity (Qiu *et al.* 2004) and V-H^+-ATPase activity (Batelli *et al.* 2007) in a SOS3-independent manner, possibly by SOS3-like Ca^{2+}-binding proteins (SCaBP) that target it to the tonoplast. Salt stress can also induce the accumulation of ABA, which, by means of ABI1 and ABI2, can negatively regulate SOS2 or SOS1 and NHX1 (Ohta *et al.* 2003).

onion epidermis cells transiently expressing an NtC7-GFP (*g*reen *f*luorescent *p*rotein) fusion protein. Its transcripts were found to accumulate rapidly and transiently within 1 h upon treatments with not only wounding but also salt and osmotic stresses (Tamura *et al.* 2003).

The knowledge on how Na^+ is sensed is still very limited in most cellular systems. Theoretically, Na^+ can be sensed either before or after entering the cell, or both (Figure 1.4). Extracellular Na^+ may be sensed by a membrane receptor, whereas intracellular Na^+ may be sensed either by membrane proteins or by any one of the many Na^+-sensitive enzymes in the cytosol (Zhu 2003). In spite of the molecular identity of Na^+ sensor(s) remaining elusive, the plasma membrane Na^+/H^+ antiporter SOS1 (*s*alt *o*verly *s*ensitive *1*) is a possible candidate (Shi *et al.* 2000). The *SOS1* gene encodes a transmembrane protein with similarities to plasma membrane Na^+/H^+ antiporters from

bacteria and fungi and the steady-state level of its transcript is up-regulated by NaCl stress (Shi *et al.* 2000). Transgenic plants showed substantial up-regulation of *SOS1* transcript levels upon NaCl treatment, suggesting post-transcriptional control of *SOS1* transcript accumulation. Undifferentiated callus cultures regenerated from transgenic plants were also more tolerant of salt stress, which was correlated with reduced Na^+ content in the transgenic cells (Shi *et al.* 2003). When expressed in a yeast mutant deficient in endogenous Na^+ transporters, SOS1 was able to reduce Na^+ accumulation and improve salt tolerance of the mutant cells, and confocal imaging of a SOS1-GFP fusion protein in transgenic *Arabidopsis* plants indicated that SOS1 is localized in the plasma membrane (Shi *et al.* 2002).

The SOS pathway was discovered when three salt-overly-sensitive mutants (*sos1*, *sos2*, and *sos3*) were characterized in a genetic screen designed to identify components of the cellular machinery that contributes to salt tolerance in *Arabidopsis*. SOS2 is predicted to encode a Ser/Thr type protein kinase with an N-terminal catalytic domain similar to that of the yeast SNF1 (sucrose non-fermenting 1) kinase (Liu *et al.* 2000) and *SOS3* encodes a Ca^{2+} sensor protein that shares significant sequence similarity with the calcineurin B subunit from yeast and neuronal calcium sensors from animals (Liu and Zhu 1998). SOS1 has been shown to be an output or target of the SOS pathway whose activity is controlled by SOS2/SOS3. *SOS1* expression was up-regulated in response to NaCl stress and this up-regulation is abated in Δ*sos3* or Δ*sos2* deletion mutant plants (Shi *et al.* 2000). The SOS1 ion antiporter, the SOS2 protein kinase, and its associated Ca^+ sensor SOS3 constitute a functional module, with SOS1 being the phosphorylation substrate for the SOS2/SOS3 kinase complex (Quintero *et al.* 2002).

Besides the implication of SOS2 in the regulation of V-H^+-ATPase activity in *Arabidopsis* (Batelli *et al.* 2007), recent evidences have also demonstrated that the tonoplast Na^+/H^+ exchanger is also a target of the SOS pathway, being regulated by the SOS2 kinase (Qiu *et al.* 2004), and the autophosphorylation of Ser 228 of SOS2 seem to be important for its function under salt stress (Fujii and Zhu 2009). In *sos1* deletion mutants, vacuolar Na^+/H^+ exchange activity is significantly higher, while in *sos2* deletion mutants this activity is strongly reduced. Activated SOS2 protein added *in vitro*

increased tonoplast Na^+/H^+ exchange activity in vesicles isolated from mutants lacking SOS2, but did not have any effect on activity in vesicles isolated from wild-type, *sos1* or *sos3* (Qiu *et al.* 2004). There can be coordination of activity between the exchangers on the tonoplast and plasma membranes; when the activity of one exchanger is missing or reduced, the activity of the other may be enhanced to compensate for the lost activity. This compensation could provide an adaptive mechanism to enable the plant to maintain the low levels of intracellular Na^+ required for growth (Qiu *et al.* 2004). Yamaguchi and co-workers (Yamaguchi *et al.* 2003) have shown that the deletion of the C-terminal domain of AtNHX1 resulted in a dramatic increase in the relative rate of Na^+/H^+ transport. In a more recent work, it was shown that the C-terminal can interact with a vacuolar calmodulin-like protein (AtCaM15) in a Ca^{2+}- and pH-dependent manner (Yamaguchi *et al.* 2005). The pH-dependence of the interaction between AtCaM15 and AtNHX1 could suggest the presence of pH-dependent signalling components in the vacuole.

1.3. Sugar and polyol transport in plants

1.3.1. Membrane transporters and loading and unloading of photoassimilates

The study of the mechanisms and regulation of sugar transport into plant sink tissues is of utmost importance since 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. In fact, up to 80% of the carbon photosynthetically fixed in the leaf is exported through the plant vascular system to sink organs, including roots and storage and developing organs (Chiou and Bush 1998, Williams *et al.* 2000). The topic of carbon fixation and translocation to sink tissues, together with the molecular mechanisms of sugar transmembrane transport, metabolism and regulation have been extensively studied, and several in-depth reviews have been published recently (Conde *et al.* 2007d, Conde *et al.* 2011b, Slewinski 2011, Davies *et al.* 2012).

Sucrose, produced through photosynthesis in the mesophyll of mature leaves, is the main carbohydrate used for long distance transport, although some plants use

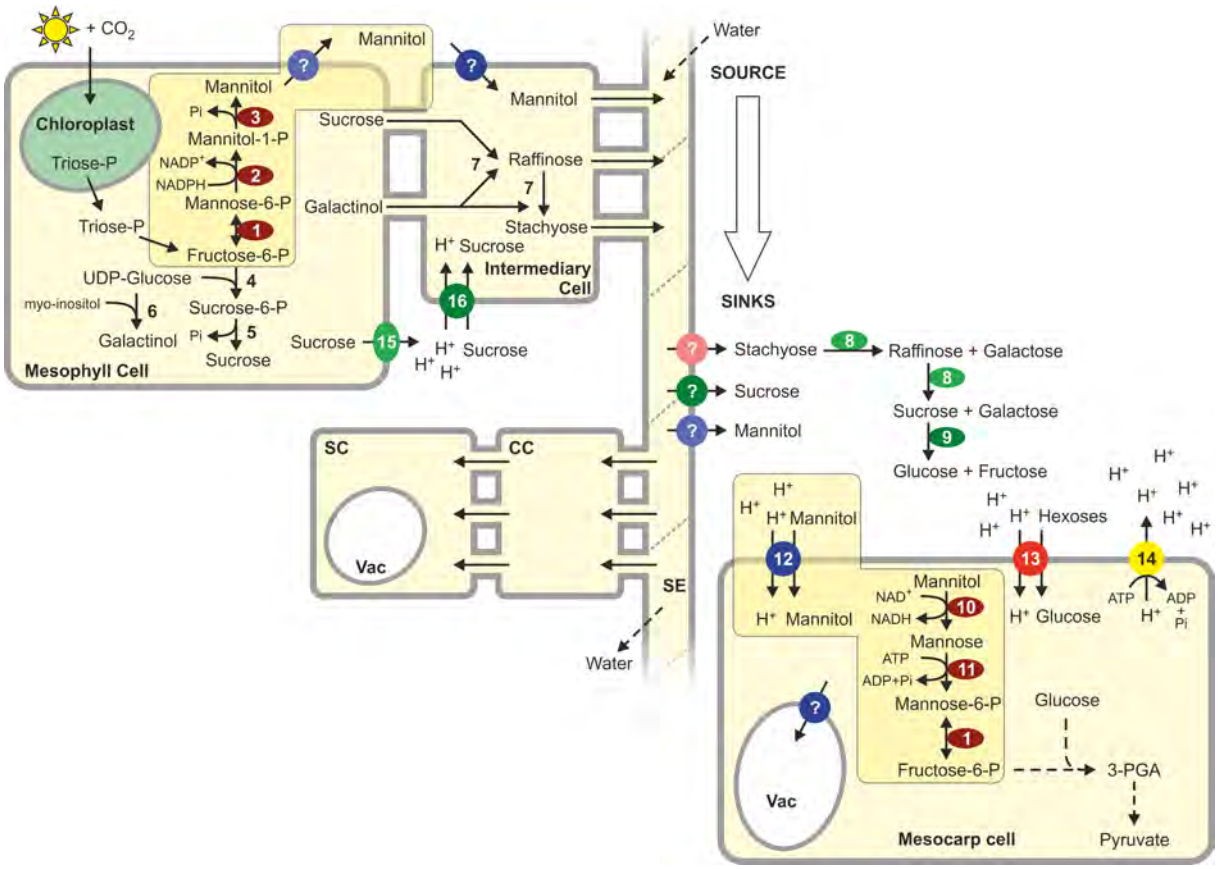

Figure 1.5. Translocation and transmembrane transport of photoassimilates in *O. europaea*, including mannitol biosynthesis, transport and catabolism. Phloem loading of stachyose and raffinose occurs *via* the symplastic pathway by "polymer trapping". Sucrose and galactinol, synthesized in mesophyll cells (MC), diffuse into intermediary cells (IC) and are used in the synthesis of raffinose and stachyose. Mannitol is synthesized *via* mannose-6-phosphate reductase (2) in the mesophyll cells of mature leaves. The RFOs are transferred to the sieve elements (SE) *via* plasmodesmata but are too large to diffuse back to the mesophyll (polymer trapping). Sucrose and mannitol may also be loaded into the phloem by the symplastic pathway, or, alternatively, *via* the apoplast. Apoplastic loading requires sucrose and mannitol export from the mesophyll (15) and reuptake into the SE-CC complex (16). This sugar efflux from the mesophyll was recently shown to be mediated by SWEETs, a new class of sugar transport proteins. Hydrostatic pressure drives phloem sap movement toward sink tissues. Apoplastic phloem unloading implies the existence of yet unidentified oligosaccharide, sucrose or polyol exporters in sink tissues. Import of photoassimilates into olive mesocarp cells may occur symplastically through plasmodesmata or may be mediated by sugar carriers. In the apoplast, α-galactosidase (8) catalyzes the hydrolysis of stachyose and raffinose into sucrose and galactose. By the action of cell wall-bound invertases (9), sucrose is hydrolyzed to glucose and fructose. These hexoses, together with galactose, are transported by a monosaccharide/H^+ symporter, OeMST2 (13), into mesocarp cells. Similarly, mannitol uptake from the apoplast into mesocarp cells is mediated by a mannitol/H^+ symporter, OeMaT1 (12). Mannitol entering the cells is oxidized to mannose *via* mannitol dehydrogenase, OeMTD1 (10). Mannitol biosynthesis is indicated in the yellow box on the upper left while mannitol catabolism is delimited in the yellow box on the lower right. (1) phosphomannose isomerase; (2) mannose-6-phosphate reductase; (3) mannose-1-phosphate phosphatase; (4) sucrose-6-phosphate synthase; (5) sucrose-6-phosphate phosphatase; (6) galactinol synthase; (7) galactinol transferases (raffinose synthase and stachyose synthase); (8) α-galactosidase; (9) invertase; (10) *O. europaea* mannitol dehydrogenase 1 (OeMTD1); (11) hexokinase; (12) *O. europaea* mannitol transporter 1 (OeMaT1); (13) *O. europaea* monossacharide transporter 2 (OeMST2); (14) plasma membrane H^+-ATPase; (15) putative SWEET-like sucrose uniporter; (16) putative *O. europaea* sucrose transporter. Adapted from Conde *et al.* 2008, Conde 2007, Stoop *et al.* 1996 and Lalonde *et al.* 1999.

raffinose, stachyose, verbascose or polyols as the main soluble carbohydrates in the phloem. After its synthesis, sucrose can move to the SE-CCC (sieve element-companion cell complex, part of the phloem) from mesophyll cells symplastically, diffusing from cell to cell *via* plasmodesmata, in a process called symplastic loading. Alternatively, sucrose can be exported from mesophyll cells to the apoplast and cross several plasma membranes before being loaded into the sieve elements. Sugar efflux into the apoplast was an unknown process until recently, when a new class of proteins with a role on sucrose efflux was identified (more on these below, Chen *et al.* 2012). Sucrose loading into the SE-CCC from the apoplast occurs actively by means of a plasma membrane sucrose/H^+ symporter, a process known as apoplastic loading (Figure 1.5). According to Munch's mass flow hypothesis, sucrose, as the major osmotically active constituent in the phloem, also provides the driving force for translocating all other compounds in the phloem sap (reviewed by Conde *et al.* 2007d).

In sink organs, sucrose can be released from the phloem apoplastically and symplastically, with the unloading pathway depending on species, organ or tissue, and development stage (Turgeon and Wolf 2009). Symplastically connected sink tissues have direct access to transported sucrose and other sap compounds, while symplastically isolated tissues have sucrose delivered to the apoplast by a putative sucrose exporter, whose protein or gene has not yet been identified, but is likely to belong to a new class of recently identified sugar uniporters named SWEETs, as discussed by Chen and co-workers (Chen *et al.* 2012; more on these below, section 1.3.3.). Cells in sink tissues then either import sucrose directly *via* sucrose transporters (DSTs) or, alternatively, sucrose can be hydrolysed to glucose and fructose by cell wall-bound invertases (CW-INV) and taken up *via* monosaccharide transporters (MSTs, Figure 1.5).

Several plant MSTs and DSTs have been studied from a molecular and functional perspective, but much less is known about sugar transporters in ligneous species, including *O. europaea.* The *Arabidopsis* gene *AtSTP1* (*A. thaliana* sugar transporter protein *1*) was the first sugar transporter gene identified in higher plants. After being heterologously expressed in an hexose transport-null (*hxt*-null) mutant yeast, it was functionally characterised as an hexose:proton symporter (Sauer *et al.* 1990).

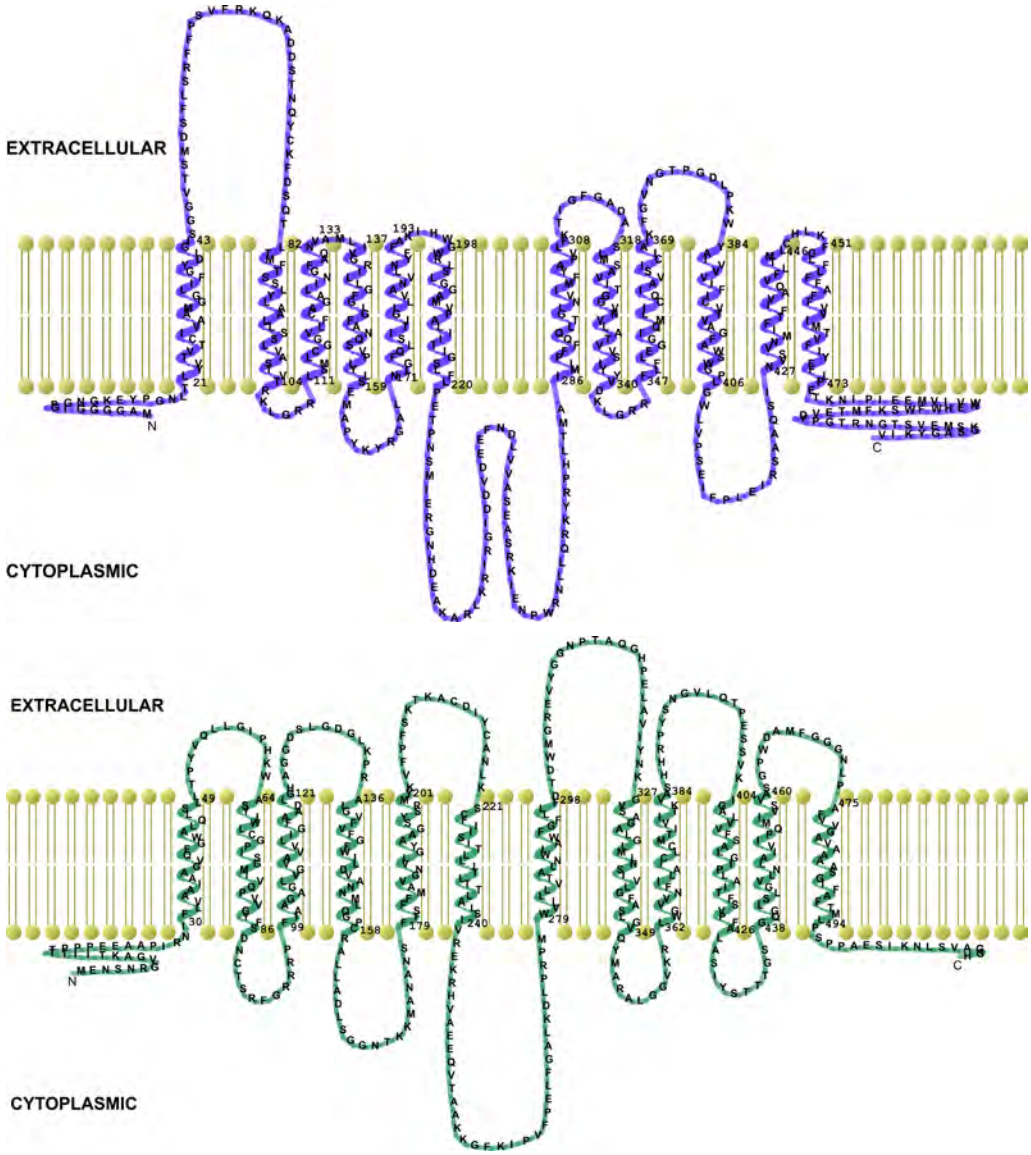

Figure 1.6. Topological model of the *Nicotiana tabacum* monosaccharide/H^+ symporter NtMST1 (top) and the *Plantago major* disaccharide/H^+ symporter PmSUC2 (bottom). The positions of the transmembrane helices were determined by the membrane protein topology prediction web service TMHMM 2.0 (Krogh *et al.* 2001) and displayed using the transmembrane protein plotting Java program TMRPres2D (Spyropoulos *et al.* 2004). The amino acid sequence is displayed along the protein traces while the numbers indicate the amino acid position where the transmembrane helices either start or stop.

MSTs, which are found in all domains of life, have highly conserved structures, and belong to the Major Facilitator Superfamily (MFS). All MST proteins characterized so far have 12 hydrophobic, transmembrane-spanning domains separated by cytoplasmic and extracellular loops, with cytosolic N- and C-terminal domains, which interact to form a central pore that allows sugars and polyols to cross hydrophobic membranes (Figure 1.6, reviewed by Büttner and Sauer 2000).

The *Arabidopsis* genome consists of 53 homologous sequences encoding MSTs,

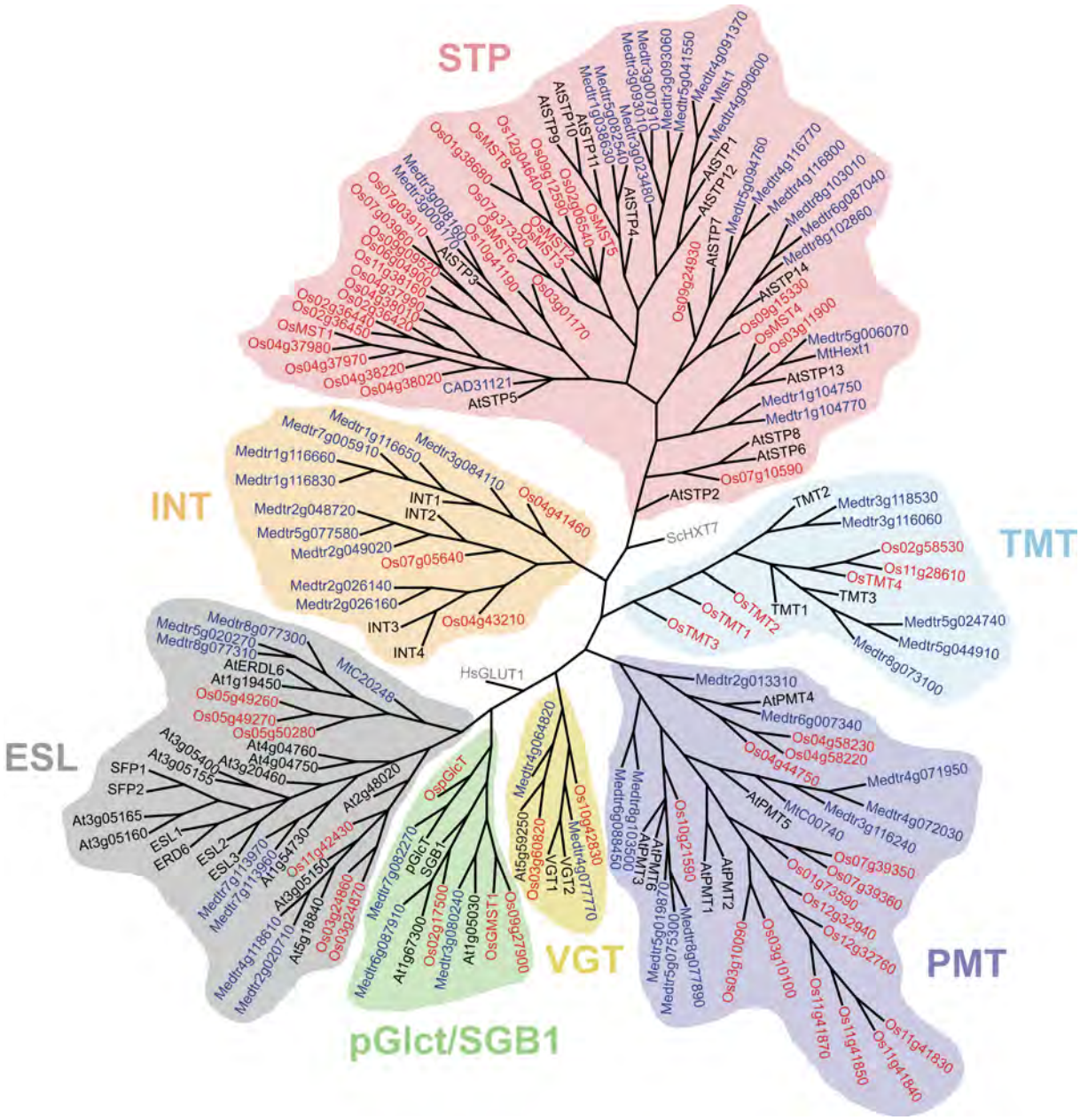

Figure 1.7. Phylogenetic tree of the monosaccharide transporter (MST) family in *Arabidopsis thaliana* (in black), *Medicago truncatula* (in blue) and *Oryza sativa* (in red). The tree separates the MST family into seven subfamilies (highlighted in colours) using 183 plant accessions, with one human and one yeast transporter (in grey) used as outgroups. Tree members are named according to their latest denomination, genomic locus or accession number. Adapted and reproduced from Doidy *et al.* (2012).

dispersed into 7 distinct clusters, two large and five small sub-families: ERD6-like and STP (*e*arly *r*esponse to *d*ehydration and *s*ugar *t*ransporter *p*roteins, respectively), the two larger ones, with the smaller ones being the pGlucT/SGB1 (*p*lastidic *gluc*ose *t*ransporter/*s*uppressor of *G*-protein *b*eta *1*), INT (*in*ositol/cyclic polyol *t*ransporter), PMT (linear *p*olyol/*m*onosaccharide *t*ransporters, recently renamed from PLT, *p*o*l*yol *t*ransporters), TMT (*t*onoplast *m*onosaccharide *t*ransporters) and VGT (*v*acuolar

*g*lucose *t*ransporters) (Figure 1.7, reviewed by Slewinski 2011).

Heterologous expression in yeasts and/or *Xenopus* oocytes has shown that all the transporters characterised so far are energy-dependent H^+ symporters. Expression of MSTs is highly regulated by sugar availability and in response to pathogen attack (Conde *et al.* 2006, Azevedo *et al.* 2006, respectively) or after wounding (Meyer *et al.* 2004). For instance, in the unicellular green alga *Chlorella kessleri* and in *V. vinifera* berries, some hexose carrier genes are induced by the substrate, while in *Chenopodium rubrum* they are constitutively expressed and not sensitive to sugar levels (reviewed by Conde *et al.* 2006, Conde *et al.* 2011b, Agasse *et al.* 2009, Davies *et al.* 2012).

1.3.2. Polyol transporters

In some plant species, polyols - the reduced form of aldoses and ketoses - are direct products of photosynthesis in mature leaves, together with sucrose. Polyols are widespread in all living organisms as carbon skeletons and energy sources (Noiraud *et al.* 2001b). Accordingly, mannitol has been detected in over 100 vascular plant species of several families including the Apiaceae (celery, carrot, parsley), Oleaceae (olive, privet) and Rubiaceae (coffee) (Table 1.1, Zimmerman and Ziegler 1978, Noiraud *et al.* 2001b). Due to its reduced nature, mannitol may be more advantageous for plants than sucrose: the initial step of mannitol oxidation is catalysed by NAD^+-dependent mannitol dehydrogenase (MTD) which regenerates NADH (Figure 1.5, lower right yellow box), thus allowing for more ATP production than in the catabolism of glucose (Pharr *et al.* 1995). Moreover, polyols may mimic the water structure and form an artificial sphere of hydration around macromolecules at low cell water potentials, as a result of the water-like hydroxyl groups of the sugar-alcohols, thus acting as compatible solutes and osmoprotectants. Consequently, they may provide tolerance to salinity or drought (reviewed by Conde *et al.* 2008, Conde *et al.* 2011b).

In mature leaves, which are autotrophic source tissues, mannitol is synthesised from mannose-6-phosphate by a mannose-6-phosphate phosphatase and a NADPH-dependent mannose-6-phosphate reductase (Figure 1.5, upper left yellow box). In heterotrophic sink tissues, it is either stored or oxidised to mannose through the

Table 1.1. List of polyols detected in the phloem sap of plant species from different families. Adapted from Zimmerman and Ziegler (1978) and Noiraud *et al.* (2001b).

Type of polyol	Family name	Plant species
Galactitol (dulcitol)	Celastraceae	*Celastrus orbiculata*
	Juglandaceae	*Juglans regia*
	Prunoideae	*Prunus padus*
	Rosaceae	*Cotoneaster hupehensis*
Mannitol	Apiaceae	*Apium graveolens*
		Daucus carota
	Bignoniaceae	*Catalpa bignonioides*
	Oleaceae	*Fraxinus americana*
		Olea europaea
	Rubiaceae	*Coffea* spp.
	Scrophulariaceae	*Paulownia tomentosa*
Ononitol	Aizoaceae	*Mesembryanthemum crystallinum*
	Fabaceae	*Medicago sativa*
Pinitol	Fabaceae	*Medicago sativa*
Sorbitol	Plantaginaceae	*Plantago major*
		Plantago maritima
	Prunoideae	*Prunus padus*
	Rosaceae	*Cotoneaster hupehensis*
		Malus domestica
		Prunus cerasus
		Prunus persica
		Pyrus spp.

action of MTD and used as a carbon and energy source (Figure 1.5, lower right yellow box). The absence of polyol synthesizing enzymes in sink organs, where polyols were detected, is in favour of long distance transport of these compounds. Another proof of long-distance transport is the detection of polyols in the phloem sap of polyol producers (Table 1.1, reviewed by Noiraud *et al.* 2001b). The elucidation of the role played by mannitol not only as a 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 polyol-metabolizing plants, such as *O. europaea* (reviewed by Conde *et al.* 2008, Conde *et al.* 2011b).

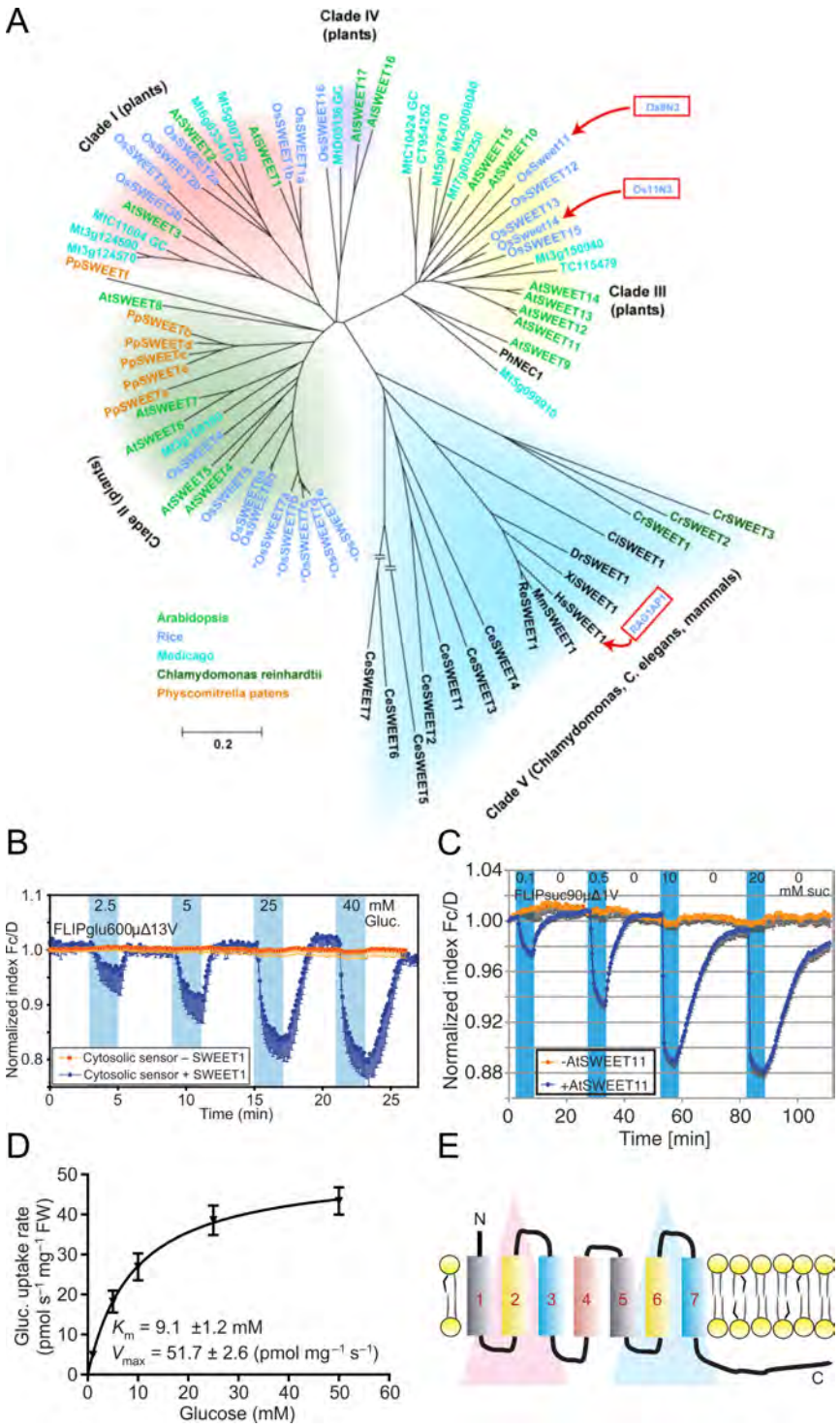

Figure 1.8. (A) Phylogenetic tree of the SWEET superfamily of *Arabidopsis thaliana* (At, light green), *Oryza sativa* (Os, blue), *Medicago truncatula* (Mt, cyan), *Chlamydomonas reinhardtii* (Cr, dark green) and *Physcomitrella patens* (Pp, orange), including members from *Caenorhabditis elegans* (Cs), mice (*Mus musculus*, Mm) and humans (*Homo sapiens*, Hs). Identification of glucose transport activity for AtSWEET1 (B) and sucrose transport activity for AtSWEET11 (C) by co-expression with a cytosolic FRET sugar sensor in HEK293T cells. Orange line indicates cells expressing sensor alone; blue line indicates cells co-expressing sensor and AtSWEET protein. (D) Initial uptake rates of [^{14}C]glucose by AtSWEET1 in a *hxt*-null *S. cerevisiae* strain. (E) Topological model of SWEET sugar uniporters based on hydrophobicity plots, evidencing the duplication of a 3 transmembrane helix protein which fused together *via* transmembrane helix 4 in a 3+1+3 configuration. Adapted and reproduced from Chen *et al.* (2010) and Chen *et al.* (2012).

In contrast to sucrose and monosaccharide transporters, much less is known about plant polyol transporters. The first cDNA encoding a mannitol transporter of a higher plant was identified and characterized in celery (Noiraud *et al.* 2001a) and named *AgMaT1* (*Apium graveolens mannitol transporter 1*). In the past years, polyol transporters from *Prunus cerasus* (cherry, *PcSOT1* and *PcSOT2*, Gao *et al.* 2003), *P. major* (broadleaf plantain, *PmPLT1* and *PmPLT2*, Ramsperger-Gleixner *et al.* 2004), *Malus domestica* (apple, *MdSOT3-MdSOT5*, Watari *et al.* 2004), *A. thaliana* (*AtPLT5* and *AtINT4*, Klepek *et al.* 2005 and Schneider *et al.* 2006, respectively) and an additional polyol carrier from celery (*AgMaT2*, Juchaux-Cachau *et al.* 2007) have been cloned and characterized (reviewed by Slewinski 2011), all being members of either the PMT or INT subfamily of MST proteins (Figure 1.7).

1.3.3. SWEET transporters: a newly identified class of sugar uniporters

As mentioned previously, despite being an essential process for the exchange of carbon and energy between cells of multicellular organisms, including plants and animals (Stümpel *et al.* 2001, Hosokawa *et al.* 2002, Lalonde *et al.* 2004), the mechanisms of sugar efflux from cells remained unknown until recently. Many cellular processes and development stages depend on this sugar efflux in order to complete successfully: for example, the maturation of pollen and development of the pollen tube requires carbohydrates and other nutrients to flow from the tapetum (Ma 2005) and stylar cells (Ylstra *et al.* 1998), respectively; carbohydrates exuded by plant roots (Walker *et al.* 2003) can feed microorganisms in the soil (known as the rhizosphere effect, Bürgmann *et al.* 2005); in young mice, the flux of glucose to pancreatic β-cells is essential to maintain serum glucose levels (Thorens *et al.* 2000); and the concentration of mannose in the blood of mammals (in the range of 50 - 100 µM) has been recently shown to be due to its efflux from cells by an yet unknown process (Sharma and Freeze 2011).

Recently, using optical glucose sensors, Chen and co-workers (Chen *et al.* 2010) identified and characterised a new class of sugar transporters, which differ significantly from the Major Facilitator Superfamily (MFS, including MSTs and DSTs). The SWEET

superfamily of proteins are low-affinity transporters which function as uniporters, and can be found in the genome of a wide variety of organisms. They can be split into five subclades (Figure 1.8A), with 17 members in *A. thaliana* and *V. vinifera*, 21 in *O. sativa*, which group into four different subclades, and a fifth subclade grouping the 7 homologues in *Caenorhabditis elegans* and the single copy present in the genome of mammals, such as humans and mice (Chen *et al.* 2010). SWEETs are small proteins, less than 300 aa in size, with 7 transmembrane helices, and are predicted to form a pore which can function bidirectionally, mediating both sugar influx and efflux in a pH-independent manner (consistent with a uniport transport mechanism). Its 7 transmembrane helices are thought to have resulted from an ancient duplication of a 3 transmembrane helix protein which fused together *via* transmembrane helix 4 in a 3+1+3 configuration (Figure 1.8E, Chen *et al.* 2010).

AtSWEET1 was characterized as a low affinity uniporter, with a K_m of 9 mM glucose (Figure 1.8D), functioning as a facilitator mediating both influx and efflux of glucose at the plasma membrane (Figure 1.8B). Other candidates, namely AtSWEET4, AtSWEET5, AtSWEET7, AtSWEET8 and AtSWEET13, also mediated glucose uniport, while, for example, AtSWEET2 was unable to mediate glucose transport. Besides glucose, additional work by Chen and co-workers (Chen *et al.* 2012) showed that AtSWEET10 to 15 were able to transport sucrose (Figure 1.8C). The fact that double *atsweet11;12* mutants had higher amount of accumulated sugars, a high expression level of *AtSWEET11* and *AtSWEET12* in source leaves, and localization of GFP fusion proteins in cells along leaf veins led authors to suggest that SWEETs are involved in apoplastic phloem unloading by exporting sucrose from mesophyll cells which are then taken up by sucrose/H^+ symporters into the SE-CC complex (Chen *et al.* 2012), and could be responsible for the first-order “diffusion-like” kinetics mentioned in the next section below.

1.3.4. Photoassimilate transport in O. europaea

O. europaea is an emblematic species and one of the most important fruit crops in the Mediterranean basin. Over 750 million olive trees are cultivated worldwide, with

about 95% located in the Mediterranean basin. *O. europaea* has also a wide distribution in Portugal, with a cultivated area of 343,200 ha (FAOSTAT, 2011), predominantly in the centre and southern areas. It represents an important economic and environmental species, making Portugal the ninth major olive-producing country in the world according to the statistics division of the Food and Agriculture Organization of the United Nations (FAOSTAT, 2011). Olive oil has a well-balanced composition of fatty acids, with small amounts of palmitate, and is highly enriched in the monoionic acid oleate, which makes it both fairly stable against auto-oxidation and suitable for human health (Conde *et al.* 2008). However, minor components, in particular phenolics, give a key contribution to the oil's high oxidative stability, colour and flavour, which makes olive oil unique among other vegetable oils.

The physiology of the olive tree is also very peculiar. It is one of the few species able to synthesise both polyols (mannitol) and oligosaccharides (raffinose and stachyose) in the leaves, which are exported, together with sucrose, to sink tissues (Figure 1.5, reviewed by Conde *et al.* 2008, Conde *et al.* 2011b). 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, together with mannitol (Marsilio *et al.* 2001).

Previous studies developed in our laboratory tested cell suspensions of *O. europaea* cv. 'Galega Vulgar' for their capacity to use sucrose, lactose, glucose, galactose, fructose, mannitol and glycerol as the sole carbon and energy sources. Mannitol and glucose were able to be successfully used as the sole carbon and energy sources, while lactose and glycerol were unable to promote cell growth. To estimate the initial uptake rates of D- or L-[^{14}C]glucose, a protocol routinely used to study solute transport in yeasts (Gerós *et al.* 1999, Silva *et al.* 2004) was optimized, and 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 1.9) associated with a first-order diffusion-like kinetics. The linear component of total glucose uptake was more evident for D-[^{14}C]glucose concentrations higher than 2 mM. The monosaccharide carrier exhibited high affinity (K_m ≈ 50 μM) and was able to transport D-glucose, D-fructose, D-galactose, D-xylose, 2-deoxy-D-glucose and

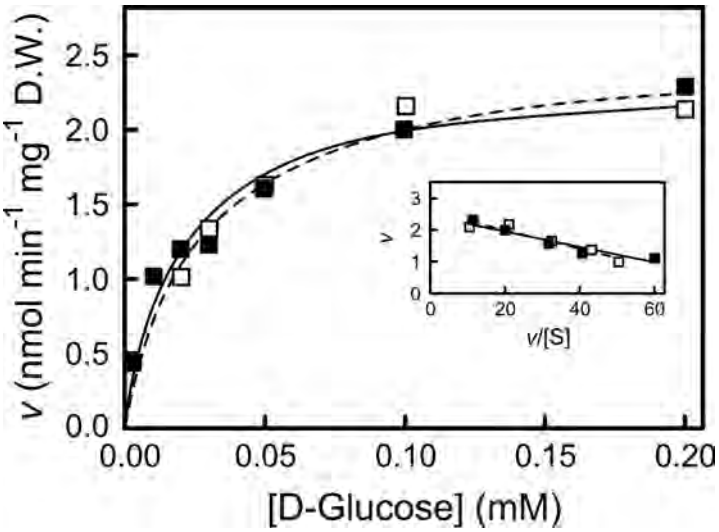

Figure 1.9. Initial uptake rates of protons (■) and D-[^{14}C]glucose (□), at pH 5.0, by *O. europaea* suspension-cultured cells grown with 0.5% (w/v) glucose. Initial alkalinisation rates of the extracellular medium were measured upon addition of glucose to cell suspensions weakly buffered with 10 mM potassium phosphate. The same cell suspensions were used to measure initial D-[^{14}C]glucose uptake. Inserts: Eadie-Hofstee plots of the initial uptake rates of protons and D-[^{14}C]glucose. Adapted and reproduced from Oliveira *et al.* (2002).

3-*O*-methyl-D-glucose, but not D-arabinose, D-mannitol or L-glucose (Oliveira *et al.* 2002). To study the energetics of the monosaccharide transport system, 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 (Oliveira *et al.* 2002). Glucose uptake was associated with proton uptake, which also followed Michaelis-Menten kinetics (Figure 1.9). The non-metabolisable glucose analogue 3-*O*-methyl-D-glucose (3-*O*-MG) was used to study the accumulative capacity of the transporter; the transport of radiolabelled 3-*O*-MG was accumulative (40-fold, at pH 5.0) and the protonophore CCCP strongly inhibited sugar accumulation. The involvement of a monosaccharide:proton symporter with a stoichiometry of 1:1 was then postulated (Oliveira *et al.* 2002). However, in cells cultivated with 3% (w/v) glucose, the uptake of D-[^{14}C]glucose followed first-order kinetics, not compatible with the involvement of a saturable transporter. The involvement of passive diffusion of the sugar was therefore proposed, a conclusion that was later disproved, as reported in the results section.

1.4. Research objectives

P. euphratica has been used as a model plant to study resistance against salt and osmotic stresses, with recent studies having characterized the tonoplast and the plasma membrane ATPases (Ma *et al.* 2002 and Yang *et al*. 2007, respectively), and six Na^+/H^+ antiporters (Ye *et al.* 2009), homologues of the *Arabidopsis* tonoplast exchanger *AtNHX1* to *AtNHX6*, were characterized and published in databases. Some reports have shown that, in *P. euphratica*, there is no significant difference in the concentration of Na^+ in the different subcellular compartments under NaCl stress (Gu *et al.* 2004). In the present study, confocal and epifluorescence microscopy techniques were employed to investigate if Na^+ is indeed accumulated in the vacuole in response to salinity. Furthermore, the activity of a tonoplast Na^+/H^+ exchange system and of the vacuolar proton pumps V-H^+-ATPase and V-H^+-PPase were evaluated, ultimately to contribute to the elucidation of the mechanisms of salt tolerance in *P. euphratica*. The results of this study are presented and discussed in Chapter 2.

Since ancient times, the olive tree (*O. europaea*), an evergreen drought- and moderately salt-tolerant species, has been cultivated for its oil and fruit in the Mediterranean basin (Loumou and Giourga 2003). Over the last decade, the biochemical and molecular mechanisms of sugar and polyol transport and utilization in *O. europaea* have been studied by our research group, aiming towards contributing to the understanding of sugar partitioning and its role in plant development and response to abiotic stress (see the review by Conde *et al.* 2011b). The work reported in Chapter 3 was developed in tight collaboration with Carlos Conde in the scope of both PhD dissertations and has already been published (Conde *et al.* 2007a, Conde *et al.* 2007b, Conde *et al.* 2007c). More recent work in olive was performed in a close collaboration with the PhD student Artur Conde and has also been published in co-authorship (Conde *et al.* 2011c). Therefore, to keep the consistency of the overall work, there may be some overlap of approaches and results with the previously mentioned papers and Ph.D. research thesis, with the contribution of each author being clearly identified in the cover page of each chapter.

After studying the main carbohydrates produced in leaves of *O. europaea* treated with salt by HPLC analysis, where mannitol was found to be the main soluble

photoassimilate (section 3.3.1.), the mechanisms of mannitol transport and utilization were characterized. For the first time in plants, it was shown that mannitol transport is regulated by means of salt-mediated changes in the transcription of mannitol carrier(s) (section 3.3.2.).

Besides mannitol, it was also found that glucose was an important soluble carbohydrate in olive leaves and, thus, subsequent work was performed to investigate the molecular and biochemical mechanisms of glucose utilization by olive cells. In section 3.3.3., the cloning and functional characterization of a monosaccharide transporter (*OeMST2*) is described, together with its expression pattern in fruits during ripening. The non-saturable transport of glucose and other organic solutes has been reported for a variety of cell types, including plant cells (see section 1.3.3.), and was also observed in the present study in olive cells. In the last part of section 3.3.3., the nature of this diffusive glucose uptake component was investigated in detail.

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Chapter 2

Role of tonoplast proton pumps and Na^+/H^+ antiport system in salt tolerance of *Populus euphratica* Oliv.

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Author contributions: HG, RMT and PS raised the hypothesis underlying this work. PS, ARF, RMT and HG designed the experiments. PS performed the experiments. PS analysed the data. PS and HG wrote the paper. HG and RMT directed the study.

Abstract

Populus euphratica has been used as a plant model to study resistance against salt and osmotic stresses, with recent studies having characterized the tonoplast and plasma membrane ATPases, and sequences of Na^+/H^+ antiporters, homologues of the *Arabidopsis* tonoplast AtNHX1, having been published in databases. In the present work, we show that *P. euphratica* suspension-cultured cells are highly tolerant to high 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. At a salt concentration of 300 mM, cells were unable to grow but remained highly viable up to 17 days after subculture. Addition of a 1 M NaCl pulse to unadapted cells did not promote a significant loss in cell viability within 48 h. In tonoplast vesicles, purified from cells cultivated in the absence of salt and from salt-treated cells, the vacuolar H^+-pyrophosphatase (V-H^+-PPase) seemed to be the primary tonoplast proton pump, however, there appears to be a decrease in V-H^+-PPase activity with exposure to NaCl, in contrast to the sodium-induced increase in the activity of the vacuolar H^+-ATPase (V-H^+-ATPase). Despite reports that there is no significant difference in concentration of Na^+ in *P. euphratica*, in the different cell compartments under NaCl stress, in the present study, confocal and epifluorescence microscopy observations using a Na^+-sensitive fluorescent probe showed that suspension-cultured cells subject to a salt pulse accumulated Na^+ in the vacuole when compared with control cells. Concordantly, a tonoplast Na^+/H^+ exchange system is described, whose activity is up-regulated by salt and, indirectly, by a salt-mediated increase of V-H^+-ATPase activity.

2.1. Introduction

The overabundance of sodium in soil and soil-solutions is a limiting factor to plant growth over large parts of the world. Excessive salinity imposes two stress factors on plants: an osmotic component that results 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 (Blumwald *et al.* 2000). Sodium toxicity is caused mainly by the similarity of the Na^+ and K^+ ions to plant transporters and enzymes. Plant cells typically maintain a high K^+/Na^+ ratio in their cytosol with relatively high K^+, in the order of 100-200 mM, and low Na^+, of about 1-10 mM (Higinbotham 1973). Thus, the efficient exclusion of Na^+ excess from the cytoplasm and vacuolar Na^+ accumulation are the main mechanisms for the adaptation of plants to salt stress. This is typically carried out by transmembrane transport proteins that exclude Na^+ from the cytosol in exchange for H^+, a secondary transport process which is energy-dependent and driven by the proton-motive force generated by the plasma membrane H^+-ATPase (P-H^+-ATPase, Serrano 1989), and by the vacuolar membrane H^+-ATPase (V-H^+-ATPase) and H^+-pyrophosphatase (V-H^+-PPase) (Rea and Sanders 1987, Rea and Poole 1993).

P. euphratica is the only tree species that occurs naturally from the semiarid areas of Northwest China to western Morocco and Spain (Browicz 1977). It is a halophytic plant, tolerating salt and drought stresses (Kang *et al.* 1996, Watanabe *et al.* 2000, Chen *et al.* 2002, Chen *et al.* 2003), as well as temperature extremes (Ferreira *et al.* 2006, Silva-Correia *et al.* 2012) and has recently been used as a model plant to study plant defense mechanisms against salt stress. In *Arabidopsis*, a vacuolar Na^+/H^+ antiporter (AtNHX1), homologue to the yeast antiporter NHX1, was cloned and functionally expressed in *S. cerevisiae* (Gaxiola *et al.* 1999). Since then, several NHX homologues have been characterized in plants (Xi *et al.* 2002, Fukuda *et al.* 2004, Wu *et al.* 2004, Saqib *et al.* 2005, Hanana *et al.* 2007). In the case of *P. euphratica*, nucleotide databases display six NHX sequences, *PeNHX1* to *PeNHX6*, with accession numbers FJ589739, FJ589740, FJ589741, FJ589742, FJ589743, GQ324700, respectively. In spite of recent work showing that Na^+ concentration in the vacuole is not too different from that in the cytosol (Gu *et al.* 2004, Ottow *et al.* 2005a), microscopy and biochemical

data are provided in the present study suggesting the involvement of a salt-inducible Na^+/H^+ exchange activity in the tonoplast of *P. euphratica* cultured cells.

2.2. Materials and Methods

2.2.1. Cell suspensions and growth conditions

Cell suspensions of *P. euphratica* Oliv. were maintained in 250 mL flasks on a rotatory shaker at 100 rpm, in the dark, at 25 °C in Murashige and Skoog (MS) medium (Murashige and Skoog 1962), supplemented with 2.5% (w/v) sucrose and 1.1 µM 6-benzylaminopurine (BAP) and 2.7 µM 1-naphthaleneacetic acid (NAA), as described by Gu *et al.* (2004). Cells were subcultured every fifteen days by transferring 10 mL aliquots into 50 mL of fresh medium. Growth was monitored by determination of dry weight. Aliquots of 1-5 mL were filtered through pre-weighted GF/C filters (Whatman, Clifton, NJ, USA). The samples were washed with deionized water and weighted after 24 h at 80 °C. Sugar consumption was monitored by HPLC, with L-arabinose as the internal standard.

2.2.2. Determination of cell viability

Fluoresceine diacetate (FDA) staining was applied to estimate cell viability. A concentrated stock solution of FDA (500 mg mL^{-1}, Sigma, St. Louis, MO, USA) was prepared in dimethyl sulfoxide (DMSO). For the staining protocol, suspension-cultured cells (1 mL) were incubated with 10 µL of FDA stock solution in the dark for 10 min at room temperature. Cells were observed under a Leitz Laborlux S epifluorescence microscope with a 50 W mercury lamp and appropriate filter settings. Images were acquired with a 3-CCD color video camera (Sony, DXC-9100P), a frame grabber (IMAGRAPH, IMASCAN/Chroma P) and software for image management and archival storage (AxioVision Version 3.0, Carl Zeiss Vision, Germany).

2.2.3. Determination of Na^+ accumulation and intracellular localization

To determine the intracellular accumulation of Na^+, cells were washed twice in MS medium. A concentrated stock solution of Sodium Green (5 mM, Molecular Probes, Eugene, OR, USA) was prepared in DMSO prior to use. One mL of a cell suspension was incubated with 10 µM (final concentration) of the fluorescent Na^+ probe. After incubation in the dark for 10 min at room temperature, cells were washed twice in MS medium and observed under an Olympus FluoView FV1000 confocal laser scanning microscope (Olympus, Germany) with appropriate filter settings. To determine the intracellular localization of Na^+ in the vacuole, cells were pre-incubated with 0.1% (w/v) of Neutral Red 5 min prior to the addition of the fluorescent Na^+ probe. After incubation of both dyes, cells were washed twice in MS medium and observed under a Leica DM5000B fluorescence microscope (Leica Microsystems, Wetzlar, Germany) equipped with a 100 W mercury lamp (AF6000, Leica Microsystems, Wetzlar, Germany) and appropriate filter settings. Visible and epifluorescence images were acquired with Leica DFC350 FX digital camera and Leica Microsystems LAS AF software, version 2.0 (Leica Microsystems, Wetzlar, Germany).

2.2.4. Isolation of vacuolar membrane vesicles

Vacuolar membrane vesicles were isolated from *P. euphratica* suspension-cultured cells by differential centrifugation in a sucrose gradient (Façanha and de Meis 1995, Façanha and de Meis 1998, Queirós *et al.* 2009). Cells (40 - 50 g fresh weight) were harvested, centrifuged at 3,000x*g* for 1 min, washed twice with deionized water, and suspended in 100 mL of ice-cold buffer containing 250 mM sucrose, 2 mM EDTA (pH 8.0), 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 70 mM Tris-HCl (pH 8.0), 3 mM $MgCl_2$, 100 mM KCl, 0.1% (w/v) BSA and 0.2% (w/v) polyvinylpolypyrrolidone (PVPP). The mixture was homogenized with an Ultra-Turrax T25 device (IKA® WERKE, Janke and Kumkel IKA, Germany) for 3 min at 24,000 rpm, on ice, and the homogenate was filtered through a layer of cheesecloth and centrifuged at 3,500x*g* for 10 min. The supernatant was centrifuged once more at 10,000x*g* for 10 min and then at 100,000x*g* for 30 min (Beckman 70Ti rotor). The

pellet was resuspended in 8 mL ice-cold resuspension buffer (15% [v/v] glycerol, 1 mM DTT, 1 mM EDTA, 1 mM PMSF and 20 mM Tris-HCl [pH 7.5]). The suspension was layered over a 32% and 46% (w/v) discontinuous sucrose gradient and centrifuged at 80,000x*g* for 3 h in a Beckman SW 28 rotor. In addition to sucrose, the gradient solutions contained 20 mM Tris-HCl buffer (pH 7.6), 1 mM EDTA, 1 mM DTT and 1 mM PMSF. The vesicles sedimenting at the 0% - 32% interface were collected, diluted with 4 volumes of resuspension buffer, and centrifuged at 100,000x*g* for 30 min (Beckman 70Ti rotor). The pellet was resuspended in the resuspension buffer described above. The vesicles were then frozen under liquid nitrogen and stored at -80 ºC until use. Protein concentration was determined by the method of Lowry (Lowry *et al.* 1951), with BSA as the standard.

2.2.5. Proton pumping activity of V-H$^+$-ATPase and V-H$^+$-PPase

Proton-pumping measurements were determined by measuring the fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA) using a Perkin-Elmer LS-5B Luminescence Spectrometer (Perkin-Elmer, Buckinghamshire, England, UK), as described earlier (Queirós *et al.* 2009). The excitation wavelength was set at 415 nm and the emission wavelength was set at 485 nm. After the addition of tonoplast vesicles (30 µg) to 2 mL of buffer containing 10 mM MOPS-Tris (pH 7.2), 2 µM ACMA, 5 mM $MgCl_2$, and 100 mM KCl, the reaction was started by addition of either ATP or PP_i at appropriate concentrations, and the rate of the initial fluorescence quenching was recorded. The Mg-PP_i complex is the actual substrate for the V-H$^+$-PPase (Rea and Poole 1985). The optimal concentration of Mg^{2+} in the assay medium was previously adjusted to values between 2 - 5 mM. The addition of 1.5 mM NH_4Cl, a H^+ uncoupler, abolished the H^+ gradient formed by either ATP or PP_i hydrolysis. All experiments were performed at 25 ºC. The H^+ pumping activity was measured by the linear initial slope of fluorescence quenching (expressed as Δ%F min^{-1} mg^{-1} protein). The results were analysed by computer-assisted nonlinear regression analysis (GraphPad Prism 4.0, San Diego, CA, USA). By this method, H^+ pumping kinetics best fitting to the experimental initial acidification curves were determined, corresponding to the quenching of ACMA fluorescence, and estimates for the kinetic parameters were then obtained.

2.2.6. Study of the activity and specificity of the tonoplast Na^+/H^+ antiport system

For measurements of the rate of dissipation of the pH gradient, tonoplast vesicles were energized by the V-H^+-PPase with the addition of a saturating concentration of PP_i (Queirós *et al.* 2009). After reaching a fluorescence quenching steady-state, aliquots of the desired salt solutions were added, and the initial rates of fluorescence recovery were recorded, corresponding to the Na^+/H^+ antiport activity (expressed as $\Delta\%F\ min^{-1}\ mg^{-1}$ protein). The time used to calculate initial rates of recovery was 15 s. The specificity of Na^+/H^+ antiport was evaluated by adding various salts to dissipate the pH gradient in vacuolar membrane vesicles. All experiments were performed at 25 ºC.

2.2.7. Determination of V-H^+-ATPase and V-H^+-PPase hydrolytic activity

The determination of V-H^+-ATPase and V-H^+-PPase hydrolytic activity was conducted as described earlier (Queirós *et al.* 2009). Briefly, the reaction was started by the addition of 60 µg of tonoplast membrane to 1.5 mL of reaction medium containing 50 mM MOPS-Tris (pH 7.2), 3 mM $MgSO_4$, 100 mM KCl and the substrate (ATP or PP_i) at the desired concentration. After 10 and 20 min, 0.5 mL was added to ice-cold 3% (w/v) trichloroacetic acid (TCA), mixed, and the release of Pi from either ATP or PPi determined colorimetrically (Fiske and Subbarow 1925). For the determination of V-H^+-ATPase activity, Pi release was measured with and without KNO_3 and the difference between these two activities was attributed to the V-H^+-ATPase.

2.3. Results

2.3.1. Growth in batch cultures with NaCl and impact of salt on cell viability

P. euphratica suspension-cultured cells were cultivated in the dark, at 25 ºC, in MS medium with 2.5% (w/v) sucrose, in the absence of salt and in the presence of 150 and 300 mM NaCl. In all experimental conditions, extracellular sucrose was completely hydrolyzed within 5 d and growth/maintenance occurred along with glucose and fructose consumption (not shown). Biomass production and cell viability were

monitored at the end of the growth period (Figure 2.1A). Cell viability was assessed by FDA, which is permeable to the intact plasma membrane and is converted into a green fluorescent dye, fluorescein, by a function of internal esterases, showing green color in viable cells. Cells cultivated without salt reached a biomass value of 11 mg mL^{-1} D.W. 17 days after subculture, while cells grown in the presence of 150 mM NaCl reached a maximal population size of 9 mg mL^{-1} D.W. In both cases, growth arrest occurred only after monosaccharide depletion (not shown) and cell viability remained close to 100%. In the case of cell cultures supplemented with 300 mM NaCl, only 15% of both glucose and fructose were consumed (not shown) and the final population size was very low, suggesting that the assimilated carbon and energy source was being redirected not towards cell growth and proliferation but instead to the maintenance of homeostasis in the presence of a high NaCl concentration, such as the biosynthesis of compatible solutes and/or the activation of plasma membrane and tonoplast proton pumps and antiport systems. The surprising observation that *P. euphratica* cell suspensions remained highly viable 17 days after subculture in media with 300 mM NaCl (71 % viability) led us to assess in more detail their capacity to resist to salt upon addition of NaCl pulses up to 1 M concentration. Cells were cultivated in media with 2.5% (w/v) sucrose without salt, aliquots were collected at mid-exponential growth phase and 0.5 to 1 M of NaCl was added.

Figure 2.1B shows that *P. euphratica* suspension cells remained close to 100% viable 24 h after a 1 M NaCl pulse, although displaying a smaller size and a denser cytoplasm when compared to control cells. Forty-eight hours after the salt pulse, cells started to form aggregates and viability decreased to around 70%. Control cells remained viable throughout the experiment.

2.3.2. Activity of tonoplast proton pumps and Na^+/H^+ exchange

Tonoplast vesicles were isolated from *P. euphratica* suspension cells in the absence of salt and in the presence of 50 and 150 mM NaCl. In these tonoplast fractions, both the ATPase hydrolytic and H^+ pumping activities, at pH 7.2 (pH optimum of V-H^+-ATPase), were inhibited less than 5% by 0.1 mM vanadate (inhibitor of P-ATPase). Also,

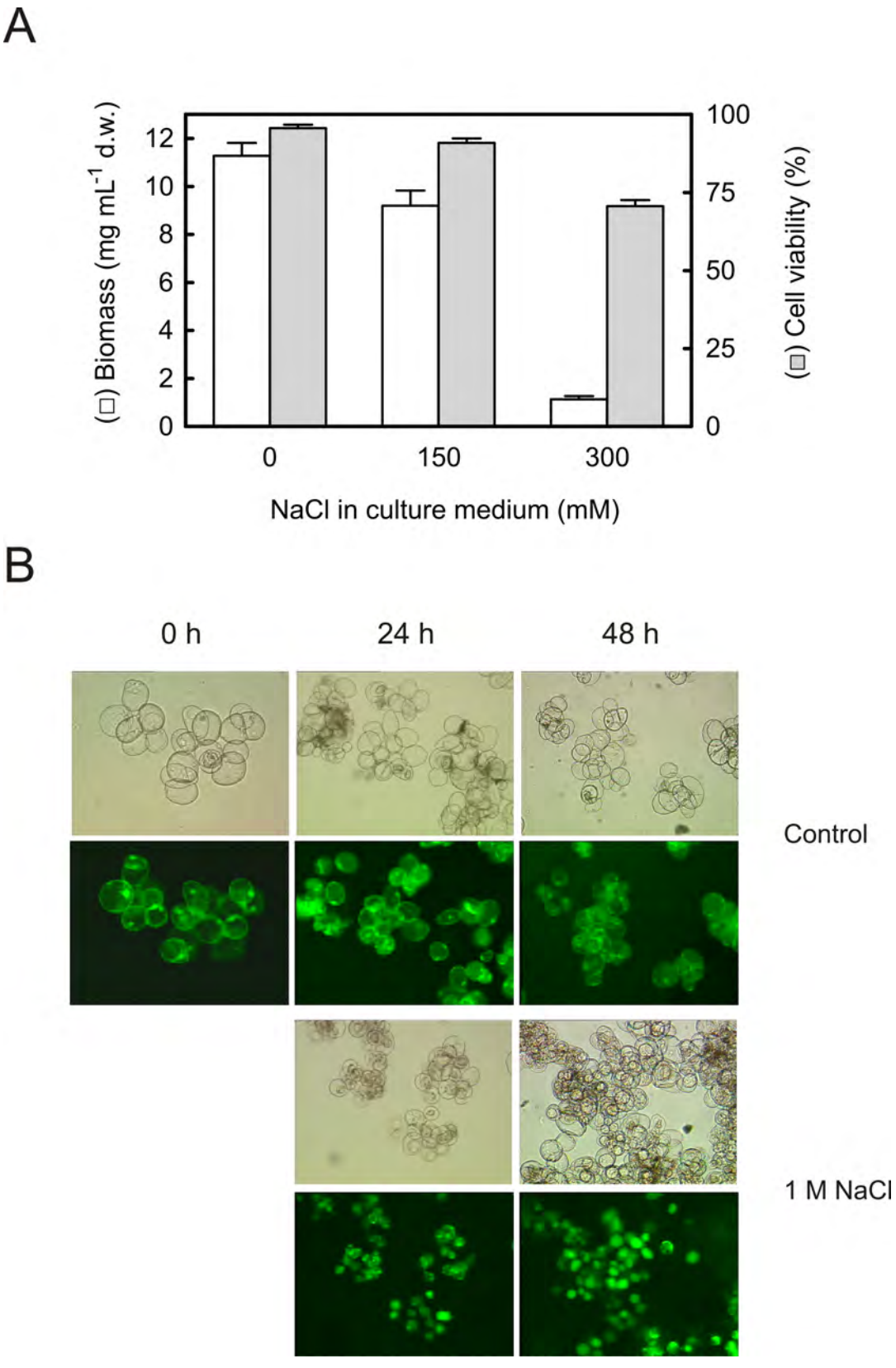

Figure 2.1. (A) Final population size and cell viability of *P. euphratica* suspension-cultured cells, 17 days after subculture in mineral media with 2.5 % (w/v) sucrose, in the absence of salt and in the presence of 150 and 300 mM NaCl (error bars denote SE, *n* = 2) and (B) viability assays after the addition of 1 M NaCl to cells cultivated in the absence of salt. Fluorescence was measured after incubation with FDA (green fluorescence).

the F-ATPase (mitochondrial and plastid) inhibitor azide (2 mM NaN_3) hardly inhibited ATPase hydrolytic activity, at the same pH condition. Conversely, 50 mM nitrate (in the form of KNO_3) inhibited almost completely both the ATPase hydrolytic and H^+ pumping activities. These results strongly suggest that the vesicles used in this study consist mainly of vacuolar membrane.

Figure 2.2 shows the PP_i-dependent (Figure 2.2A) and ATP-dependent (Figure 2.2B) H^+ pumping activities across tonoplast vesicles, as measured by the fluorescence quenching of ACMA, determined at substrate saturating concentrations. Both NH_4Cl and CCCP (not shown) promptly recovered ACMA fluorescence, demonstrating that a pH gradient had been generated. In this biological system, the V-H^+-PPase is able to generate a pH gradient 3-fold greater than the V-H^+-ATPase at 10-fold less substrate concentration: the V_{max} value for the V-H^+-PPase H^+ pumping was 936 Δ%F min^{-1} mg^{-1} protein (Figure 2.2C) and the V_{max} for V-H^+-ATPase was 248 Δ%F min^{-1} mg^{-1} protein (Figure 2.2B); conversely, the K_m value of V-H^+-PPase H^+ pumping estimated from the Michaelis-Menten plot (Figure 2.2C) was determined to be 3.9 µM, while the K_m for the V-H^+-ATPase is 0.65 mM ATP (Ma *et al.* 2002). Figure 2.2D shows the dependence of the initial velocities of PP_i hydrolysis by V-H^+-PPase on the substrate concentration. From the Michaelis-Menten plot, the following kinetic parameters were estimated: V_{max} = 172 nmol PP_i min^{-1} mg^{-1} protein, K_m = 45.8 µM PP_i. By comparing H^+ pumping activity with hydrolytic activity we may observe that there is a higher coupling efficiency (H^+ pumping/PP_i hydrolysis) at lower substrate concentrations. Following the analysis of the initial velocities of H^+ pumping at saturating PP_i concentrations (Figure 2.2A), in tonoplast vesicles from suspension-cultured cells subject to 50 mM NaCl, the V_{max} of the V-H^+-PPase decreased around two-fold, to 528 Δ%F min^{-1} mg^{-1} protein, while in tonoplast vesicles from cells cultivated in the presence of 150 mM NaCl, a recovery to levels close to the control, 771 Δ%F min^{-1} mg^{-1} protein, was observed.

The hydrolytic activity of the V-H^+-PPase (measured at 0.1 mM PP_i) followed a pattern similar to the H^+ pumping, with a slight decrease in vesicles from cells cultivated in the presence of 50 mM NaCl when compared with the control, and a subsequent recovery of hydrolytic activity in vesicles from cells grown with 150 mM NaCl (Figure 2.3). However, the most dramatic change occurred in the V-H^+-ATPase hydrolytic

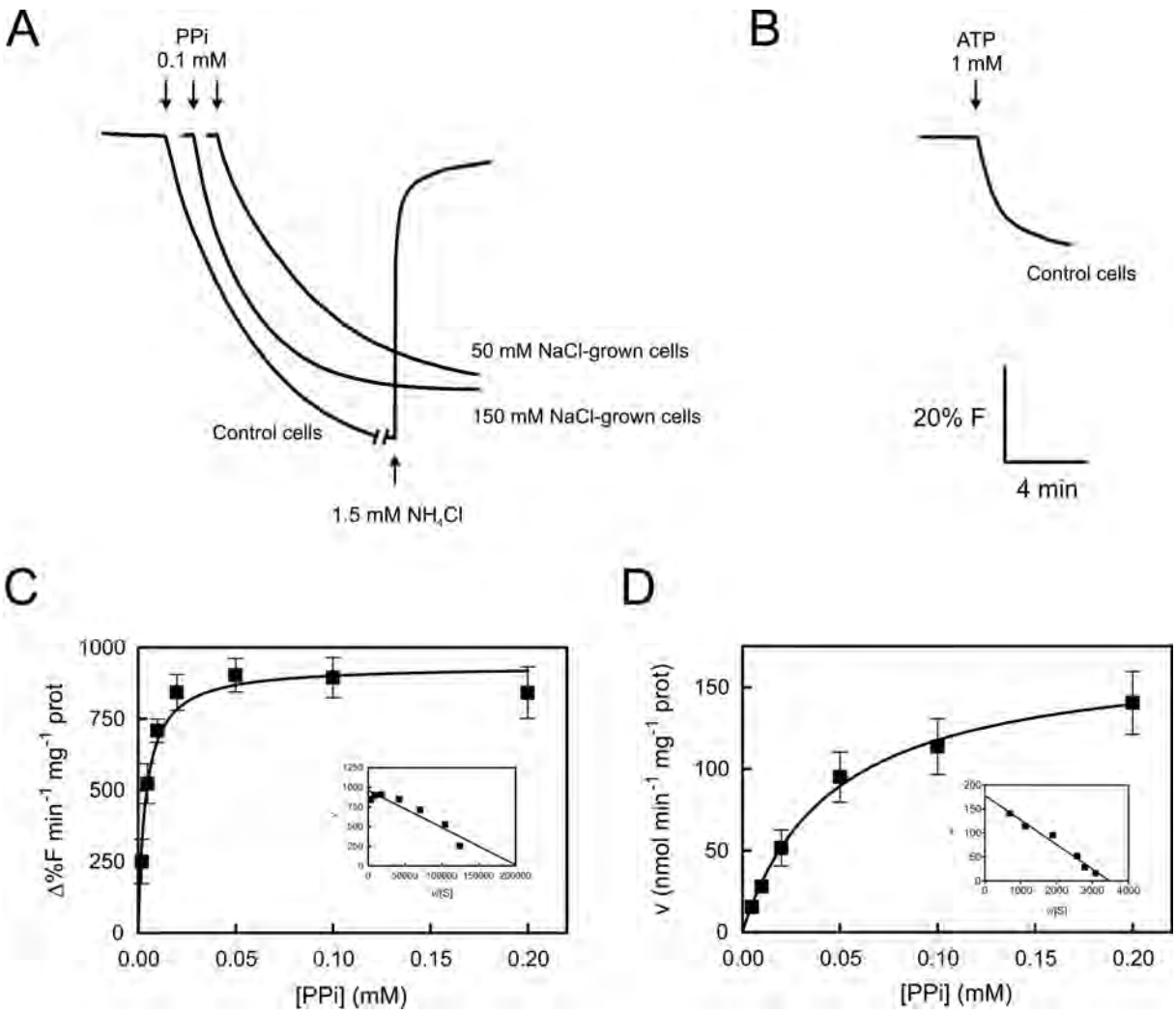

Figure 2.2. Proton pumping activity of (A) V-H$^+$-PPase in tonoplast vesicles isolated from *P. euphratica* suspension-cultured cells grown in the absence of salt (control) and in the presence of 50 and 150 mM NaCl and of (B) V-H$^+$-ATPase in suspension cultures grown in the absence of salt. The accumulation of H$^+$ inside the vesicles was determined by measuring the fluorescence quenching of ACMA (Façanha and de Meis 1998). Addition of 1.5 mM NH_4Cl promoted the recovery of fluorescence. Initial velocities of (C) proton pumping and (D) hydrolytic activity of the *P. euphratica* V-H$^+$-PPase. *Insert:* Eadie-Hofstee plot of the initial H$^+$ pumping rates and PP_i hydrolysis, respectively. Error bars denote SE, n = 3.

activity (measured at 1 mM ATP), which increased in vesicles from cells treated with salt, with the highest value measured in vesicles from cells grown in the presence of 150 mM NaCl (Figure 2.3), confirming the results of Ma and co-workers (Ma *et al.* 2002) whose studies showed that *P. euphratica* cell suspensions respond to salt stress by increasing both the V-H$^+$-ATPase hydrolytic and H$^+$ pumping activities.

It has been hypothesized that the V-H$^+$-PPase may be directly inhibited by salt due to the similarly of Na^+ to K^+, a cofactor of the enzyme (Rea and Poole 1993). In the present work, the inhibition of *P. euphratica* V-H$^+$-PPase by NaCl was inferred by the

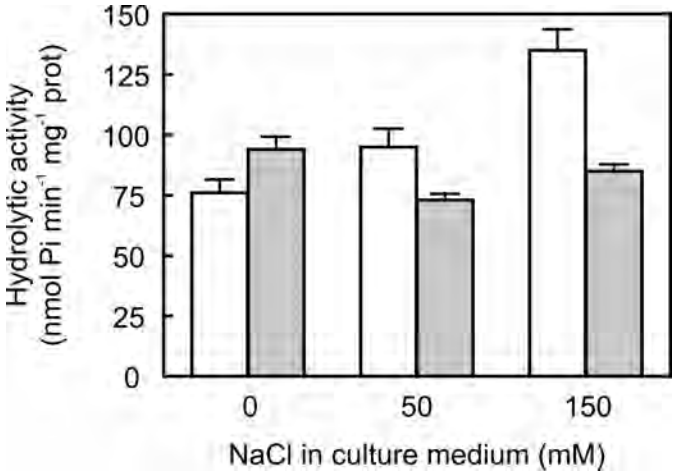

Figure 2.3. Hydrolytic activity of the V-H⁺-ATPase (□) and V-H⁺-PPase (■) in tonoplast vesicles isolated from *P. euphratica* suspension-cells cultivated in the absence of salt and in the presence of 50 and 150 mM NaCl. Error bars denote SE, $n = 2$.

effect of different salt concentrations added before activation of the proton pump with 0.1 mM PP_i (saturating concentration). The initial velocities of proton pumping were recorded in the first seconds and compared with the value obtained in the absence of salt, an estimate of the V_{max} of the enzyme. As can be seen in Figure 2.4, NaCl decreased H^+ pumping and the inhibition kinetics obeyed to an exponential relation. The concentration necessary to reduce the V_{max} of proton pumping by 50% (IC_{50}) allowed the expression of Na^+ toxicity on V-H⁺-PPase, and a IC_{50} = 158 mM and IC_{50} = 124 mM were estimated in vesicles from cells cultivated in the absence and in the presence of 150 mM NaCl, respectively.

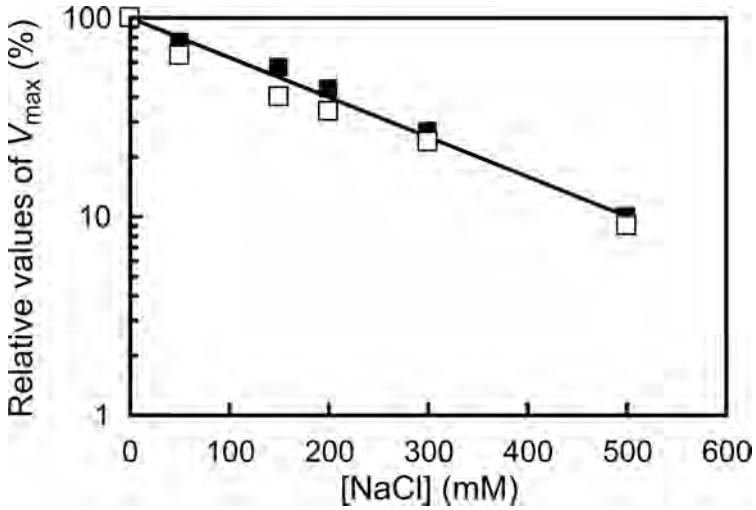

Figure 2.4. Effect of NaCl on proton pumping activity of the *P. euphratica* V-H⁺-PPase: dependence of H^+ pumping activity on NaCl concentration in the reaction mixture in tonoplast vesicles isolated from cells cultivated in the absence of salt (■) and in the presence of 150 mM NaCl (□).

The ability of Na^+ to dissipate a pre-established pH gradient was used to study the involvement of a Na^+/H^+ exchange transport system in tonoplast vesicles from *P. euphratica*. The ΔpH was generated by the V-H^+-PPase, as it showed a greater efficiency in generating and maintaining a pH gradient across tonoplast vesicles than the V-H^+-ATPase, and NaCl may stimulate the activity of V-H^+-ATPase (Rea and Poole 1985). After the pH gradient reached a steady-state, aliquots of salt were added to achieve 50 - 400 mM concentrations in the assay medium, which falls within the range used to study the *Arabidopsis* Na^+/H^+ exchanger AtNHX1 in acid-loaded lipid vesicles (Venema *et al.* 2002), and the rate of fluorescence recovery was recorded. As can be seen in Figure 2.5, the addition of NaCl to tonoplast vesicles caused the dissipation of a pre-formed pH gradient, measured as the recovery of ACMA fluorescence. Moreover, the initial rates of fluorescence recovery were much more pronounced in vesicles isolated from salt-grown suspension cultures (Figure 2.5B and C) than in vesicles from cells cultivated without salt (Figure 2.5A), with the following values calculated upon the addition of 400 mM NaCl to the reaction medium in control, 50 mM and 150 mM NaCl-grown cells: 63.7 $\Delta\%F\ min^{-1}\ mg^{-1}$ protein, 166 $\Delta\%F\ min^{-1}\ mg^{-1}$ protein (2.6-fold increase) and 392 $\Delta\%F\ min^{-1}\ mg^{-1}$ protein (6.1-fold increase), respectively. In tonoplast vesicles isolated from 150 mM NaCl-grown cells, fluorescence recovery increased almost linearly for NaCl concentrations up to 400 mM, however, when Na_2SO_4 was used in the range of 100 - 600 mM, an evident saturation was observed, and an apparent K_m of 373 mM Na^+ was estimated (Figure 2.5E).

The possibility that part of the fluorescence recovery observed after salt addition may be due to the direct inhibition of V-H^+-PPase proton pumping activity by Na^+, as shown above, cannot be discarded. However, this effect is similar in membranes from control cells and from salt-stressed cells (Figure 2.4), therefore not influencing the overall interpretation of the results. In the same way, we cannot exclude that part of the inhibition of V-H^+-PPase proton pumping after salt addition may be attributed to the dissipation of the proton gradient through the Na^+/H^+ antiport system, although the initial velocities of proton pumping were measured in the first few seconds after activation of the enzyme, when the ΔpH magnitude might not be sufficient to measure antiport activity.

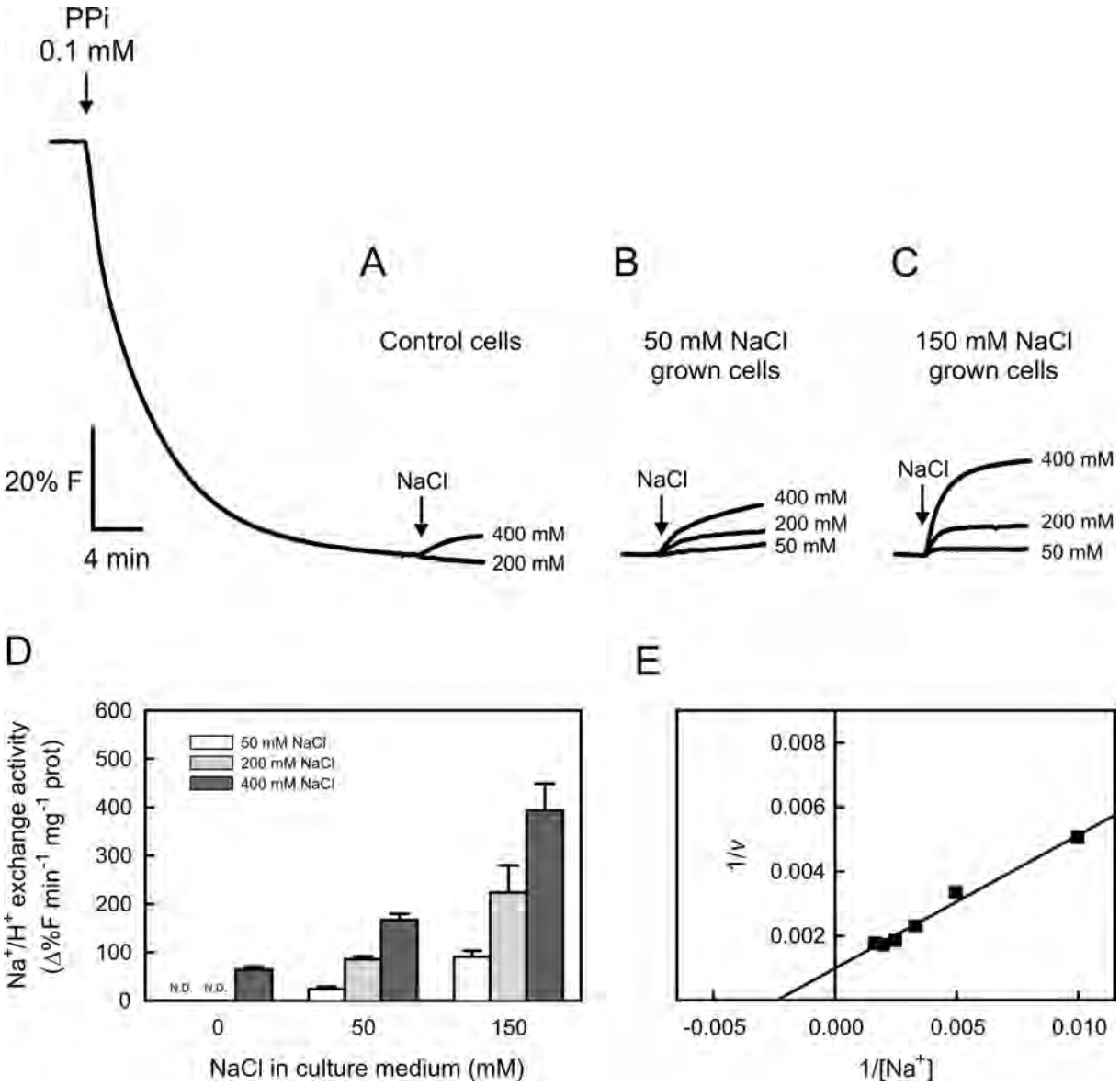

Figure 2.5. Dissipation of the PP_i-dependent H^+ gradient upon addition of 50 mM to 400 mM NaCl (final concentrations) to tonoplast vesicles isolated from *P. euphratica* suspension-cultured cells grown (A) in the absence of salt and (B) in the presence of 50 mM and (C) 150 mM NaCl. (D) Initial velocities of fluorescence recovery upon addition of NaCl to tonoplast vesicles. (E) Lineweaver-Burk plot of the initial velocities of Na^+-induced proton dissipation upon addition of aliquots of Na_2SO_4 to tonoplast vesicles of 150 mM NaCl grown cells. Error bars denote SE, *n* = 3. N.D., not detected.

In order to investigate the ion specificity of the measured Na^+/H^+ exchange activity, different salts were added after the ΔpH had been generated by the V-H^+-PPase. Vesicles isolated from suspension-cultured cells grown in the presence of 150 mM NaCl were used, where the exchange activity was highest. Besides NaCl (427 Δ%F min^{-1} mg^{-1} protein), other sodium salts (Na_2SO_4 and Na-gluconate, 502 Δ%F min^{-1} mg^{-1} protein and 557 Δ%F min^{-1} mg^{-1} protein, respectively) were also able to dissipate the pre-established pH gradient with similar initial velocities, and lithium (in the form of

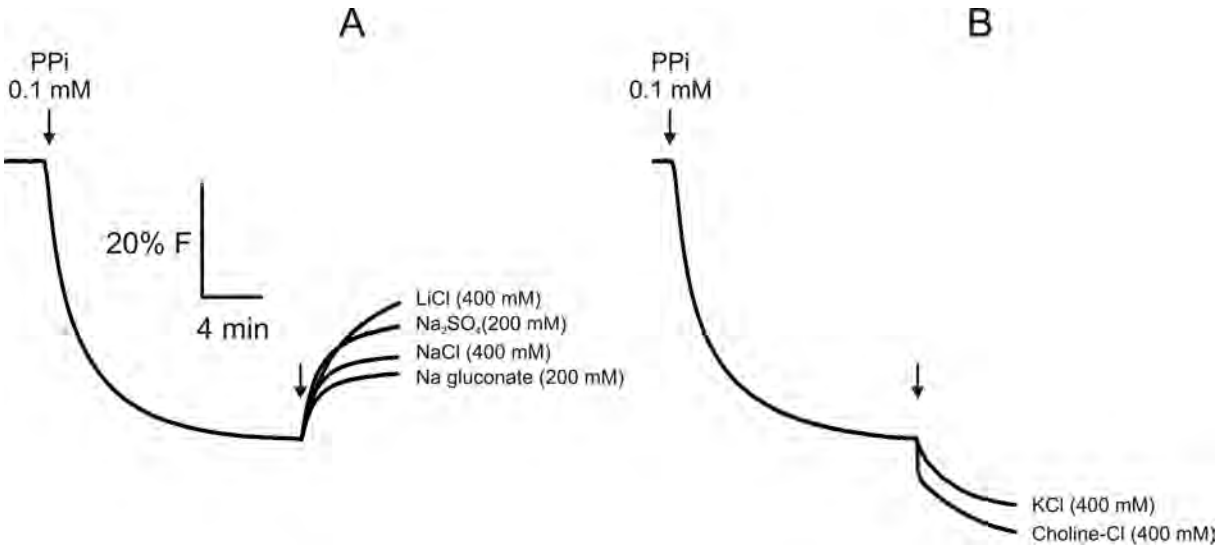

Figure 2.6. Ion specificity of the tonoplast H^+-coupled Na^+ exchanger in vesicles isolated from *P. euphratica* suspension-cultured cells grown in the presence of 150 mM NaCl.

LiCl, 304 Δ%F min^{-1} mg^{-1} protein), a cation with a similar charge and ionic radius, also dissipated the ΔpH, although with a lower initial velocity (Figure 2.6A). The addition of KCl had an opposite effect, increasing the fluorescence quenching of ACMA (Figure

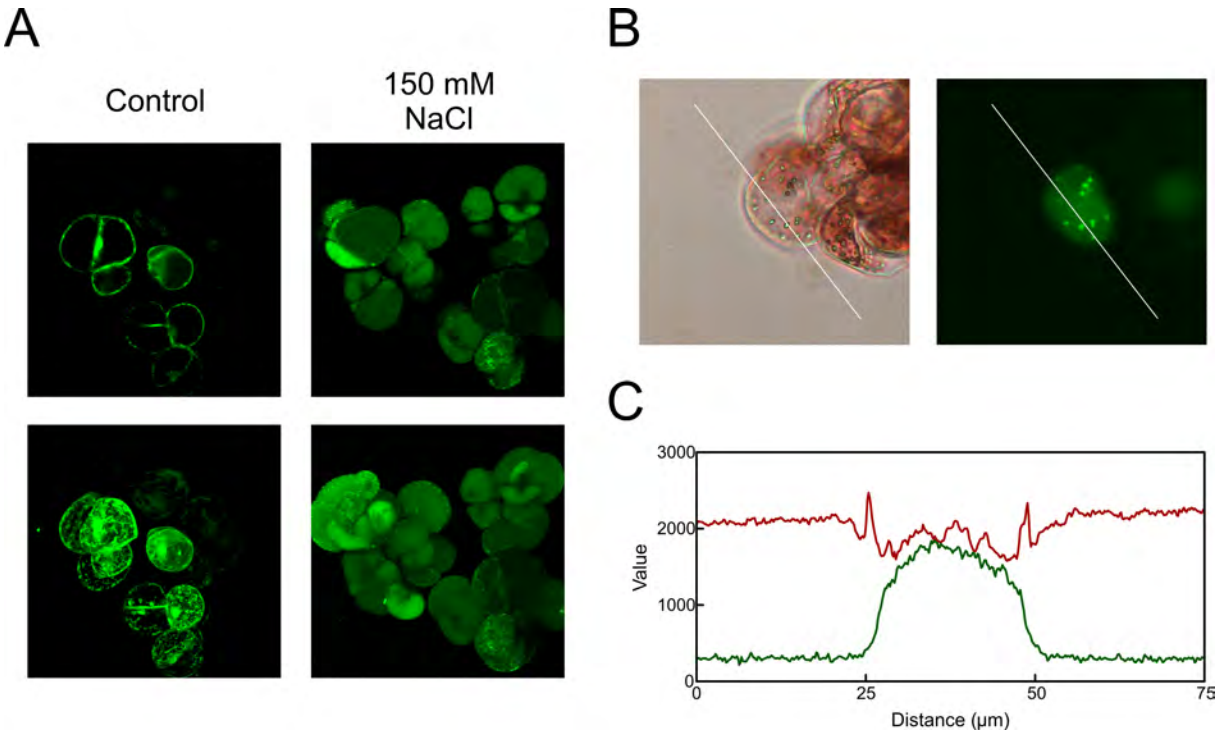

Figure 2.7. Na^+ accumulation in *P. euphratica* suspension cells. (A) Cells were stained with Sodium Green and observed under a confocal microscope. Top row: single section; bottom row: maximum Z projection of 20 sections covering approximately 30 µm. (B) Co-localization of Sodium Green fluorescence and Neutral Red in *P. euphratica* suspension cells subject to a 150 mM NaCl pulse for 24 h, observed under a fluorescence microscope. (C) Pixel intensity plot along the line shown in (B) of Sodium Green (green line) and Neutral Red (red line).

2.6B), probably due to the stimulating effect of K^+ on the V-H^+-PPase (Rea and Poole 1985) and/or the accumulation of Cl^- in the vacuole *via* anion channels. The addition of choline chloride had the same effect (Figure 2.6B), possibly through the stimulation of V-H^+-PPase H^+ pumping to compensate the electrical depolarization of the tonoplast vesicles caused by the accumulation of the negatively charged chloride anions.

Many studies have shown that vacuolar Na^+/H^+ antiporter activity is enhanced by salt stress, but few direct links has been built between this increased activity and Na^+ sequestration into vacuole. In order to determine whether Na^+ accumulates inside the vacuole in *P. euphratica* suspension cells, both control and cells subject to 150 mM NaCl treatment for 24 h were stained with the florescent Na^+ probe Sodium Green. Figure 2.7A shows that control cells show only fluorescence on the periphery, while green fluorescence in 150 mM treated cells seems to be distributed throughout the inside of most cells, suggesting Na^+ compartmentalization inside the vacuole. To more accurately co-localize the green Na^+ fluorescence to the vacuole, aliquots of salt-stressed cells were double-stained with Sodium Green and Neutral Red, a pH-sensitive dye that stains acidic organelles in red, such as the vacuole. Figure 2.7B and C show that the green fluorescence co-localizes with the vacuoles stained by Neutral Red.

2.4. Discussion

The following evidences support that *P. euphratica* cells are highly tolerant to high salinity in the extracellular medium: (i) cells were able to grow with up to 150 mM NaCl in the culture medium, with a slight reduction of the final population size, when compared to the control cells in the absence of salt; (ii) when the salt concentration in the culture medium was doubled to 300 mM, cells were unable to grow but remained highly viable up to 17 days after subculture; and (iii) pulses of 1 M NaCl did not promote a significant loss in cell viability 48 h after addition to unadapted cells. Gu *et al.* (2004) also studied the impact of salt in the growth of *P. euphratica* cell suspensions and reported that 479 mM NaCl induced extensive cell death after 4 subcultures. The effect of salt on cell viability was also studied in suspension-cultured cells obtained from the

moderately salt-tolerant tree *O. europaea* cultivated either with sucrose or mannitol as the sole carbon and energy source (Conde *et al.* 2007b). Cells growing with sucrose were unable to survive a 500 mM salt pulse after 24 h, whereas cells grown in a medium supplemented with mannitol showed increased viability. It was proposed that the higher resistance to salt is due, at least in part, to the intracellular accumulation of this osmoprotectant *via* a polyol/H^+ symport system, whose activity is increased by NaCl which transcriptionally up-regulates *OeMaT1* (*O. europaea mannitol transporter 1*). Although it is recognized that suspension-cultured cells may not be close to normal 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 (Vera-Estrella *et al.* 1999, Xia *et al.* 2002, Conde *et al.* 2006, Conde *et al.* 2007a,b,c).

The maintenance of internal cellular ion homeostasis is important for all living organisms. Salt stress creates ion imbalances, causing inhibition of K^+ uptake by roots and thus changing the internal Na^+/K^+ ratio. In plants, this ratio can be restored either by pumping excess Na^+ out of the cell by means of a SOS1-like (Shi *et al.* 2000) and/or NhaD-like (Ottow *et al.* 2005a) Na^+/H^+ antiporter at the plasma membrane level, or by sequestration of Na^+ and Cl^- into the vacuole, or both. Besides detoxifying the cytoplasm, this accumulation can allow plants to use NaCl as an osmoticum, driving water into the cells. As referred to in the Introduction, the *Arabidopsis* vacuolar Na^+/H^+ antiporter (AtNHX1), homologue to the yeast antiporter NHX1, was cloned, and its expression in *S. cerevisiae* Δ*nxh1* mutants restored its phenotype (Gaxiola *et al.* 1999). In addition, overexpression of this gene in *Arabidopsis* resulted in plants able to grow in soil watered with up to 200 mM NaCl (Apse *et al.* 1999). Since then, several NHX genes have been characterized in other plants, such as cotton (*Gossypium hirsutum*, Wu *et al.* 2004), wheat (*Triticum aestivum*, Saqib *et al.* 2005), beet (*Beta vulgaris*, Xia *et al.* 2002), grape berry (*Vitis vinifera*, Hanana *et al.* 2007) and rice (*Oryza sativa*, Fukuda *et al.* 2004). In this work, we present biochemical data corroborating the involvement of Na^+/H^+ exchange activity in *P. euphratica* cell suspensions at the tonoplast level, whose salt-inducible activity increased 6-fold in NaCl-treated cell suspensions over cells cultivated in the absence of salt. The data obtained are particularly relevant since

six sequences for *P. euphratica* NHX genes (*PeNHX1*, FJ589739, *PeNHX2*, FJ589740, *PeNHX3*, FJ589741, *PeNHX4*, FJ589742, *PeNHX5*, FJ589743, *PeNHX6*, GQ324700), homologues to the *Arabidopsis* exchangers *AtNHX1-6*, are already published in nucleotide databases. Indeed, Ye and co-workers (Ye *et al.* 2009) identified these six NHX isoforms and demonstrated that they could compensate, at least in part, the phenotype of a salt-sensitive yeast strain lacking NHX1, and all isoforms were up-regulated in roots after 6 h of 200 mM NaCl treatment, suggesting that these genes may be responsible for the observed biochemical activity.

The electrogenic H^+ pumps V-H^+-ATPase and V-H^+-PPase are major components of the vacuolar membrane of plant cells (reviewd by Maeshima 2001). Up to now, vacuolar membranes prepared from all plant species exhibit V-H^+-PPase activity in addition to V-H^+-ATPase activity, with the noticeable exception of lemon, where the V-H^+-PPase is absent (Müller *et al.* 1996). We also found that, in *P. euphratica* suspension cells, both V-H^+-ATPase and V-H^+-PPase generate and maintain the electrochemical gradient across the vacuolar membrane. In this system, the V-H^+-PPase seems to be able to generate and maintain a higher pH gradient across the vacuolar membrane than the V-H^+-ATPase, at PP_i concentrations in the micromolar range. This is similar to the germinating tissue of mung bean (*Vigna radiata*, Nakanishi and Maeshima 1998) and cowpea (*Vigna unguiculata*, Otoch *et al.* 2001), where the V-H^+-PPase is the main H^+ pump, due to the high availability of PP_i, produced as a by-product of several metabolic processes. The V-H^+-PPase also appears to be the main H^+ pump acidifying the vacuole of grape berry mesocarp cells, as measured in tonoplast vesicles from grape tissues (Terrier *et al.* 2001) and in tonoplast vesicles and intact vacuoles from cell suspensions obtained from grape berry *calli* (Cabernet Sauvignon Berry cells, Fontes *et al.* 2010). Suspension-cultured cells, growing exponentially, and thus with a high metabolic rate, probably also have a high concentration of PP_i and therefore show a higher V-H^+-PPase activity than V-H^+-ATPase activity (Pfeiffer 1998).

It has been described that both V-H^+-ATPase and V-H^+-PPase activity can be regulated by salt. Induction of protein synthesis by salt has been described as an important mode of regulation of V-H^+-ATPase activity, in spite of some authors having proposed that post-translational modifications of the V-H^+-ATPase can also occur

in response to salt (Barkla and Pantoja 1996). In the halophyte *Suaeda salsa*, the hydrolytic and H^+ pumping activity of the V-H^+-ATPase increased two-fold in plants treated with 200 mM NaCl, when compared to control plants (Qiu *et al.* 2007). The same result was found in *Mesembryanthemum crystallinum*, where both V-H^+-ATPase H^+-transport activity and ATP hydrolytic activity were found to be two-fold higher in vesicles isolated from leaves of plants treated with 200 mM NaCl, when compared with the activity measured in control plants of the same age (Barkla *et al.* 1995). Regarding *P. euphratica*, studies performed previously by Ma *et al.* (2002) showed that cell suspensions treated with 50 mM NaCl increased both the hydrolytic activity and H^+ pumping activity of the tonoplast V-H^+-ATPase, when compared with control cells. Results obtained in our work correlate well with those, with a 1.8-fold increase in the V-H^+-ATPase hydrolytic activity in cells grown in the presence of 150 mM NaCl, when compared with the control (Figure 2.3).

Besides evidence suggesting that the V-H^+-PPase may be regulated developmentally or by tissue-specific signals, several studies have also focused on the regulation of the V-H^+-PPase activity by growth in NaCl-containing medium (Barkla and Pantoja 1996). In contrast with the general sodium-induced increase in V-H^+-ATPase activity, there appears to be a decrease in V-H^+-PPase activity with exposure to NaCl, as it was observed in tonoplast vesicles from barley roots and *M. crystallinum.* This appears to be case of the *P. euphratica* V-H^+-PPase, with a slight decrease of both H^+ pumping and hydrolytic activities in cells cultivated in the presence of 50 or 150 mM NaCl, when compared to the control. However, exceptions to this Na^+-induced decrease in V-H^+-PPase activity have also been described in NaCl-adapted cells of *A. pseudoplatanus* and NaCl treated *Daucus carota* cells, where the activity of the V-H^+-PPase increases over control cells (Barkla and Pantoja 1996). We could also observe in our lab that both the V-H^+-ATPase and V-H^+-PPase activities are twice as high in 150 mM NaCl-adapted cells of *Solanum tuberosum* than in an unadapted cell line (Queirós *et al.* 2009).

According to the data of Barkla *et al.* (1995), V-H^+-ATPase H^+ transport measured in tonoplast vesicles from the halophyte *M. crystallinum* was stimulated directly by the presence of 50 mM Cl^- in the reaction medium, in both control and salt-treated

plants, whereas H^+ transport of the V-H^+-ATPase of the salt-sensitive *Kalanchoe daigremontiana* was inhibited in the presence this ion (White and Smith 1989). This may be due to an adaptation of salt-tolerant plants to NaCl stress, where a greater permeability of the tonoplast to Cl^- can allow it to accumulate in the vacuole down its electrical gradient, dissipating an inside-positive membrane potential and thus stimulating the formation of a ΔpH through V-H^+-ATPase and V-H^+-PPase activity (Bennet and Spanswick 1983). Chloride channels have already been identified and cloned in plants (Plant *et al.* 1994, Lurin *et al.* 1996) and, in yeast, mutants lacking the gene *GEF1*, encoding a chloride channel, are more susceptible to cation toxicity (Gaxiola *et al.* 1998). Our results showed an enhanced ability of the V-H^+-PPase to create a H^+ gradient in the presence of Cl^- (Figure 2.6B). This could be due to the accumulation of this anion down its electrical gradient *via* a CLC-like chloride channel present in the tonoplast of *P. euphratica*. In fact, results by Chen *et al.* (2002) showed that, in salt-stressed *P. euphratica*, young root cortical cells accumulated Cl^- in the vacuoles when compared with control plants. In *P. euphratica* suspension-cultured cells subjected to 200 mM NaCl, a higher amount of Cl^- was found in the vacuole than in the cytoplasm and cell wall (Gu *et al.* 2004). Chloride accumulation into the vacuole may allow the maintenance of a higher tonoplast H^+ gradient that can be used in cation detoxification and an increase in osmotic pressure (Gaxiola *et al.* 1999).

The Na^+/H^+ group of antiporters has long attracted attention in relation to salt tolerance in plants (Maeshima 2001). Our results showed that Na^+/H^+ exchange activity is negligible in cells grown without salt; activity is induced abruptly when suspension cells were grown with salt, indicating an important role of this antiporter in Na^+ detoxification. Concordantly, preliminary work by Ottow *et al.* (2003) showed a fast and significant increase in *PeNHX1* and *PeNHX2* transcripts, as measured by quantitative RT-PCR, in response to severe salt stress, therefore supporting the involvement of a concentrative Na^+/H^+ antiporter mediating Na^+ uptake into the vacuole. In addition, recent work by Ye *et al.* (2009) identified an additional four isoforms of the NHX family of genes in *P. euphratica* (*PeNHX1-6* in total) and showed that they were able to rescue a yeast strain lacking NHX1 (Δ*nxh1*). Furthermore, all six isoforms were up-regulated in roots following 6 h of 200 mM NaCl treatment, and onion epidermal cells transformed by particle bombardment with a PeNHX3-GFP fusion protein displayed

green fluorescence localized to the tonoplast (Ye *et al.* 2009), supporting a role for these genes in salt exclusion from the cytosol to the vacuole in *P. euphratica*.

In agreement with these biochemical and molecular observations, confocal and epifluorescence microscopy analysis performed in the present study using a Na^+-sensitive probe revealed that suspension-cultured cells subject to a salt pulse accumulated Na^+ in the vacuole when compared with control cells. Distinct conclusions were drawn by Gu *et al.* (2004), who found no significant difference in the sequestration of Na^+ in the different cell compartments under NaCl stress, although its level increased in all cell compartments as the NaCl stress level increased, as measured by electron microscope dispersive X-ray microanalysis. Nevertheless, it may be noticed from the study that, at the tested external concentrations of 51 mM, 137 mM and 225 mM NaCl, there is a consistent increase of sodium in the vacuole when compared to the cytosol of about 80%, 23% and 24%, respectively. Additionally, in a study with *P. euphratica* plantlets, it was concluded that there is apoplastic Na^+ accumulation, but not vacuolar accumulation, as a response mechanism against salinity (Ottow *et al.* 2005a); however, 9 weeks after 150 mM NaCl treatment, plantlets showed a higher accumulation of Na^+ in the vacuole than in the cytosol. Therefore, we may not discard the hypothesis that the discrepancy between our conclusion that Na^+ is accumulated in the vacuole and those reported above is due to the different sensitivities of the Na^+ detection methods used, and that Na^+ accumulation in the vacuole may be tissue specific or dependent on salt level and/or on the physiological/developmental state of the plant.

In the present work, Na^+-induced fluorescence recovery signals were measurable at very high Na^+ concentrations, revealing the involvement of a tonoplast H^+/cation antiporter with a very low affinity (K_m = 373 mM Na^+). Similarly, very high concentrations of NaCl (10 - 500 mM) were used to measure cation-dependent H^+ exchange activity of AtNHX1 (Venema *et al.* 2002). This very low-affninity Na^+/H^+ exchange system is likely to be physiologically relevant to *P. euphratica* under salt stress, since very high NaCl concentrations, in the molar range, have been measured in the apoplast (Ottow *et al.* 2005a) and in the cytosol (Gu *et al.* 2004). The exchanger AtNHX1 from *Arabidopsis* catalyses low affinity Na^+ transport (K_m = 42 mM Na^+, Venema *et al.* 2002), a value similar to that found in *Mesembryanthemum crystallinum* (K_m = 44 - 51 mM Na^+, Barkla

et al. 1995) and *Solanum tuberosum* (K_m = 40 - 69 mM Na^+, Queirós *et al.* 2009). The gene *VvNHX1*, encoding a vacuolar cation/H^+ antiporter from *V. vinifera*, was recently cloned and characterized by Hanana *et al.* (2007). VvNHX1 displays low affinity K^+/H^+ and Na^+/H^+ exchange activities (K_m = 12.8 and 40.2 mM, respectively). The possibility raised that the Na^+/H^+ antiporter of *P. euphratica* may accept K^+ deserves further investigation. If this were the present case, the Na^+-dependent H^+ dissipation observed would be much more pronounced in the absence of KCl, which was introduced in the assay medium to stimulate the H^+ pumping activity of the V-H^+-PPase. However, since the addition of 400 mM KCl promoted an additional stimulating effect of H^+ pumping activity after the H^+ gradient had been generated (Figure 2.6B), contrarily to the fluorescence recovery signal observed upon NaCl addition (Figures 2.5 and 2.6A), the *P. euphratica* cation/H^+ antiport system proposed in this work seems to not transport K^+, or, at least, this cation may be transported with much lower affinity than Na^+.

As a whole, the coordinated activities of the tonoplast Na^+/H^+ antiport system proposed in this work, together with the extrusion of salt through sodium antiporters at the plasma membrane level, should contribute towards the active reduction of Na^+ in the cytosol in *P. euphratica*. The characterization at a molecular level of the *P. euphratica* tonoplast antiporters, as it was carried out for the plasma membrane SOS-like (Wu *et al.* 2007) and NhaD-like Na^+ antiporters (Ottow *et al.* 2005b), has been recently accomplished by Ye and co-workers (Ye *et al.* 2009), advancing our understanding of salt stress resistance in *P. euphratica*. Proteomic analysis using highly enriched vacuolar preparations has been undertaken and published for *Arabidopsis* and barley vacuoles. These studies have revealed novel tonoplast transporters and their role in important cell functions, including salinity tolerance (for review, see Martinoia *et al.* 2007). Also, osmotic adjustments resulting in a net increase of compatible solutes in the cytoplasm has been reported in *P. euphratica* in response to salt (Watanabe *et al.* 2000), as described in olive cells, which dramatically increase mannitol uptake *via* salt-induced transcription of the corresponding transporter gene (Conde *et al.* 2007b) and repression of the gene encoding the first mannitol oxidation step (Conde *et al.* 2011). An additional mechanism possibly involved in the response of *P. euphratica* to salt may be inferred from the observed reduction of the cell size after a salt pulse, suggesting an osmotically induced reduction in surface area in response to the

decrease of extracellular water activity, possibly *via* an endocytic internalization of the cell wall, as already reported in other plants species and cell models (Kubitscheck *et al.* 2000, Bahaji *et al.* 2003), and more recently in *Arabidopsis* root tip cells in response to salt stress (Leshem *et al.* 2007). The *P. euphratica* suspension-cultured cells can be adapted to high salinity and might also provide a good experimental system for investigation of these hypotheses.

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Chapter 3

Olea europaea as a moderately salt-tolerant tree: studies on sugar and polyol metabolism

Part of the work presented in this chapter has been published:

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Abstract

Olive tree is one of the oldest cultivated plants and has been historically associated with Mediterranean climates. In the present study, heterotrophic sink models, such as olive cell suspensions and fruit tissues, and source leaves were used for analytical, biochemical and molecular studies. Uptake of mannitol in heterotrophic cell suspensions of *Olea europaea* was shown to be mediated by a 1:1 polyol:H^+ symport system with a K_m of 1.3 mM mannitol and a V_{max} of 1.3 nmol min^{-1} mg^{-1} D.W. Addition of 100 - 500 mM NaCl to cultured cells enhanced the capacity of the polyol:H^+ symport system and the amount of *OeMaT1* (*O. europaea* mannitol transporter *1*) transcripts. Mannitol-grown cells remained viable 24 h after a 250 and 500 mM NaCl pulse, whereas extensive loss of cell viability was observed in sucrose-grown cells. *OeMaT* transcripts increased throughout maturation of olive fruits, suggesting that an OeMaT is involved in the accumulation of mannitol during olive fruit ripening. The kinetic parameters of mannitol dehydrogenase, the first enzyme involved in mannitol metabolism, were determined in cells growing in mannitol (K_m = 54.5 mM mannitol, V_{max} = 0.47 mmol h^{-1} mg^{-1} protein), and the corresponding cDNA was cloned and named *OeMTD1* (*O. europaea* mannitol dehydrogenase *1*). MTD activity and *OeMTD1* expression were repressed after Na^+, K^+ and polyethylene glycol (PEG) treatments, in both mannitol- and sucrose- grown cells. In contrast, salt and drought significantly increased mannitol transport activity and *OeMaT* expression. Taken together, these studies support that olive trees cope with salinity and drought by coordinating mannitol transport with intracellular metabolism. Subsequent studies on the molecular mechanisms of glucose utilization by olive cells, which was found as the main important soluble sugar in the leaves and fruits of *O. europaea*, led to the cloning and functional characterization of a monosaccharide transporter, named *OeMST2* (*O. europaea* monosaccharide transporter *2*). Heterologous expression of this gene in a *Saccharomyces cerevisiae* strain deficient in glucose uptake confirmed the involvement of a H^+/monosaccharide transporter. Transcript levels of *OeMST2* increased during fruit maturation, suggesting that OeMST2 takes part in the massive accumulation of monosaccharides in olive fruits. In the present work, we also investigated the nature of the diffusive glucose

transport in *O. europaea* cell cultures. The measurement of [^{14}C]glucose transport by cells and membrane vesicles in the presence of specific inhibitors, the measurement of activation energies of glucose uptake, among other biochemical approaches, led us to demonstrate that the low-affinity, high-capacity, diffusion-like glucose uptake in olive cells occurs through a channel-like structure whose transport capacity may be regulated by intracellular protonation and phosphorylation/dephosphorylation. This diffusive uptake system was reported in several organisms but its biochemical nature has remained elusive.

3.1. Introduction

As referred to in Chapter 1, *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 the 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 the 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.

Phloem unloading may take place either symplasmically *via* plasmodesmata or apoplasmically 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-bound invertases (CW-INV) 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 the account of the successful cloning of several genes 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). Most transporters characterized so far are plasma membrane carriers, functioning as energy-dependent H^+ symporters.

As mentioned in Chapter 1, polyols (or sugar alcohols), the reduced form of aldoses and ketoses, can be either cyclic (cyclitols) or linear (alditols), and are present in all living forms (Bieleski 1982). In contrast to sucrose and monosaccharide transporters, little is known regarding the identity and regulation of polyol transporters, either in sink or in source tissues in higher plants. The first cDNA encoding a mannitol transporter of a higher plant was identified and characterized in the celery phloem (Noiraud *et al.* 2001a). This cDNA (*AgMaT1*, *Apium graveolens* mannitol transporter *1*) was used to establish a heterologous expression system in yeast cells. Mannitol biosynthesis may confer several potential advantages, including more efficient carbon use (Stoop *et al.* 1996), resistance against oxidative stress (Smirnoff and Cumbers 1989, Williamson *et al.* 1995, Jennings *et al.* 1998) and salt tolerance. Concordantly, the concentration of mannitol in celery grown in hydroponic nutrient solution progressively increases as the total salinity of the growth solution increases (Stoop and Pharr 1994). Increased mannitol accumulation in leaves was also observed in plants irrigated with 300 mM NaCl, as a consequence of a massive shift in partitioning of fixed carbon into mannitol instead of sucrose (Everard *et al.* 1994). The strong water stress tolerance of *Fraxinus excelsior* is, in part, related to an accumulation of malate and mannitol (Guicherd *et al.* 1997), and, in plants subjected to drought stress, the mannitol content of the leaf xylem sap increases (Patonnier *et al.* 1999). Additional evidence for a role for mannitol in salinity tolerance was obtained when *Nicotiana tabacum*, *Populus tomentosa* and other plants were genetically engineered to synthesize mannitol through introduction of an *Escherichia coli* mannitol-1-phosphate dehydrogenase (*mtlD*), which catalyzes the biosynthesis of mannitol from fructose, resulting in more salt-tolerant plants (Tarczynsky *et al.* 1993, Hu *et al.* 2005). In *Arabidopsis*, *mtlD* gene transfer and expression enhanced seed germination under salinity conditions (Thomas *et al.* 1995).

Polyol transporters belong to two sub-families (PMT and INT) of the MST family of transporter proteins (see Introduction, section 1.3.1., and Figure 1.7). Several plant MSTs have been characterised functionally and molecularly, however, not as much is currently known about these sugar transporters in ligneous species, including *O. europaea.* After the identification of *Arabidopsis* transporter gene *AtSTP1* (*A. thaliana* sugar transporter protein *1*), it was heterologously expressed and functionally characterised as an hexose:proton symporter in an *hxt*-null mutant *Saccharomyces*

cerevisiae strain (Sauer *et al.* 1990).

In the first part of the work, we determine the main soluble carbohydrates present in leaves from whole plants treated with salt, confirming the presence of mannitol together with glucose, among others. Next, we report the cloning and functional characterisation 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. Furthermore, the present work also characterizes a mannitol transporter expressed in cultured cells and intact fruits of *O. europaea*. For the first time in plants, it is shown that mannitol transport is regulated by means of salt-mediated changes in the transcription of mannitol carrier(s). Altogether, the results showed that transmembrane transport of mannitol is a critical step in terms of osmotic adjustments and productivity in *O. europaea*. In addition, we performed a detailed biochemical characterization of a NAD^+-dependent MTD from olive, identified its potential coding cDNA (*OeMTD1*) and provided a solid body of evidence that indicates that its activity is tightly coordinated with mannitol transport capacity, in order to regulate the cellular mannitol pool, which proved to be critical for olive cells to be able to cope with salinity and drought conditions.

As referred to in Chapter 1, a previous study (Oliveira *et al.* 2002) showed that glucose uptake into suspension-cultured cells of *O. europaea* follows biphasic kinetics, due to a high affinity glucose/H^+ symporter system superimposed on a linear component, which was more evident at higher sugar concentrations. The nature of this non-saturable, diffusion-like mechanism, able to sustain both cell growth and metabolism, is also investigated in this work.

3.2. Material and Methods

3.2.1. Cell suspensions and growth conditions

Cell suspensions of *O. europaea* L. var. Galega Vulgar were grown in 250 mL flasks on a rotary shaker at 100 r.p.m., in the dark, at 25 ºC, on modified Murashige and Skoog (MS) medium (Murashige and Skoog 1962, modified as in Oliveira *et al.*

2002), supplemented with 0.5% or 3% (w/v) glucose, 0.5%, 1% or 2% (w/v) mannitol, 1% or 2% (w/v) sucrose, or 0.5% (w/v) mannitol plus 0.5% (w/v) glucose. Cells were sub-cultured weekly by transferring 10 mL aliquots to 70 mL of fresh medium. When necessary, cell growth was monitored by determination of dry weight. Aliquots of 1 - 5 mL were filtered through pre-weighted GF/C filters (Whatman, Clifton, NJ, USA). The samples were washed with deionized water and weighted after 24 h at 80 °C.

To induce salt stress, NaCl or KCl were added at a final concentration of 250 mM, and to mimic osmotic stress, polyethylene glycol (PEG-8000) was added at a final concentration corresponding to the osmotic potential of 250 mM of both salts (Money 1989). The stress treatments were undertaken for a 24 h period at the mid-exponential growth phase of the cells.

3.2.2. Transport studies of radiolabelled substrates in suspension-cultured cells

Harvested cells were centrifuged, washed twice with ice-cold modified MS medium without sugar at pH 4.5, and resuspended in the same medium at a final concentration of about 5 mg D.W. mL^{-1}. To estimate the initial uptake rates of radiolabelled sugars and polyols, 1 mL of cell suspension was added to 10 mL flasks, with shaking at 100 rpm. After 2 min of incubation at 25 ºC, the reaction was started by the addition of 40 µL of an aqueous solution of radiolabelled substrate at the desired specific activity and concentration. The specific activities were defined according to the final concentration of the carbohydrate in the reaction mixture, as follows: 500 dpm $nmol^{-1}$ (0.1 to 2 mM), 100 dpm $nmol^{-1}$ (5 to 20 mM) for mannitol and 500 dpm $nmol^{-1}$ (0.02 to 2 mM), 100 dpm $nmol^{-1}$ (5 to 10 mM) and 10 dpm $nmol^{-1}$ (20 to 100 mM) for L- and D-glucose. Sampling times were 0, 60 and 180 s, time periods during which the uptake was linear.

Washing, radioactivity measurements and calculations were performed as described by Conde *et al.* (2006). Briefly, the reaction was stopped by dilution with 5 mL 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, Loughborough, UK). The radioactivity

was measured in a Packard Tri-Carb 2200 CA liquid scintillation counter (Packard Instruments Co., Inc., Rockville, MD, USA). Results were corrected for non-specific binding of labelled sugars to the filters and/or cells, by diluting the cells with 5 mL ice-cold modified MS medium without sugar, before the addition of the labelled sugar.

Competition between labelled substrates and other sugars and/or polyols was tested by running competitive uptake kinetics. Inhibition of radiolabelled sugar or polyol transport by non-labelled sugars and polyols was assayed by adding simultaneously the labelled and non-labelled substrate. The concentration range of labelled mannitol varied from 0.1 to 2 mM, while the concentration of labelled L- or D-glucose varied from 0.02 to 0.5 mM, and the final concentration of the unlabelled competitor was at least 10-fold higher than the K_m value estimated for the transport system.

The data of the initial uptake rates of labelled substrates were analyzed by a computer-assisted non-linear regression analysis (GraphPad Prism 4.0 software; San Diego, CA, USA). By this method, the transport kinetics best fitting to the experimental initial uptake rates were determined, and estimates for the kinetic parameters were then obtained. Substrate uptake is presented as mean values ± SE and *n* denotes the number of independent experiments. D-[U-^{14}C]glucose (290 - 305 mCi mmol^{-1}), L-[1-^{14}C]glucose (55 mCi mmol^{-1}) and D-[1-^{14}C]mannitol (59 mCi mmol^{-1}) were obtained from Amersham Biosciences (Little Chalfont, UK).

3.2.3. Determination of mannitol dehydrogenase activity

Protein extraction and determination of OeMTD1 activity were carried out as described by Stoop and Pharr (1993). *O. europaea* suspension-cultured cells were harvested, as described above, and ground using a chilled mortar and pestle in an approximately 1:1 (v/v) powder:buffer ratio. The protein extraction buffer contained 50 mM MOPS (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM dithiothreitol (DTT) and 1% (v/v) Triton X-100. The homogenates were then centrifuged at 20,000x*g* for 20 min and the supernatants collected and maintained on ice and used in the enzymatic assays. MTD activity assays were performed at room temperature (25 ºC), except for the evaluation of the effect of heat on MTD activity, in

a total volume of 1 mL. The reaction mixture contained enzyme extract, 100 mM BTP (Bis-Tris propane, pH 9.0, or the desired pH in the case of the evaluation of pH effect on MTD activity), 2 mM NAD^+, and D-mannitol at the desired final concentration. The reduction of NAD^+ was evaluated spectrophotometrically at 340 nm. All reactions were initiated by the addition of mannitol. For the assessment of the effects of temperature and pH on OeMTD1 activity, 200 mM D-mannitol was used to ensure the measurement of the V_{max} of the enzyme. Total protein concentrations of the extracts were determined by the method of Bradford (1976), using BSA as a standard.

3.2.4. Cloning of an O. europaea *mannitol transporter gene (*OeMaT1*)*

To identify potential cDNA sequences encoding mannitol transporters in *O. europaea*, degenerated primers were designed based on conserved regions of plant polyol transporters. The sequences of the primers were as follows: forward, OeMaTY5' [5'-TTT TAG CTT CAA TGA MTT CM-3'] and reverse, OeMaTY3' [5'-CAA YTC TTT CCA CAC WGC-3']. Reverse transcriptase-PCR (RT-PCR) was performed on RNA extracted from suspension-cultured cells exhibiting high mannitol transport activity. The amplified 501 bp cDNA was cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) according to the manufacturer's instructions, sequenced and submitted to GenBank (accession number, DQ059507) and subsequently named *OeMaT1*.

3.2.5. Cloning of an O. europaea *mannitol dehydrogenase gene (*OeMTD1*)*

In order to identify and clone putative cDNA sequences encoding *MTD*s in *O. europaea*, conserved regions of several plant *MTD*s were identified and subsequently used to design degenerated primers. The sequences of the primers were as follows: forward, OeMTDY5' [5'-CCN GGN CAY GAR ATH GTN-3'] and reverse, OeMTDY3' [5'-CAT YTC YTG NGT YTC YTT-3']. Reverse transcriptase-PCR (RT-PCR) was performed on mRNA extracted from suspension-cultured cells grown with 2% (w/v) mannitol as carbon and energy source. An 816 bp cDNA sequence was amplified and cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) according to

the manufacturer's instructions and subsequently sequenced, submitted to GenBank (accession number, EF470250) and named *OeMTD1*.

3.2.6. Cloning of an O. europaea *monosaccharide transporter gene (*OeMST2)

To isolate the *OeMST2* clone, degenerated primers were designed from conserved regions of plant hexose transporter cDNA sequences obtained from GenBank. The sequences of the primers were as follows: forward, OeMST2Y5' [5'-TYT CHG GWG GWG TIA CIT CHA TG-3'] and reverse, OeMST2Y3' [5'-GGI CCC CAI GAC CAI GCR AA-3']. Reverse transcriptase-PCR (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 the Clontech SMART RACE cDNA Amplification Kit (Clontech Laboratories, CA, USA). The primers were designed according to the partial *OeMST2* sequence isolated: *OeMST2* 5'-RACE primer [5'-TGC TGC GCT TGT TGC AAG CTT TGG-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 the 3'-RACE products. The forward primer (defined on the 5'-RACE product) and the reverse primer (defined on the 3'-RACE product) were, respectively, [5'-ACT TTT AGC TAC CAA AAT GGC CG-3'] and [5'-ACC ATT GCC ATT CTC CAA TTC AA-3']. The PCR product was cloned into the pGEM-T easy vector (Promega, Madison, WI, USA) according to the manufacturer's instructions and sequenced. This approach resulted in the isolation of an 1816 bp sequence subsequently named *OeMST2* (submitted to GenBank, accession number, DQ087177).

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

OeMST2 cDNA was excised from the pGEM-T easy vector by a *Not*I restriction enzyme digestion. The pDR195 vector was linearized by a *Not*I digestion and the extremities generated were dephosphorylated using the SAP enzyme (shrimp alkaline phosphatase) to prevent religation, following the manufacturer's recommendations (Fermentas, Life Sciences, Vilnius, Lithuania). The *OeMST2* cDNA fragment was inserted in the corresponding restriction sites of the pDR195 yeast shuttle vector and the construction checked by sequencing. The pDR195-*OeMST2* construction and the empty pDR195 plasmid were used to transform EBY.VW4000 *S. cerevisiae* strain by a PEG-based method (Dohmen *et al.* 1991). The EBY.VW4000 strain is unable to transport glucose, due to multiple mutations on the hexose transporters (Wieczorke *et al.* 1999), but it can grow on maltose medium. The growth phenotype of the transformants was tested on YNB media depleted of URA and containing either glucose or maltose.

3.2.8. Transport tests in S. cerevisiae

S. cerevisiae strains EBY.VW4000, carrying either pDR195-*OeMST2* or an empty pDR195 vector, were grown in YNB maltose medium at 30 ºC to an OD_{600} 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 D.W. mL^{-1}. To estimate initial uptake rates of radiolabelled sugar, a protocol previously employed to measure carbohydrate uptake to yeast cells was used (Geros *et al.* 1999, Silva *et al.* 2004). Briefly, 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 radiolabelled sugar with 8000 dpm $nmol^{-1}$ (D-[^{14}C]glucose and L-[^{14}C]glucose) or 16000 dpm $nmol^{-1}$ (D-[^{14}C]fructose) at the desired concentration. Potential sugar competitors or metabolic inhibitors were added to the reaction mixture 10 s prior to the addition of radiolabelled glucose. The reaction was stopped by dilution with 5 mL 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 (OptiPhase HiSafe II, LKB Scintillation Products, Loughborough, UK) and the radioactivity was measured as indicated previously. D-[U-^{14}C]glucose (290 - 305 mCi mmol^{-1}), L-[U-^{14}C]glucose (55 mCi mmol^{-1}) and D-[U-^{14}C]fructose (316 mCi mmol^{-1}) were obtained from Amersham Biosciences (Little Chalfont, UK).

3.2.9. RNA gel blot analysis

Total RNAs from olive suspension-cultured cells were isolated by phenol extraction combined with a 2 M LiCl precipitation step (adapted from Howell and Hull 1978), and total RNAs from olive fruits, harvested at green, cherry and black stages, were isolated using the hot borate method adapted from Wan and Wilkins (1994). This method produced satisfying yields of good quality RNA from small samples of phenolic-containing tissues. RNA blot analysis was performed for *OeMST2*, *OeMaT1* and *OeMTD1* as described in Conde *et al.* (2006), using partial [^{32}P]*OeMST2*, [^{32}P] *OeMaT1* and [^{32}P]*OeMTD1* probes, respectively.

Briefly, 20 µg of each RNA sample were separated by formaldehyde-agarose gel electrophoresis and transferred onto Hybond N membrane (Amersham Biosciences, Little Chalfont, UK), or, in the case of the dot blot for *OeMTD1* expression, transferred directly onto the membrane using a pipette. For RNA blotting analysis, the 501 bp cloned fragment of the mannitol transporter gene *OeMaT1* and the 816 bp fragment of the mannitol dehydrogenase gene (*OeMTD1*) were randomly ^{32}P radiolabelled (“Prime-a-gene”, Promega, Madison, WI, USA), while a gene specific [^{32}P]*OeMST2* cDNA probe obtained from the 3’ untranslated region of the 1816 bp monossacharide transporter gene *OeMST2* was used. Hybridized RNA blots were revealed on autoradiographic films and by imaging (BioRad Personal Molecular Imager FX, BioRad Laboratories, Hercules, CA, USA).

3.2.10. Sugar and polyol quantification by HPLC analysis

Leaves from 6 month old olive plants, irrigated periodically with either water, 100 mM NaCl or 250 mM NaCl, were ground in liquid nitrogen and the frozen powder homogenised in ethanol/H_2O and boiled for 10 min at 80 ºC. After centrifugation of the suspension for 5 min at 15000x*g*, the compounds in the supernatant were measured by HPLC, after evaporation under a N_2 flow and resuspension in water. The quantification of mannitol and other soluble carbohydrates in leaf extracts and in the culture medium was performed in a HPLC system from Gibson (132 RI Detector) using a HyperRez H^+ column (Hypersil), at a flow rate of 0.5 mL min^{-1}, with 2.5 mM H_2SO_4 as the mobile phase. Before each experiment, the column was balanced for 30 min at 30 ºC. The standard solution contained glucose, sucrose, fructose, mannitol, raffinose and acetate (internal standard), all at 0.5% (w/v). The samples were diluted 1:1 in an acetate solution of 1% (w/v), and 25 µL of the standard solution was injected to calibrate the column, followed by the injection of the samples.

3.2.11. Determination of cell viability

Fluorescein diacetate (FDA) and propidium iodide (PI) double staining was used to evaluate cell viability, as described by Jones and Senft (1985). Stock solutions of FDA (500 mg mL^{-1}, Sigma, St. Louis, MO, USA) and PI (500 mg mL^{-1}, Sigma, St. Louis, MO, USA) were prepared in DMSO and water, respectively. For the double staining protocol, 1 mL of cell suspension was incubated with 10 µL of FDA stock solution and 1 µL of PI stock solution for 10 min at room temperature in the dark. Stained cells were observed under a Leica DM5000B microscope (Leica Microsystems, Wetzlar, Germany) equipped with a 100 W mercury lamp (AF6000, Leica Microsystems, Wetzlar, Germany) and appropriate filter settings. Visible and epifluorescence images were acquired with Leica DFC350 FX digital camera and Leica Microsystems LAS AF software, version 2.0 (Leica Microsystems, Wetzlar, Germany).

3.2.12. Determination of 2-NBDG accumulation

To determine the entry of the fluorescent glucose analogue 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino)-2-deoxy-D-glucose (2-NBDG), cells were washed twice in a modified MS medium without sugar. A concentrated stock solution of 2-NBDG (100 mM, Molecular Probes, Eugene, OR, USA) was prepared in water. Three mL of a 6-day old *O. europaea* cell suspension were incubated with 10 mM of the fluorescent glucose analogue. At selected times, after the beginning of incubation, a 1 mL aliquot was sampled and washed five times in ice-cold 2-NBDG-free modified MS medium by centrifugation, resuspended in 1 mL of the same medium and observed under a Leica DM5000B fluorescence microscope, as described in the previous section.

3.2.13. Determination of endocytic vesicle formation

To assess the presence of endocytic vesicle formation in suspension-cultured cells of *O. europaea*, the styryl FM dye N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino) styryl)pyridinium dibromide (FM1-43) was used as described earlier (Emans *et al.* 2002). A concentrated stock solution of FM1-43 (1 mM, Molecular Probes, Eugene, OR, USA) was prepared in water. Three mL of a 6-day old *O. europaea* cell suspension were incubated on ice with 5 µM FM1-43 for 15 min. The cells were then washed three times in ice-cold marker-free modified MS medium by centrifugation, resuspended in 3 mL of the same medium, and placed on an orbital shaker at 100 rpm, 25 ºC. Immediately after incubation, and after 10 min and 14 h, a 1 mL aliquot was removed and observed under a Leica DM5000B fluorescence microscope, as described before.

3.2.14. Quantification of ROS production in response to salt

The work regarding the determination and quantification of ROS in olive cell suspensions benefited from the help and assistance of Dr. Herlânder Azevedo, Ph.D.

3.2.14.1. Detection of total intracellular ROS

To determine the overall oxidative stress state of cells, the cell-permeable non-fluorescent probe 2',7'-dichlorodihydrofluorescin diacetate (H_2DCFH-DA, Molecular Probes, Invitrogen, OR, USA) was used, as described previously (Parsons *et al.* 1999, Allan *et al.* 2001, Azevedo *et al.* 2009). After entering cells, H_2DCFH-DA is deacetylated by nonspecific cellular esterases to 2',7'-dichlorofluorescin (H_2DCFH) which, when in the presence of oxidative agents, oxidizes to 2',7'-dichlorofluorescein (DCF), a highly fluorescent and detectable end product (Cathcart *et al.* 1983).

After the addition of salt to *O. europaea* cell cultures, 1 mL aliquots were removed periodically, and 10 µL of a 20 µM H_2DCFH-DA solution was added to the sample. After incubation at room temperature for 30 min, in the dark, with agitation, the sample was centrifuged at 8,000x*g* for 5 min. The supernatant was recovered and the relative fluorescence was measured using a Perkin-Elmer LS-5B Luminescence Spectrometer (Perkin-Elmer, Buckinghamshire, England, UK), with the excitation wavelength set at 488 nm and the emission wavelength set at 525 nm.

3.2.14.2. Detection of $O_2^{\cdot -}$

The production of intracellular superoxide radical ($O_2^{\cdot -}$) in response to salt stress imposition was quantified as described in Able *et al.* (1998), by measuring the reduction of the tetrazolium dye sodium 3'-(1-[phenylamino-carbonyl]-3,4-tetrazolium)-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate (XTT, Molecular Probes, Invitrogen, OR, USA) to a soluble formazan. Briefly, before the addition of salt, 0.5 mM XTT (final concentration) was added to cell suspensions, followed by incubation at room temperature in the dark, under shaking. Aliquots were removed periodically along time, and the absorbance of the supernatant measured at 470 nm, in order to quantify the reduced XTT form.

3.3. Results

3.3.1. Sugar partitioning in olive source tissues

To confirm the main carbohydrates that are assimilated in *O. europaea* cv. Galega Vulgar leaves and, in parallel, assess the effect of salt stress on sugar partioning, potted trees were irrigated for 6 months with water (control), 100 mM NaCl and 250 mM NaCl, and the soluble carbohydrate content in the leaves was evaluated by HPLC. Results showed that mannitol accounts for up to 60% of the total detected sugars. The irrigation with increased NaCl concentrations negatively impacted plant growth (Figure 3.1), and the proportion of mannitol in leaves increased to 53.7% and 56.5 % in plants treated with 100 mM NaCl and 250 mM NaCl, respectively, when compared to non-salt irrigated plants (43.5%, Figure 3.1). This is in good agreement with the analysis of olive leaf sugar composition performed by Cataldi *et al.* (2000) in which it was shown that mannitol is an important photoassimilate (41%), together with glucose (49.2%), while sucrose (2.9%) and various oligosaccharide precursors (galactose, raffinose, stachyose) represent a minor part of leaf sugar content in leaves of the cultivar Coratina. In another study, Drossopoulos and Niavis (1988) reported that mannitol may account for 82 - 92% of total sugars in leaves. Mannitol has also been detected in olive phloem sap exudates (reviewed by Conde *et al.* 2008), indicating that photoassimilated mannitol is translocated through the phloem to olive sink tissues, where it assumes a critical importance, particularly in the pulp of the olive fruit. Altogether, result confirmed that mannitol is an important photoassimilate that may have an important role in the response of plants to salt.

Subsequent studies were performed in suspension-cultured olive cells as sink models, which allowed the characterization of sugar and polyol transport mechanisms at biochemical and molecular levels, and to perform studies on the effect of salt on polyol transport and metabolism.

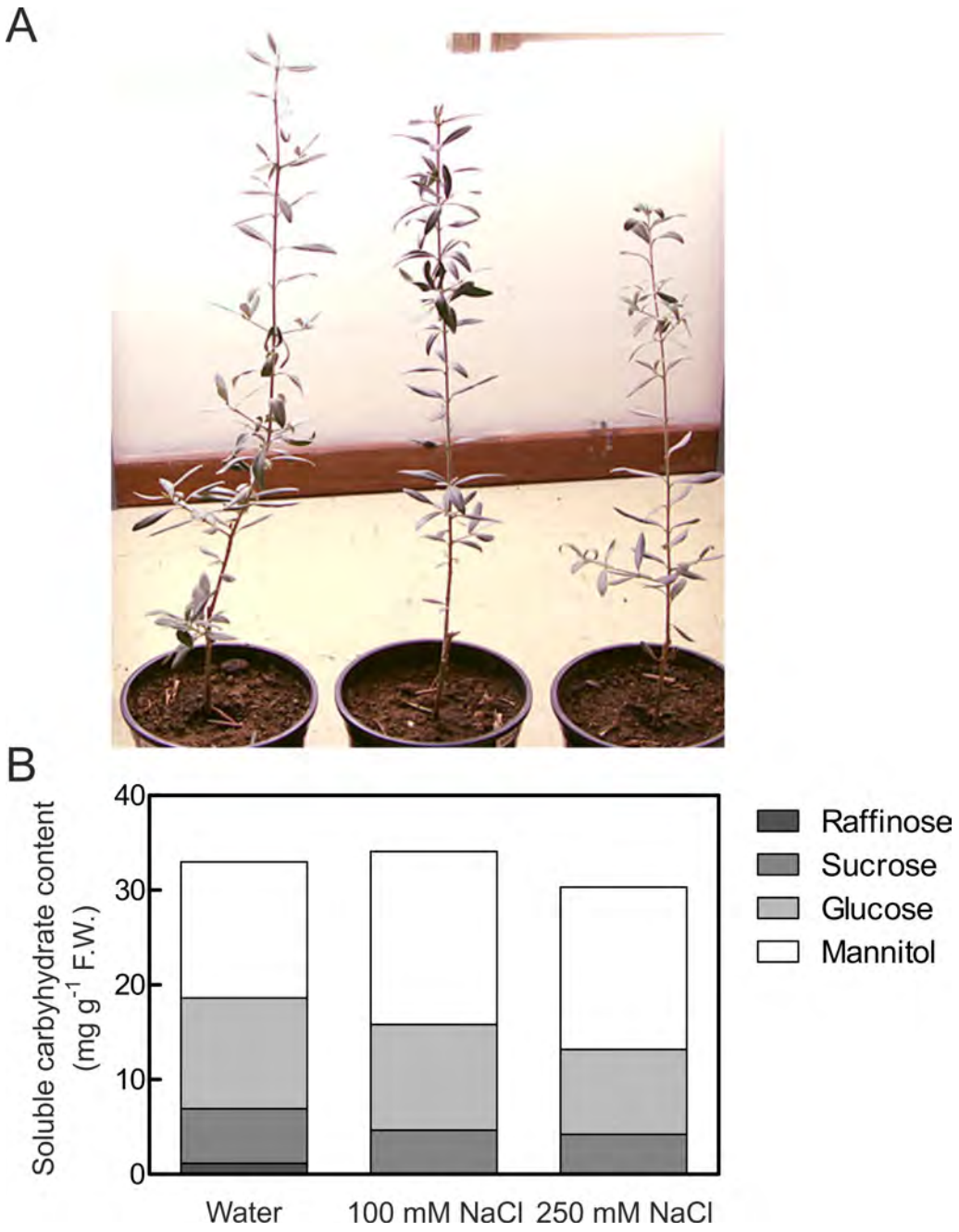

Figure 3.1. Six months old potted olive plants, irrigated periodically (twice weekly) with water or salted water (A) and corresponding soluble carbohydrate content in leaves, as determined by HPLC (B).

3.3.2. Mannitol transport and metabolism in olive cells - effect of salt stress

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 ± 0.15 mM mannitol and V_{max} = 1.29 ± 0.04 nmol mannitol min^{-1} mg^{-1} D.W. (Figure 3.2A). Competition experiments showed that dulcitol, sorbitol and xylitol competitively inhibited mannitol uptake, whereas glucose and sucrose did not. To study the energetics

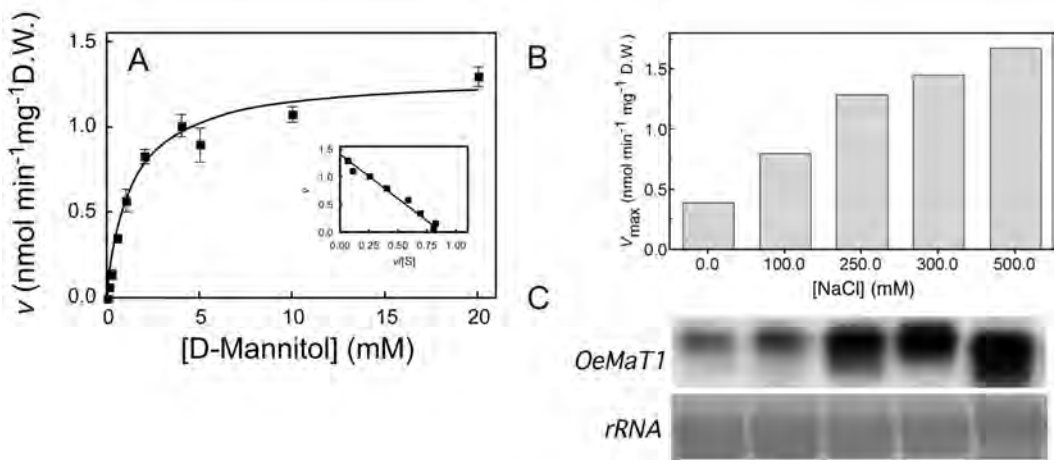

Figure 3.2. Mannitol transport by suspension-cultured cells of *O. europaea* cultivated with 1% (w/v) mannitol. (A) Initial uptake rates of D-[^{14}C]mannitol, at pH 4.5, by cells collected at the end of exponential growth phase. (B) Effect of the concentration of NaCl on the V_{max} of mannitol transport and (C) *OeMaT1* expression in *O. europaea* suspension-cultured cells collected at mid-exponential growth phase, 24 h after addition of salt. In each lane, 50 µg of RNA were used. *Insert:* Eadie-Hofstee plot of the initial uptake rates of D-[^{14}C]mannitol.

of mannitol transport in *O. europaea* cell suspensions, the dependence of the polyol uptake on the value of external pH was measured. The V_{max} was highest at pH 4.5, with little activity remaining above pH 5.5. Moreover, the addition of 50 µM of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) inhibited mannitol uptake by over 60%. These, and other evidences, suggested the involvement of a transport system with a stoichiometry of 1 mannitol:1 proton (see Conde *et al.* 2007b for all results described).

To identify the cDNA sequence encoding an *O. europaea* mannitol:H^+ symport system, degenerated primers corresponding to conserved regions of polyol transporters were used, and RT-PCR was performed on mRNA extracted from *O. europaea* suspension-cultured cells exhibiting high mannitol transport activity. This allowed the cloning of a 501 bp cDNA *OeMaT1* (accession number, DQ059507) with extensive homology with the celery mannitol transporter *AgMaT2*. Remarkably, mannitol transport activity and the expression of *OeMaT1* severely increased simultaneously upon the addition of NaCl (Figure 3.2B, C).

Figure 3.3 shows the expression of *OeMaT* during olive ripening as measured by northern-blot. *OeMaT* is mostly expressed in mature olives (black stage). Given that mannitol is an important photoassimilate accumulated in the fruit, these data support

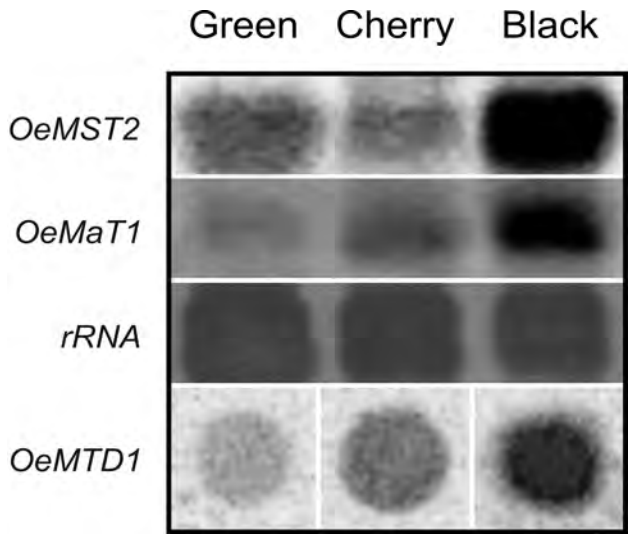

Figure 3.3. Analysis of the expression of *OeMST2* and *OeMaT1* (Northern blot) and *OeMTD1* (dot blot) during *O. europaea* fruit ripening: green, cherry and black.

an important role of polyol transporters in mannitol unloading.

To correlate mannitol transport with the rate of intracellular mannitol conversion, the activity of mannitol dehydrogenase (MTD) was characterized and measured in crude extracts of *O. europaea* cells growing in mannitol (2%, w/v) at different temperatures, ranging from 20 to 50 ºC, and pH values, ranging from 7.5 to 10.5. The activity was subsequently compared with the activity measured at 25 ºC and pH 9 in homogenates from cells growing in 2% (w/v) sucrose (Figure 3.4), allowing for the correlation with gene expression in both growth conditions. The kinetic parameters, at 25 ºC and pH

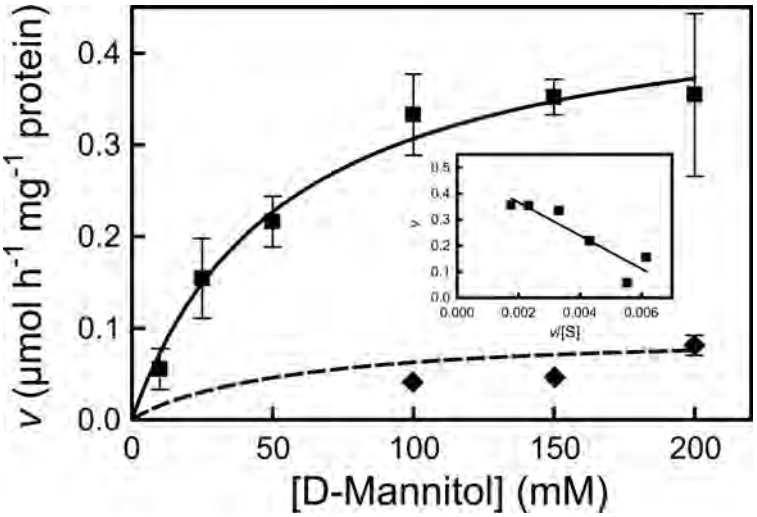

Figure 3.4. Mannitol dehydrogenase (MTD) activity, measured at 25 ºC and pH 9.0, in extracts of *O. europaea* suspension-cultured cells cultivated up to the mid-exponential growth phase with 2% (w/v) mannitol (■) and 2% (w/v) sucrose (◆). *Insert:* Eadie-Hofstee plot of the initial D-mannitol oxidation rates. Error bars denote the SD from the mean, *n* = 3.

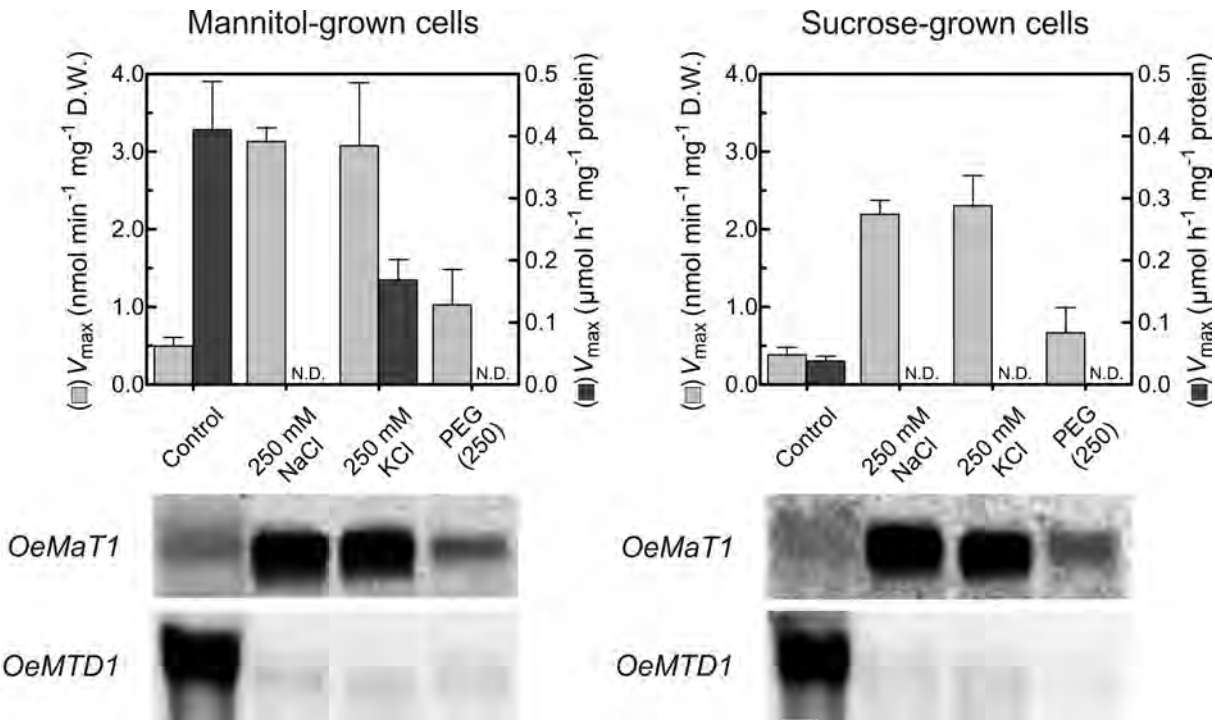

Figure 3.5. Effect of NaCl, KCl and PEG on the V_{max} of mannitol transport and the V_{max} of mannitol oxidation in *O. europaea* cells growing in mannitol or in sucrose, and expression of *OeMaT1* and *OeMTD1*. Cells were collected at mid-exponential growth phase and subjected to salt and drought treatment during 24 h. N.D., not detected. Error bars denote the SD from the mean, n = 3. In each lane, 50 µg of RNA were used.

9.0, were as follows: K_m, 54.5 ± 22.7 mM mannitol; and V_{max}, 0.47 ± 0.07 µmol mannitol h^{-1} mg^{-1} protein. The corresponding cDNA was cloned and named *OeMTD1* (accession number, EF470250). In both mannitol- and sucrose-grown cells, *OeMTD1* expression was compared with MTD activity, *OeMaT1* expression and carrier-mediated mannitol uptake (Figure 3.5). Results showed that non-treated mannitol-grown cells display basal mannitol transport activity and *OeMaT1* transcript levels, together with a high MTD activity and high level of *OeMTD1* transcription (Figure 3.5, left panel). Conversely, in non-treated sucrose-grown cells, both the mannitol transporter and MTD activities are kept at basal levels, but *OeMTD* expression is high (Figure 3.5, right panel).

The addition of NaCl (250 mM) to cells growing in mannitol caused a severe decrease in MTD activity associated with a repression of *OeMTD1* transcription. A similar repression of *OeMTD1* transcription was observed after addition of KCl, but the activity of the enzyme was not completely repressed (V_{max} = 0.17 ± 0.03 µmol mannitol h^{-1} mg^{-1} protein). In contrast, mannitol transport activity paralleled the high increase of *OeMaT1* transcript levels in both situations. To mimic drought conditions without the

ionic cytotoxic component, PEG, with an equivalent osmotic pressure to that of 250 mM NaCl, was added to the cell cultures (Money 1989). PEG caused cells growing in mannitol to repress *OeMTD1* transcription, and, consequently, MTD activity was also reduced to non-detectable levels. However, the up-regulation of *OeMaT1* expression and activity was less significant than under salt stress conditions.

To assess the physiological role of mannitol in salt stress tolerance in *O. europaea*, cell viability was studied after salt addition to cells growing in either mannitol or sucrose. Cell aliquots were collected from each medium at mid-exponential growth phase, and 250 mM NaCl or 250 mM KCl were added. Cell viability was assessed after 24 h by fluorescein diacetate (FDA) and propidium iodide (PI) staining. FDA is permeable to the intact plasma membrane and is converted to a green fluorescent dye, fluorescein, by a function of internal esterases, showing green colour in viable cells. In contrast, PI is impermeable to the intact plasma membrane. Damaged cells, having pores on the plasma membrane, incorporate the dye, which binds to genomic DNA and generates red fluorescence (Jones and Senft 1985). Figure 3.6 shows that a large population of mannitol-grown cells remained viable 24 h after a 250 mM NaCl or KCl pulse. Cells growing in sucrose, however, seemed to be much more sensitive than mannitol-grown

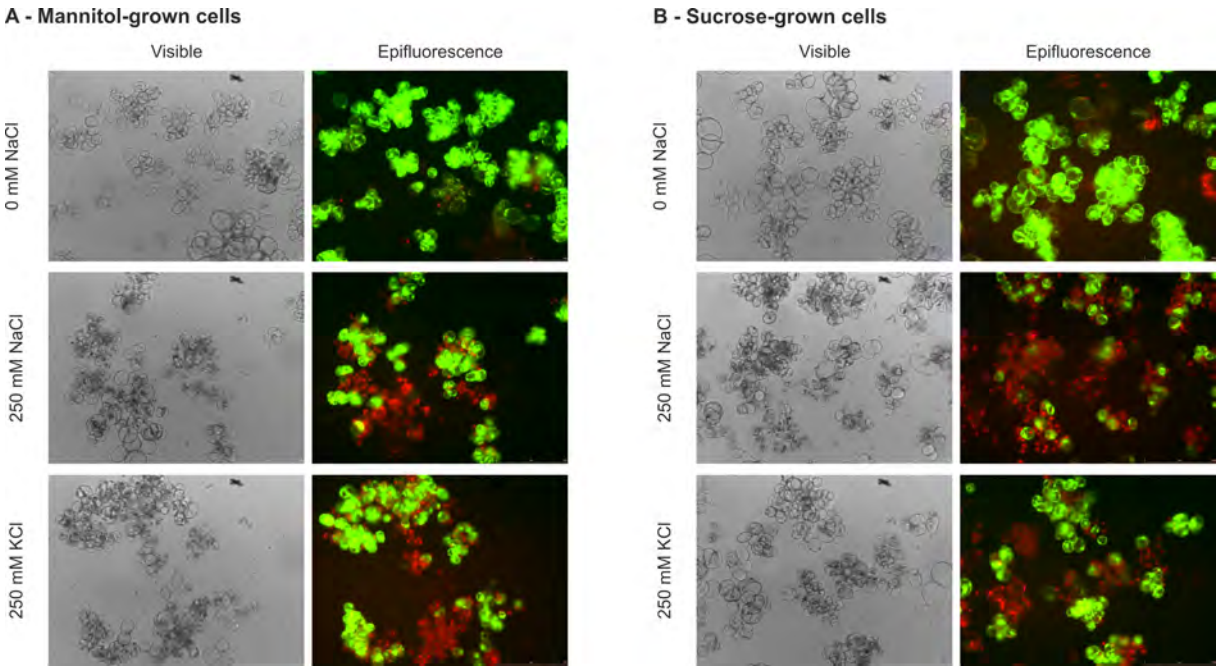

Figure 3.6. Cell viability assays in *O. europaea* suspension-cultured cells cultivated with (A) mannitol and (B) sucrose, 24 h after the addition of 250 mM NaCl or 250 mM KCl. Fluorescence was measured after incubation with fluorescein diacetate (FDA, green fluorescence) and propidium iodide (PI, red fluorescence).

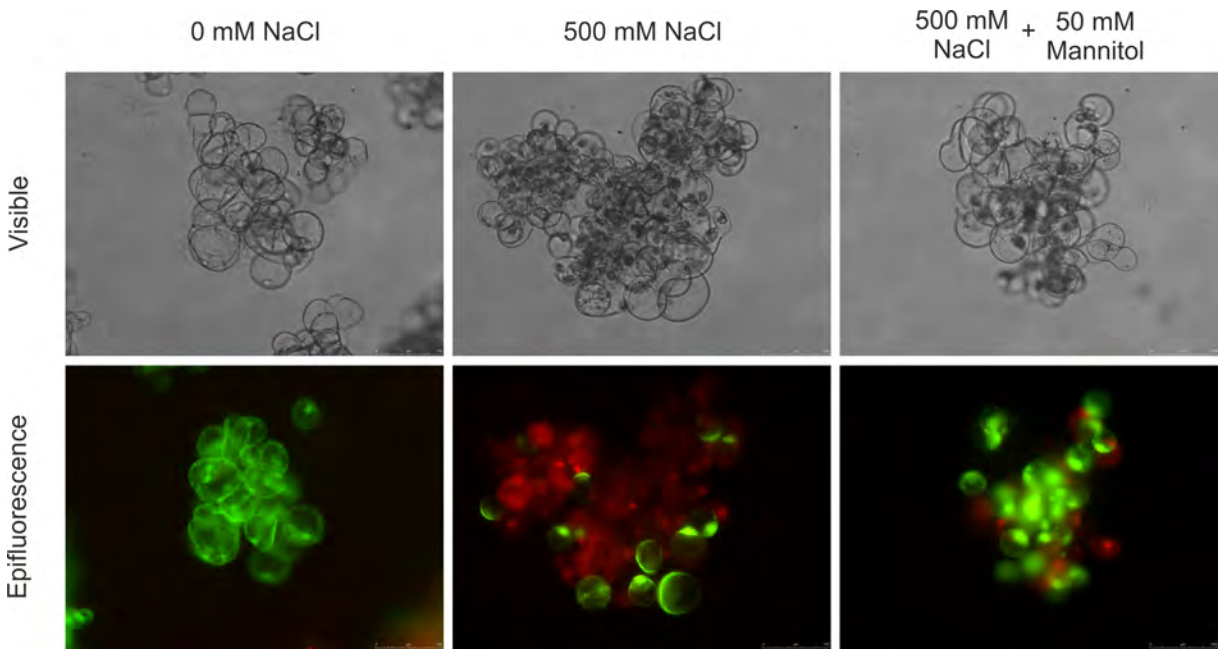

Figure 3.7. Protective role of mannitol from the toxic effect of NaCl in *O. europaea*. Cell viability assays in suspension-cultured cells, cultivated with sucrose in the absence of salt (0 mM NaCl, control), 24 h after the addition of 500 mM NaCl or the addition of 500 mM NaCl plus 50 mM mannitol. Fluorescence was measured after incubation with fluorescein diacetate (FDA, green fluorescence) and propidium iodide (PI, red fluorescence).

to the deleterious effect of both salts, and showed extensive loss of cell viability, as evaluated by the increase in PI red fluorescence and the loss of green flourescence after the same treatment. A similar result was obtained with glucose-grown cells (not shown).

When the effects of NaCl, KCl and PEG were evaluated in cells growing in sucrose, there was also a substantial increase of mannitol transport activity and of *OeMaT1* transcript levels, together with a decrease of MTD activity from basal levels to total repression (Figure 3.5, right panel). *OeMTD1* transcription was strongly repressed in all experimental stress conditions. Furthermore, addition of mannitol to sucrose-grown cells substantially protected them from the deleterious effect of NaCl: the reduction of cell viability in cells incubated in the presence of 500 mM NaCl during 24 h (>90%) diminished in cells incubated simultaneously with 500 mM NaCl and 50 mM mannitol (≈50%,), as assessed after incubation of cells with the fluorescent dyes FDA and PI (Figure 3.7).

Cell viability was also assessed in batch cultures along growth, with sucrose or mannitol as the sole carbon and energy source, in the absence of salt and in the

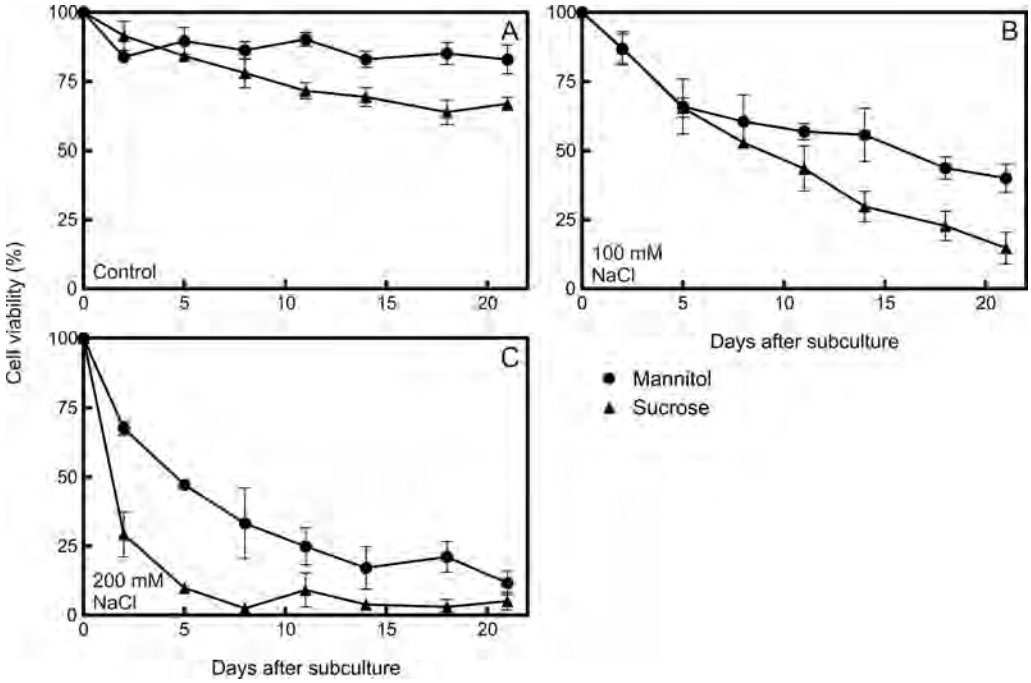

Figure 3.8. Viability of *O. europaea* cells along exponential growth, cultivated in medium supplemented with 2% (w/v) mannitol (●) and 2% (w/v) sucrose (▲) as the sole carbon and energy source, in absence of salt or in the presence of 100 or 200 mM NaCl. Error bars denote the SD from the mean, $n = 3$.

presence 100 and 200 mM NaCl. For that, cell aliquots were taken every two days and labelled with FDA. As shown in Figure 3.8, the increase of salt concentration in the medium negatively impacted cell viability in a dose-dependent manner, but the effect was much less evident in mannitol-grown cells. Interestingly, cell viability was also higher in cells growing in mannitol without salt when compared to sucrose (Figure 3.8A), possibly due to the role of mannitol as an hydroxil radical scavenger (Smirnoff and Cumbers 1989).

3.3.2.1. ROS homeostasis and the protective role of mannitol

In order to study the ROS homeostasis during salt stress, *O. europaea* suspension-cultured cells were cultivated in either sucrose or mannitol in the absence or in the presence of 150 mM NaCl. Cell aliquots were withdrawn from the culture and incubated with the fluorescent probe H_2DCFDA (see Material and Methods) before measuring its fluorescence in a spectrofluorometer to quantify total intracellular ROS production. Results show that salt stressed cells have higher oxidative status than control cells (Figure 3.9A), but the two different carbon sources did not cause

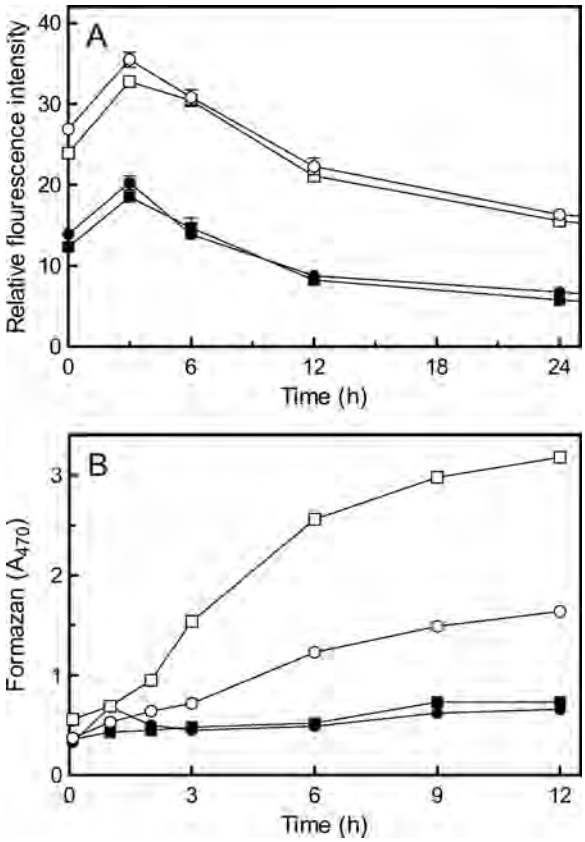

Figure 3.9. Determination of the oxidative stress status in *O. europaea* cell suspensions cultivated in medium supplemented with 2% (w/v) mannitol in the absence of salt (●) and after the addition of a 150 mM NaCl pulse (○), and 2% (w/v) sucrose in the absence of salt (■) and after the addition of the same salt concentration (□). (A) Quantification of the overall oxidative stress state of cell suspensions during a 24 h period by means of the fluorescence produced by the reaction product of H_2DCFDA. (B) Measurement of superoxide radical ($O_2^{\cdot-}$) production by the quantification of XTT reduction to a soluble formazan at 470 nm during a 12 h period.

observable differences. To quantify intracellular superoxide radical, the tetrazolium dye XTT was added to 50 mL of cell cultures, immediately before the addition of 150 mM NaCl, and 1 mL samples were removed to quantify the formazan production during 12 h. Results showed a substantial increase in formazan production after addition of salt to sucrose-grown cells, which was markedly prevented in mannitol-grown cells (Figure 3.9B).

Endocytosis has only recently been shown to occur in plant cells (Emans *et al.* 2002) and can be responsible for the formation of intracellular ROS (Leshem et al. 2007). Most studies have been done using protoplasts, due to the selective permeability of the plant cell wall that hinders most markers from staining the plasma membrane. Here, the involvement of endocytosis as a mechanism of cell volume

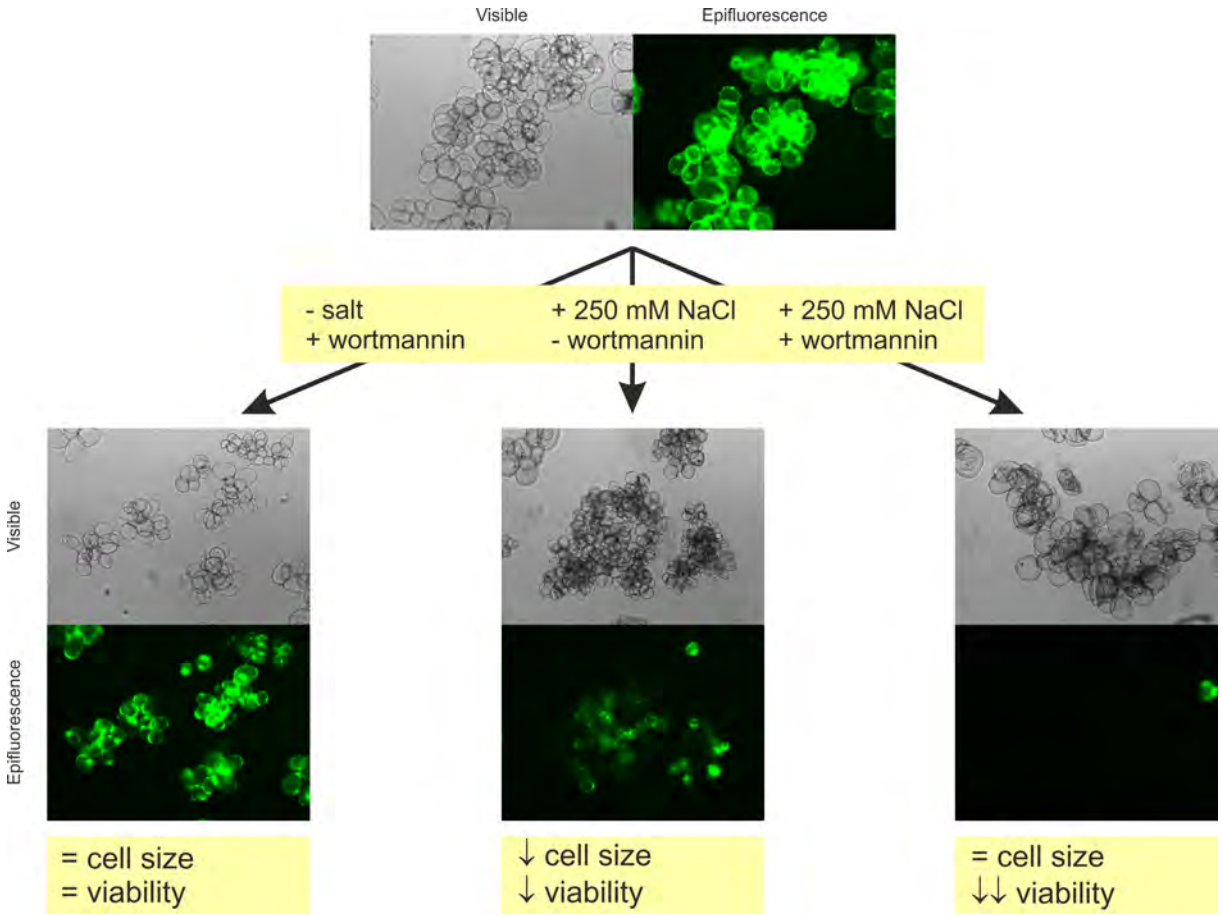

Figure 3.10. Osmotic adjustment by endocytosis-mediated cell contraction of *O. europaea* cell suspensions grown in mannitol. Cell viability and size was measured in the absence of salt (*top*) and in the presence of 33 µM of the endocytic inhibitor wortmannin alone (*left*), 250 mM NaCl alone (*middle*) and 250 mM NaCl plus wortmannin (*right*), 24 h after the addition of each chemical. Fluorescence was measured after incubation with fluorescein diacetate (FDA, green fluorescence).

adjustment providing tolerance against osmotic stress was clarified. The incubation of cells with 250 mM NaCl for 24 h promotes a decrease of the cell volume, but not a significant loss of viability measured by FDA (Figure 3.10). The endocytic inhibitor wortmannin (33 µM) alone does not affect the viability of the cells in the absence of salt. In cells incubated with 250 mM NaCl, wortmannin prevents the decrease of the cell volume with a consequent loss of viability. This suggests the involvement of osmotic adjustment by endocytosis-mediated cell contraction.

The membrane soluble family of styryl FM dyes have been used to successfully monitor endocytic vesicles formation in neurons, yeasts and fungi, among others, and have recently been used in intact, walled plant cells. In the present study, the formation of endocytic vesicles was clearly shown in olive cells after incubation with FM1-43 under a confocal microscope (see the next section, Figure 3.15), although additional

experiments would be necessary to measure the rate of endocytic vesicles formation in salt-stressed and control cells.

3.3.3. Evidences for carrier- and channel-mediated sugar transport in olive cells

As referred to in the Introduction (section 1.3.4.), 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

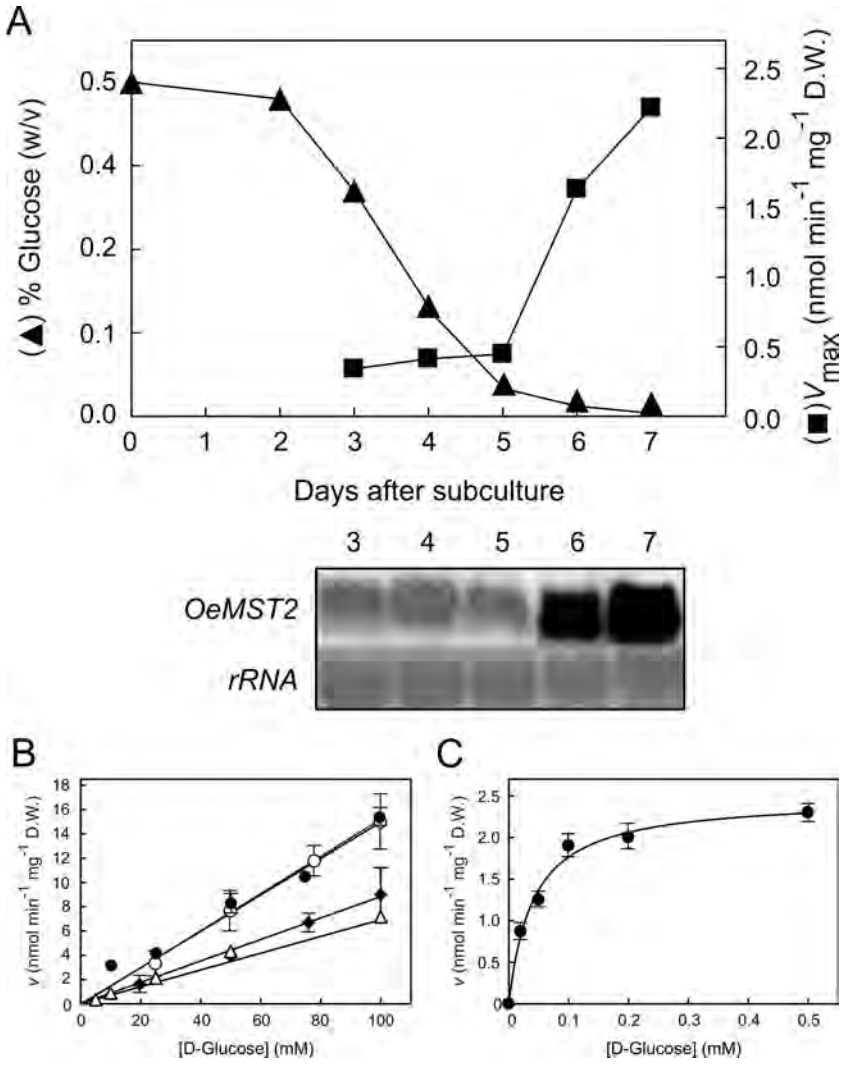

Figure 3.11. Diffusive and saturating glucose transport in suspension-cultured cells of *O. europaea*. In cells collected from the culture medium up to day 5 after subculture (glucose-sufficient cells, A), uptake is linear with respect to D-[^{14}C]glucose concentration (up to 100 mM, B, ●). As glucose is depleted from the culture medium, the activity of a saturating monosaccharide transport system becomes apparent (C) together with *OeMST2* transcription (A, lower box). Transport experiments of D-[^{14}C]glucose were performed at 25 ºC in the absence (●) and in the presence of 1 mM $HgCl_2$ (◆), 5 µM staurosporine + 20 mM propionic acid, pH 6 (△).Transport experiments of L-[^{14}C]glucose were performed at 25 ºC (○). Error bars denote the SD from the mean, *n* = 3.

mode when high external glucose concentrations are present. Figure 3.11 shows these two modes of glucose incorporation in *O. europaea* suspension-cultured cells growing in 0.5 % (w/v) glucose. Cells collected from the medium up to day 5 after subculture, when the sugar concentration in the medium is above 0.05% (Figure 3.11A), display linear uptake kinetics up to 100 mM glucose (glucose-sufficient cells, Figure 3.11B). As glucose is depleted from the culture medium, 5 days after subculture (Figure 3.11A), the activity of a saturating monosaccharide transport system becomes apparent (glucose-starved cells, Figure 3.11C).

To identify any potential cDNAs encoding MSTs, 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 959 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 in order to obtain the full-length cDNA sequence. This approach resulted in the isolation of a 1,816 bp sequence, subsequently named *OeMST2* (*O. europaea* monosaccharide transporter *2*, accession number, DQ087177). *OeMST2* has a 1,569 bp open reading frame, potentially encoding a protein of 523 aa residues, with a predicted molecular mass of 57.6 kDa and a pI of 8.76 (Figure 3.12)

The expression of *OeMST2* was studied in suspension-cultured cells in the conditions depicted in Figure 3.11, in order to investigate if it is involved in the observed saturating sugar transport component (Figures 3.11C). Although detectable since day 3, *OeMST2* mRNA increased abruptly after day 5, when the glucose concentration of the medium decreased below 0.025% (w/v), with the highest value 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-[^{14}C]glucose revealed that the activity of the H^+-dependent monosaccharide transport system started to increase from basal levels at day 5, and the maximum transport activity was detected at day 7 (Figure 3.11A). This transient sequential phenomenon reflects a close relationship between *OeMST2* transcript and glucose transport activity.

Expression of this *OeMST2* in an *hxt*-null *S. cerevisiae* strain unable to take up

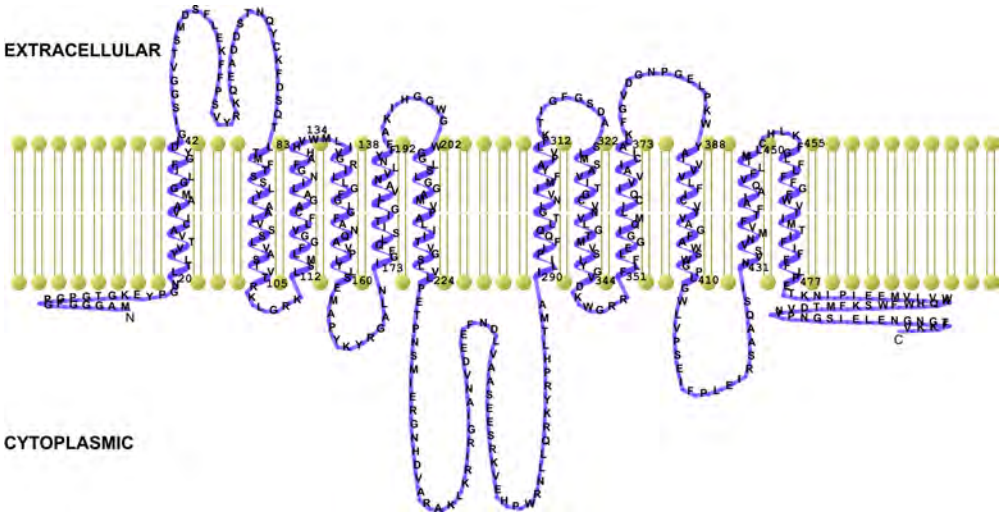

Figure 3.12. Topological model of the *O. europaea* monosaccharide/H^+ symporter OeMST2 (accession number DQ087177). The positions of the transmembrane helices were determined by the membrane protein topology prediction web service TMHMM 2.0 (Krogh *et al.* 2001) and displayed using the transmembrane protein plotting program TMRPres2D (Spyropoulos *et al.* 2004). The amino acid sequence is displayed along the protein traces and the numbers indicate the amino acid position where the transmembrane helices start or stop.

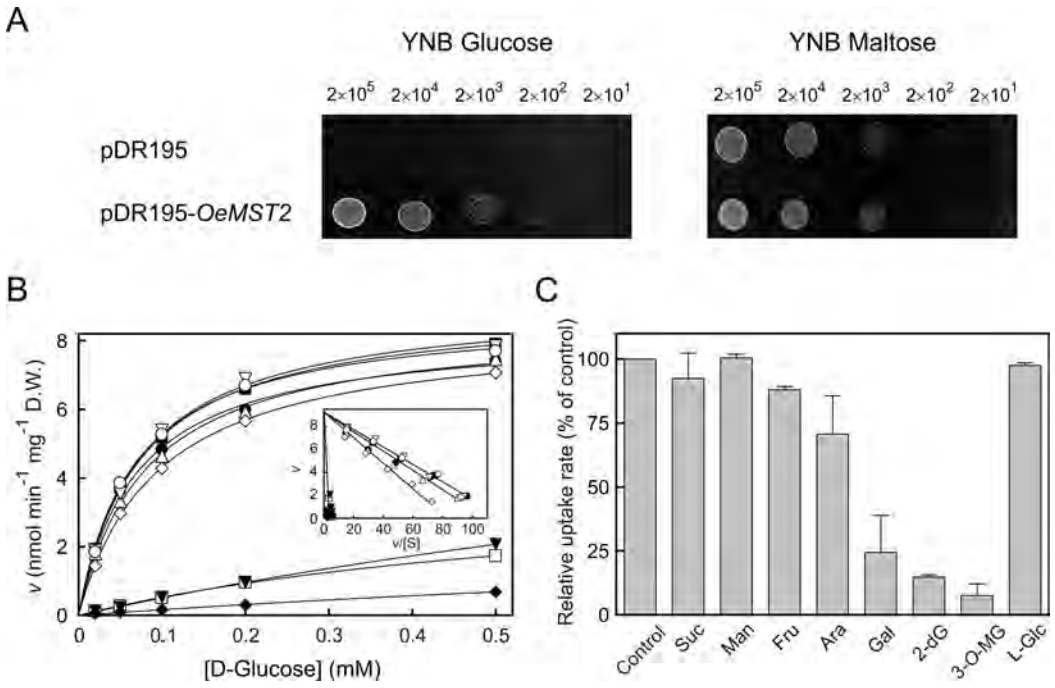

Figure 3.13. Functional expression of the *OeMST2* cDNA in the *S. cerevisiae* EBY.VW4000 mutant defective for monosaccharide uptake and specificity of substrate uptake mediated by OeMST2. (A) Growth phenotype of the mutant complemented by the empty vector (pDR195) and the vector carrying *OeMST2* (pDR195-*OeMST2*) on media containing either 0.1 mM maltose or glucose as the sole carbon and energy source. Numbers above the pictures indicate the approximate amount of yeast cells plated. (B) Uptake of 0.02 to 0.5 mM D-[^{14}C]glucose (■) challenged by an excess (2 mM) of unlabelled sugar: sucrose (◇), D-mannitol (▽), D-fructose (●), D-arabinose (△), D-galactose (□), 2-deoxy-D-glucose (▼), 3-*O*-methyl-D-glucose (◆) and L-glucose (○). *Insert:* Eadie-Hofstee plot of the initial uptake rates of D-[^{14}C]glucose. (C) Effect of several mono- and disaccharides, mannitol and glucose analogues on the initial uptake rates of 0.5 mM D-[^{14}C]glucose. Error bars represent SD from the mean, n = 3.

glucose (EBY.VW4000, Wieczorke *et al.* 1999) restored its capacity to grow (Figure 3.13A) and to transport glucose (Figure 3.13B). The encoded protein showed high affinity for D-glucose (K_m = 25 ± 2 µM) 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 (Figure 3.13B and C). 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 3.3).

As referred to above, 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 (Figure 3.11B). To study the involvement of mediated transport, activation energies from the initial glucose uptake rate at different temperatures by intact cells and plasma membrane vesicles were estimated. From the corresponding Arrhenius plots, the following values were obtained for intact cells and plasma membrane vesicles, respectively: 4 and 7 kcal mol^{-1}. In addition, $HgCl_2$ inhibited both the linear component of sugar uptake in sugar sufficient cells (Figure 3.11B) and plasma membrane vesicles (not shown, see Conde *et al.* 2007c), suggesting a protein-mediated transport. Diffusive uptake of glucose was also inhibited by propionic acid (Figure 3.11B), suggesting that this putative protein can be regulated by cytosolic pH changes, much like the gating of some aquaporins, and stimulated (237 %) by the protein kinase inhibitor staurosporine.

To investigate the possible involvement of endocytosis in glucose uptake by glucose-sufficient cells, the initial uptake rates of 0.02 to 100 mM D-[^{14}C]glucose were measured, after pre-treatment with wortmannin or NH_4Cl, two well-known inhibitors of the endocytic pathway, and both compounds failed to inhibit glucose transport. Likewise, the uptake of the non-metabolizable glucose analog 3-*O*-methyl-D-[U-^{14}C]glucose was not inhibited by wortmannin. In addition, fluorescent microscopy studies showed that, 10 min after incubation, the amount of 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl amino]-2-deoxy-D-glucose (2-NBDG) incorporated intracellularly in the presence of wortmannin did not decrease over the control (Figure 3.14). These results suggest that endocytosis does not contribute significantly to glucose uptake in glucose-sufficient cells, at least within short incubation periods.

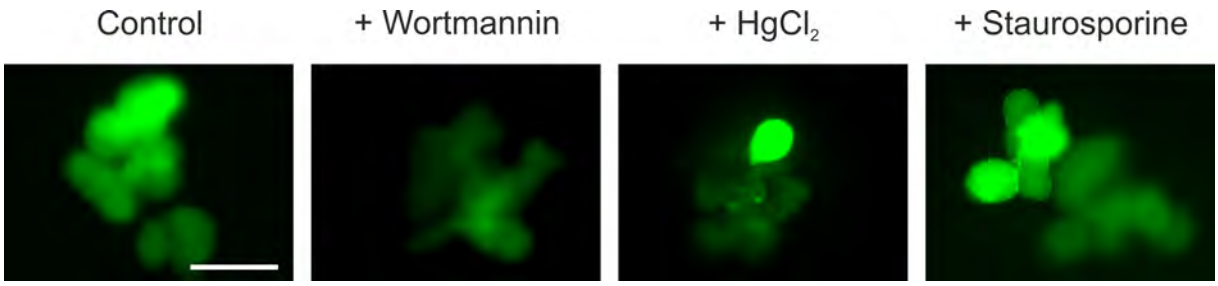

Figure 3.14. Fluorescent micrograph of suspension-cultured cells after incubation with 5 mM of the fluorescent glucose analogue 2-NBDG, for 14 h at 25 °C. Fluorescence was measured after careful washing 6 times with medium without fluorescent sugar to remove unabsorbed substrate. Bar = 100 µm.

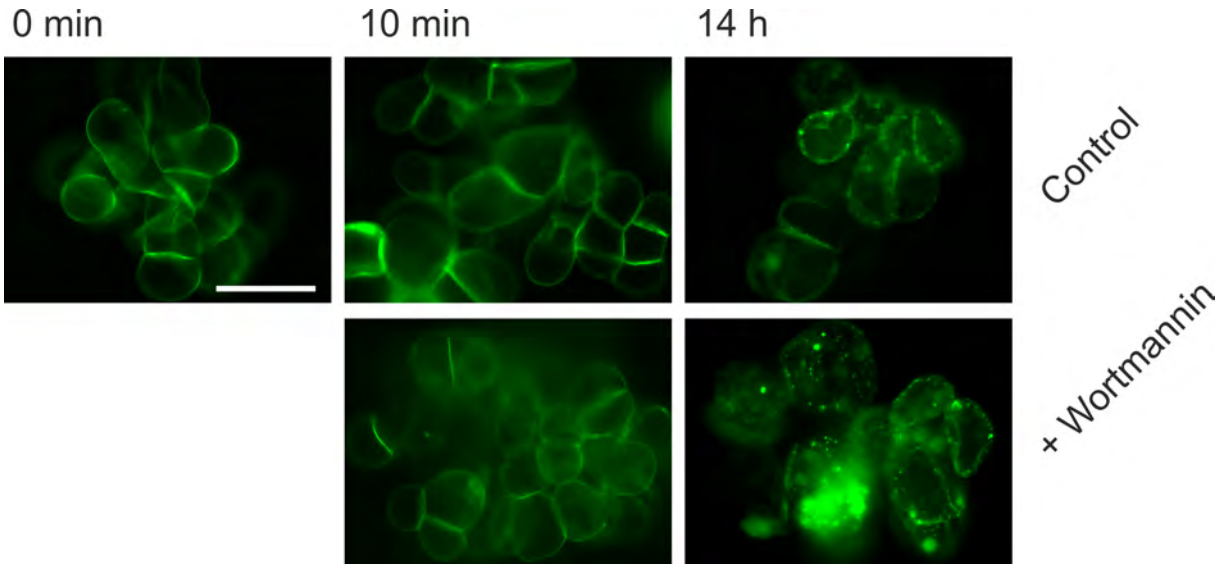

Figure 3.15. Micrograph showing the internalization of the fluorescent endocytosis marker FM1-43 into suspension-cultured cells, after 10 minutes and 14 hours, at 25 ºC. After incubation with FM1-43 for 15 minutes on ice, the cells were washed 3 times with ice cold, marker-free medium supplemented with 25 mM glucose, transferred to an orbital shaker at 25 ºC, and imaged at the indicated times. Bar = 100 µm.

To further investigate the possible involvement of endocytosis on sugar uptake in glucose-sufficient cells of *O. europaea*, the formation of endocytic vesicles was monitored with the fluorescent cell membrane marker N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl)pyridinium dibromide (FM1-43) (Kubitscheck *et al.* 2000, Emans *et al.* 2002). Figure 3.15 depicts the progression of the membrane marker into the cytoplasm after plasma membranes had been labelled with 5 µM FM1-43, and cells resuspended in a marker-free medium containing 25 mM glucose. The formation of endocytic vesicles, which might carry the sugar inside the cells, was clearly demonstrated; however this mechanism appears to proceed at a very slow rate and only becomes apparent after several hours of incubation with the fluorescent

marker. Wortmannin appeared to affect normal vesicle formation, but the phenomenon of membrane trafficking was still visible in the presence of this inhibitor.

3.4. Discussion

3.4.1. Relevance of mannitol in source and sink tissues of O. europaea

HPLC analysis of the total soluble carbon in leaves of potted olives revealed that mannitol is main soluble carbohydrate in leaves, in agreement with previous studies (Cataldi *et al.* 2000, Drossopoulus and Niavis 1988). In addition, when plants were treated with up 250 mM NaCl, there was clear shift in carbohydrate partitioning to mannitol, from 43.5% in control plants to 56.5% in salt-treated plants (Figure 3.1B), suggesting a key role of mannitol in salt stress response. As reported elsewhere, mannitol has also been detected in olive phloem sap exudates (reviewed by Conde *et al.* 2008). Unlike glucose, whose concentration falls steadily as the mesocarp accumulates storage lipids, mannitol content of olive pulp increases during maturation, reaching values of 8 mg g^{-1} D.W. in the fully ripe olive (Marsilio *et al.* 2001). The elucidation of the role played by mannitol, not only as a carbon and energy source for plant growth, but also as a protecting osmolyte against drought, soil salinity and the resulting oxidative stress, may also have an important practical agricultural dimension, besides its interest from the scientific standpoint, allowing for the enhancement of the yield potential of *O. europaea*.

3.4.2. Mannitol transport and mannitol dehydrogenase activities are involved in the response of O. europaea *to salt and osmotic stresses*

The saturable transport observed in *O. europaea* cells (Figure 3.2A) involves a polyol:H^+ symport system with a stoichiometry of 1 mannitol:1 proton, as indicated by the following observations: (i) the addition of mannitol to weakly buffered cell suspensions is associated with a transient alkalinization of the extracellular medium; (ii) the V_{max} of proton uptake is similar to the V_{max} of carrier-mediated D-mannitol uptake and depends on the extracellular pH; (iii) dissipation of the proton-motive force by 50 µM CCCP

significantly inhibited the initial velocities of D-mannitol uptake (63 %); and (iv) mannitol transport was inhibited 43 % by 10 mM TPP^+, suggesting that the $\Delta\Psi$ is an important component of the proton-motive force involved in mannitol accumulation. Proton dependence and substrate affinity (K_m = 1.3 mM mannitol) are in good agreement with the data obtained for the celery mannitol transporters *AgMaT1* and *AgMaT2* (Noiraud *et al.* 2001a and Juchaux-Cachau *et al.* 2007, respectively). In this polyol-producing plant, different membrane transport steps have been studied, from phloem loading to phloem unloading and storage in parenchyma cells. The cloned mannitol transporter gene *AgMaT1* gave yeast cells the ability to grow on mannitol, and a K_m value of 0.34 mM for mannitol uptake was obtained (Noiraud *et al.* 2001a), which correlates well with the value determined in plasma membrane vesicles isolated from phloem strands of celery (K_m = 0.64 mM, Salmon *et al.* 1995). Similarly, a K_m of 1.8 mM was obtained for *AgMaT2* expressed in yeast (Juchaux-Cachau *et al.* 2007). The involvement of a co-transport with protons was proposed for both polyol transporters, since the uptake of mannitol was almost abolished by CCCP and was maximal at an acidic pH. Also, K_m values of ≈1 mM were obtained in storage parenchyma discs of celery leaves and in plasma membrane vesicles isolated from parenchyma cells (Keller 1991 and Salmon *et al.* 1995, respectively). In contrast, mannitol transport in vacuoles of celery parenchyma cells seems to be mediated by facilitated diffusion, since it was neither stimulated by energization with ATP and PPi nor impaired by the dissipation of the proton-motive force (Greutert *et al.* 1998).

This study provides a description of how the coordination between the mannitol transport and oxidation steps operating in olive suspension-cultured cells is crucial for salt and drought stress tolerance in *O. europaea*. Although a significant amount of information is already available on the involvement of mannitol in the tolerance of plants to abiotic stress, much less is known about the regulation of transport activity and metabolism in relation to gene expression and its contribution to the tolerance process. Following transmembrane transport, the oxidation of mannitol to mannose, catalysed by a NAD^+-dependent MTD, is the first step of mannitol metabolism in heterotrophic tissues (Figure 1.5, Stoop *et al.* 1996). MTD activity measured in homogenates from cultured cells grown with mannitol was substantially higher (up to 10-fold) than in homogenates from cells growing in sucrose, but the transcription of *OeMTD1* was high

in both conditions (Figure 3.4 and 3.5), suggesting post-transcriptional…

The analysis of Figure 3.5 regarding OeMTD activity and *OeMTD* expression in control cells, cultivated with mannitol and sucrose as the sole carbon and energy sources, corroborating that OeMTD is regulated at both a transcriptional and post-transcriptional level. Remarkably, after Na^+, K^+ and PEG treatments in both mannitol- and sucrose-grown cells, gene transcription was completely abolished and, consequently, a strong reduction of mannitol oxidation activity was observed, suggesting that these stress factors signal an inhibition of *OeMTD* at the transcriptional level.

Carrier-mediated mannitol transport was up-regulated at a transcriptional level by NaCl, KCl and PEG, in both mannitol- and sucrose-grown cells. In all experimental conditions, there was an increase of the transcript levels of *OeMaT1* and an increase of the V_{max} of carrier-mediated mannitol transport over the control that paralleled the repression of *OeMTD* expression. Taken together, these observations suggest that olive cells display an integrated response that leads to a nearly universal reaction to salt and osmotic stresses: intracellular osmolyte accumulation. This is thought to allow for osmotic adjustment, in order to compensate for the decrease of external water potential and allow for oxidative detoxification (Shen *et al.* 1997b). The stress signaling pathways involved seem to be epistatic over sugar-mediated regulation of gene expression, as this highly integrated response occurred even in the absence of extracellular mannitol (Figure 3.5). Hence, the addition of mannitol to cells growing in sucrose substantially alleviated the damage caused by salt, as shown by fluorescence microscopy with vital fluorescent stains (Figure 3.7).

In agreement with the protective role of mannitol, it was shown that, after a salt pulse, a high percentage of mannitol-grown cells remained viable 24 h after the addition of 250 (Figure 3.6A) and 500 mM NaCl (Conde *et al.* 2007b), contrasting with the dramatic decrease of cell viability in sucrose-grown cells (Figure 3.6B). Similarly, the loss of cell viability during growth of cells cultivated with sucrose was higher in the presence of NaCl than that of cells grown in mannitol in the same conditions (Figure 3.8). In accordance with these results, the growth rate of heterotrophic celery cell suspensions cultivated with sucrose was much more inhibited by NaCl than that of mannitol-grown cells, although it was demonstrated that both types of cells accumulated

soluble sugars to the same osmotic potential (Pharr *et al.* 1995).

In the present work, conducted with heterotrophic cells as sink models, we have studied how cells coordinate the activity of mannitol/H^+ symport with the activity of NAD^+-dependent mannitol dehydrogenase at the gene expression and protein activity level, under salt and drought stress. By adjusting mannitol transport and intracellular metabolism, the olive tree should be able to cope with increased salinity and drought, typical of the Mediterranean basin. As a result, the increase in the concentration of mannitol in leaves of salt-treated olive plants indicates that it may function primarily as a compatible solute, as was reported in the adaptation to salinity of *Phillyrea latifolia* (Tattini *et al.* 2002). Furthermore, in celery, root zone salinity increased the activity of M6PR (mannitol-6-phosphate reductase) up to 6-fold in leaves and promoted changes in photosynthetic carbon partitioning from sucrose to mannitol, facilitating its accumulation in leaf tissues, providing improved stress tolerance (Everard *et al.* 1994). Similarly, in peach (*Prunus persica*), the *in vitro* activity of aldose-6-phosphate reductase, the key enzyme in sorbitol synthesis, increased linearly in response to drought stress, as did the partitioning of newly fixed carbon into sorbitol and its extrusion and concentration in the phloem sap (Escobar-Gutiérrez *et al.* 1998). In addition, leaf cells of *Plantago major* displayed an increase in sorbitol content together with a drastic decrease in the transcription of sorbitol dehydrogenase (*PmSDH1*) in response to treatment with salt (Pommerrenig *et al.* 2007). A significant shift in photosynthetic carbon partitioning to mannitol under salt or drought stress has also been reported in leaves of *O. europaea*, confirming the results obtained in the present study in the cultivar Galega Vulgar that mannitol can act as a potential osmoregulator in leaf mesophyll (Tattini *et al.* 1996, Gucci *et al.* 1996, Gucci *et al.* 1997, Gucci *et al.* 1998, Dichio *et al.* 2009, Melgar *et al.* 2009, Remorini *et al.* 2009, Cimato *et al.* 2010).

Our preliminary results also showed that, in *O. europaea*, mannitol may also function as an antioxidant osmoprotectant against oxidative stress resulting from salt stress (Figure 3.9). Similar results have been described regarding ROS production as a response to salt and drought stress, and even solar irradiance (Melgar *et al.* 2009, Remorini *et al.* 2009, Cimato *et al.* 2010). Mannitol accumulation has been recently suggested to protect salt-treated leaves in full sunshine from heat stress-

induced oxidative damage to a greater extent than leaves growing under partial shading (Cimato *et al.* 2010). Concordantly, mannitol has been described as a potent scavenger of hydroxyl radicals that may result from salt or drought stress (Shen *et al.* 1997a, Shen *et al.* 1997b). Furthermore, the antioxidant function of mannitol may be to shield susceptible thiol-regulated enzymes, such as phosphoribulokinase, thioredoxin, ferredoxin and glutathione, from inactivation by hydroxyl radicals (Shen *et al.* 1997b).

In light of our results, the coordination of mannitol transport *via* OeMaT1 together with the significantly lowered mannitol oxidation *via* OeMTD1 suggests a function of mannitol in salt or drought stress tolerance, whether resulting from excessive concentration of Na^+ or K^+ ions, or from actual low water availability. However, it is not yet completely clear if the abiotic stress-induced accumulation of osmoprotectant compounds such as mannitol may, by itself, directly allow an increased tolerance, or if it is just a participant in a much more complex and intricate mechanism of stress tolerance by acting synergistically with other key intervenients. Given the key importance of mannitol accumulation in abiotic stress tolerance, a coordination of transport and oxidation seems reasonable in plant sink cells, and its tight regulation is critical in allowing the regulation of mannitol pool size, as demonstrated in the present work.

The production of ROS in response to salt stress has been documented in plants (reviewed by Zhu 2001), which can damage intracellular components and lead to subsequent cell death (Levine *et al.* 1994). Recently, the production of ROS in endosomes formed as a response to salt stress has been shown in *Arabidopsis* root tip cells and cell suspensions (Leshem *et al.* 2007). Endocytosis and the production of ROS were shown by the authors to be dependent on the phosphatidylinositol 3-kinase (PI3K) pathway, and were inhibited by wortmaninn, a specific inhibitor of PI3K. In *O. europaea* suspension cells, incubation with salt seemed to promote a reduction in cell size, possibly by endocytosis-mediated internalization of the cell wall, while cells incubated with salt plus wortmannin seemed to maintain the same size as the control, while suffering a drastic loss in cell viability (Figure 3.10). The inability of cells to reduce the intracellular volume in the presence of salt and wortmaninn, and the inhibition by wortmannin of ROS production, which has a known signalling role in biotic and abiotic stress (Foyer and Noctor 2005), might account for the drastic loss in cell viability.

The continued study of the signalling pathways responsible for responses to salt, drought and other abiotic stresses may allow for the treatment of plants with exogenous compounds, such as mannitol and other osmoprotectants and antioxidants, without resorting to genetic manipulation, thus avoiding the introduction of genetically manipulated plants in nature. The protective role of mannitol when supplemented to salt-stressed sucrose-grown cells of *O. europaea* (present study) or its enhancing effect on several antioxidant enzymes of wheat, which does not produce mannitol, under high salinity conditions (Seckin *et al.* 2009), confirms that this research topic calls for a continuous investment from the scientific community.

3.4.3. OeMST2 *encodes a monosaccharide transporter expressed throughout olive fruit maturation*

Besides mannitol, olive tree also translocates sucrose as well as RFOs, which are hydrolysed into sucrose, and ultimately monosaccharides such as galactose, glucose and fructose. Therefore, the transport of these monosaccharides into sink tissues is an important step in carbohydrate partitioning in *O. europaea*. This part of the work describes the cloning of a full-length cDNA sequence of a MST from *O. europaea* and the characterization of the corresponding gene product by heterologous expression in the yeast *S. cerevisiae*. *OeMST2* was isolated from RNA extracted from olive fruits, an organ that accumulates high concentrations of glucose, fructose and galactose. The amino acid sequence shares a high overall homology with other MST sequences identified in plants, sharing the highest homology with the *N. tabacum* monosaccharide transporter 1 (*NtMST1*). 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 (Figure 3.12).

Heterologous expression of *OeMST2* in an *hxt*-null strain of *S. cerevisiae* confirmed its function as a high affinity, broad specificity, monosaccharide:H^+ symporter (Figure 3.13) 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 μM (reviewed by Büttner and Sauer 2000). A striking

feature of *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 (Figure 3.3), suggesting that transport of monosaccharides and their compartmentation by OeMST2 are important to allocate this source of carbon and energy. A differential expression of the mannitol carrier *OeMaT1* was also observed during olive fruit maturation (Figure 3.3). Taken together, these results support the involvement of an apoplastic step in the unloading of both sugars and polyols in *O. europaea*, and that both mannitol and sugar carriers play an important role during olive fruit ripening.

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. Here, evidence is provided showing that, in *O. europaea* cells, alterations in glucose levels have a pronounced effect on *OeMST2* expression and on proton-coupled glucose transport activity (Figures 3.11A). *OeMST2* transcription is negatively regulated by high glucose levels. When external glucose decreases to residual levels, a sharp increase in *OeMST2* expression and consequently in monosaccharide:H^+ transport system activity is observed (Figure 3.11A).

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 the developing fruits and, finally, the regulation of *OeMST2* by its substrate in suspension-cultured cells contributed to a better understanding of sugar transport in *O. europaea* and, more generally, in fleshy fruits.

3.4.4. A mercury-sensitive channel mediates the diffusional component of glucose transport in olive cells

Numerous transport studies carried out with many unicellular and multicellular organisms, including plants, have yielded complex multiphasic uptake kinetics for

organic and mineral nutrients (Oliveira *et al.* 2002, Conde *et al.* 2006, Conde *et al.* 2007b, Völker *et al.* 1997, Reinhardt *et al.* 1997, Wille *et al.* 1998, Crawford and Glass 1998, Chen and Halkier 2000, Fan *et al.* 2001). Although the interpretation of these data is often difficult, complex transport kinetics may result from the coexistence of multiple transport systems, allowing the uptake of a given nutrient over a broad range of substrate concentrations (Ludewig and Frommer 2002). In general, a family of genes, rather than an individual gene, exists in plant genomes for each transport function. As mentioned in the Introduction (section 1.3.1.), seven clusters were recognized in the MST superfamily, with 53 and 65 putative MSTs in the *Arabidopsis* and rice genomes, respectively (Johnson *et al.* 2006, Johnson and Thomas 2007). In many cases, complex transport kinetics of disaccharides, monosaccharides and amino acids are resolved into one or two saturable components superimposing a linear component. The nature of this diffusive component is still elusive, and mechanisms such as free diffusion across the membrane lipids or passage through integral membrane proteins, possibility proteinaceous channels, have been proposed.

Three main approaches have been used to establish whether the uptake of non-electrolytes occurs either as passive permeation through the lipid bilayers or through channels. The first one is the use of chemical reagents that bind to proteins and may inhibit transport, thus suggesting the involvement of a protein component. The second approach consists in the determination of the activation energy (E_a). When the measured E_a is low, the molecule is predominantly transported through a channel, and when it is high, it is predominantly transported through the lipid bilayer (Reinhardt *et al.* 1997, Wille *et al.* 1998, Tsukaguchi *et al.* 1998, Dordas *et al.* 2000). The third approach is the heterologous expression of transporter in yeast or *Xenopus* oocytes. In this way, it was shown that homologues of aquaporins may mediate the transport of urea, glycerol and other nonelectrolytes (Ishibashi *et al.* 1994, Mulders *et al.* 1995, Echevarria *et al.* 1996, Tsukagushi *et al.* 1998). In particular, the aquaporin AQP9 of rat liver, which defined a new evolutionary branch of the major intrinsic protein family (MIP), mediates the transport of some non-charged solutes such as carbamides, polyols, purines and pyrimidines, together with water, in a mercury-sensitive manner and with activation energies of about 7 kcal mol^{-1} (Tsakaguchi *et al.* 1998).

Glucose uptake into suspension-cultured cells of *O. europaea* may follow a biphasic kinetics, due to a high affinity glucose/H^+ symporter system superimposed on a linear component that is more evident at higher sugar concentrations. In a low-sugar medium (0.5% glucose, w/v), both transport modes operate. However, in a high sugar medium (3% glucose, w/v), the saturable component was absent (Figure 3.11B) due to catabolic repression of the high affinity system, and glucose was exclusively absorbed by a non-saturable mechanism able to sustain both cell growth and metabolism. Here we investigated the nature of this diffusive glucose uptake component.

Several mechanisms or a combination of them could be responsible for diffusive uptake, namely: (i) non-specific permeation by free diffusion, (ii) involvement of a carrier with very low affinity, (iii) passage of the solute through a hydrophilic protein channel, and (iv) endocytosis-mediated incorporation. Fluid-phase endocytosis was recently suggested to be a parallel uptake system for sugars in a wide variety of heterotrophic cells (Baroja-Fernandez *et al.* 2006, Etxeberria *et al.* 2005a, Etxeberria *et al.* 2005b). In most experiments, this conclusion was based on the inhibition of sugar uptake by wortmannin, together with confocal imaging of fluorescent probes. In olive cells, however, both wortmannin and NH_4Cl failed to inhibit D-glucose uptake within 3 min, and wortmannin did not prevent uptake of 3-*O*-methyl-D-[U-^{14}C]glucose (not shown, see Conde *et al.* 2007c), suggesting that endocytosis is not the preferential mechanism accounting for the observed transport activity.

Altogether, out results suggest that facilitated diffusion through a mercury-sensitive hydrophilic channel mediates glucose uptake in olive suspension-cultured cells. As discussed by Conde *et al.* (2010), the inhibitory effect of mercury indicates the existence of an integral membrane protein involved in this sugar uptake, most likely a channel instead of a transporter, because: (i) glucose transport linearly depended on sugar concentration up to 100 mM, a concentration for which it would be very unlikely to find a mediated transporter with physiological relevance, (ii) uptake rates of both D-glucose and L-glucose were equivalent, although this glucose enantiomer is not recognized by sugar permeases, (iii) glucose counter-transport, indicative of the activity of a membrane transporter, was absent in glucose-sufficient cells exhibiting linear sugar uptake, but present in sugar-starved cells displaying activity for a saturable-

monosaccharide transport system, (iv) 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, similar to the values described for free diffusion of glucose in water (Stein 1986) and for the permeation of water through aquaporins (Tsukaguchi *et al.* 1998) and significantly lower than the activation energy for simple diffusion of glucose across phospholipids vesicles, of 12 kcal mol^{-1}, and (v) propionic acid caused a sharp decrease in the diffusive uptake, suggesting that this putative protein can be regulated by cytosolic pH changes, much like the gating of plasma membrane intrinsic protein (PIP) in *Arabidopsis* roots during anoxia stress (Tournaire-Roux *et al.* 2003, Törnroth-Horsefield *et al.* 2006).

The low activation energy values calculated from the first order rate constants, both in intact cells and plasma membrane vesicles (4 and 7 kcal mol^{-1}, respectively), are similar to the values described for the free diffusion of glucose in water (Stein 1986) and for the movement of water through aquaporins (Tsakaguchi *et al.* 1998). They are significantly lower than the value obtained for the free diffusion of glucose across phospholipids vesicles, 12 kcal mol^{-1}, which is similar to those reported in the literature (Macey 1979). These results excluded free diffusion of glucose as the sole mechanism accounting for the linear sugar uptake by sugar-sufficient cells. This conclusion was strengthened by the observation that mercury inhibits both the uptake of D-[^{14}C]glucose and 3-*O*-methyl-D-[^{14}C]glucose and the incorporation of the fluorescent glucose analogue 2-NBDG.

These set of evidences 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. 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 (Chaudhuri *et al.* 2008). Interestingly, more recently, a new class of sugar transporters, which are low-affinity glucose uniporters, were identified, characterised and named SWEETs (Chen *et al.* 2010). The SWEET family contains 17 members in *Arabidopsis*. The biochemical properties of *AtSWEET1* are markedly

similar to the unidentified transport activity characterised in roots by Chauduri *et al.* (2008). However, *AtSWEET1* expression in roots is low, implicating other *AtSWEET* paralogues for this function (Chen *et al.* 2010).

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Chapter 4

Conclusions and perspectives

4.1. Conclusions and perspectives

Our team has been devoted to the topic of membrane transport and plant-environment interactions, and important agricultural crops in Portugal, including grapevine (*Vitis vinifera* L.) and olive (*Olea europaea* L.), have been the focus of research. The salt resistant model tree *Populus euphratica* Oliv. (Jansson and Douglas 2007) was also a target of research on abiotic stress resistance, in particular salt stress. The investigation conducted has been funded by Portuguese and European research projects, in collaboration with leading national and international researchers and laboratories. In addition, the group has benefited from the collaborative efforts of several PhD and Post-doc researchers, funded by the Portuguese Foundation for Science and Technology (FCT). Up to now, a large part of the research outcomes have been predominantly of a biochemical and molecular nature, but always aiming towards ecophysiological applications. For instance, the understanding of the mechanisms and regulation of sugar and polyol transport into sink tissues of grapevine and olive has an important basic and applied relevance, since sugar accumulation in the fruit is a key step in fruit ripening. Massive sugar transport and compartmentation into olive mesocarp cells starts at the beginning of the ripening process and continues until harvest. Likewise, the study of the impact of environmental stresses, in particular drought, salt and heat on fruit growth and ripening, and plant development in general, is of great practical significance, particularly in the context of the ongoing climate changes and human population growth. Olive tree is one of the oldest cultivated plants and has been historically associated with Mediterranean climates. Climate change has already caused significant shifts in agricultural practices and climate models predict that future trends will continue to impose dramatic physiological changes in plants.

In what concerns the work developed in Chapter 2 on *P. euphratica*, we aimed to contribute to the elucidation of the biochemical mechanisms involved salt response, in particular, to understand if and how sodium is accumulated in the vacuole. In this context, confocal and epifluorescence microscopy techniques were employed to investigate sodium intracellular compartmentation. In addition, the activity of a tonoplast Na^+/H^+ exchange system and of the vacuolar proton pumps V-H^+-ATPase and V-H^+-PPase were evaluated. As reported in the Introduction (section 1.2), the ability to compartmentalise

salt into the vacuoles is an important step towards the maintenance of ion homeostasis inside the cell. The first plant tonoplast Na^+/H^+ antiporter, *AtNHX1*, was isolated from *Arabidopsis* (Apse *et al.* 1999, Gaxiola *et al.* 1999) and several studies have shown that exposure to salt up-regulates Na^+/H^+ antiport activity, suggesting a role of the exchanger in salt tolerance; however, there has been very few direct links made between this increased activity and Na^+ sequestration into the vacuole. In the present study, biochemical data corroborated the involvement of Na^+/H^+ exchange activity in *P. euphratica* cell suspensions at the tonoplast level, whose activity increased 6-fold in NaCl-treated cell suspensions. In agreement with these observations, confocal and epifluorescence microscopy analyses using a Na^+-sensitive probe showed that suspension-cultured cells subjected to a salt pulse accumulated Na^+ in the vacuole. As reported in the Discussion section of Chapter 2, distinct conclusions were drawn by Gu *et al.* (2004), who found no significant difference in sequestration of Na^+ in the different cell compartments under NaCl stress. However, recent studies by Sun *et al.* (2012) confirmed an increase in sodium content in the vacuole using a fluorescent Na^+ probe, in line with the results presented here.

The electrogenic proton pumps V-H^+-ATPase and V-H^+-PPase are major components of the vacuolar membrane of plant cells (reviewed by Maeshima 2001, Martinoia *et al.* 2007). In tonoplast vesicles purified from cells cultivated in the absence of salt and from salt-stressed cells, the V-H^+-PPase seemed to be the primary tonoplast proton pump; however, there appears to be a decrease in V-H^+-PPase activity with exposure to NaCl, in contrast to the sodium-induced increase in the activity of the vacuolar H^+-ATPase. The increase of both the pH gradient across the tonoplast and the Na^+/H^+ antiport activity in response to salt strongly suggested that Na^+ sequestration into the vacuole contributes to salt tolerance in *P. euphratica*. In this context, the study of the activity and regulation of V-H^+-PPase and V-H^+-ATPase, which energize the tonoplast, the plasma membrane P-type H^+-ATPase, which creates a proton gradient across the plasma membrane, and the activity of tonoplast and plasma membrane Na^+ antiporters (NHX and SOS1, respectively) has contributed considerably to the elucidation of the molecular mechanisms of plant response to salinity. Furthermore, several molecular approaches have been undertaken to clone the corresponding genes and to study how they are regulated. In particular, as reported in the Introduction

(section 1.2.3), the identification of the genes encoding the plasma membrane SOS1 and the vacuolar NHX antiporters, and the recent progresses in the elucidation of the SOS signalling pathway (Mahajan *et al.* 2008) were particularly important, and have led to the successful improvement of plant salt tolerance through genetic manipulation. These sort of approaches, by altering the genetic makeup of plants, is nowadays a current practice, namely by the USA-based multinational agricultural biotechnology corporation Monsanto. However, the more generalized application of transgenic technology to all plants of agricultural interest is still a matter of great controversy.

The sequence of the genome of *Populus trichocarpa* was recently released (Tuskan *et al.* 2006), and 98% of *P. euphratica* ESTs were found in the *P. trichocarpa* genome (Brosché *et al.* 2005). As discussed by the authors, this implies that *P. euphratica* does not contain specific genes that confer a higher resistance to salt, but rather, the difference in salt resistance resides in the expression and regulation of a different set of genes that make it more adapted to growth in saline conditions (Brosché *et al.* 2005). The study of which genes are expressed and how they are regulated will promote more consistent advancements in the discovery of the responses of this model tree to drought and salinity.

Still in the context of the study of plant responses to abiotic stress, our work progressed towards the study of stresses responses of *O. europaea*. In particular, we wanted to explore and elucidate some key biochemical and molecular steps involved in the partitioning of sugars and polyols, and how polyols may enhance salt and drought stress resistance. As previously mentioned, polyols are the reduced form of aldoses and ketoses, having been detected throughout all living organisms. In *O. europaea* leaves, mannitol was found to be the main soluble carbohydrate, followed by the monosaccharide glucose. The monosaccharide fructose was not detected, probably because it acted as precursor for mannitol biosynthesis. Contrarily to the observed in most plants, sucrose is almost absent in mature leaves of *O. europaea* (5.8%). Whether or not reducing sugars like glucose are transported in phloem is a matter of controversy (van Bel and Hess 2008, Liu *et al.* 2012). When plants were irrigated with saline water, an increase in the proportion of mannitol content in leaves was observed, corroborating previous results in other olive cultivars, as discussed in Chapter 3, and

suggesting a key role of this carbohydrate in salt stress response of olive.

Considering that mannitol is the main translocated soluble carbohydrate in *O. europaea*, an important aim of our work consisted in the study of its catabolism in sink tissues. In this context, particular relevance was given to the membrane transport and to the first intracellular oxidation step. Transport experiments with radiolabelled mannitol showed that a polyol:H^+ symport system operates in *O. europaea* heterotrophic cultured cells with a K_m of about 1 mM. Subsequent work led to the cloning of a cDNA sequence of a mannitol carrier which was named *OeMaT1*. Remarkably, mannitol transport activity and the expression of *OeMaT1* increased simultaneously upon the addition of NaCl, suggesting, for the first time in plants, that mannitol transport is regulated by means of salt-mediated changes in the transcription of mannitol carrier(s). In parallel experiments, salt strongly repressed mannitol dehydrogenase activity measured in cell extracts and down-regulated *OeMTD1*. As discussed, this should allow for the intracellular accumulation of mannitol in order to compensate for the decrease of external water activity, thus conferring a response mechanism to salinity in *O. europaea.* Indeed, as discussed by Conde *et al.* (2011), a broader and more in-depth knowledge of the sensing mechanisms and signalling pathways involved in responses to environmental stresses such as salt, drought, cold or heat, and the cross-talk between these different pathways could certainly make possible the treatment of plants with exogenous compounds such as mannitol, glycine betaine, proline, trehalose and other osmoprotectants and antioxidants, to name a few, avoiding the introduction in nature of genetically engineered plants. Thus, when mannitol was exogenously applied to wheat under salt stress, a plant which is not able to synthesize this polyol, a significant increase in the activity of antioxidant enzymes was found, protecting wheat against the toxic effects of salt (Seckin *et al.* 2009). Similar protective effects of osmolytes were described for maize, upon the exogenous application of glycine-betaine (Nawaz and Ashraf 2007). Also, the exogenous application of trehalose, a sugar also found in *O. europaea*, improved abiotic stress tolerance in *Arabidopsis* and maize (Bae *et al.* 2005 and Zeid 2009, respectively). This topic has been thoroughly reviewed by Conde *et al.* (2011).

The role of polyols in plant response to drought is being currently researched

in our laboratory in grapevine by the PhD student Artur Conde, in collaboration with the lab of Manuela Chaves (ITQB). A GC-TOF-MS analysis revealed that several polyols are present in grapevine mature leaves and berry mesocarp cells in significant concentrations and that sorbitol and galactinol accumulate during water deficit in the final maturation stages of berry development. Also, sorbitol oxidation to fructose *via* sorbitol dehydrogenases (VvSDHs) in mesocarp is clearly inhibited in the full mature stage of grape berries under drought, and mannitol dehydrogenase (VvMTD) activity is also severely repressed in the mature and full mature stages under water deficit conditions (Conde *et al.* 2013, unpublished). A similar metabolomic analysis would give important insights on the effect of drought in olive fruit composition. In fact, genomic and metabolomic techniques are already being applied in the context of industrial olive oil production in the EU: an European project named Oliv-Track was established to allow the profiling of DNA and metabolites in olive oil, in order to determine the cultivars that originated the oil, including adulterations and mislabelling of origin, in effect enabling the traceability of olive oil from the olive groves to the final consumer product (Agrimonti *et al.* 2011).

Subsequent studies on the molecular mechanisms of glucose utilization by olive cells, which was found as an important soluble sugar in the leaves and fruits of *O. europaea*, led to the cloning and functional characterization of a monosaccharide transporter (*OeMST2*). Expression of this cDNA in a mutant *S. cerevisiae* strain deficient in glucose transport restored its capacity to grow and to transport glucose. These approaches of heterologous expression of plant transporters in yeasts has yielded clear-cut information about the real nature of MSTs after the pioneer experiments of Sauer and collaborators in the nineties in the algae *Chlorella kessleri* (Sauer *et al.* 1990). When the expression of *OeMST2* was studied in olive fruits, it was shown that transcript levels increased during fruit maturation, confirming that *OeMST2* catalyses the membrane transport process of hexoses during sugar unloading in the fruits. Several mono- and disaccharide transporters have been cloned up to now and its kinetic properties and regulation have been studied in heterologous systems. This work is of great importance to understand fruit development and ripening and its response to environmental challenges.

An important objective of our study was to uncover the nature of the non-saturable diffusion-like transport of glucose across the plasma membrane of olive cells. As reported previously, in a variety of cell types including plant cells, sugars, polyols and other organic solutes may be incorporated according to first-order kinetics, but the real nature of this transport mechanism has been elusive. In the present work, solid evidences pointed towards the existence of a proteinaceous channel mediating the diffusive uptake of sugars in plant cells. The simultaneous transport of water and sugars through such hydrophilic channel would enable rapid cellular uptake or exit of sugar with minimal osmotic perturbation; hence, it is potentially an important mechanism during fruit ripening, when high amounts of sugars and water are accumulated. In *Arabidopsis* root tips, it was also suggested that sugar influx may be mediated by a channel-like proton-independent transport system (Chauduri *et al.* 2008), supporting our model. The recent discovery of the new class of sugar transporters named SWEET (Chen *et al.* 2010) provided new insights on the nature of diffusion-like kinetics of solute transport. The identification and functional characterization of a channel or a transporter is not simple, especially in higher organisms, including plants. As discussed by Conde *et al.* (2010), the involvement of several transport systems with overlapping functions and specificities, their complex regulation by both transcriptional and post-translational mechanism and, in some cases, their atypical kinetics, are just the primary reasons hindering the assignment of a transport activity to a specific transporter or channel.

As a whole, this research work provided relevant contributions in the field of plant solute transport in response to environmental stress, with particular emphasis on the plasma membrane transport of photoassimilates and tonoplast transport of protons and salt. This area of research is important to understand plant physiology and its adaptation to their surrounding environment, and ultimately manipulate productivity, through genetic manipulation and breeding of new cultivars, and selection of the more resistant and productive ones.

4.1.1. The new avenue of genetic improvements

As discussed by Rugini *et al.* (2011), there is currently an increasing demand in terms of quantitative and qualitative olive production, and the olive crop improvement can have a key role in guiding production agriculture toward sustainability. Modern goals for olive breeding strive towards a new plant model, in which reduced size, reduced apical dominance, and constant and high productivity in terms of fruit and oil are the main features. Additionally, it is desirable that cultivars express multiple innovative traits, including self-fertility, suitable fruit composition, as well as tolerance to environmental factors, such as salinity and drought. Currently, although more than 1,000 cultivars are available, originated mostly from selections made by growers, none of them possesses all the desirable traits that could be introgressed into cultivars by conventional breeding or by gene transfer (Rugini *et al.* 2011). Thus, the exploitation of the olive tree genetic background and diversity to help cope with stress is an important scientific topic of great practical implications.

To achieve this goal, genomic sequences for olive will enable researchers to explore the breadth of genetic diversity present within the species and within the breeding germplasm, as discussed by Muleo *et al.* (2012), using high throughput methods of resequencing, based on NGS (*n*ext *g*eneration *s*equencing) technologies. This will give access to all types of variations, namely SNPs (*s*ingle *n*ucleotide *p*olymorphisms), small insertion/deletions and structural variants (large insertion/deletions). It will allow a better assessment of the relationships among the different accessions, of the geographical patterns of distribution of genetic variation and of the genetic consequences of olive trees domestication. It will finally form the basis for the development of unique molecular marker assays. In addition, the sequencing of the olive genome is currently being carried out by an Italian-led consortium (OLEA, http://genomes.cribi.unipd.it/olive/ [accessed 28/05/2013], Muleo *et al.* 2012) as well as an independent Turkish-led consortium (http://olivegenome.karatekin.edu.tr/ [accessed 28/05/2013]). However, in the absence of the sequence of the complete olive genome, the application of NGS technologies to transcripts isolated from different tissues (Muñoz-Mérida *et al.* 2013), at different developmental stages, such as during fruit ripening (Alagna *et al.* 2009, Galla *et al.* 2009) and/or biotic and abiotic stress imposition, can

be used to build databases (such as Olea ESTdb, http://454reads.oleadb.it/, Alagna *et al.* 2009) and enable the discovery of new genes and their function and regulation.

Novel approaches, such as sequencing of the whole genome (as mentioned) and large scale transcriptome analysis (RNA-seq and microarrays) of important agricultural crops can help identify genes involved in desirable traits easier and faster, moving towards the implementation of appropriate genetic engineering strategies, including genetic transformation with *Agrobacterium*, for improving biotic and abiotic stress tolerance, and ultimately productivity and fruit quality.

4.2. References

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