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Plant adaptation to environmental stress

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Membrane Transport, Sensing and Signaling in Plant Adaptation to Environmental Stress

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Abstract

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. Among the plant arsenal to keep homeostasis are efficient stress sensing and signaling mechanisms, plant cell detoxification systems, compatible solute and osmoprotectant accumulation and a vital rearrangement of solute transport and compartmentation. The key role of solute transport systems and signaling proteins in cellular homeostasis is addressed in the present work. The full understanding of the plant cell complex defense mechanisms under stress may allow for the engineering of more tolerant plants or the optimization of cultivation practices to improve yield and productivity, which is crucial in the present time as food resources are progressively scarce.

Keywords:

Compatible solute, environmental stress, ion homeostasis, membrane transport, proton pumps, sensing and signaling

1. Introduction

Almost half of the irrigated land and about 20% of the world's farmable land are affected by drought and salinity, which constitute major problems to agricultural production, affecting plant growth and productivity worldwide (Bartels and Sunkar 2005, Rengasamy 2006, Sahi et al. 2006, Silva and Gerós 2009). Understanding ion homeostasis mechanisms and plant tolerance to drought and salinity overall is, therefore, of crucial importance and generates one of the major research topics nowadays. The maintenance of biological membrane potential, the activities of several enzymes and appropriate osmolyte concentration/cell volume regulation are all dependent on intracellular Na^+ and K^+ homeostasis. In normal conditions, plant cells maintain a high K^+/Na^+ ratio in the cytosol with relatively high K^+ levels, of about 100 mM to 200 mM, and low levels of Na^+: 1 mM to 10 mM (Higinbotham 1973). A correct regulation of ion flux is required for cells to maintain the concentrations of toxic ions low and to accumulate essential ions. Plant cells employ primary active transport, mediated by H^+-ATPases, and secondary transport, via co-transporters and channels, to maintain the characteristic high K^+/Na^+ ratio in the cytosol.

High salt levels inflict two stress components on plants: an osmotic factor arising from the lowered water availability provoked by high osmotic pressure in the soil, and also an ionic stress as a consequence of a solute imbalance, increasing the levels of Na^+ and Cl^- in the cytosol and changing the intracellular K^+/Na^+ ratio (Blumwald et al. 2000). Sodium toxicity arises mainly from the resemblance of the K^+ and Na^+ ions to plant enzymes and transporters. For instance, Na^+ stress disrupts K^+ uptake by root cells. Additionally, when Na^+ is incorporated inside the cells and accumulates to high levels, it exerts severe toxic effects towards enzymes, causing an impairment of the metabolism (Hasegawa et al. 2000). The yeast HAL2 nucleotidase and its ortholog AtAHL of the *Arabidopsis* HAL2-like family are paradigmatic examples of targets of Na^+ toxicity as it antagonizes Mg^{2+} at the active site of the

enzyme, ultimately leading to an impairment of methionine biosynthesis and RNA processing (Murguía et al. 1996; Gil-Mascarell et al. 1999). This disturbance of the ion homeostasis results in molecular and cellular damage and whole-plant growth cessation or even cell death. Thus, the regulation of ion transport by salt-stress sensing and signaling provides a model case for understanding the general regulation of ion homeostasis in plant cells. Excessive Na^+ has to be effluxed or compartmentalized in the vacuole. However, contrarily to animal cells, plant cells do not have Na^+-ATPases or Na^+/K^+-ATPases and, instead, rely on H^+-ATPases and H^+-pyrophosphatases to create a proton-motive force that drives the transport of all other ions and metabolites, including Na^+ and K^+. A wide variety of transporters of H^+, K^+ and Na^+ have been characterized over the last decades (reviewed by Zhu 2003).

Plants can recognize abiotic stresses and trigger proper responses involving changes in metabolism, growth and development. Drought and salt stresses are frequent threats and may affect most habitats, so plants have evolved several strategies and adaptive mechanisms to tolerate these rough conditions, providing different degrees of tolerance to different plants. Recognized stress-tolerant plants display efficient mechanisms of stress sensing, signal transduction and gene expression programs, or even different metabolic pathways (Bartels and Sunkar 2005). However, a sort of genetic program for tolerance also seems to be present in non-tolerant plants, as their gradual acclimation results in a gain of stress-resistance. These sensitive plants might need a slow and steady adaptation for the proper expression of genes responsible for this phenomenon (Zhu 2001).

Biotechnological approaches and traditional plant breeding methods have been frequently used, among other efforts, to improve the tolerance to salinity and drought of economically important plants (Flowers 2004, Karrenberg et al. 2006). Crucial towards these efforts are the fundamental studies developed in *Arabidopsis,* which has been an essential

model plant for assessing the function of individual stress-related genes, given its proneness to genetic manipulation and the accessibility of knock-out mutant plants.

This review deals with several aspects concerning ion homeostasis, from its general features to the more detailed level, such as the activity and regulation of cation transporters to maintain ion homeostasis, the importance of osmolyte synthesis for protein and membrane stabilization, redox control and scavenging of reactive oxygen species (ROS), and the role that remarkable salt-sensing and signaling mechanisms play in the achievement of ion homeostasis and, therefore, abiotic stress tolerance.

2. Plant cell detoxification strategies towards homeostasis

In order to achieve salt and drought tolerance, three related plant activities are essential. First, damage must be prevented or lessened. Second, homeostasis must be re-established in the new, stressful conditions. Third, even if at a lowered rate, plant growth must be resumed following the modifications at the cellular and whole-plant levels (Zhu 2001; Amudha and Balasubramani, 2011). The toxic effects caused by ion and osmotic homeostasis disruptions need to be quickly neutralized at the cellular level, so stress-tolerant plants have evolved efficient detoxification mechanisms.

Plant adaptation to salinity and drought is a complex process, involving far more changes than just an attenuated growth. It implies, at the cellular level, regulation of gene expression, such as those encoding for transporter proteins highlighted later in this paper (i.e. H^+ pumps and Na^+/H^+ antiporters), transient increases in ABA concentration, accumulation of compatible solutes and protective proteins, increased levels of antioxidants and suppression of energy-consuming pathways (Bartels and Sunkar 2005; Chaves et al 2009). All these changes

at the cellular level are critical to restore ion homeostasis after the imbalance caused by any given abiotic stress. By restoring ion homeostasis, plants will tolerate more easily those stresses.

High salt stress, besides imposing toxic effects in the activities of various enzymes, in the integrity of cellular membranes, in nutrient acquisition and in the photosynthetic mechanisms, also generates reactive oxygen species (ROS) (Skopelitis et al. 2006). ROS by themselves are a significant cause of damage to a plant in a salt/osmotic stress environment. As a response, plants trigger the production of several stress proteins and compatible osmolytes, among other complex molecular changes (Zhu et al. 1997). Indeed, many of the stress proteins and osmolytes with unknown functions probably play a role in detoxifying plants by scavenging ROS or preventing them from damaging cellular structures, in a similar manner to some key enzymes and osmoprotectants already known to be involved in oxidative protection (Zhu 2001). Plant engineering towards increased biosynthesis of osmolytes that are active ROS scavengers such as mannitol, proline, ononitol, trehalose, fructans, ectoine and glycinebetaine was shown to have a positive effect in abiotic stress tolerance through oxidative detoxification (Shen et al. 1997). The contribution of osmoprotectants towards cellular homeostasis will be further approached in more detail in this chapter.

Transgenic plants overexpressing proteins like superoxide dismutase, ascorbate peroxidase, glutathione peroxidase and glutathione reductase significantly display increased salt and osmotic stress tolerance, as a consequence of a partial neutralization of ROS (Allen et al. 1997; Roxas et al. 1997). A similar gain-of-tolerance outcome was observed after the engineering of tobacco regulatory protein NPK1, a mitogen-activated protein (MAP) kinase. Corroborating the role of NPK1 in oxidative stress response, its *A thaliana* orthologue ANP1 is activated by H_2O_2 and starts a phosphorylation cascade with the involvement of two stress MAPKs, AtMPK3 and AtMPK6 (Kovtun et al. 2000). The HOG1 MAPK pathway plays a

key role in abiotic stress tolerance in yeast (de Nadal et al. 2002) and increasing evidence suggests a vital role of the MAPK pathway in oxidative protection in stressed plants (Bartels and Sunkar 2005). Astonishingly, at least 20 MAPK, 10 MAPKK and 60 MAPKKK genes have been identified in *Arabidopsis* based on sequence similarities (Riechmann et al. 2000, Ichimura et al. 2002). However, the increased levels of ROS as a consequence of a given environmental stress are not always a negative factor to plants. Indeed, ROS are also critical in plant growth regulation, hormone transduction, and have a pivotal role in abiotic stress signaling towards a defensive response (Foreman et al. 2003; Einset et al, 2007; Miller et al. 2008; Jaspers and Kangasjärvi, 2010). For instance, disruption by mutagenesis of extracellular ATP-induced and plasma membrane NADPH oxidase-mediated ROS production impairs the activation of Ca^{2+} channels and transcription of MAP kinase3 gene, resulting in a less effective stress response (Demidchik et al. 2009). Additionally, ROS signalling through the activation of Ca^{2+} channels in also required for possible plant mechanotransduction (Mori and Schroeder, 2004).

Late embryogenesis abundant (LEA) proteins are extremely hydrophilic proteins first identified in plants belonging to 7 different groups, and whose intracellular accumulation allows for a more efficient acquisition of tolerance to desiccation by protein and membrane stabilization (Chakrabortee et al. 2007; Battaglia et al. 2008; Hand et al. 2011). The accentuated expression of LEA proteins like barley HVA1 and stress-related transcription factors such as CBF/DREBs in transgenic plants are good examples of detoxifying roles played by either the actual expressed proteins or their downstream target proteins. A characteristic of this type of detoxification effect is its remarkable lack of specificity to a given abiotic stress. Transgenic plants overexpressing these proteins have improved tolerance to salinity, drought and freezing stress (Liu et al. 1998). The CBF/DREB transcription factors are able to bind to the DRE/CRT element of the promoters of some stress-related responsive

genes, leading to an expression of these target genes regardless of stress (Stockinger et al. 1997; Liu et al. 1998). Despite the scarce information until now, the biochemical function of these activated genes, mainly LEAs and specifically dehydrins (LEA class 2), is being progressively understood. One of them, the *A. thaliana* COR15A, is up-regulated under freezing conditions, when low water availability arises as a consequence, and seems to exert a "cryoprotective" role by interacting with cellular membranes to prevent the formation of impaired membrane structures (Steponkus et al. 1998). Findings in the anhydrobiotic nematode *Aphelenchus avenae* shed some light in the way that LEA proteins may act. The LEA protein identified in *A. avenae* acts synergistically with the accumulation of trehalose in response to dehydration, by stabilizing the water-replacing hydration envelopes, often named as organic bioglass, which this osmolyte forms to stabilize the cell's content (Browne et al. 2002). Similar function has been described for the group 3 member LEA1 protein in *A. avenae* (Chakrabortee et al. 2007). Indeed, LEA proteins are significantly hydrophilic and resistant to denaturation by heat, prompting suggestion that they help to avoid damage by water stress, by acting as hydration buffers, molecular chaperones, ion sinks or membrane stabilizers (Crowe et al. 1992; Dure 1993; Hundertmark and Hincha, 2008). In proving the importance of LEA proteins in plants, 51 LEA protein encoding genes were recently identified in *A. thaliana*, with significant sequence diversity within them, various intracellular locations, and also different expression patterns (Hundertmark and Hincha, 2008). According to the authors, the high fraction of retained duplicate genes and the consequent functional diversification suggest that they allow for an evolutionary advantage for an organism under multiple stressful environmental conditions. Overexpression of the barley *HVA1* gene, which leads to a significant constitutive accumulation of the HVA1 protein in roots and leaves of transgenic rice plants, provided tolerance to salt and drought stresses (Xu et al. 1996). HVA1 overexpression in transgenic wheat, mulberry and creeping bentgrass (*Agrostis stolonifera*),

provided similar results (Sivamani et al. 2000; Chandra Babu et al. 2004; Fu et al. 2007; Lal et al. 2008). In *Arabidopsis*, a constitutive expression of the wheat group 3 LEA-L2 protein allowed for a significant increase in the freezing tolerance of plants acclimated to cold (N'Dong et al. 2002). Accentuated salt and freezing tolerance was achieved in transgenic bacteria overexpressing the soybean PM2 protein, and in yeast by constitutive expression of barley's HVA1 and wheat's TaLEA3 protein (Zhang et al. 2000; Yu et al. 2005; reviewed by Battaglia et al. 2008).

In *A. thaliana*, ERD10 and ERD14 (early response to dehydration) are members of the dehydrin family that also accumulate in response to cold, drought and salt stresses. These proteins are intrinsically disordered proteins (IDPs), unfolded, that have potent molecular chaperone activity *in vitro* on several substrates due to an 'entropy transfer' mechanism, and also interact with phospholipid vesicles through electrostatic forces (Kovacs et al. 2008a,b). These unfolded and highly entropic proteins are thus crucial in the gain of tolerance process to environmental stresses.

3. Sensing and signaling environmental stress

Sensing of salt and dehydration stresses, and of high or low temperatures, is of utmost importance in the process of achieving cellular homeostasis in plants. The sensing mechanisms allow for the activation of multiple signaling cascades responsible for the triggering of various cellular responses and, together, stress sensing and signal transduction form crucial adaptive mechanisms in the tolerance to the negative effect of multiple environmental stresses. Plants suffering from dehydration under high salinity and drought, as well as low-temperature conditions trigger the biosynthesis of abscisic acid (ABA) which activates a significant set of genes induced by drought, salt and cold (Boudsocq and Laurière,

2005). Probably the most iconic gene up-regulated by ABA is *AtNHX1* that encodes the vacuolar Na^+/H^+ exchanger in *A. thaliana* (Shi and Zhu, 2002).

Abscisic acid is indeed a universal plant hormone involved in several developmental processes and environmental stress responses of plants. Recently, two plasma membrane ATP-binding cassette (ABC) transporters with remarkable ABA-receptor properties have been identified in *Arabidopsis* giving further insight into the influx/efflux mechanism of ABA and providing information on how ABA is transported from cell to cell in plants – the pleiotropic drug resistance transporter AtPDR12/ABCG40 (Kang et al. 2010) and AtABCG25 (Kuromori et al. 2010). The first is responsible for the uptake of ABA and is implicated in the drought tolerance process, as the stomata of loss-of-function *atabcg40* mutants closed significantly slower in response to ABA, resulting in lowered tolerance to drought (Kang et al 2010). The latter was expressed mainly in vascular tissues at the plasma membrane and displays ATP-dependent ABA efflux transport activity (Kuromori et al. 2010). According to the authors, *AtABCG25*-overexpressing plants revealed higher leaf temperatures, pointing to the influence of this transporter on stomatal regulation and suggesting its involvement in the intercellular ABA signaling pathway, with a crucial role in ABA-mediated plant responses to environmental stresses. Apparently, following an environmental stimulus, ABA induces the interaction of PYR/PYL/RCAR and PP2C proteins, resulting in PP2C inhibition and SnRK2 activation, an ABA-mediated signalling mechanism that may function in both the cytosol and nucleus (reviewed by Umezawa et al 2010; see references within).

Over the last few years we have gained new insights into the mechanisms involved in the sensing of osmotic and salt stress in plants, even if further discoveries are still expected.

3.1. Osmosensing

In yeast, hyperosmotic stress is sensed by two types of osmosensors, SLN1 and SHO1, which signal the HOG (high-osmolarity glycerol) MAPK pathway (Bartels and Sunkar 2005). In plants, drought stress may be sensed in part by stretch-activated channels and by transmembrane protein kinases, such as two-component histidine kinases (Urao et al. 1999) and wall-associated kinases (Kohorn 2001). Indeed, in *Arabidopsis* the SLN1 homologue ATHK1 acts as osmosensor and sends the stress signal to a MAPK cascade downstream. Recently, direct genetic evidence was found demonstrating that ATHK1 is not only involved in the water stress response in early vegetative stages of plant growth but also plays a distinctive role in the regulation of desiccation processes during seed formation (Wohlbach et al. 2008). Heterologous expression of the *ATHK1* cDNA in the yeast double mutant, which lacks SLN1, suppressed cell death in high-salinity media and triggered the high osmolarity glycerol response 1 (HOG1) mitogen-activated protein kinase (MAPK) (Urao et al. 1999). In *Arabidopsis*, MEKK1 and AtMPK3, AtMPK4 and AtMPK6 are also activated in the signalling process towards osmotic stress tolerance (Mizoguchi et al. 1996; Ichimura et al. 2000; Droillard et al. 2002). The activation of AtMPK3 and AtMPK6 is also triggered by oxidative stress, such as previously mentioned. Also, the activity of the plant histidine kinase cytokinin response 1 (Cre1) is regulated by changes in turgor pressure in a similar manner to yeast's SLN1, which prompts it as a probable candidate for sensing osmotic stress in plants (Reiser et al. 2003). The gene *NtC7* from tobacco encodes a receptor-like protein functioning in osmotic adjustment whose membrane location was confirmed in onion epidermis cells transiently expressing an NtC7-GFP fusion protein. NtC7 transcripts rapidly increase after not only salt and osmotic stress treatments but also after wounding (Tamura et al. 2003). Moreover, other MAPKs are activated by osmotic stresses in tobacco and several have also been correlated to drought stress signaling in *Medicago* (reviewed by Jonak et al. 2002).

3.2. Salt sensing and signaling

The understanding of how Na^+ is sensed is still very limited in most cellular systems. In theory, Na^+ can be sensed either before or after entering the cell, or both. Extracellular Na^+ may be sensed by a membrane receptor, whereas intracellular Na^+ may be sensed either by membrane proteins or by any of the several Na^+-sensitive enzymes in the cytoplasm (Zhu 2003). Even though the molecular identity of Na^+ sensors remains elusive, the plasma-membrane Na^+/H^+ antiporter SOS1 (SALT OVERLY SENSITIVE1) is a probable candidate (Shi et al. 2000). The transport activity of SOS1 is essential for Na^+ efflux from *Arabidopsis* cells (Qiu et al. 2002; Quintero et al. 2002), but, additionally, its unusually long cytoplasmic tail is thought to be involved in Na^+ sensing (reviewed by Zhu 2003).

The AtSOS1 transporter (Fig. 1) is a good example of a membrane transporter protein with atypical dual functions of solute transport and sensing, a phenomenon that has been increasingly observed (reviewed by Conde et al. 2010). The SOS1 gene encodes a transmembrane protein with significant identities to plasma membrane Na^+/H^+ antiporters from bacteria and fungi, and NaCl stress strongly up-regulates a usually constant level of gene transcription (Shi et al. 2000). Undifferentiated callus cultures regenerated from transgenic plants were also more salt-tolerant, a fact correlated with reduced Na^+ content in the transgenic cells (Shi et al. 2003). When expressed in a yeast mutant devoid of endogenous Na^+ transporters, *SOS1* was able to reduce Na^+ accumulation and improve salt stress tolerance of the mutant cells (Shi et al. 2002). The SOS pathway was found out when three *salt-overly-sensitive* mutants (*sos1*, *sos2* and *sos3*) were characterized in a genetic screen designed to identify components of the cellular mechanisms that contribute to salt tolerance in *Arabidopsis*. SOS2 is serine/threonine protein kinase with an N-terminal catalytic domain in a similar manner as in the yeast SNF1 kinase, and *SOS3* encodes a Ca^{2+} sensor protein sharing a high sequence similarity with the calcineurin B subunit from yeast and neuronal Ca^{2+} sensors from animals (Liu and Zhu, 1998; Liu et al. 2000; reviewed by Silva and Gerós 2009).

SOS1 has been demonstrated to be a target of the SOS pathway, whose signalling is controlled by SOS2/SOS3. *SOS1* transcription up-regulated in response to salt stress but this positive regulation does not occur in *sos3* or *sos2* mutant plants (Shi et al. 2000). SOS1 cation transporter, the SOS2 protein kinase, and its associated Ca^{2+} binding/sensor myristoylated SOS3 indeed make up a functional module, where SOS1 is the phosphorylation substrate for the SOS2/SOS3 kinase complex (Quintero et al. 2002).

Also critical in salt stress sensing and signalling is the MAPK signalling pathway. The MAPK cascade follows a MAPKKK–MAPKK–MAPK module connected in a wide diversity to upstream sensors/receptors and targets downstream by phosphorylation and/or physical interaction. Indeed, in every plant species, MAPKs have either a TEY or TDY phosphorylation domain at the active site, and specifically TDY MAPKs also have long C-termini. TEY MAPKs have been extensively studied in several plants, such as tobacco, *A. thaliana*, tomato, parsley, rice and *Medicago* (reviewed by Nakagami et al. 2005). In *Arabidopsis*, in addition to osmotic stress, MEKK1, AtMPK3, AtMPK4 and AtMPK6 are also critically activated by salinity. In fact these elements of the MAPK pathway-mediated response appear not to be significantly stress-specific, given that they are also stimulated by low temperatures, and even by touch and wounding (Mizoguchi et al. 1996; Ichimura et al. 2000; Droillard et al. 2002). Moreover, MEKK1 triggers the activity of MKK4 and MKK5 that in turn mediate flagellin signaling (Asai et al. 2002). Additionally, MKK2 also plays a role in salt and cold stress as an activator of MPK4 and MPK6 as shown in transient protoplast assays (Teige et al. 2004). Remarkably, *mkk2-null* mutant *Arabidopsis* plants show severe hypersensitivity to salt and low-temperature stresses, whereas MKK2-overexpressing plants are significantly more salt and cold stress-tolerant. The extensive research on the MAPK pathway has revealed that in salt and cold stress a MEKK1–MKK2–MPK4/MPK6 module is commonly observed (reviewed by Nakagami et al. 2005). However, insights on the

various roles of the MAPK pathway and the diversity of its elements are still being obtained as only recently, for instance, a novel and unexpected MKK9-MPK3/6 cascade that phosphorylates and stabilizes EIN3 in ethylene signaling has been described in *Arabidopsis* (Yoo et al. 2008). In wheat, TMKP1 was recently the first MKP of this plant to be characterized and plays an active role in controlling the subcellular localization of TMKP3 and TMKP6 and ultimately in prompting plant cell responses to salt and drought stress (Zaïdi et al. 2010).

3.3. Ca^{2+} in sensing and signaling

In plant cells, Ca^{2+} acts as a second messenger connecting a wide range of extracellular stimuli with various intracellular responses (Snedden and Fromm, 1998, 2001; DeFalco et al. 2010). Several major classes of Ca^{2+} sensors have been characterized in plants. These classes are calmodulin, calcineurin B-like proteins (CBLs) and CBL-interacting protein kinases (CIPKs), often designated as calcium dependent protein kinases (CDPKs) when acting as a CBL-CIPK complex (Yang and Poovaiah 2003; Bouché et al. 2005). The sensing and the signaling of calcium, as well as in other ions like Na^{+}, are intimately interconnected and cannot be dissociated as the sensing event immediately implies the transmission of the perceived signal, and the signaling event implies that a signal was sensed. Thus many times signal transducers are also mentioned as sensors, which from this point of view cannot be considered incorrect. Several lines of evidence suggest that all these three classes of Ca^{2+} sensors are involved in stress signal transduction (Snedden and Fromm 2001; Luan et al. 2002; Zhu 2000). The involvement of Ca^{2+} signaling in response to osmotic and ionic stress, even if well documented, is still being progressively understood. For instance, recently in *Arabidopsis* the CPK21, a newly identified CIPK, was demonstrated to be biochemically activated *in vivo* in response to hyperosmotic stress (Franz et al. 2010). This osmotic stress-related protein has been suggested to have a N-terminal EF-hand pair with a role in calcium

sensing determinant that controls the specificity of CPK21 function. Moreover, salt stress originates a fast and transient increase in cytosolic Ca^{2+} that in turn triggers many signal transduction pathways, such as the previously referred SOS and MAPK involved in ion channel activity, the regulation of enzymatic activity, and gene transcription. This results in a wide variety of cellular responses (Snedden and Fromm 1998, 2001) and mediates salt adaptation, all leading to ion homeostasis (Bressan et al. 1998; Liu and Zhu 1998; Serrano et al. 1999; Serrano and Rodriguez-Navarro 2001). Moreover, whole-plant cytosolic [Ca^{2+}] measurements have demonstrated that the intensity of the salt stress and the magnitude of cytosolic [Ca^{2+}] increase are directly correlated (Tracy et al. 2008). The involvement of Ca^{2+} in the re-establishment of cellular homeostasis has to be tightly regulated as the spatial and temporal dynamics of the Ca^{2+} signal encodes the response to different osmotic stresses (Knight and Knight, 2001). For instance, in response to salt, osmotic and low temperature stresses, the alterations in cytosolic Ca^{2+} levels were cell-type specific in *Arabidopsis* root cells (Kiegle et al. 2000; reviewed by Bartels and Sunkar 2005.

Many Ca^{2+} sensors and signal transducers involved in environmental stress tolerance have been recognized throughout the years. For instance, the calcium sensor CBL1 has a role in mediating multiple stress responses by interacting with several CIPKs (Albrecht et al. 2003). The sensor CBL9 is closely related to CBL1 but plays a specific role in different signalling mechanisms including response to osmotic stress and ABA (Pandey et al. 2004). Abscisic acid-dependent and independent stress responses in *Arabidopsis* are controlled alternatively by a complex formation of CBL1 or CBL9 with the kinase CIPK1 (D'Angelo et al. 2006). The Ca^{2+} sensors CBL1 and CBL9 upon interaction with CIPK are described to regulate the K^+ channel AKT1 and the uptake of this cation into *Arabidopsis* roots and stomatal guard cells (Li et al. 2006; Xu et al. 2006; Cheong et al. 2007). The aperture of guard cells is also regulated by the action of CPK3 and CPK6 (Mori et al. 2006). Recently, the

calcium sensor CBL10 has also been found to interact with the SOS2 (CIPK24) kinase, thus contributing to salt tolerance by regulating ion homeostasis in *A. thaliana* (Kim et al. 2010).

The major physiological role played by Ca^{2+}-ATPases, also designated as Ca^{2+} pumps, is to restore and keep homeostasis by pumping Ca^{2+} out of the cytosol to end a signaling occurrence, and it is critical in all eukaryotic cells and not only during environmental stress conditions (Sze et al. 2000). Both animal and plant cells use two distinct types of Ca^{2+}-ATPases, type IIA and type IIB. The expression of genes encoding type IIA Ca^{2+} -ATPase, and enzyme activity in tomato, soybean, tobacco and *Physcomitrella patens* was demonstrated to be triggered by salt stress (Wimmers et al. 1992; Chung et al. 2000; Qudeimat et al. 2008). The consequence of up-regulating the Ca^{2+} pump in response to salinity is thought to provide an adaptive response. The soybean Ca^{2+}-ATPase1 was up-regulated by NaCl but not by KCl and mannitol, indicating that specific Ca^{2+} signals trigger the enhancement in the gene expression (Chung et al. 2000). The *Arabidopsis* Ca^{2+}-ATPase isoform 4 (ACA4), a calmodulin-regulated Ca^{2+}-ATPase, has also been reported to be part of the Ca^{2+}-dependent signal transduction pathway associated with salt stress (Geisler et al. 2000a). According to the authors, *Arabidopsis* seedlings treated with increasing concentrations of NaCl for 24 h demonstrated a dose-dependent increase in *ACA4* gene expression, and, additionally, when N-terminal truncated ACA4 was heterologously expressed in yeast, it conferred increased salt tolerance to its host (Geisler et al. 2000b; reviewed by Bartels and Sunkar 2005). Phosphorylation or dephosphorylation events also tightly and specifically regulate the activity of Ca^{2+}-ATPase (reviewed by Kudla et al. 2010, see references within).

3.3.1. Role of Ca^{2+} in cold sensing and signaling

In cold stress conditions, Ca^{2+} also plays a vital role as messenger in a low temperature signal transduction pathway. Modification in cytosolic Ca^{2+} levels is a primary step in a

temperature sensing mechanism, enabling the plant to tolerate low temperatures more effectively. In both *Arabidopsis* (Knight et al. 1996; Polisenski and Braam 1996) and alfalfa (Monroy and Dhindsa 1995) cytoplasmic Ca^{2+} levels increase rapidly in response to low temperature, largely due to an influx of Ca^{2+} from extracellular stores. Contrarily to salt stress, cold stress induces only a monophasic $[Ca^{2+}]_{cyt}$ increase in the four types of *Arabidopsis* root cells, without noticeable temporal difference, suggesting that all cells sense temperature changes simultaneously (Kiegle et al. 2000). Cold-induced increased Ca^{2+} levels possess circadian modulations that are more significant during the mid-photoperiod (Dodd et al. 2006). Calcium is responsible for an increased expression of several cold-induced genes including the *CRT/DRE* controlled *COR6* and *KIN1* genes of *Arabidopsis* (Monroy et al. 1993; Knight et al. 1996; Monroy and Dhindsa 1995). For instance, Ca^{2+} chelators such as BAPTA and Ca^{2+} channel blockers such as La^{3+} inhibited the cold-induced influx of Ca^{2+} and resulted in a lowered expression of the cold-inducible *Cas15* gene, impairing the capacity of alfalfa to acclimate to a cold environment. Interestingly, *Cas15* expression can be induced at the high temperature of 25°C by treating the cells with A23187, a Ca^{2+} ionophore that causes a rapid influx of this divalent cation (Monroy and Dhindsa 1995; reviewed by Mahajan and Tuteja 2005). Moreover, the calcium sensor CBL1 is negatively regulated by low temperatures, as CBL1-overexpressing *Arabidopsis* plants have reduced tolerance to freezing, but increased salt and drought tolerance (Cheong et al. 2003).

In *A. thaliana*, the CBF cold response pathway also plays a vital part in cold tolerance, being characterized by a fast induction by cold of genes encoding the *CBF1-3* transcription factors, followed by the expression of the CBF gene regulon. Calmodulin binding transcription activator (CAMTA) proteins, like the positive regulator of *CBF2* expression in *Arabidopsis* CAMTA3, are transcription factors involved in cold acclimation by establishing

a bridge between low temperature calcium/calmodulin signaling and a cold-regulated gene expression (Doherty et al. 2009).

3.3.2. Heat sensing and signaling – yet another role of Ca^{2+}

Besides the ability to sense salt, osmotic and low temperature stresses, plants have sensing mechanisms capable to detect high temperature stress. Even though the existence of a "plant thermometer" has not been recognized, it is suggested that changes in membrane fluidity play a key role in sensing and influencing gene expression not only under freezing, but also high temperatures. Therefore, sensors are probably located in microdomains of membranes, which are capable of detecting physical phase transition, eventually leading to conformational changes and/or phosphorylation/dephosphorylation events when temperature changes (Plieth 1999). According to this, a model for temperature sensing and regulation of heat shock responses should integrate detectable membrane modifications. Indeed, membranes have their lipid composition adapted to the growth temperatures of the plant. Corroborating a key heat-sensing role played by membranes, a specific and transient Ca^{2+} influx across the plasma membrane has been demonstrated during heat (Gong et al. 1998, Liu et al. 2006, Saidi et al. 2009, Wu and Jinn 2010). Concordantly, benzyl alcohol, a membrane fluidizer, induces a rapid influx of Ca^{2+} and a progressive heat-shock proteins (HSPs) expression culminating in developed thermotolerance within days (Saidi et al. 2005, 2009; Suri and Dhindsa, 2008). Moreover, calmodulins (CaMs) are also involved in heat signaling and ultimately HSP expression as demonstrated by the increased expression of CaM3 and CaM7 during heat-shock (Liu et al. 2005, Zhang et al. 2009). The activation of CaMs allows for their interaction with calcium-dependent kinases such as AtCBK3, ultimately leading to the activation of HSFs. Thus, changes in membrane fluidity and a Ca^{2+}-CaM-kinase pathway are responsible for the activation/phosphorylation of HSFs, leading to increased heat tolerance (Fig. 2) (reviewed by Saidi et al. 2010). Additionally, increased heat-shock tolerance can be

obtained by heat-induced higher levels of other metabolites like nitric oxide (NO) and hydrogen peroxide (H_2O_2), which may activate similar signaling cascades or other molecular mechanisms (Hua et al. 2009).

The expression and activity of heat stress-responsive transcription factors (HSFs) is thus probably altered by changes in the proportion of saturated and unsaturated fatty acids when the temperature threshold responsible for the induction of a heat shock response is attained. Moreover, stiffness of the thylakoid membranes is suggested to invoke altered expression profiles of heat shock genes, HSPs being significantly up-regulated. This suggests that a temperature sensing mechanism may be located on the thylakoid membrane (Horváth et al. 1998). The prospect for a role of the thylakoid membrane as a high-temperature sensor is physiologically crucial, because it is highly susceptible to temperature increases due to its highly unsaturated constitution and the presence of photosystems, which are very susceptible to temperature alterations (Sung et al. 2003; reviewed by Wahid et al. 2007).

3.4. Protein misfolding in heat perception and HSF- and HSP-mediated response

Similarly to other environmental stresses, heat shock results in the accumulation of misfolded or unfolded proteins and several downstream consequences for cellular homeostasis and growth. This protein misfolding phenomena is not exclusive of plants, as its also observed in yeast, for instance (Geiler-Samerotte et al. 2011). The characterization of *Arabidopsis HSFA2* knockout and overexpressing plants has revealed that heat-shock transcription factor HSFA2 is a regulator of the cytoplasmic protein response (CPR) after heat-induced accumulation of misfolded proteins (Sugio et al. 2009). Under high temperatures, misfolded or unfolded proteins also accumulate in the ER of plant cells, triggering an unfolded protein response. Protein unfolding results in a response where the perceived ER stress is opposed by the up-regulation of the expression of genes encoding

components of the protein folding machinery, such as HSP90 or other HSPs and chaperones, or of the ER-associated degradation mechanism (Cha et al. 2010; reviewed by Liu and Howell. 2010). In *A. thaliana*, ER stress is sensed and stress signals are thus transduced by transcription factors such as HSFA2 (Charng et al. 2007; Sugio et al. 2009), which are activated and mobilized under abiotic stress conditions like heat. After heat-induced activation, HSPs in general, not only HSP90 but particularly small HSPs, display a huge role in protecting plant cell machinery in various organelles during high temperature conditions (Banzet et al. 1998; Liu and Shono, 1999; Soto et al. 1999; reviewed by Saidi et al. 2010). For HSF- and HSP- mediated stress responses in plant cells, the protein unfolding or misfolding is thus a very important signal.

4. The role of ion transport and compartmentation in cellular homeostasis during stress

One of the most important parts in the complex and remarkable ability to tolerate an environmental stress such as salinity and drought, always intimately connected, is played by the wide variety of modifications in ion transport inside and outside the cell.

4.1. Na^+ homeostasis

Sodium is deleterious to many organisms, except for halotolerant ones, such as halobacteria and halophytes, which possess specific mechanisms that maintain low concentrations of intracellular Na^+. In halophytes, the accumulation of Na^+ in the cytoplasm is prevented by inhibiting its influx across the plasma membrane and instead by promoting its efflux or sequestration into the vacuole (Hasegawa et al. 2000). The activity of most enzymes is negatively affected by high salt concentrations due to perturbation of the hydrophobic-

electrostatic balance between the forces maintaining protein structure. However, toxic effects on cells occur even at moderate salt concentrations of about 100 mM, unveiling specific salt toxicity targets (Serrano 1996). Apoplastic enzymes from halophytes have been shown *in vitro* to be remarkably salt-insensitive, coping with NaCl concentrations up to 500 mM (Thiyagarajah et al. 1996).

As previously mentioned, Na^+ toxicity arises not only due to toxic effects of Na^+ in the cytosol, but also because of the impairment of K^+ homeostasis, probably due to competition of Na^+ for K^+ binding sites. Ion transporters have long been known to play a key role in ion homeostasis (Hasegawa et al. 2000; Blumwald et al. 2000; Apse and Blumwald 2002; Zhu 2003). Under salt/drought stress, to avoid excessive Na^+ accumulation in the cytosol and reach ion homeostasis, plant cells exhibit three major mechanisms: restriction of Na^+ permeation and uptake catalyzed Na^+ transporters; sequestration of Na^+ into the vacuole; and efflux of excess sodium, with symplastic Na^+ being transported back to the apoplast through plasma membrane Na^+/H^+ antiporters (reviewed by Bartels and Sunkar 2005).

4.1.1. Na^+ influx

Sodium is transported between plant cells through the high-affinity K^+ transporter HKT1, through low-affinity cation transporter (LCT1) and through non-selective cation channels benefiting from the significant negative membrane potential across the plasma membrane (Amtmann and Sanders 1999; Rus et al.2001, Máser et al. 2002). Also, in several plant species such as rice, Na^+ leakage into the transpiration stream via the apoplast is responsible for a vast part of Na^+ entry into plants (Yeo et al. 1999). Sodium currents that are mediated by non-selective cation channels are also partially sensitive to calcium signaling, as demonstrated by the inhibition of Na^+ uptake by roots caused by Ca^{2+} (Tester and Davenport 2003). It remains, however, to be fully understood if the regulation of the activity of non-

selective cation channels by Ca^{2+} is direct or indirect via intracellular signalling cascades (Zhu 2003).

The *Arabidopsis* AtHKT1 protein mediates Na^+ influx when heterologously expressed in yeast and *Xenopus oocytes* (Uozumi et al. 2000). AtHKT1 is in fact the best-characterized member of class-1 HKTs in *A. thaliana*, and its mediation of Na^+ transport is actually well established (Møller et al. 2009) although its main role is currently believed to be in regulating Na^+ transport through xylem vessels to shoots due to its significant presence in the membrane of parenchyma cells (Máser et al. 2002; Sunarpi et al. 2005; Hauser and Horie 2010; Kronzucker and Britto 2011). Mutation in AtHKT1 suppresses the hypersensitivity of *sos3* mutants (Rus et al. 2001) suggesting that the wild-type SOS3 and other components of the SOS regulatory pathway can restrain the activity of AtHKT1 as a Na^+ intake transporter (Bartels and Sunkar 2005).

4.1.2. Na^+ efflux

At a first sight, the efflux of Na^+ in individual cells is not logical in multicellular organisms like plants, as the extrusion of Na^+ could negatively impact the surrounding cells (Zhu 2003). Thus, Na^+ efflux needs to be considered in specific tissues and in a whole-plant context. Transgenic rice plants expressing the moss sodium pumping ATPase PpENA1 display increased biomass production under salt stress and are severely more salt-tolerant (Jacobs et al. 2011). In *Arabidopsis*, Na^+ efflux is catalyzed by the plasma membrane Na^+/H^+ antiporter encoded by the previously mentioned SOS1 gene (Shi et al. 2000, 2002; Qiu et al. 2002; Quintero et al. 2002). This transmembrane protein is an electroneutral Na^+/H^+ exchanger that is specific to Na^+, being unable to transport Li^+ or K^+ (Qiu et al. 2002, 2003). Activity of the SOS1 promoter is detected ubiquitously in virtually all tissues, but its greatest activity occurs in root epidermal cells, particularly at the root tip, and in cells bordering the

vascular tissue throughout the plant (Shi et al. 2002). This SOS1 expression pattern, together with the results of ion analysis in *sos1* mutant plants, suggests several roles for SOS1: Na^+ efflux into the root medium; time gaining for Na^+ storage in the vacuole by slowing down Na^+ accumulation in the cytoplasm; and the control over long-distance Na^+ transport between roots and leaves by loading and unloading Na^+ into and from the xylem and phloem. The function of SOS1 in long-distance transport is important for coordination between transpirational Na^+ flow and the vacuolar sequestration of Na^+ in leaves. As previously mentioned, the transcript level of SOS1 is up-regulated at a transcriptional level by salt stress (Shi et al. 2000, 2003). Indeed, increased expression of SOS1 results in improved ion homeostasis and salt tolerance in transgenic *Arabidopsis* (Shi et al. 2003; Zhu 2003).

4.2. K^+ homeostasis

A high cytosolic K^+/Na^+ ratio is important for the normal functioning of cellular metabolism and, of course, plant growth and productivity. Under normal conditions of nutrient availability, about 80% of K^+ intake by plants happens through the action of two major systems, KUP/HAK/KT and AKT (Kronzucker and Britto 2011). They catalyze high- and low-affinity uptake, respectively (Hirsch et al. 1998; Rubio et al. 2008; Szczerba et al. 2009). Backup uptake mechanisms such as the non-characterized cation and K^+ transporting families CHX and KEA, respectively, may provide additional K^+ influx capacity at higher external K^+ concentrations (Pardo et al. 2006; Pyo et al. 2010). Both KUP/HAK/KT and AKT potassium uptake systems are severely inhibited by Na^+ (Fu and Luan 1998; Britto et al. 2010). In theory, under salt stress, Na^+ competes with K^+ for influx into roots. In salinity, not only the transcript amounts of several K^+ transporter genes are either down- or up-regulated so that plants can keep the uptake of K^+ under excessive Na^+ availability, but also post-

translational regulation is vital for K^+ homeostasis. For example, in the common ice plant, salt-stress significantly increases the expression of KMT1, a member of the AKT/KAT family, and of several HAK/KUP genes, whereas on the other hand transcript levels of MKT1, also part of the AKT/KAT family, are down-regulated to provide efficient K^+ transport (Su et al. 2001, 2002). In parallel, post-translational regulation of HAK/KUP transporters tightly influence their activities towards homeostatic K^+ levels. Also, gene expression of the *Arabidopsis* root K^+-transporter AtKC1 is up-regulated by salt (Pilot et al. 2003). Similarly, in barley, the amount of *HvHAK1* transcripts is enhanced by K^+ deprivation and transiently by exposure to high salt levels (Fulgenzi et al. 2008). However, the authors also showed that the yeast protein phosphatase PPZ1 as well as the halotolerance HAL4/HAL5 kinases negatively regulate the HvHAK1-mediated K^+ transport in *HvHAK*-expressing yeast cells. Recently, functional studies on the *Puccinellia tenuiflora* high-affinity potassium transporter PutHKT2;1 suggested a possible role in salt stress tolerance by allowing for the K^+ uptake at low K^+ and high Na^+ external levels, thus maintaining a high K^+/Na^+ ratio in these adverse conditions (Ardie et al. 2009).

The activity of K^+ channels are known to be regulated by protein kinases (Li et al. 1998) and phosphatases (Cherel et al. 2002). Whether these proteins are influenced or somehow directly or indirectly regulated by salt stress is yet to be clarified. In *Eucalyptus camaldulensis*, two Na^+-K^+ co-transporter HKT1 homologs display intrinsic osmosensing capabilities when expressed in *Xenopus oocytes* (Liu et al. 2001). Their Na^+- and K^+-transport activities are enhanced by a downshift in extracellular osmolarity.

As previously referred to, the *A. thaliana* plasma membrane SOS1 protein is a specific Na^+/H^+ antiporter responsible for Na^+ efflux and regulates its distribution between root and shoot. Surprisingly, however, it also interacts with K^+ influx mechanisms by roots, suggesting an influence of the SOS signaling pathway in K^+ homeostasis. Concordantly, *Arabidopsis sos*

mutant plants display a growth impairment under K^+-limiting conditions (Zhu et al. 1998). The involvement of the SOS pathway in K^+ uptake and homeostasis is possibly indirect, because in theory the severe inhibition of Na^+ efflux in *sos* mutant plants can lead to an accumulation of cytoplasmic Na^+ that may be repressive to less specific K^+-uptake transporters. Under K^+-limiting conditions, inhibitory levels of cytoplasmic Na^+ may emerge in *sos* mutant plants, even when grown in media without extra NaCl addition (Zhu et al. 2003).

Additionally, two CHX isoforms, AtCHX17 and AtCHX23, have been shown to affect K^+ homeostasis and the control of chloroplast pH, respectively (Cellier et al. 2004; Song et al. 2004; Pardo et al. 2006). In parallel with the recently discovered KEA family of K^+ transporters, the CHX family still needs further characterization studies to fully understand its role in K^+ homeostasis.

4.3. H^+ transporters in ion homeostasis

The plasma membrane H^+-ATPase as well as vacuolar H^+-ATPase and H^+-PPase (pyrophosphatase) play key roles in ion homeostasis re-establishment. The proton gradient generated by plasma membrane H^+-ATPases is the driving force for Na^+ transport by SOS1. Accordingly, gene expression of several H^+-ATPases is significantly up-regulated in response to salt stress (Niu et al. 1993).

Plant plasma membrane H^+-ATPases (PMAs) can also be activated at a post-translational level by phosphorylation of the penultimate residue of the C-terminus - a threonine (Thr) - and the subsequent binding of regulatory 14-3-3 proteins, thus transforming a dimer into a hexamer (Svennelid et al. 1999; Kanczewska et al. 2005). Conversely, they are

negatively regulated when phosphorylation events occur in two residues of the H^+-ATPase binding site of 14-3-3 protein instead of the actual binding of the protein (Duby et al. 2009). However, surprisingly in *N. tabacum* suspension-cultured cells, binding of 14-3-3 proteins was not necessary for the activation of PMA2 as phosphorylation in Thr889 was sufficient for activation when PMA2 was also phosphorylated in the penultimate residue (Piette et al. 2011). Phosphorylation regulation of PMAs is critical in the context of an abiotic stress. For instance, cold stress of culture cells or leaf tissues of tobacco lowered the phosphorylation of the penultimate Thr of PMA2, whereas the phosphorylation of the respective residue of PMA4 remained unaltered, suggesting that PMA2 and PMA4 are differentially regulated by phosphorylation (Bobik et al. 2010a). Moreover, a cytosol acidification of BY2-tobacco cells results in increased phosphorylation of PMA2, thus demonstrating not only the impact that cytosolic pH changes have in stress signaling but also the concomitant role of the H^+-ATPase in cell pH homeostasis after an imbalance caused by a given environmental stress (Bobik et al. 2010b). Under heavy metal stress, such as excessive aluminum conditions, an up-regulation of the plasma membrane H^+-ATPase phosphorylation is also observed, in addition to its gene expression and translation in the root tip of *Glycine max* (Shen et al. 2005).

The transport of H^+ into the vacuole generates proton motive force across the tonoplast allowing for the action of Na^+/H^+ antiporters (Hasegawa et al. 2000; Blumwald et al. 2000; reviewed by Bartels and Sunkar 2005 and by Silva and Gerós 2009). In several plant models the V-H^+-PPase seems, in fact, to be able to generate and keep across the vacuolar membrane a higher pH gradient than the V-H^+-ATPase at PPi concentrations in the micromolar range (Nakanishi and Maeshima 1998; Queirós et al. 2009). In general, V-H^+-PPase activity is high in young tissues, whereas V-H^+-ATPase activity is relatively constant during maturation and growth (Martinoia et al. 2007). In *Arabidopsis* plants without *AHA4* gene, which encodes an ATPase, salt stress provoked an increase in Na^+ levels and, on the contrary, less K^+

accumulation in the leaves. It is believed that AHA4 functions in the control of Na^+ flux across the root endodermis, possibly by partially disrupting the activity of other pumps (Vitart et al. 2001). In the halophyte *Suaeda salsa*, the main mechanism to cope with high salt levels seems to be an up-regulation of V-H^+-ATPase (Wang et al. 2001). In tonoplast vesicles isolated from leaves, both ATP-hydrolytic and H^+ pumping activity of the V-H^+-ATPase increased two-fold in leaves treated with 200 mM NaCl, when compared with control leaves (Qiu et al. 2007). Indeed, a general sodium-induced increase in V-H^+-ATPase activity in plant response to salt-stress has been observed (Vera-Estrella et al. 1999; Otoch et al. 2001; reviewed by Silva and Gerós 2009).

Similarly, in salt-adapted cell line of *Solanum tuberosum*, the activity of V-H^+-PPase increased about three-fold in comparison to cells cultivated in the absence of salt (Queirós et al. 2009). Moreover, the transcripts encoding V-H^+-PPase are thought to be positively regulated by salt-stress in maize and bean plants (Marivet et al. 1994), and in *Arabidopsis* the overexpression of the H^+-pyrophosphatase, AVP1, was shown to improve salt and drought tolerance (Gaxiola et al. 2001). This fact further confirms that the generation of a H^+ gradient across the tonoplast is an additional driving force for vacuolar sodium compartmentation catalyzed by NHX transporters and, therefore, salt tolerance. The recent observations in *Populus euphratica* by Silva and co-workers (2010) have provided further evidence that corroborate this theory. Thus, the coordination between Na^+ tonoplast antiport and increased activity of V-H^+-ATPase and H^+-pyrophosphatase are crucial for sequestering Na^+ in cellular compartments and therefore to regain ion homeostasis during salt, drought or even temperature stress.

4.4. Na^+ compartmentation in the vacuole is critical for ion homeostasis

The vacuoles of plant cells are widely diverse in size, form, content, and functional dynamics and play key roles in plant growth, development and stress responses (Paris et al. 1996; Martinoia et al. 2007). They have recognized functions in protein turnover, ion and pH homeostasis, turgor pressure maintenance, sequestration of toxic compounds and pigmentation. The vacuolar membrane, or tonoplast, controls the movement of inorganic and organic solutes to and from the cytoplasm through a wide range of pumps, carriers, ion channels and receptors, but these proteins are generally less well known than the corresponding plasma membrane proteins (Shimaoka et al. 2004; Neuhaus 2007; reviewed by Silva and Gerós 2009).

Vacuolar sequestration of Na^+ not only lowers its concentration in the cytoplasm, but also contributes to osmotic adjustment in order to maintain water absorption from saline solutions (Silva and Gerós 2009). Mitochondria and plastids are other organelles with the ability to accumulate some Na^+ and therefore contribute to the compartmentation of Na^+ (Zhu 2003). NHX transporters catalyze transport of Na^+ into the vacuole in exchange of protons. The first plant Na^+/H^+ antiporter, *AtNHX1*, was molecularly characterized from *Arabidopsis* by functional genetic complementation of a yeast mutant devoid of endosomal Na^+/H^+ activity, and its overexpression suppressed its salt-hypersensivity phenotype (Apse et al. 1999; Gaxiola et al. 1999). Since then, many Na^+/H^+ antiporter genes have been characterized in plants such as rice (Fukuda et al. 1999), *Atriplex gmelini* (Hamada et al. 2001), *B. vulgaris* (Xia et al. 2002), *Brassica napus* (Wang et al. 2003), cotton (Wu et al. 2004), wheat (Yu et al. 2007), and grapevine (Hanana et al. 2007; reviewed by Silva and Gerós 2009). NHX homologous or transgenic overexpression has been confirmed to provide salt stress tolerance in a wide variety of plant species (reviewed by Kronzucker and Britto 2011). Under salt stress, the up-regulation of *NHX* gene expression is generally synchronized with increased Na^+/H^+ antiport activity, protein abundance and regulation at the protein level (Hamada et al.

2001; Parks et al. 2002; Queirós et al. 2009; reviewed by Silva and Gerós 2009). Additionally, overexpression in *A. thaliana* of NHX genes from yeast and some plant species resulted not only in increased salt tolerance but also in more significant ROS scavenging and higher photosynthetic activity under stress (Liu et al. 2010). A link between osmotic stress and vacuole sequestration of Na^+ has also been demonstrated in *Arabidopsis* where osmotic stress activates the synthesis of ABA, which in turn up-regulates the expression of *AtNHX1* (Shi and Zhu 2002). In addition, the SOS pathway appears to also regulate the activity of vacuolar Na^+/H^+ antiporters. There is strong evidence proving that the coordination of activity between the exchangers on the tonoplast and plasma membranes and the C-terminus of AtNHX1 facing the vacuolar lumen may indeed have a key role in the regulation of protein activity by interaction with calmodulin. (Yamaguchi et al. 2003, 2005; Qiu et al. 2004). Other modes of regulation of vacuolar antiporters may occur, similarly to what happens in sugar beet Na^+/H^+ exchanger 1 (BvNHX1), where a MYB transcription factor exerts a role in the regulation (Adler et al. 2010).

Tonoplast Cl^- channels are also likely to play a role in plant cell ion homeostasis during salt/drought stress. Chloride channels have already been isolated and characterized in plants (Lurin et al. 1996). Recently in rice, OsCCC1, a member of the cation-Cl^- cotransporter (CCC) family has been shown to play a significant role in K^+ and Cl^- homeostasis and rice plant development (Kong et al. 2011). Yeast mutants knocked out on the gene *GEF1,* which encodes a chloride channel, are more prone to the toxic effects of cations (Gaxiola et al. 1998). More recently, *OsClC1* and *OsClC2*, two tonoplast Cl^- transporter genes from rice, were identified and functionally characterized in yeast (Nakamura et al. 2006). The expression level of *OsClC1*, but not of *OsClC2*, was increased upon salt-stress. It has been demonstrated that young root cortical cells of salt stressed *P. euphratica* accumulated more Cl^- in the vacuoles than control plants, and a higher amount of Cl^- was detected in the vacuole

instead of the cytoplasm and cell wall in suspension-cultured cells treated with 200 mM NaCl (Chen et al. 2002). This suggests an adaptation of salt-tolerant plants to salt stress because a greater Cl^- permeation of the tonoplast may allow for its compartmentation in the vacuole down its electrical gradient, dissipating an inside-positive membrane potential and therefore inducing the formation of a higher ΔpH through V-H^+-ATPase and V-H^+-PPase activity. This mechanism could be used in Na^+ and other cation detoxification and also in the balance of osmolarity by their rescue into the vacuole (Gaxiola et al. 1999; reviewed by Silva and Gerós 2009).

Thus, the astonishing capacity to sequester excessive Na^+ (Fig. 3), and other ions, into the vacuole, is a key resource that plant cells possess to overcome environmental stresses such as salinity, and re-establish ion homeostasis under these rough conditions.

5. Osmoprotectant and compatible solute synthesis and transport

Living organisms, from simple microbes to complex animals and plants, synthesize compatible solutes in response to drought stress (Burg et al. 1996). Sugars, polyols (or sugar alcohols), amino acids and glycine betaine are all non-toxic compounds that are frequently accumulated as compatible solutes in plants. They accumulate preferably in the cytoplasm at high concentrations under osmotic stress not interfering with the normal cellular metabolism (Chen and Murata 2002). These molecules play a role in turgor maintenance and osmotic balance but they are also involved in cell structure protection from stress. Compatible solutes are in most cases also osmoprotectants. Osmoprotectants are small neutral molecules non-toxic to the cell at molar concentration that stabilize proteins and cell membranes against the disturbing or denaturing effect of stress on cellular functions (Yancey et al. 1982). Ongoing investigation has shown that engineering the introduction of osmoprotectant synthesis

pathways is a potential strategy for improving abiotic stress tolerance of crop plants that are not able to naturally synthesize and accumulate osmoprotectants (Rathinasabapathi 2000).

Mannitol is the most widely distributed polyol in nature, and has been reported in more than 100 species of vascular plants of several families, including the Rubiaceae, Oleaceae and Apiaceae. It enhances tolerance to water-deficit stress primarily through osmotic adjustment, but also due to its osmoprotectant character (Noiraud et al. 2001; Conde, et al. 2008). Mannitol osmoprotectant function probably involves the scavenging of hydroxyl radicals and/or the stabilization of macromolecules. In tobacco, mannitol protects ferredoxin, thioredoxin, glutathione and the thiol-regulated enzyme phosphoribulokinase from the severe negative impact of hydroxyl radicals (Shen et al. 1997). In tobacco and wheat, the introduction of a mannitol-1-phosphate dehydrogenase (*mtlD*) *Escherichia coli* gene resulted in more water stress-tolerant plants (Tarczynski et al. 1993; Abebe et al. 2003; reviewed by Valliyodan and Nguyen 2006). In the moderately salt tolerant plant *Olea europaea*, mannitol is indeed an osmoprotectant besides being an important carbon and energy source. A NaCl treatment to suspension-cultured cells of *O. europaea* significantly increased the activity of the polyol:H^+ symport system and the levels of *OeMaT1* (*Olea europaea* mannitol transporter 1) transcripts, whereas, in contrast, it strongly repressed mannitol dehydrogenase (OeMTD) activity. The coordination between these proteins provides intracellular accumulation of mannitol (Conde et al. 2007) (Fig. 4). Therefore, the improvement of salt tolerance in plants could be achieved by the increased production of osmolytes in addition to the stress proteins that protect or alleviate damage caused by salt stress (Rengasamy 2006; Silva and Gerós 2009).

Moreover, targeted synthesis of osmolytes in the chloroplast, one of the main sites of ROS production, by placing a signal sequence in front of the engineered enzymes has been demonstrated to result in better plant cell protection against an abiotic stress (Shen et al.

1997). In fact, transgenic plants with engineered and enhanced osmoprotectant production have improved tolerance not only to salt stress, but also to other abiotic stresses such as heat and drought, chilling and freezing, which also generate ROS. This is clearly shown in glycine betaine-producing plants (Alia et al. 1998; Holmstrom et al. 2000): *Arabidopsis* plants engineered with a modified bacterial gene encoding a choline oxidase accumulated glycine betaine in chloroplasts coped better with salt, cold and heat stresses than wild-type plants (Sakamoto and Murata, 2000). Myo-inositol and D-ononitol are also known to protect intracellular components such as membranes and enzymes from abiotic stress-induced ROS. Transgenic tobacco overexpressing the inositol methyl transferase gene (IMT1) from the ice plant *M. crystallinum* have increased salt/drought stress tolerance due to a higher accumulation of the methylated form of inositol, D-ononitol (Sheveleva et al. 1997).

Trehalose, a non-reducing disaccharide present in many different organisms that functions as reserve carbohydrate, also acts as osmoprotectant during abiotic stress in several plants, protecting membranes and proteins from denaturation or instability (Goddijn and van Dun 1999). In rice, a stress-inducible controlled trehalose overproduction by trehalose phosphate synthase (*TPS*) gene overexpression resulted in drought tolerance without any negative effect on plant growth or productivity (Garg et al. 2002). Also playing important roles in plant osmotic stress tolerance are raffinose-family oligosaccharides, such as raffinose, stachyose and galactinol, as shown in observations in *A. thaliana* (Taji et al. 2002). Also, fructan metabolism and accumulation allows for higher plant tolerance to drought and cold stresses, as demonstrated in sugar beet and tobacco (Pilon-Smits et al. 1995, 1999; Vereyken et al. 2003). Another well-known osmoprotectant/compatible solute in plants is proline. The involvement of proline in the response to water deficits has been demonstrated in several plants such as tobacco, *A. thaliana* and rice through the engineering of increased proline

biosynthesis enzymes (Kavi Kishor et al. 1995; Roosens et al. 2002; Yamada et al. 2005; Vallyiodan et al. 2006).

Thus, the genetic engineering of metabolic pathways for the production of osmolytes such as mannitol, raffinose-family oligossacharides, fructans, trehalose, ononitol, proline, or glycinebetaine, among others, might increase resistance to drought and facilitate the re-establishment of ion homeostasis, even though the mechanism by which these osmolytes provide protection is still presently being investigated.

6. Is there a future for the use of osmolytes in agricultural practices?

A deepened knowledge of the sensing mechanisms and the signaling pathways involved in responses to environmental stresses like salt, drought, cold or heat, and the cross-talk between these different pathways could definitely make possible the treatment of plants with exogenous compounds, such as mannitol, glycinebetaine, trehalose, proline and other osmoprotectants and antioxidants, without recurring to genetic manipulation and avoiding the introduction in nature of genetic engineered plants. Indeed, mannitol applied exogenously to salt-stressed wheat, a plant unable to synthesize this polyol, significantly increased its salt tolerance mainly by stimulating the activity of antioxidant enzymes (Seckin et al. 2009). Also, in maize, an exogenous application of glycibetaine alleviates the adverse effects of salt stress by modulating water relations (Nawaz and Ashraf, 2007), and in tomatoes it enhances tolerance to chilling not only by protecting membranes and macromolecules directly, but also by inducing antioxidant enzymatic mechanisms (Park et al. 2006). Trehalose has also been shown to improve abiotic stress tolerance when exogenously applied to *Arabidopsis* and maize (Bae et al. 2005; Zeid 2009). In tobacco BY-2 suspension-cultured cells, proline also improves tolerance to salinity by up-regulating antioxidant enzymes, and more significantly

than glycinebetaine (Hoque et al. 2007). Remarkably, even exogenous nitric oxide (NO) alleviates NaCl-induced salt/drought and oxidative stresses in *A. thaliana* (Zhang et al. 2010). Thus, a carefully planned use of protective compounds during the treatment and regular practices of plant crops should not be ruled out in the future, and on the contrary should be regarded as a relatively economical and practical way to improve plant growth and productivity in the increasingly rough environmental conditions faced in the context of the ongoing natural/anthropogenic climate changes.

7. Conclusion

Plants have remarkably evolved mechanisms to maintain cellular homeostasis under salinity, drought, cold, freezing or intense heat. The number and complexity of the mechanisms responsible for plant ion homeostasis and the intricate way they are regulated and interconnected, either at the cellular level or at the whole-plant level, is remarkable. The involvement of sophisticated and tightly regulated stress sensing mechanisms, efficient signal transduction pathways, efflux or compartmentation of toxic ion systems and key detoxification strategies, such as osmoprotectant accumulation, illustrate how fascinating the story of plant ion homeostasis is under environmental stress. This area of research has been deeply explored and new knowledge has led to some successful improvements in plant stress resistance through genetic manipulation. However, the introduction in nature of genetic engineered plants is still controversial. Thus, the continued study of the intricate signaling pathways involved in the plant response to environmental stress is a promising area of research, which may ultimately lead to improvements of the yield potential through plant treatment with exogenous compounds, therefore without recurring to genetic manipulation. In the context of the ongoing climate changes there is no doubt that the study of plant ion

homeostasis is more important than ever, which will certainly be the subject of further research in the future.

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Legends to figures

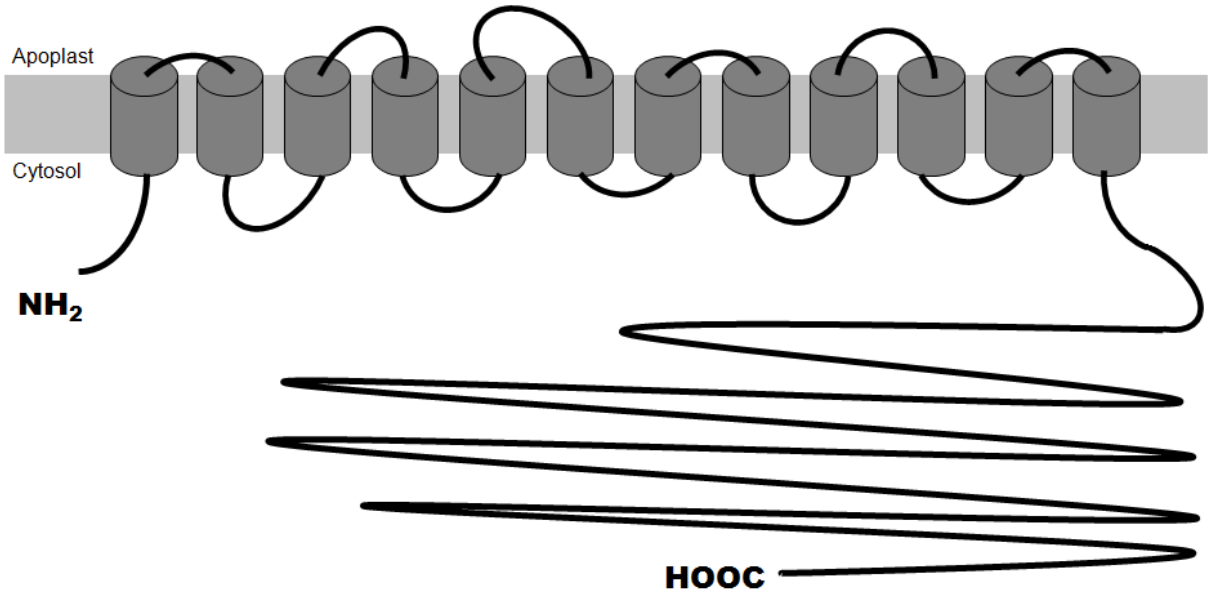

Figure 1 – Topological model of the *Arabidopsis* plasma membrane Na^+/H^+ antiporter AtSOS1, a 12 transmembrane domain protein with the N-terminal and C-terminal both located at the cytosol. In addition to its antiporter activity, the unusually long cytosolic C-terminal tail of AtSOS1 is thought to be involved in the sensing of Na^+. The model was constructed and adapted according to the work of Shi and co-workers (2000).

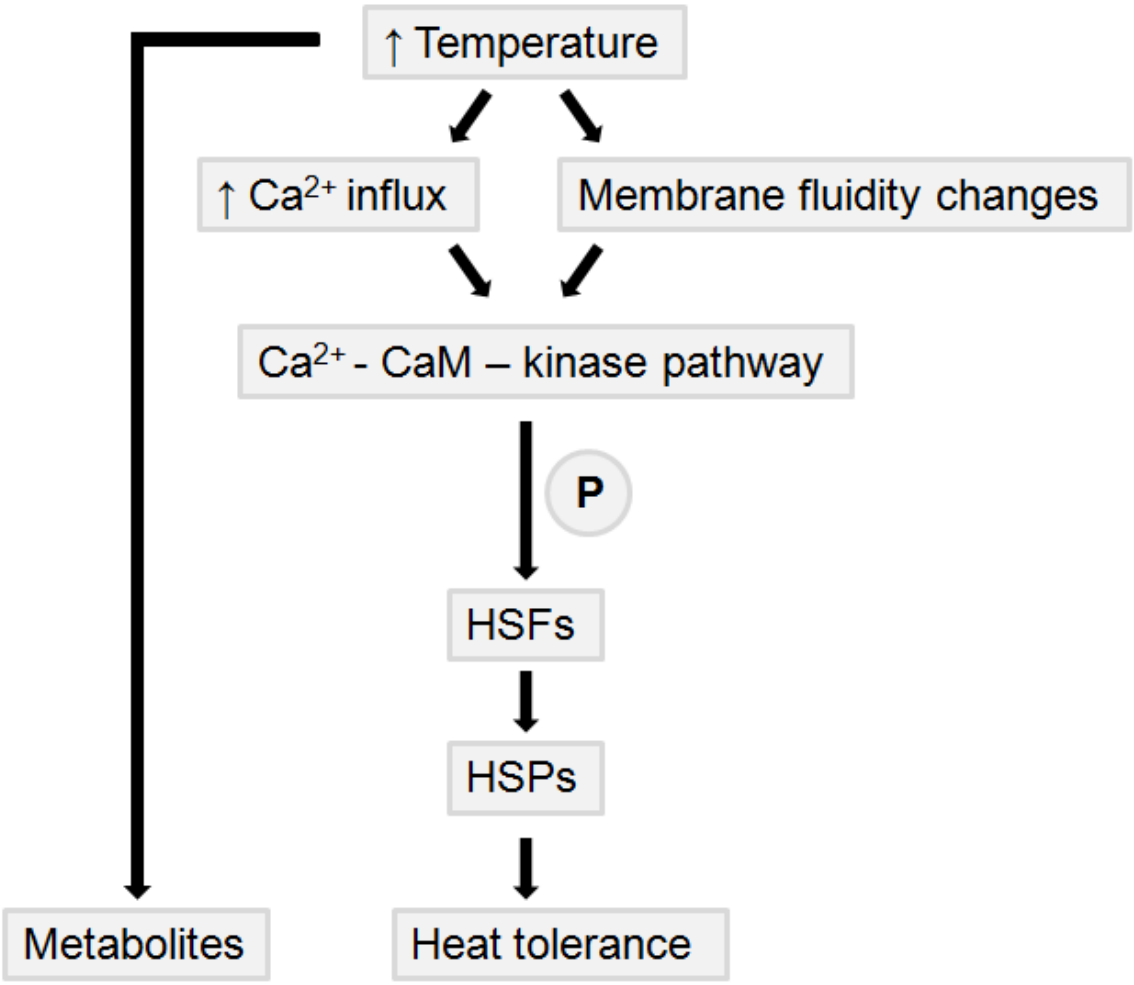

Figure 2 - Schematic representation of major plant sensing and signalling mechanisms that ultimately lead to heat tolerance. Temperature increase alters membrane fluidity and stimulates a transient Ca^{2+} influx by up-regulating putative Ca^{2+} channels. In turn, Ca^{2+} activates calmodulins (CaM) that interact with CaM-binding protein kinases or other heat-activated protein kinases, thus inducing the phosphorylation (P) and activation of heat-shock transcription factors (HSFs). The expression and activity of heat-shock proteins (HSPs) are then up-regulated by the activity of HSFs, and plant cell tolerance to high temperatures is increased. In parallel, the levels of several heat-responsive metabolites like H_2O_2 and nitric oxide (NO) are also increased, possibly triggering the activation of similar pathways or other molecular mechanisms (reviewed by Saidi et al. 2010).

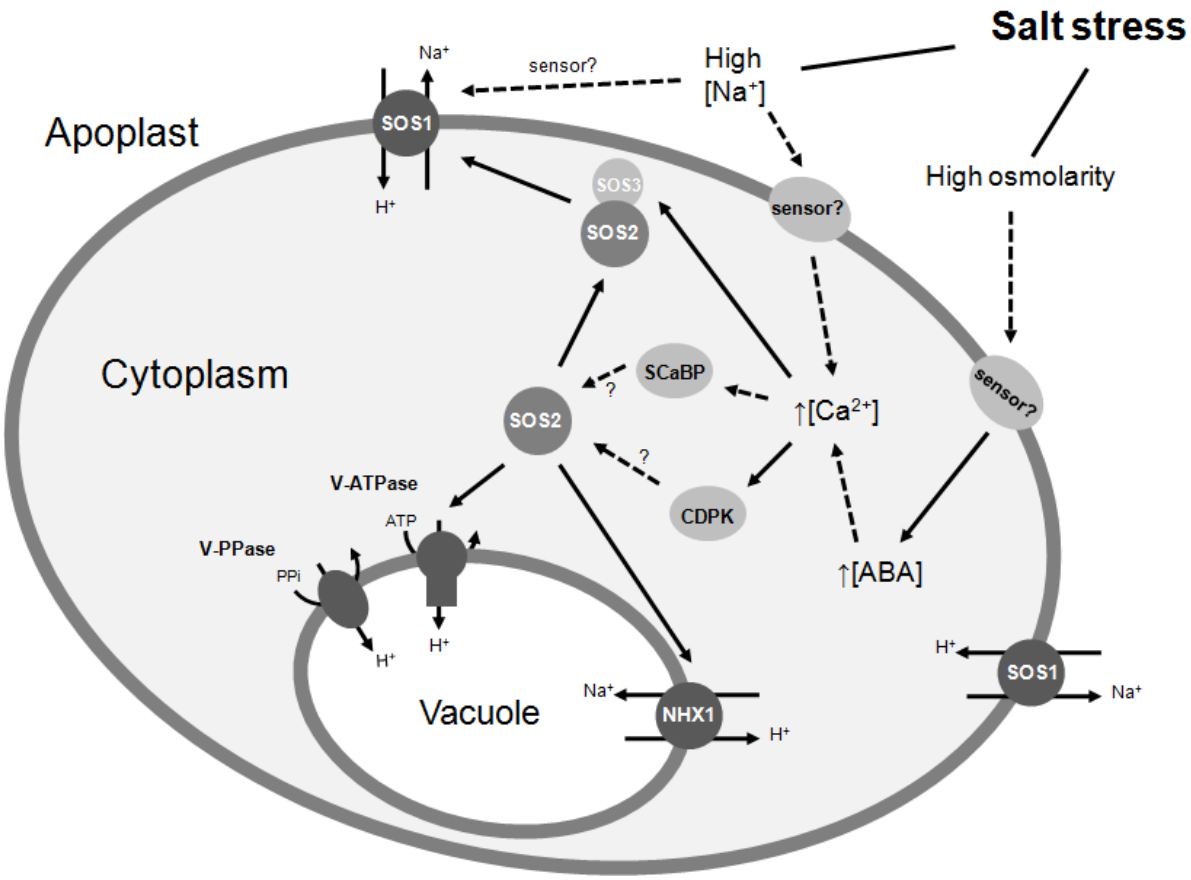

Figure 3 - Signaling pathways involved in Na^+ efflux and compartmentation in *Arabidopsis* under salt stress. Excessive Na^+ and high osmolarity are separately detected by so far unidentified plasma membrane sensors that in turn up-regulate cytosolic Ca^{2+} concentration. This increase is sensed by Ca^{2+}-binding protein SOS3 which activates SOS2. The active SOS3-SOS2 protein complex phosphorylates the plasma membrane Na^+/H^+ antiporter SOS1, resulting in a higher Na^+ extrusion capacity. SOS2 also regulates V-H^+-ATPase and NHX1 antiport activity in a SOS3-independent way, possibly via CBL-CIPK protein complexes (CDPKs) or other SOS3-like Ca^{2+}-binding proteins (SCaBP). The osmotic stress arising from high salinity also triggers the accumulation of ABA, which indirectly regulates SOS1 and NHX1 towards ion and osmotic homeostasis.

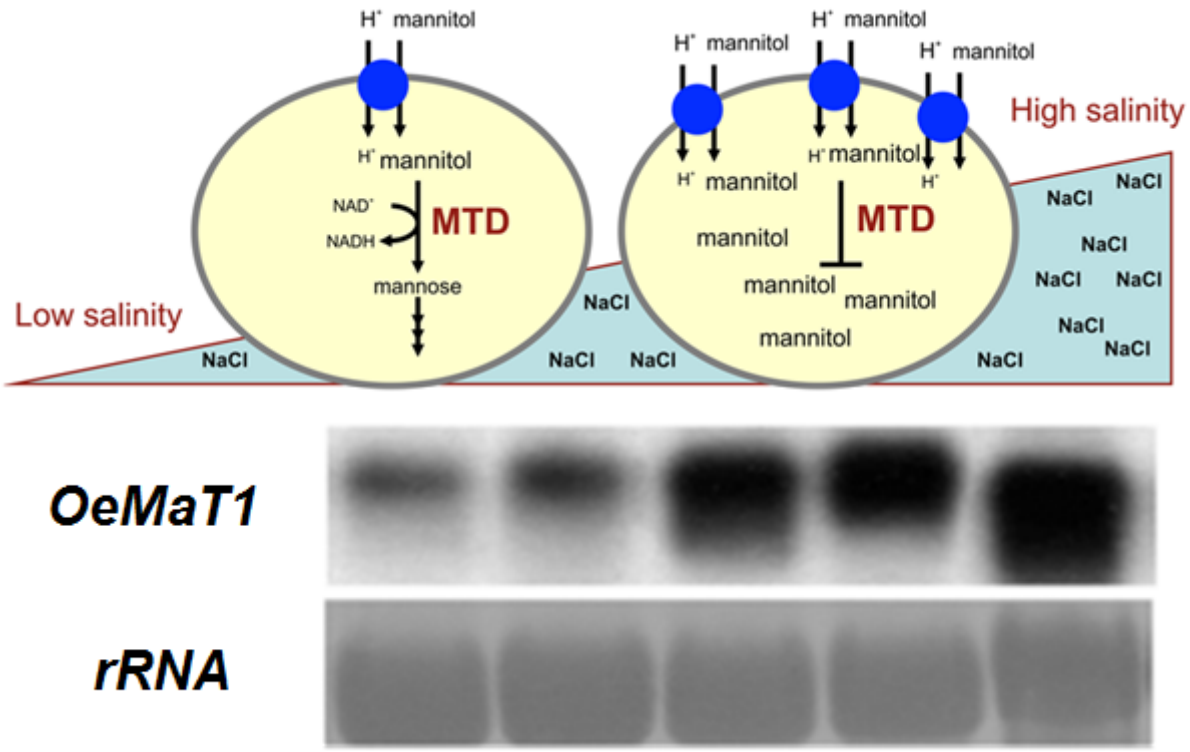

Figure 4 - Regulation of mannitol transport and metabolism as a mechanism providing salt tolerance in *O. europaea*. *OeMaT1* expression was assessed by Northern Blot (adapted from Conde et al. 2007).