Doctoral Thesis

Molecular Physiological Study on the Adaptive Mechanisms to Salinity Stress in Egyptian Rice Cultivars

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Abbreviations

DW	Dry weight
RWC	Relative water content
ELR	Electrolyte leakage ratio
EC	Electrical conductivity
HKT/HAK	High affinity K transporter
KORC	Potassium outwardly rectifying channel
KIRC	Potassium inward rectifying channel
NHX	Na^+/H^+ antiporter
SOS	Salt overly sensitive
NSCCs	Non selective cation channels
CNGCs	Cyclic nucleotide gated channels
LCT	Low affinity cation transporter
PMP3	Plasma membrane protein 3
Lti-6a/b	Low temperature induced 6a/b
PCR	Polymerase chain reaction
qRT-PCR	Quantitative real-time polymerase chain reaction
cDNA	Complementary deoxyribonucleic acid
C_T	Threshold cycle
ΔC_T	Change in threshold cycle
MTs	Metallothioneins
ROS	Reactive oxygen species

IPTG	Isopropyl β-D-1-thiogalactopyranoside
OD	Optical density
NBT	Nitro-blue tetrazolium
O_2^-	Oxygen superoxide

Contents

Chapter 1

General introduction

1.1 Soil salinity

Soil salinization is one of the factors causing serious soil degradations, which can arise from natural causes and anthropogenic activity, such as irrigation in arid and semi-arid regions. Saline soils contain a variety of salts, including Na_2SO_4, NaCl, $MgSO_4$, $CaSO_4$, $MgCl_2$, KCl and Na_2CO_3, but are normally dominated by NaCl, which causes most of the salt problems for higher plants in nature (Flowers *et al.,* 1977). Saline soils can be generally found in arid regions, estuaries, and coastal fringes, which are dominated by Na^+ ions with electrical conductivity (EC) of more than 4 dS/m that corresponds to approximately 40 mM NaCl (USDA-ARS 2008; Munns and Tester 2008). It has been estimated that more than 800 million hectares of land throughout the world are salt affected, accounting for more than 6% of the world's total land area (FAO, 2008).

1.2 Salt stress, Na^+ toxicity, and its effects on plant growth

Soil salinity is one of the major constrains affecting plant growth and results in reduction of crop yield throughout the world (Flowers, 2004; Cuartero *et al.,* 2006). It is estimated that if the current scenario of salinity stress would persist, up to 50 % of present cultivated land for agriculture could be lost by 2050 (Wang *et al.,* 2003). Generally, salt stress causes both osmotic stress and ionic stress. Osmotic stress is triggered by an excess accumulation of salts in the soil, and ionic stress is caused by the over-accumulation of salts in the cells. These stresses can individually or simultaneously affect the physiological status of plants (Lefèvre *et al.,* 2001; Ueda *et al.,* 2003). Na^+ is very harmful in cells for most plants when it is present in the cytosol at a concentration higher than the adequate level (1-10 mM) (Munns and Tester, 2008). Beyond this range, Na^+ interferes with many cellular functions. K^+ is one of the essential and most abundant monovalent cations in cells, and needs to be maintained within 100-200 mM range in cytosol for

efficient metabolic functioning (Cuin *et al.,* 2003). As a co-factor in cytosol, K^+ activates more than 50 enzymes, which are very susceptible to high cytosolic Na^+ and high Na^+/K^+ ratios (Munns *et al.,* 2006). Therefore, apart from low cytosolic Na^+, maintenance of a low cytosolic Na^+/K^+ ratio is also critical for the function of cells (Zhu *et al.,* 1998). Under salt stress conditions, Na^+ often competitively inhibits the uptake of K^+ leading to an imbalance in Na^+/K^+ ratios within plant cells since Na^+ and K^+ are physico-chemically similar monovalent cations, thus competing for the binding sites of many enzymes (Tester and Davenport, 2003). High uptake of Na^+ and leakage of K^+ result in an imbalance in the Na^+/K^+ ratio in the cytosol, which, in turn, leads to many imbalances in the cellular enzymatic reactions. An unfortunate consequence of salinity stress in plants is the excessive generation of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radical (Foyer *et al*., 1994; Mittler, 2002). The excess production of ROS during salinity stress results from impaired electron transport processes in chloroplast and mitochondria as well as from pathways such as photorespiration. This is supported by reports, which demonstrate that the oxygenase activity of Rubisco (which carries out the first step of the photorespiratory pathway) gets significantly enhanced under conditions of salinity stress (Sivakumar *et al*., 2000). Under normal growth conditions, the production of ROS in the cell is as low as 240 μM s^{-1} $O_2^{\bullet -}$ and the steady state level of H_2O_2 in chloroplasts is 0.5 μM (Mittler, 2002; Polle, 2001). However, under conditions of abiotic stress such as salinity, the disrupted cellular homeostasis leads to the production of ROS like $O_2^{\bullet -}$ to a level as high as 720 μM s^{-1} and H_2O_2 to a level as high as 15 μM (Mittler, 2002; Polle, 2001). H_2O_2 at a concentration of 10 μM in chloroplasts has been reported to cause a net reduction in photosynthesis in plants by over 50% (Kaiser, 1979). The main toxicity of superoxide and hydrogen peroxide has been attributed to their ability to initiate cascade reactions that result in the production of hydroxyl radicals and other

destructive species such as lipid peroxides (Noctor and Foyer, 1998) Hydroxyl radicals are very reactive and can damage vital cellular macromolecules, e.g., via denaturation of proteins, mutation of DNA and peroxidation of lipids.

1.3 Mechanisms of Na^+-uptake in plants

Under salt stress, a plant root is the first organ to combat high Na^+ in the soil solution. The root tip in particular seems to be the major nutrient uptake region rather than the elongated mature segments of the root (Golldack *et al.,* 2003). This nutrient uptake occurs primarily in the plasma membrane of root epidermal cells including root hairs. The uptake of Na^+ proceeds through the same path like any other mineral nutrient taken up by cells through the plasma membrane of either the root epidermal cells or cortical cells. Structurally the plant plasma membrane is a lipid bilayer, which surrounds cell cytoplasm and, in principle, is semi-permeable to solutes. There are, however, many transport proteins, specific for one or a group of solutes, spanning the lipid bilayer of the plasma membrane and facilitate the movement of solutes in and out of the cytosol. In plant cells, the plasma membrane potential is negative inside (-120 to - 200 mV), but Na^+ is a positive ion (Munns and Tester, 2008). Therefore, Na^+ mainly enters the cytosol through the passive way. When it enters in epidermal cells or cortical cells, Na^+ may follow a symplastic pathway or apoplastic pathway before it encounters the endodermis layer. Since the endodermis with the Casparian strip is an effective barrier for apoplastic Na^+ movement (White, 2001; Karahara *et al.,* 2004), Na^+ enters it symplastically. Thus, membrane transport at the level of root epidermal and cortex cells is a critical point for the uptake or rejection of toxic ions like Na^+ from the environment. When Na^+ is eventually taken up by roots, it is loaded into the xylem for long-distance xylem transport following the transpiration stream to shoots. Finally, Na^+ reaches to all cells including metabolically active mesophyll cells in leaves. Here, the leaf mesophyll cells

conveys it to the phloem for another long-distance transport to other tissues (Sondergaard *et al.,* 2004). Thus, Na^+ may also be recirculated in different cells and/or tissues.

1.4 Transporters involved in Na^+-uptake

Different uptake mechanisms for Na^+ into plant cells have been suggested. Nonselective cation channels (NSCCs) are proposed to be the dominant pathways for Na^+-influx in many plant species (Davenport and Tester, 2000). However, the molecular identity of these NSCCs is still unknown. High-affinity potassium transporters (HKTs) were also suggested to mediate a substantial Na^+-influx in some species (Horie *et al.,* 2001; Golldack *et al.,* 2002; Gárciadeblás *et al.,* 2003). In rice, nine HKT homologues (OsHKT1-9) have been identified of which only OsHKT5 is considered as a non- functional gene, due to the existence of three stop codons in its mRNA (Gárciadeblás *et al.,* 2003). All of these functional genes encode proteins with distinct transport activities, which might be expressed in various tissues and/or organs. Horie *et al.,* (2001) suggested that OsHKT1 encodes a Na^+-transporter and OsHKT2 a Na^+/K^+-coupled transporter. Garciadeblás et al., (2003) also showed that OsHKT1 (recently, OsHKT2;1) could be a high affinity Na^+-transporter and OsHKT4 (recently, OsHKT1;1) a low affinity Na^+-transporter. SKC1 (recently, OsHKT1;5) has recently been shown to be a Na^+-transporter, but contributing to the increased ability of salt tolerance by maintaining shoot K^+ homeostasis under salt stress (Ren *et al.,* 2005). This is orthologous to the function of AtHKT1 gene in Arabidopsis, which is a Na^+-transporter, and interestingly, plays a very important role in controlling the cytosolic Na^+ detoxification (Sunarpi *et al.,* 2005). AtHKT1 (recently, AtHKT1;1) functions in mediating tolerance to salt stress by unloading Na^+ from xylem vessels to xylem parenchyma cells and thus, protecting the plant leaves from salt stress (Sunarpi *et al.,* 2005). This transporter might also be responsible for unloading of Na^+ from the phloem (Berthomieu *et al.,* 2003). Therefore, it is very likely that the HKT gene family in rice has an

important role for plant ion homeostasis even though some of the members evidently transport Na^+. Apart from these NSCCs and HKTs, other transport proteins that might be involved in mediating Na^+ influx under salinity stress are HAK/KT/KUP-type transporters, inward-rectifying potassium channels, and low-affinity cation transporters of the LCT-1 type (Golldack *et al.,* 2003).

1.5 Salinity tolerance mechanisms in plants

Many studies have revealed some of the mechanisms evolved by plants to adjust their physiology and metabolisms to salt stress. First, Na^+ entry to plants may be restricted by selective ion uptake. Second, internalized Na^+ can be stored in vacuoles. Third, Na^+ in the cytosol may be exported back to the growth medium or to apoplastic space. Fourth, the Na^+ recirculation from shoots into roots by the phloem sap, limiting Na^+ accumulation in leaves, could be important for salt tolerance. Finally, plants have also developed an antioxidant defense system to cope with oxidative stress caused by high concentration salts in soil (Zhu, 2001; Gao *et al.,* 2003).

Under saline conditions the protection of Na^+-sensitive metabolic mechanisms in cell cytosol partly depends on the ability to keep cytosolic Na^+ levels low (Carden *et al.,* 2003). For plant cells, the most important way of keeping cytosolic Na^+ concentration at a low level is to minimize Na^+-influx into the cytosol. Na^+ entry into plant cells may be restricted either by down-regulating or inactivating the Na^+-influx channels and transporters in cells. Once Na^+ enters the cytosol at a toxic level, plant cells can deal with the internal Na^+ by sequestering it either into the apoplast or into the vacuole. The SOS pathway, has been extensively studied as it plays an important role for maintaining Na^+ homeostasis in the cytosol. The plasma membrane Na^+/H^+ antiporter, SOS1, is the primary transport system responsible for cellular Na^+-efflux (Zhu, 2002; Zhu 2003). Overexpression of *SOS1* increases the salt tolerance in Arabidopsis (Zhu, 2002; Shi *et al.,* 2003).

In this species it controls Na^+ loading into the xylem of the root and thus, restricts the accumulation of Na^+ in the shoot (Shi *et al.,* 2002).

Na^+ compartmentalization into vacuoles is an efficient strategy for plant cells to cope with salinity stress (Fukuda *et al.,* 1998, 2004; Apse *et al.,* 1999; Blumwald, 2000; Tester and Davenport, 2003). Once compartmentalized into the vacuole, Na^+ is no more toxic for cells (Subbarao *et al.,* 2003) and has an advantage for growth via osmotic adjustment (Zhu, 2003; Rodriguez-Navarro and Rubio, 2006). The tonoplast Na^+/H^+ antiporter, NHX1, has been established to control the vacuolar sequestration of excess Na^+ and/or K^+ (Fukuda *et al.,* 2004).

Oxidative stress is caused by accumulation of ROS that damage membrane lipids, proteins, and nucleic acids. The protection of plants against salt-induced ROS is achieved by means of different strategies including, in particular, partial suppression of ROS production, and scavenging of ROS already produced. Scavenging of ROS is achieved by a complex interplay between different enzymatic and non-enzymatic molecules. These include compounds, such as ascorbate, glutathione, polyamines, carotenoids and metallothioneins, as well as antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and ascorbate peroxidase (APX) (Zhu, 2001).

1.6 Plant biotechnology for developing salinity tolerant plants

Conventional approaches to breeding crop plants with improved stress tolerance have thus far met with limited success because of the difficulty of breeding tolerance associated traits from diverse plant backgrounds. Hence, an increasing emphasis has been directed to molecular strategies aimed at enhancing the intrinsic ability of plants to survive under stress conditions. Current approaches proposed to date focus attention on identification of genes associated with salinity, drought and other abiotic stress resistance, followed by genetic modification of the plants

expressing genes enabling them to withstand restrictive growth imposed by unfavorable environmental conditions (Sreenivasulu *et al.,* 2007).

Agricultural biotechnology can provide powerful solutions to the problem of maintaining agricultural productivity in saline soils. Application of crop biotechnology can expedite the development of new crop varieties that would be more tolerant to physical stresses such as saline soils, and drought, in an environmentally friendly way (Arzani, 2008). However, because of the complexity of salt tolerance, it is hard to assume that transferring a single gene encoding a single specific stress protein to a transgenic plant could dramatically improve salt tolerance in plants (Wang *et al.,* 2003).

In recent years, many transgenic crop varieties have been developed through both over-production of compatible solutes, and overexpression of a vacuolar Na^+/H^+ antiporter gene in transgenic plants. For instance, transgenic crops including rice (Mohanty *et al.,* 2002), wheat (Abebe *et al.,* 2003) and tobacco (Hong *et al.,* 2000) have been generated by overexpressing compatible osmolytes synthesizing genes in these plants. The resultant transgenic plants have been reported to show better germination, seedling growth and seed production under high salt and osmotic stresses. Likewise, transgenic plants have been generated by over expressing the Na^+/H^+ antiporter gene in Arabidopsis (Apse *et al.,* 1999), tomato (Zhang and Blumwald, 2001), *Brassica napus* (Zhang *et al.,* 2001), maize (Yan *et al.,* 2004), wheat (Xue *et al.,* 2004) and tobacco (Wang *et al.,* 2004). These transgenic plants exhibited improved salt tolerance compared to their wild type progenitors.

1.7 Study rationale

With higher population densities in developing countries, there is an urgent need to increase productivity of cultivated land. This makes also understanding of environmental stress phenomena

and related tolerant mechanisms more important in countries such as Egypt. Soil salinity is one of the major problems for agriculture in semi-arid regions. In Egypt, plants are subjected to extreme climatic factors such as high temperatures and drought. Under these conditions, dissolved salts may accumulate in soils because of the insufficient leaching of ions. An accumulation of salts in upper soil layers may be also due to an unsuitable irrigation management. Challenges faced by crop plants cultivated in the presence of excess salts are disturbance of osmotic regulation, ion imbalance, and oxidative stress, which impair plant metabolism and growth. Rice is one of the most important crops in Egypt and its production plays a significant role in the strategy to overcome food shortage and improvement of self-sufficiency for local consumption and export. In 2005 season, the total rice production in Egypt reached 6.6 million tons with a national average of 10.0 tons/ha (Badawi, 2005).

An experiment has been conducted under normal and drought conditions to examine the magnitude of yield response of diverse genotypes to drought stress and to identify traits that may confer drought resistance including 18 Egyptian, six Italian and nine Chinese rice varieties (Abd Allah *et al.,* 2010). Among the Egyptian rice cultivars, Giza 178 and Giza 182 were proved to be drought-tolerant cultivars, while Sakha 104 was shown to be drought-sensitive in terms of agronomic traits. Kandil *et al.,* (2012), investigated the response of ten Egyptian rice cultivars (Giza 178, Giza 181, Giza 182, Sakha 102, Sakha 104, Sakha 105, Sakha 106, Egyptian Yasmine, Egyptian Hybrid 1, Egyptian Hybrid 2) to germination under salinity stress conditions to confirm growth performance and to examine a range of genetic variability for salinity tolerance among the rice cultivars under seven salinity levels. The results showed that rice cultivars significantly varied in means of final germination percentage, germination rate, germination index, vigor index, root and shoot lengths, root and shoot fresh weights, root dry weight, relative dry weight and seedling

height reduction. Moreover, the highest germination percentage and the enhance growth parameters under salinity stress were in Sakha 102, Sakha 106, Sakha 104 and Egyptian Yasmine cultivars, and that these cultivars were more tolerant to salinity. However, the authors did not estimate the Na^+ and K^+ contents in the studied cultivars. Another study showed the effect of salt stress on phospholipid signaling responses in the leaves of the Egyptian rice cultivars Sakha 102 and Sakha 104 (Darwish *et al.,* 2009). Under both long- and short-term exposure to salinity stress in hydroponics, Sakha 102 appeared to be salinity-sensitive, while Sakha 104 was shown to be salinity-tolerant. Moreover, the results showed that salt stress rapidly activates several lipid responses in rice leaves but that these responses do not explain the difference in salt tolerance between sensitive and tolerant cultivars. A study conducted by Shehata *et al.,* (2009) showed the development of salt tolerant rice lines through mutation breeding. That investigation dealt with the development of early maturity, non-lodging and high yielding with salinity tolerance, employing a mutation approach of gamma rays irradiation on traditional Egyptian rice cultivars; Sakha 101, Sakha 102, Egyptian Yasmine and the line, AC 1453. Four mutant lines, Sakha 101-M30, Sakha 102-M20, AC- M50 and Egyptian Yasmine M30 were developed. These mutants were tested for yield performance in the salt-affected soils with a pH 8.00 to 8.20 and EC = 6.00 to 6.50 dS m^{-1}. Mutant lines were highly promising and far better than their respective control in the areas affected by salinity conditions and produced higher paddy rice yield on salt-affected soils than the commercial cultivar, Giza 178. In addition maintaining many desirable traits, such as early maturity, non-lodging, quality traits and blast resistance, comparing with their respective control.

From all the previously mentioned reports, the response of the Egyptian rice cultivars to salinity stress has not been yet clearly established. Up to date, there are no reports or information published regarding the regulatory mechanisms of salinity stress tolerance in those cultivars.

Therefore, the objective of this study was to characterize the physiological responses and adaptation mechanisms to salinity stress in some Egyptian rice cultivars by comparing the physiological parameters, Na^+ accumulation patterns and expression profiles of the genes that encode Na^+ and K^+ transport proteins. Moreover, further elucidation of salinity-tolerance mechanisms in the salinity-tolerant cultivar through the isolation and characterization of salinity-inducible genes which might function in other tolerance pathways under salinity stress conditions, thus could be another adaptation strategy to abiotic stress in this cultivar.

1.8 Study objectives

1. Investigating the physiological responses and mode of adaptation to salinity stress in the Egyptian rice cultivars, Sakha 102 and Egyptian Yasmine by comparing the growth criteria, Na^+ and K^+ accumulation patterns and other physiological parameters under salinity stress conditions in a hydroponic culture system.

2. Elucidation of differences in the mechanisms of salinity tolerance (Na^+ and /or K^+ accumulation patterns), between the salinity-tolerant and salinity-sensitive rice cultivars by analyzing the expression profiles of the genes that encode Na^+ and/or K^+ transport proteins.

3. Further elucidation of the molecular mechanisms of salinity tolerance in the salinity-tolerant rice cultivar, through the isolation and characterization of salinity-inducible genes and examine their functional roles which could be involved in other adaptation processes under salinity stress conditions.

Chapter 2

Growth, physiological adaptation and gene expression analysis of two Egyptian rice cultivars under salt stress

2.1 Introduction

Soil salinity is a major environmental problem that has deleterious effects on world agriculture, especially in irrigated lands (Flowers, 1999). Salinity stress causes serious damage to many cellular and physiological processes including photosynthesis, nutrient uptake, water absorption, root growth, and cellular metabolism, which all lead to yield reduction (Pardo, 2010). Furthermore, excess Na^+ causes an imbalance in cellular ion homeostasis, resulting in ion toxicity (Mandhania *et al.,* 2006; Assaha *et al.,* 2013). Cellular Na^+ and K^+ homeostasis plays a fundamental role in the growth and development of higher plants. As one of the most important macronutrients in plants, K^+ is necessary for the maintenance of membrane potential and turgor pressure, activation of enzymes, regulation of osmotic pressure, stomatal movement, and tropisms (Golldack *et al.,* 2003). In contrast to animal cells, Na^+ is not an essential element for plant cells; therefore, high K^+/Na^+ ratios maintain osmotic balance in plant cells.

Genes from many functional classes including those encoding transcription factors, signal transduction, cell wall components, and membrane transporters were found to be differentially regulated in response to salt stress (Walia *et al.,* 2007; Ueda *et al.,* 2002, 2004, 2006).

The transmembrane movement of Na^+ and K^+ in plants is mediated by several types of transporters and/or channels (Yao *et al.,* 2010), and a number of transporters have been implicated in leaf Na^+ exclusion. These include members of the high-affinity K^+ transporters (HKTs), including *Arabidopsis thaliana* HKT (AtHKT1;1) and its ortholog in rice (OsHKT1;5), which retrieves Na^+ from the xylem to the surrounding parenchyma cells (Ren *et al.,* 2005; Horie *et al.,* 2009). Plasma membrane protein 3 (PMP3) is a small hydrophobic peptide that plays a role in shoot Na^+ exclusion by preventing excess Na^+ entry into the plant roots (Nylander *et al.,* 2001). In addition to Na^+ exclusion, plants may avoid toxic Na^+ accumulation in the cytosol by sequestering

excess Na^+ into vacuoles, which is a process mediated by the Na^+/H^+ antiporter (NHX1) localized in vacuolar membranes (Venema *et al.,* 2002). However, these transporters only function to counteract the activities of other transporters that are known to induce Na^+ influx into roots. This may occur, through cyclic nucleotide-gated channels (CNGCs), which are considered the dominant pathways of Na^+ influx in many plants (Roberts and Tester, 1997). Some members of the HKT family are also thought to mediate a substantial Na^+ influx in some species, including rice (Golldack *et al.,* 2003).

K^+ is the most abundant cation in plants, and it is an essential nutrient for plant growth and development. Under salinity stress conditions, plants suffer from K^+ deficiencies stemming from the competitive inhibition of its uptake by Na^+, and this often leads to high Na^+/K^+ ratios that disrupt cellular homeostasis (Tester and Davenport, 2003). However, potassium inward rectifying channels (KIRC) (e.g., AKT1) have high K^+/Na^+ selectivity and are known to activate K^+ influx upon plasma membrane hyperpolarization (Sentenac *et al.,* 1992). The activity of the AKT1 channel is important in maintaining elevated K^+/Na^+ ratios in plants. Thus, the activities of the transporters/channels involved in Na^+ exclusion and K^+ uptake are important to the improvement of salt stress tolerance in plants.

In Egypt, the total rice cultivation area is 651,323 hectare. Due to the intrusion of sea-water, most of agricultural lands in the northern Nile Delta where rice cultivation takes place are affected by different degrees of salinity (Arafat *et al.,* 2010), thus affecting rice production in growing areas in Egypt. Since the development of salt tolerant varieties has been considered as one of the strategies to increase rice production in saline prone areas (Kandil *et al.,* 2012), it is important to assess the physiological adaptation of Egyptian rice cultivars.

The traditional Egyptian rice cultivars; Sakha 102 and Egyptian Yasmine are important local rice cultivars which have superiority over other local ones due to their exclusive quality; early maturity, non-lodging and high yielding. Thus, as soil salinization is becoming increasingly challenge for agriculture in Egypt, it is imperative to seek ways of improving these cultivars to withstand salt stress. Whereas Sakha 102 has been established as a salt-sensitive cultivar (Darwish *et al.,* 2009), the response of Egyptian Yasmine has not been clearly established. Therefore, the objective of this study was to investigate the physiological responses to salinity stress in the cultivars Sakha 102 and Egyptian Yasmine and to elucidate differences in the mechanisms of salinity tolerance between the two cultivars, by comparing the physiological parameters, Na^+ and K^+ accumulation patterns and expression profiles of the genes that encode Na^+ and K^+ transport proteins under salinity stress conditions.

2.2 Materials and Methods

2.2.1 Plant material, growth conditions, and salt treatment

The Egyptian rice cultivars Sakha 102 and Egyptian Yasmine used in this study were obtained from the Field Crops Research Institute, Giza, Egypt. These genotypes were chosen from the germplasm collections based on their reputation in terms of agronomic performance. The subset of the germplasm samples included the subspecies *O. sativa* ssp. *indica* and *O. sativa* ssp. *japonica*. Seeds were surface sterilized via immersion in a 5% NaClO solution for 30 min, and were then thoroughly rinsed with distilled water. Seeds were subsequently soaked in tap water for 24 h at 28 ºC. After germination, the seeds were transferred to a nylon mesh floating on 20 L of tap water for two days. Water was then replaced with half-strength Kimura B solution (0.18 mM $(NH_4)_2SO_4$, 0.27 mM $MgSO_4 \cdot 7H_2O$, 0.09 mM KNO_3, 0.18 mM $Ca(NO_3)_2 \cdot 4H_2O$, and 0.09 mM KH_2PO_4, 20 µM NaEDTA-$Fe \cdot 3H_2O$, 6.7 µM $MnCl_2 \cdot 4H_2O$, 9.4 µM H_3BO_3, 0.015 µM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$,

0.15 µM $ZnSO_4 \cdot 7H_2O$, and 0.16 µM $CuSO_4 \cdot 5H_2O$). Twenty eight-day-old seedlings (4-5 leaf stage) were transferred to either Kimura B nutrient solution (control) or nutrient solution supplemented with 50 mM NaCl (salinity) for two weeks. The solutions were replaced every two days and the pH was adjusted to 5.0-5.5 each day. Seedlings were grown in a growth chamber under the following controlled environmental conditions: 70% relative humidity, 24 ± 2 ºC, and a 16 h photoperiod at a photosynthetic photon flux density of 250-350 µmol m^{-2} s^{-1}.

2.2.2 Measurement of fresh and dry weight, relative water content, and electrolyte leakage ratio

The fresh weight (FW) was measured following the separation of leaves, stems, and roots. For dry weight (DW) determination, leaves, stems, and roots were dried at 70°C for three days prior to being weighed. To determine the relative water content (RWC), four plants from each treatment were randomly selected and the method described by Weatherly, (1950) was implemented. Briefly, leaf samples were weighed to determine the FW. The leaf samples were then soaked in fresh deionized water for 24 h under light, and were placed on tissue paper to remove excess water. The samples were then weighed to determine the fully turgid weight (TW). Samples were next oven-dried at 70ºC for three days, and the DW was obtained. The RWC was determined using the following formula: RWC = (FW - DW)/(TW - DW) × 100.

To determine the electrolyte leakage ratio (ELR), the third leaf from the top of each plant was dissected and soaked in a bottle containing 30 mL of deionized water, and the bottles were gently shaken overnight. Conductivity of the solution was measured with an EC meter (EC1). The bottles were then autoclaved and cooled, and the conductivity of the solution was again measured (EC2). The ELR was calculated as the ratio of the conductivity before autoclaving to the conductivity after autoclaving using the following formula: ELR = (EC1/EC2) × 100.

2.2.3 Determination of Na^+ and K^+ content

The Na^+ and K^+ content in leaves, sheaths, and roots was measured using a flame photometer (ANA-135; Tokyo Photoelectric, Tokyo, Japan) according to the method of Kushizaki, (1968). Dried samples were gently agitated in 1N HCl overnight, and the content of Na^+ and K^+ was estimated from the Na^+ and K^+ standard curves.

2.2.4 Measurement of proline content

Free proline was determined as previously described by Bates et al., (1973) with some modifications. Proline was extracted from about 200 mg fresh leaves in 4 mL of 3% sulfosalicylic acid. Two mL of the extract was then mixed with 2 mL ninhydrin reagent, containing glacial acetic acid and incubated at 100 °C for 40 min. The reaction mixture was quickly cooled in an ice water bath. After cooling, 4 mL toluene was added for chromophore development. The absorbance of the chromophore was measured at 520 nm.

2.2.5 Expression analysis of genes encoding Na^+/K^+ transport proteins

Total RNA was extracted from the leaves, sheaths, and roots of the control and stressed plants using TRIzol reagent (Invitrogen, Carlsbad, CA). After digestion with DNaseI, total RNA (1 µg) was reverse-transcribed to cDNA using a ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). Quantitative polymerase chain reaction (qPCR) was conducted as previously described (Ueda *et al.,* 2013), using a Thunderbird SYBR qPCR Mix (Toyobo) and an ABI Step One Plus system (Applied Biosystems). RT-qPCR was performed using the following profile: an initial incubation at 95ºC for 1 min, followed by 40 cycles with 15 sec at 95ºC and 60 sec at 60ºC. Relative abundance of genes transcripts were calculated with the comparative $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). Expression levels were represented as fold changes relative to the respective

control. The relative expression level of the genes was normalized to the expression level of *OsUBQ-5* as an internal control (Jain *et al.,* 2006). The sequences of the primers used were listed in Table 1. The PCR products were verified via a melting curve analysis by changing the temperature from 60ºC to 95ºC to monitor dissociation of double strand DNA and by running an agarose gel electrophoresis. Amplification efficiencies (*E*) of the primers were determined using the standard curve method and calculated according to the equation $E\ (\%) = (10^{-1/\text{slope}} - 1) \times 100$. All primers showed *E* values of more than 90%.

2.2.6 Statistical analysis

The collected data were subjected to one-way analysis of variance (ANOVA) using the SPSS statistics package, version 21 (IBM Inc., USA), and the means ($n = 4$) were separated using Duncan's multiple range test at $p = 0.05$.

2.3 Results

2.3.1 Effect of salt stress on biomass production, relative water content, and electrolyte leakage ratio

After 14 days of salt treatment (50 mM NaCl), the visual symptoms of salt toxicity on leaves (whitish leaf tips, leaf rolling, dried or dead leaves) of both cultivars were photographed (Figure 2.1). The cultivar Sakha 102 appeared to be salinity-sensitive, being most of the leaves were rolled, dried or died with retarded growth (Figure 2.1A), while Egyptian Yasmine showed to be more tolerant to salinity stress and maintained healthy expanded green leaves with few whitish leaf tips and vigor growth (Figure 2.1B). The dry weight (DW) of both cultivars was affected by salt stress treatment (Figure 2.2). Under 50 mM NaCl stress conditions, the leaf DW of Sakha 102 was drastically decreased by 64% in comparison with that of Egyptian Yasmine (35%). The sheath DW was not significantly affected by salt stress in either cultivar. However, the root DW decreased by 49% in Sakha 102, but it increased by 7% in Egyptian Yasmine under salt stress conditions (Figure 2.2).

To estimate the amount of water lost under salinity conditions, the relative water content (RWC) was measured using leaf tissues. The results indicated that Egyptian Yasmine exhibited a greater potential to maintain tissue water than Sakha 102. Under 50 mM NaCl stress conditions, the RWC of Sakha 102 plants decreased from 97% in control plants to 71% in experimental plants. However, there was no significant difference in the RWC of Egyptian Yasmine between the control (88%) and salinity-treated plants (86%) (Figure 2.3A).

The electrolyte leakage ratio (ELR) was measured to examine cell membrane stability under salinity stress. The results showed that Egyptian Yasmine exhibited a lower electrolyte leakage rate (27.5%) than Sakha 102 (51.9%) in response to salt stress (Figure 2.3B). These results

indicated that Egyptian Yasmine maintained a better physiological status than Sakha 102 under salinity conditions.

2.3.2 Effect of salt stress on Na^+ and K^+ accumulation in different tissues

In both cultivars, salinity treatment led to increased Na^+ content in all tissues examined (Figure 2.4). In the leaves, the increase in Na^+ content of Sakha 102 was nearly two-times higher than that of Egyptian Yasmine (Figure 2.4A). In the sheaths, there was no significant difference in Na^+ content between the two cultivars (Figure 2.4B), but there was a slight increase in Na^+ content in the roots of Egyptian Yasmine (Figure 2.4C).

Salinity stress did not significantly affect the leaf K^+ content of the two cultivars (Figure 2.5A); however, there was a significant decrease in K^+ content in the sheaths of both cultivars (Figure 2.5B). Moreover, there was a significant increase in K^+ content in the roots of the two cultivars under salt stress conditions (Figure 2.5C). Notably, under both control and salt stress conditions, Egyptian Yasmine accumulated a higher concentration of K^+ in the roots than Sakha 102 (Figure 2.5C). Consequently, a higher Na^+/K^+ ratio was observed in the leaves, sheaths, and roots of Sakha 102 compared to Egyptian Yasmine (Figure 2.6A, B, C).

2.3.3 Effect of salt stress on proline accumulation

In response to salinity stress, a significant increase of proline content was observed in the leaves of Egyptian Yasmine (21%), while no significant change was observed in the Sakha 102 leaf proline content. Notably, under both control and salt stress conditions, Egyptian Yasmine accumulated a higher amount of proline than Sakha 102 (Figure 2.7). The greater accumulation of proline in Egyptian Yasmine might partially contribute to cellular osmotic adjustment under high salinity conditions and indicating a better physiological adaptation to salinity stress.

2.3.4 Differential expression of genes encoding Na^+/K^+ transport proteins in response to salt stress

To determine the mechanisms underlying differential Na^+ and K^+ accumulation in the salinity-tolerant Egyptian Yasmine and the salinity-sensitive Sakha 102, expression profiles of the genes encoding Na^+ and K^+ transport proteins were analyzed. Although the rice genome has divergent transport systems for monovalent cations, functionally identified Na^+ and K^+ transport proteins were chosen for gene expression analyses. The main site of Na^+ toxicity is in the shoots, where Na^+ accumulates and disrupts metabolic processes. A Na^+ transporter, OsHKT1;5 functions in the root xylem parenchyma to retrieve Na^+ from the xylem stream, thereby reducing Na^+ accumulation in the shoots (Ren *et al.,* 2005). In the present study, quantitative RT-PCR analyses showed that salt stress induced the expression of the *OsHKT1;5* gene by 4.8-fold in the roots of Egyptian Yasmine, but it was repressed by 0.3-fold in Sakha 102 (Figure 2.8A). This repression of *OsHKT1;5* in the roots may cause increased Na^+ accumulation in the leaves of Sakha 102 when under salt stress (Figure 2.4A). Also, *OsHKT1;5* was repressed in the leaves and sheaths of both cultivars.

To determine the physiological role of the OsHKT2;1 transporter in the rice cultivars under salt stress conditions, expression analyses were performed for the *OsHKT2;1* gene. We found that there was repression of *OsHKT2;1* expression in all plants parts, particularly in the roots (0.04-fold), for the two studied cultivars. Expression of *OsHKT2;1* was repressed in the sheaths of Sakha 102 (0.3-fold) and Egyptian Yasmine (0.4-fold). Furthermore, *OsHKT2;1* was also repressed in the leaves of Sakha 102 (0.8-fold) and Egyptian Yasmine (0.3-fold) (Figure 2.8B).

Plant PMP3 proteins, which are relatively small sized (approx. 56 aa), were found to be involved in the prevention of excess Na^+ entry in yeast and *Arabidopsis* (Nylander *et al.,* 2001).

The rice genome has two homologous PMP3 genes, *OsLti6a* and *OsLti6b*. In response to salinity stress, repression of *OsLti6a* expression was observed in the roots and sheaths of both cultivars (less than 1.0-fold) and in the leaves of Egyptian Yasmine (0.5-fold), while in Sakha 102 leaves, the gene expression did not change in response to salt stress (1.1-fold) (Figure 2.8C). Induced expression of *OsLti6b* in the roots of Egyptian Yasmine (2.5-fold) was observed, while the expression of the gene was unaltered in the Sakha 102 roots (1.1-fold) under salt stress (Figure 2.8D). However, salinity stress caused repression of *OsLti6b* expression in the sheaths and leaves of both cultivars (less than 1.0-fold) (Figure 2.8D). These results indicated the role of rice PMP3 genes in restricting excess Na^+ influx to the roots of Egyptian Yasmine and consequently lower Na^+ levels in its leaves (Figure 2.4A).

The vacuolar Na^+, K^+/H^+ antiporter, OsNHX1, plays an important role in the compartmentalization of the highly accumulated cytosolic Na^+ and K^+ into the vacuoles (Fukuda *et al.,* 2004). The expression of this *OsNHX1* gene was regulated by the level of Na^+ in plants (Fukuda *et al.,* 1999). In the present study, *OsNHX1* was differentially expressed in the roots and shoots of both Sakha 102 and Egyptian Yasmine under salt stress conditions (Figure 2.8E). Whereas, salinity induced the expression of *OsNHX1* highly in the leaves of Sakha 102 (5.5-fold), the gene was repressed in Egyptian Yasmine leaves (0.5-fold). This difference in *OsNHX1* induction might be due to higher Na^+ levels in the leaves of Sakha 102. In the roots, *OsNHX1* expression did not change in response to salt stress in Sakha 102 (1.0-fold), but it was repressed in Egyptian Yasmine (0.7-fold). In the sheaths, *OsNHX1* was repressed in both cultivars (0.2-fold).

The Na^+/H^+ antiporter salt overly sensitive1 (SOS1), localized in the plasma membrane, is considered a general regulator of Na^+ export from cytosol (Shi *et al.,* 2002). Our results indicated that there was a higher level of induced expression of *OsSOS1* in the Sakha 102 roots (3.5-fold)

(Figure 2.8F), which might be responsible for the relatively low Na^+ levels in its roots under salt stress; however, *OsSOS1* expression in Egyptian Yasmine roots was not induced (1.2-fold). The repression of *OsSOS1* expression was observed in the leaves (0.2-fold) and sheaths (0.4-fold) of Egyptian Yasmine under salt stress conditions (Figure 2.8F), which suggested that OsSOS1-mediated Na^+ extrusion from the cytosol may not be active in Egyptian Yasmine.

Cyclic nucleotide gated channels, such as AtCNGC1, are potential candidates for nonselective channels that contribute to the ion-conducting pathway, which allows toxic levels of Na^+ to be taken up by plants from saline soils (Maathuis and Sanders, 2001). Our results revealed differential expression of the *OsCNGC1* gene in the roots, sheaths, and leaves of both cultivars (Figure 2.8G). Under salt stress, an induction of *OsCNGC1* expression in the roots of Sakha 102 (3.2-fold) was higher than that of Egyptian Yasmine (2.5-fold). In the sheaths, *OsCNGC1* was repressed in Sakha 102 (0.4-fold), and not significantly affected in Egyptian Yasmine (1.4-fold). In the leaves, there was induction of *OsCNGC1* expression in Sakha 102 (1.8-fold), and unaltered expression in Egyptian Yasmine (1.2-fold).

Salinity stress treatment induced the expression of *OsAKT1* highly in the leaves of Egyptian Yasmine (4.3-fold) compared to the expression in Sakha 102 leaves (1.7-fold), while in roots, the expression of *OsAKT1* was induced in Egyptian Yasmine (1.6-fold), but was repressed in Sakha 102 (0.8-fold). The expression of *OsAKT1* was repressed by salt stress treatment in the sheaths of both cultivars (0.3-fold) (Figure 2.9A). These results suggested that reduced growth and impaired K^+ homeostasis in salt-stressed seedlings of Sakha 102 might be a consequence of reduced K^+ influx via OsAKT1 channels.

In the current experiment, under salt stress conditions, *OsHAK7* expression was induced in the roots in both cultivars, with no significant differences between the two cultivars (2.9-fold in Sakha

102 and 2.6-fold in Egyptian Yasmine). However, in the leaves, it was highly induced in Sakha 102 (15.7-fold) compared to Egyptian Yasmine (3.2-fold) (Figure 2.9B). In the sheaths, *OsHAK7* expression was markedly induced in Egyptian Yasmine (30-fold), but was repressed in Sakha 102 (0.2-fold) (Figure 2.9B).

Table 1. Primers used for quantitative real-time RT-PCR.

Genes	Forward primer (5´-3´)	Reverse primer (5´-3´)
OsHKT1;5	CCCATCAACTACAGCGTCCT	AGCTGTACCCCGTGCTGA
OsLti6a	CCTTCCAAGGTGATGGTGAA	CCGTCCAAAGAACCAGAAAA
OsLti6b	GCTCCAAACCGCTTCATCTA	CAAGAATTGGAGCACTCAGGA
OsHKT2;1	TGCATTCATCACTGAGAGGAG	GGTGCAGTTTCTGCAACCTC
OsNHX1	AATGATCACCAGCACCATCA	AAGGCTCAGAGGTGACAGGA
OsSOS1	ATACTGAGTGGGGTTGTTATTGC	AAAGGTAAATTTCAAAAGGTACATGG
OsAKT1	GAAACGAGCAATGCGTCAG	CTTCTCACACAGCGCTTCC
OsHAK7	TGCTGTGACACTTGGTTTCC	AAATAACAAGGCGAGCAGGA
OsCNGC1	TGCAATAGCAAAGCGATACTTG	TTTTGGCTTTTGCAACCTCT
OsUBQ5	ACCACTTCGACCGCCACTACT	ACGCCTAAGCCTGCTGGTT

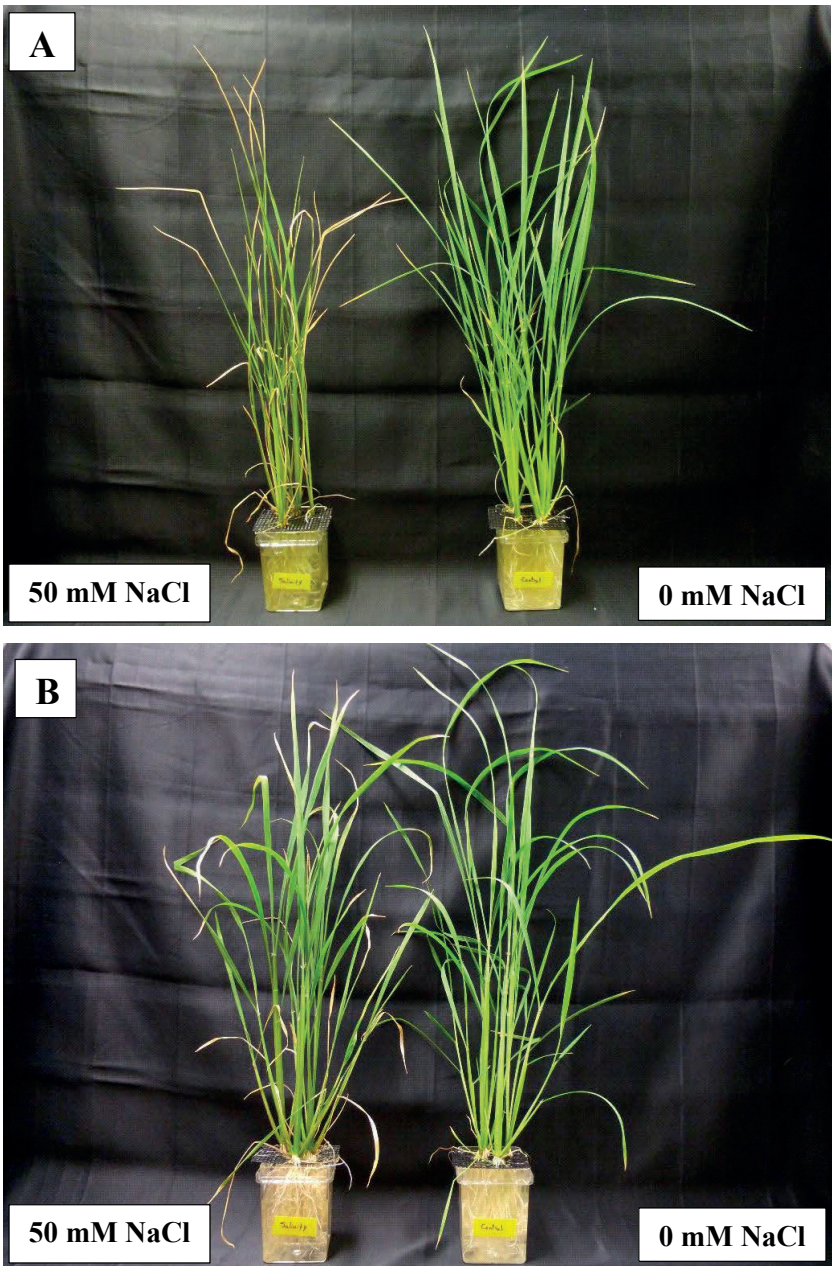


Figure 2.1. Effect of salinity stress on the growth of the rice cultivars, Sakha 102 (A) and Egyptian Yasmine (B) after 14 days treatment.

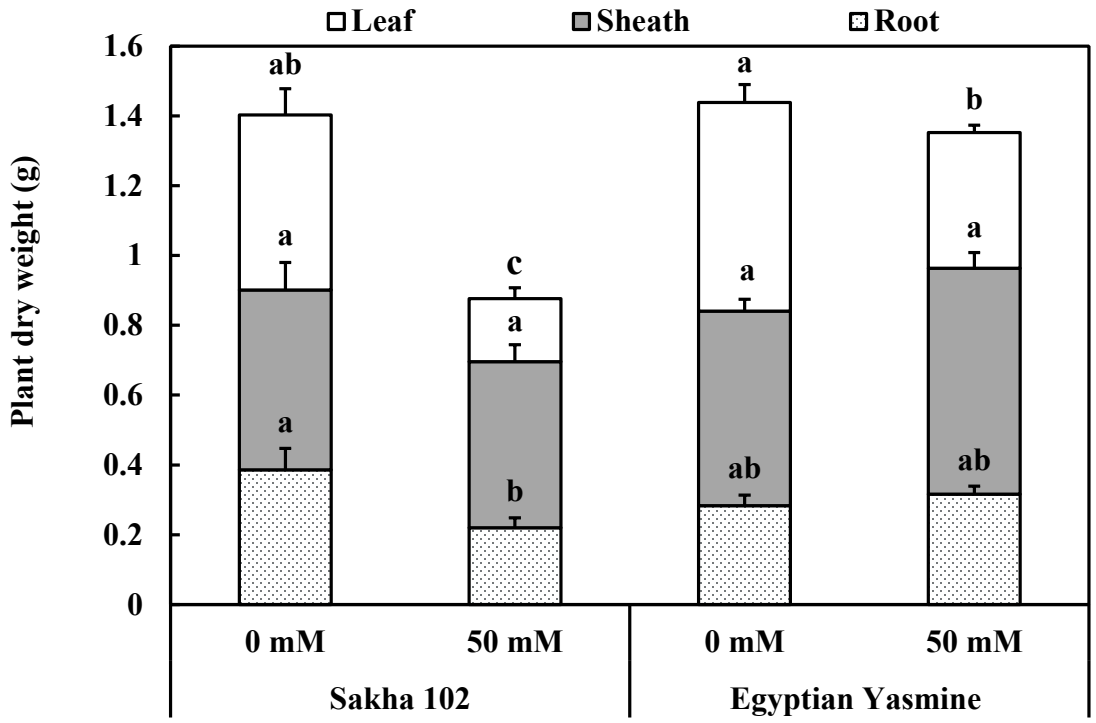


Figure 2.2. Plant dry weight (DW) of the rice cultivars Sakha 102 and Egyptian under control and salt stress condition (50 mM NaCl) for 14 days. Data represent the means of four replicates ± SE. The same letters indicate no significant differences ($P < 0.05$).

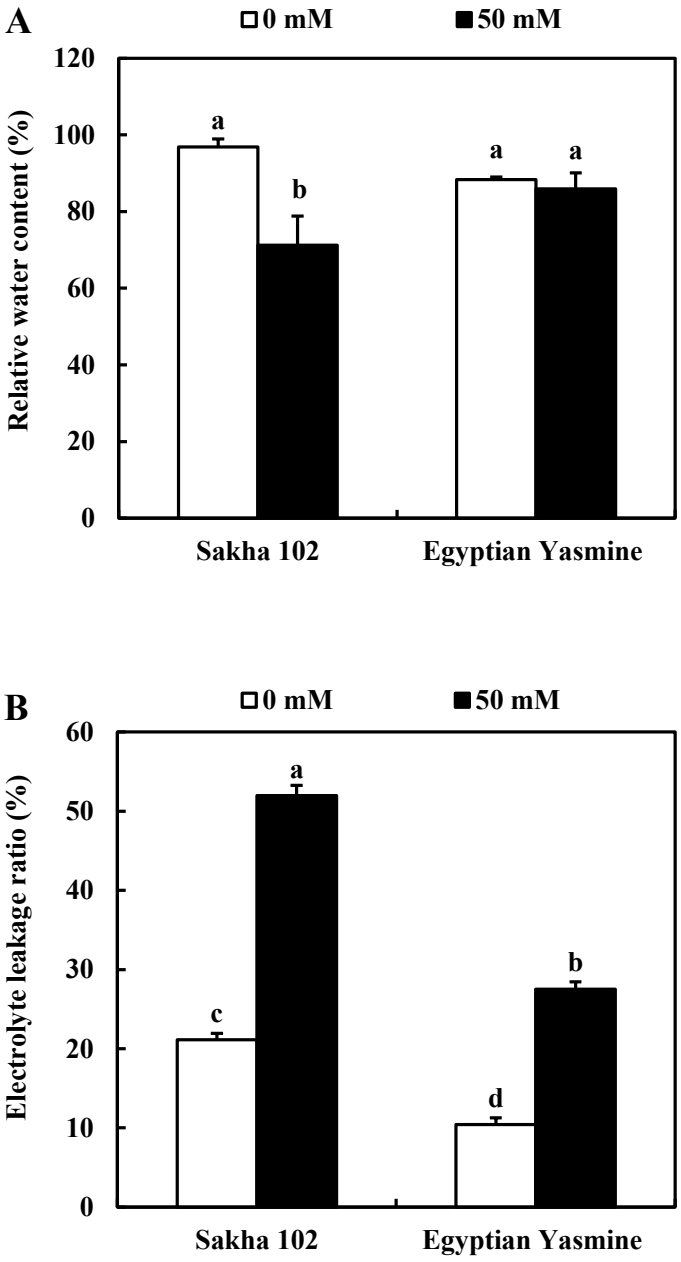


Figure 2.3. (A) Relative water content and (B) electrolyte leakage ratio (ELR) of the rice cultivars Sakha 102 and Egyptian Yasmine under control and salt stress condition (50 mM NaCl) for 14 days. Data represent the means of four replicates ± SE. The same letters indicate no significant differences ($P < 0.05$).

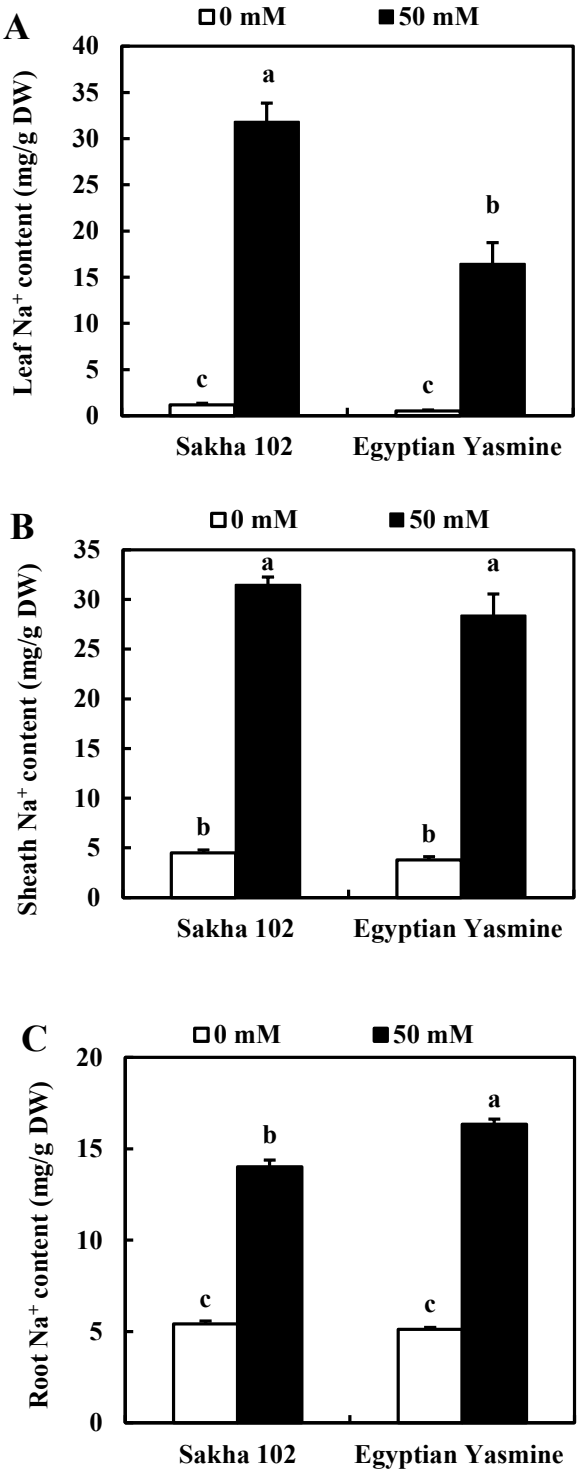


Figure 2.4. (A) Leaf Na^+ content, (B) sheath Na^+ content and (C) root Na^+ content of the rice cultivars Sakha 102 and Egyptian Yasmine under control and salt stress condition (50 mM NaCl) for 14 days. Data represent the means of four replicates ± SE. The same letters indicate no significant differences ($P < 0.05$).

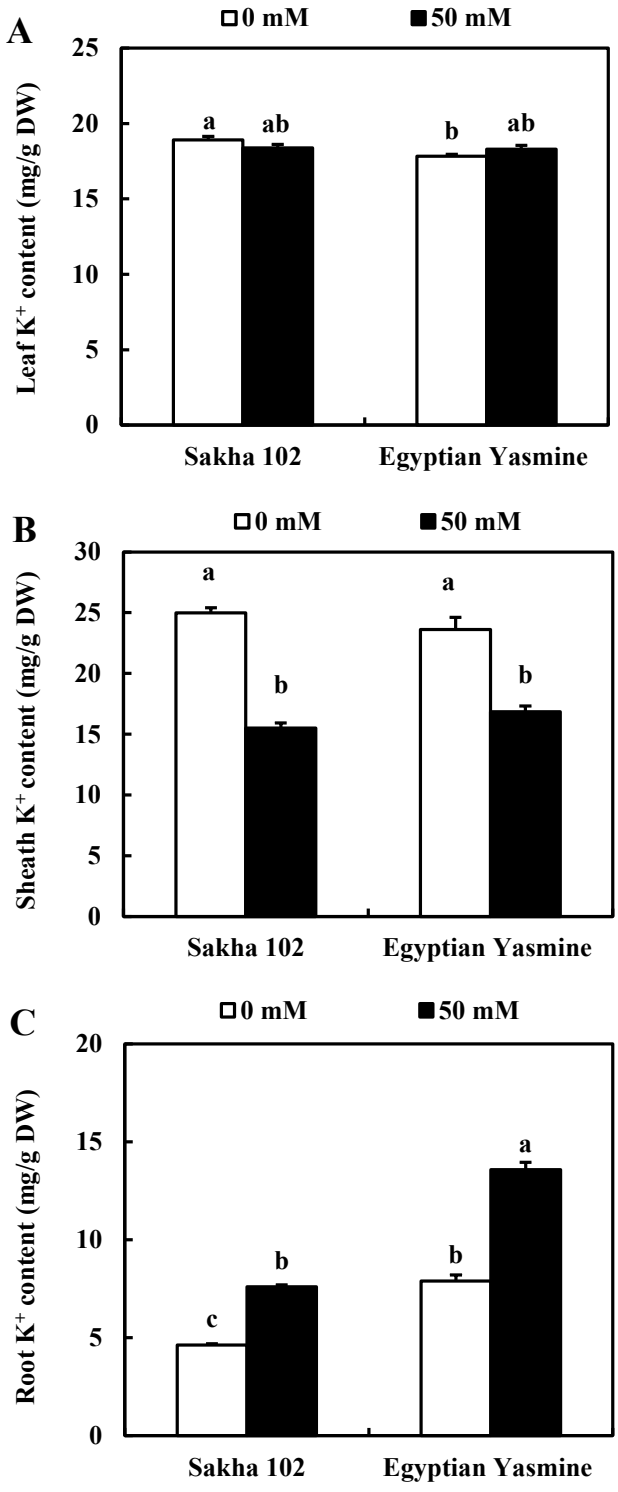


Figure 2.5. (A) Leaf K^+ content, (B) sheath K^+ content and (C) root K^+ content of the rice cultivars Sakha 102 and Egyptian Yasmine under control and salt stress condition (50 mM NaCl) for 14 days. Data represent the means of four replicates ± SE. The same letters indicate no significant differences ($P < 0.05$).

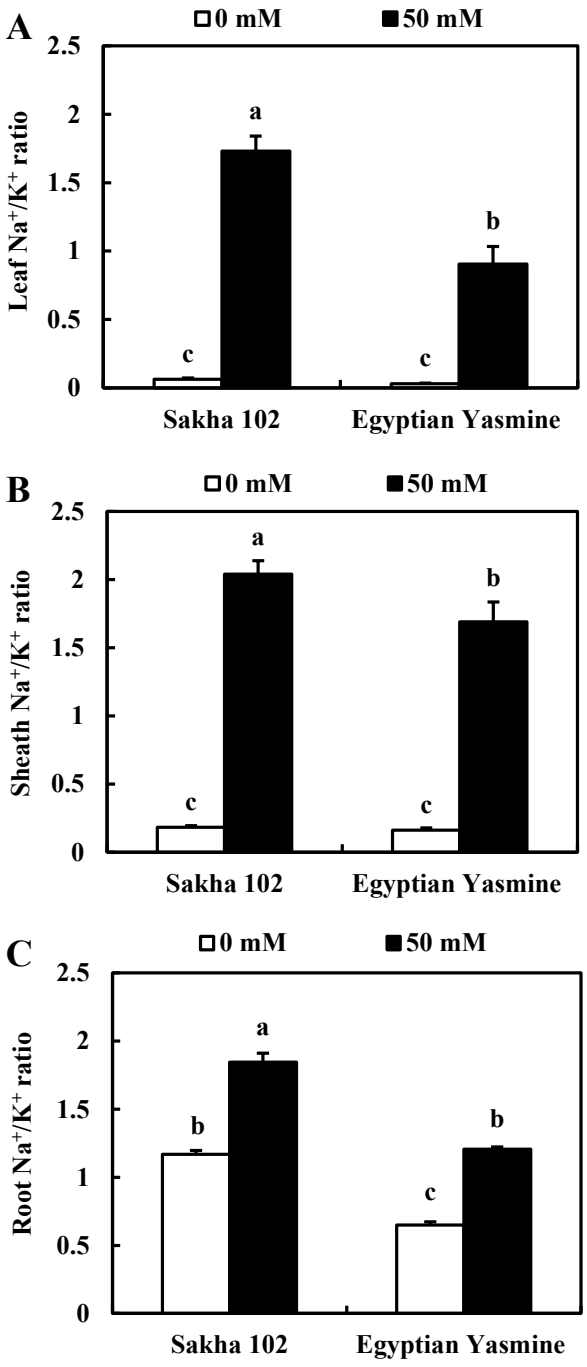


Figure 2.6. (A) Leaf Na^+/K^+ ratio, (B) sheath Na^+/K^+ ratio and (C) root Na^+/K^+ ratio of the rice cultivars Sakha 102 and Egyptian Yasmine under control and salt stress condition (50 mM NaCl) for 14 days. Data represent the means of four replicates ± SE. The same letters indicate no significant differences ($P < 0.05$).

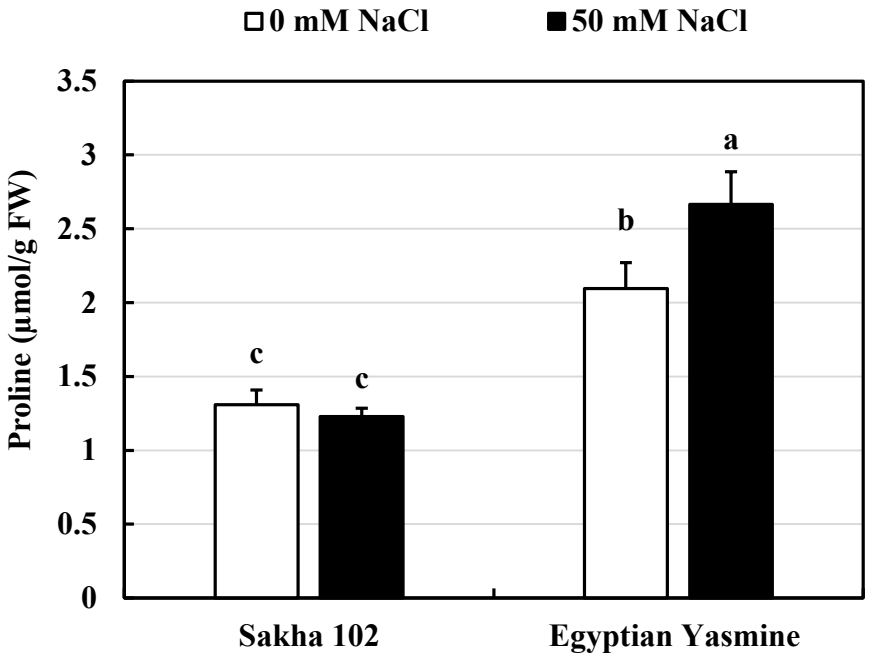


Figure 2.7. Proline content in the leaves of the rice cultivars Sakha 102 and Egyptian Yasmine under control and salt stress condition (50 mM NaCl) for 14 days. Data represent the means of four replicates ± SE. The same letters indicate no significant differences ($P < 0.05$).

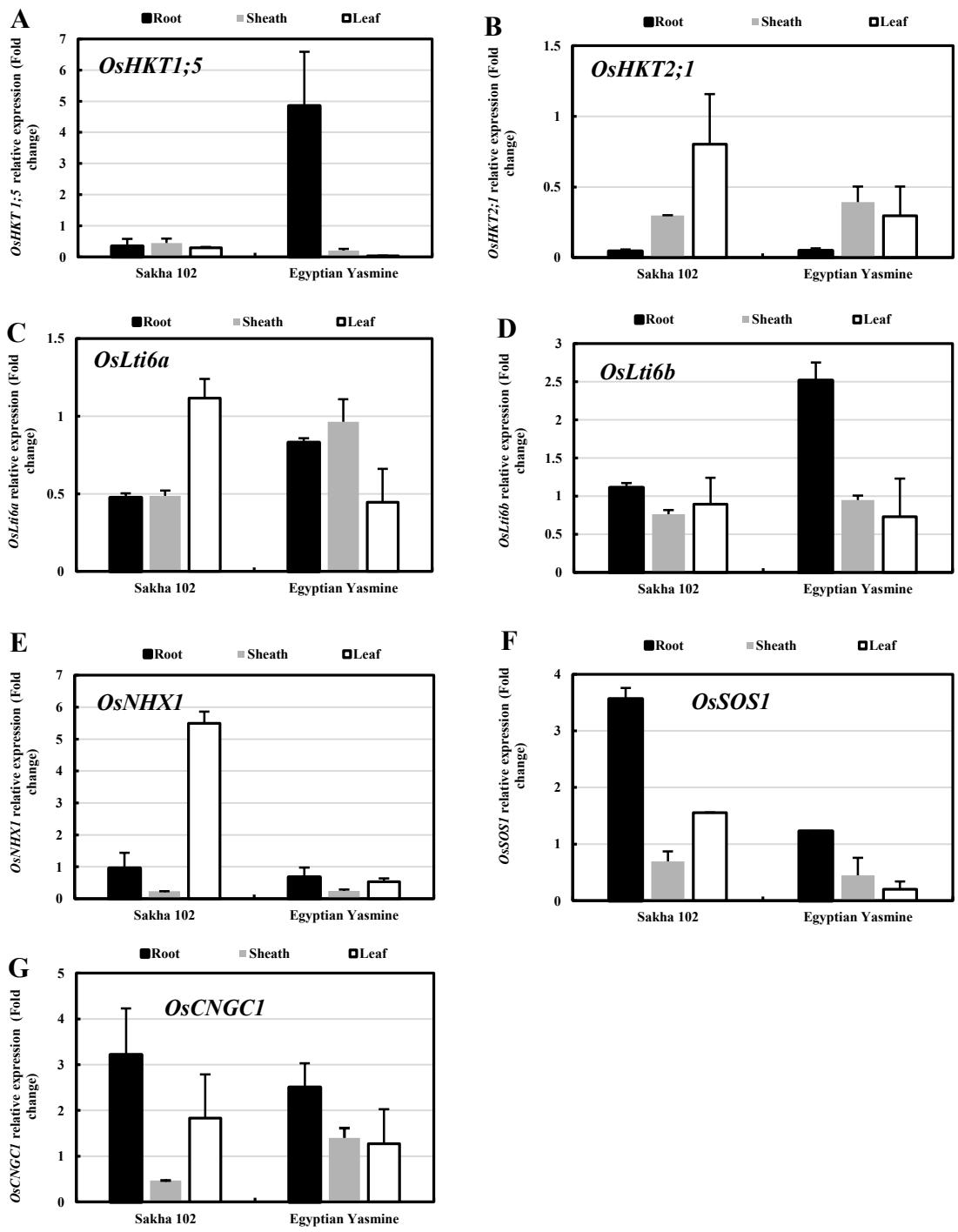


Figure 2.8. Relative expression of the genes encoding Na^+ transport proteins. (A) *OsHKT1;5*, (B) *OsHKT2;1*, (C) *OsLti6a*, (D) *OsLti6b*, (E) *OsNHX1*, (F) *OsSOS1*, and (G) *OsCNGC1* in the roots, sheaths and leaves of rice seedlings for the cultivars Sakha 102 and Egyptian Yasmine grown under control and salt stress condition (50 mM NaCl) for 14 days. Data represent the means of two independent experiments ± SD.

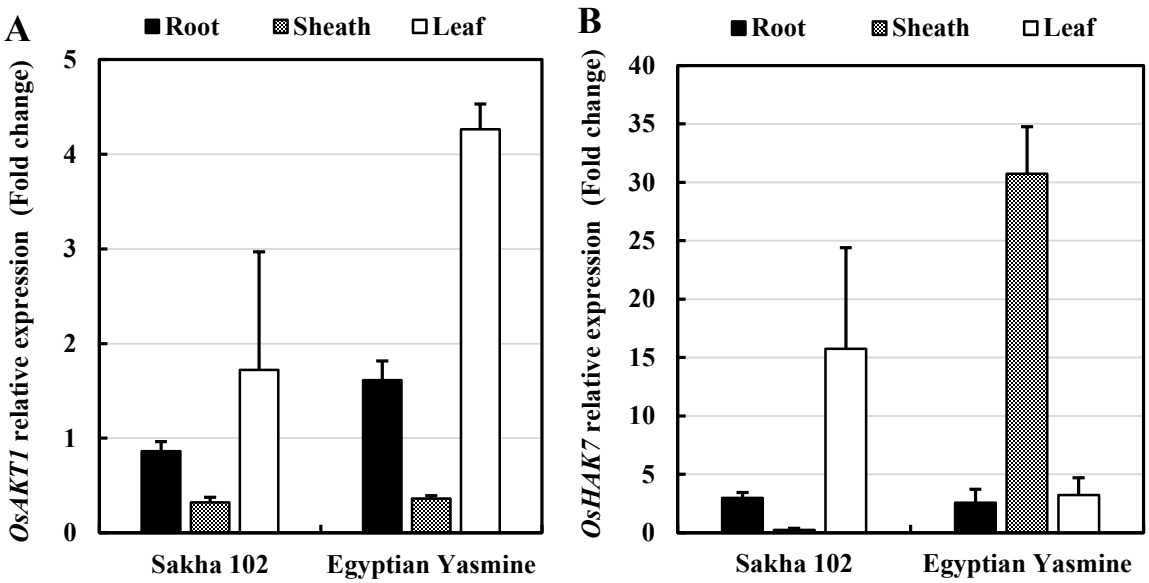


Figure 2.9. Relative expression of the genes encoding K^+ transport proteins. (A) *OsAKT1* and (B) *OsHAK7* in the roots, sheaths and leaves of rice seedlings for the cultivars Sakha 102 and Egyptian Yasmine grown under control and salt stress condition (50 mM NaCl) for 14 days. Data represent the means of two independent experiments ± SD.

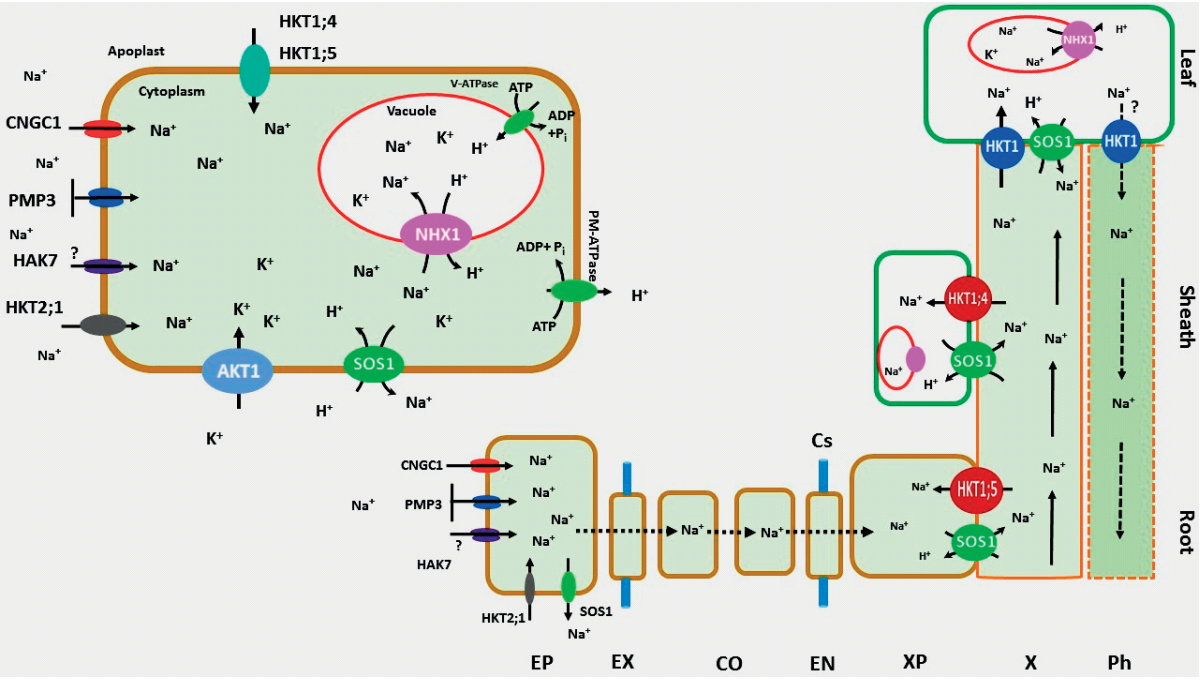


Figure 2.10. A hypothesized illustration for Na^+/K^+ uptake and transport in rice plants. The transporters and channels involved in Na^+ influx from soil to the epidermis: OsHKT2;1, HAK7, and cyclic-nucleotide gated channel (CNGC1). The plasma membrane protein 3 (PMP3) functions to restrict excess Na^+ influx. OsHKT1;5, responsible for Na^+ retrieval from the xylem to the xylem parenchyma cells in the root, and OsHKT1;4 in the shoot. Vacuolar sequestration of Na^+ and K^+ is mediated by a vacuolar Na^+/H^+ antiporter (NHX1). The Na^+ /H^+ antiporter (SOS1) responsible for efflux of Na^+ out of cells either to external medium or loading into xylem for long-distance Na^+ transport and recirculation. AKT1 channel, functions in K^+ up-take. The electrochemical potential is provided by the vacuolar H^+-ATPase (V-ATPase) and the plasma membrane (PM-ATPase). Cell types depicted include: epidermis (EP), exodermis (EX), cortex (CO), endodermis (EN), casparian stripes (Cs), xylem parenchyma (XP) xylem (X) and phloem (Ph).

2.4 Discussion

Two popular Egyptian rice cultivars, Sakha 102 and Egyptian Yasmine, were used in the present study to elucidate their mode of adaptation to salinity stress through physiological and transcriptional analyses. The two cultivars showed differential responses to salinity, and Egyptian Yasmine appeared more tolerant than Sakha 102 in that; it exhibited higher DW, RWC and proline values and lower ELR, leaf Na^+ content, and Na^+/K^+ ratios.

2.4.1 Mechanisms of Na^+ retrieval at the tissue level

Among salt-tolerant traits, the most significant plant adaptation to salinity is the ability to restrict the transport and accumulation of Na^+ in the leaves (Munns and Tester, 2008). Thus plants, such as Egyptian Yasmine, that exhibit lower leaf Na^+ content would be better adapted to salinity. This restricted transport of Na^+ to the leaf blade is often accompanied by a reduced Na^+/K^+ ratio, which is relevant for the sustainability of normal metabolic functions (Tester and Davenport, 2003). This is, because high Na^+ accumulation often interferes with K^+ functions, which results in impaired metabolic activities. Restricted transport of Na^+ or Na^+ exclusion to the leaf has been widely studied and has been shown to be under the control of various Na^+ transport proteins (Munns and Tester, 2008).

To understand the mechanisms underlying limited Na^+ transport to the leaves in Egyptian Yasmine, we analyzed the expression of *OsHKT1;5* (Figure 2.8A). Under salt stress conditions, the gene expression was markedly induced in the roots of Egyptian Yasmine, but was repressed in the roots of Sakha 102. The OsHKT1;5 transporter is localized in the roots, where it mediates Na^+ retrieval from the xylem into xylem parenchyma cells before it is transported in the transpiration stream to the shoot (Ren *et al.,* 2005; Munns and Tester, 2008). An increase in *OsHKT1;5* activity was observed in the tolerant cultivar Pokkali, but reduced in the sensitive cultivar IR29 (Walia *et*

al., 2007). Thus, it is possible that in the present study, the improved growth of Egyptian Yasmine under salt stress (Figures 2.1, 2.2) may be due to induced expression of *OsHKT1;5*, whereas, the sensitivity of Sakha 102 might be due to the repression of *OsHKT1*;5 expression, thus leading to unregulated Na^+ transport to the leaves and growth impairment (Figures 2.1, 2.2). By comparing the patterns of Na^+ accumulation in the tolerant and sensitive cultivars under salt stress conditions, Cotsaftis *et al*., (2012) reported that OsHKT1;4 (an OsHKT1;5 homolog), may participate in a Na^+ retrieving mechanism in rice leaf sheaths. Therefore, we also investigated the expression profiles of *OsHKT1;4* in both Egyptian Yasmine and Sakha 102. Under salt stress, expression of *OsHKT1;4* was not affected in the leaves, sheaths, or roots of either cultivar (data not shown), which suggests that the function of Na^+ retrieval in sheaths did not contribute to the restriction of Na^+ accumulation in the leaves of either cultivar.

2.4.2 Mechanisms associated with the restriction of Na^+ entry into cells

PMP3 proteins have been shown to control the excessive uptake of Na^+ in yeast (Navarre and Goffeau, 2000), implying that PMP3 proteins can potentially contribute to Na^+ exclusion from cells. Regulation of Na^+ entry in cells was proven by the overexpression of PMP3 homologous genes isolated from *Arabidopsis* and sheep grass (Nylander *et al.,* 2001; Inada *et al.,* 2005). In the present study, the expression of the rice PMP3 orthologs, *OsLti6a* and *OsLti6b*, was analyzed in both cultivars (Figure 2.8C, D). The results revealed marked induction of *OsLti6b* expression in the roots of Egyptian Yasmine, but it was not induced in Sakha 102 roots. These results clearly indicate that this gene may contribute to the regulation of Na^+ entry in the roots of Egyptian Yasmine, whereas such a function would be unlikely in the Sakha 102 cultivar. Just as the loss of PMP3 function facilitated Na^+ accumulation in yeast cells (Navarre and Goffeau, 2000), increased Na^+ entry may be caused by the repression of *OsLti6b* in the roots of Sakha 102.

Furthermore, another factor contributing to enhanced adaptation to salinity stress in Egyptian Yasmine relative to Sakha 102 could reside in the expression of *OsCNGC1*. This gene product is involved in Na^+ influx in the roots and has been shown to be up-regulated in the sensitive IR29 rice cultivar and down-regulated in the tolerant Pokkali cultivar (Senadheera *et al.,* 2009). Its induction in the roots of Sakha 102 in the current study indicates another facilitated Na^+ uptake route with subsequent delivery to the leaf. In contrast, its lower expression levels in Egyptian Yasmine suggest tight control of Na^+ uptake at the root level (Figure 2.8G). Although OsHKT2;1 is known to mediate Na^+ influx in yeast and plants (Garciadeblás *et al.,* 2003), it was repressed in both cultivars in the current study (Figure 2.8B). This result is in agreement with the findings of Horie *et al*., (2007), which suggested that *OsHKT2;1* was down-regulated during salt stress conditions. Therefore, this indicates that the OsHKT2;1 transporter is important to the restriction of toxic accumulation of Na^+ in both cultivars.

2.4.3 Mechanisms of Na^+ exclusion at the cell level

SOS1 antiporter has been shown to be localized at the plasma membrane of Arabidopsis, where it catalyzes Na^+/H^+ exchange (Shi *et al.,* 2002). The preferential expression of *SOS1* in cells surrounding the vasculature throughout the plants as demonstrated by the *GUS* reporter gene, suggests a role of this transporter in long distance Na^+ transport in plants, since Na^+ is transported from the root to the shoot via the xylem. In light of this function, Shi et al., (2002) observed that high levels (100 mM NaCl) of salt stress, substantially increased the concentration of Na^+ in the xylem sap of both the *sos1*-mutants and the wild-type plants over time, but the Na^+ concentration was always higher in the *sos1* plants. However, Ding and Zhu (1997) showed that, at low levels (25 mM NaCl) of salt stress, the *sos1* mutants accumulated less Na^+ than the wild type plants. These results suggest a dual role of SOS1 in Na^+ transport in plants depending on the cellular and

extracellular Na^+ environments; whereby, under moderate salt stress, SOS1 might function in loading Na^+ into the xylem for controlled delivery to the shoot and storage in leaf mesophyll cells. Whereas, under high salinity stress, SOS1 would function in Na^+ retrieval from the xylem to prevent over accumulation of Na^+ in the transpiration stream (Shi *et al.,* 2002). In addition, the *Oryza sativa* SOS1 (OsSOS1) has been shown to complement the function of SOS1 in the *sos1* mutant of Arabidopsis, indicating the conservation of the salt overly sensitive pathway in rice as well (Martínez-Atienza *et al.,* 2007). In the present study, under 50 mM NaCl stress, the expression of *OsSOS1* in the roots of Egyptian Yasmine was unaltered, but was markedly enhanced in the Sakha 102 roots (Figure 2.8F). This indicates that *OsSOS1* may not be involved in the adaptation of Egyptian Yasmine to salinity, whereas, its induction in Sakha 102 in response to salt treatment might imply a role in long-distance Na^+ transport from root to shoot. Thus, when *OsSOS1* is up-regulated, it facilitates Na^+ loading into the xylem, and Na^+ is controllably delivered to the leaves and subsequently compartmentalized into the vacuoles, presumably through the OsNHX1 activity, leading to high leaf Na^+ levels and lower Na^+ levels in root (Figure 2.4A,C).

Generally, under high salinity conditions, plants respond by accumulating the excess Na^+ in vacuoles, away from the cytosol, and this compartmentalization is under the control of the vacuolar Na^+/H^+ antiporter (NHX) (Munns and Tester, 2008). Arabidopsis AtNHX1 was shown to catalyze Na^+/H^+ and K^+/H^+ exchange with similar affinity (Venema *et al.,* 2002). The dual affinity of AtNHX1 for Na^+ and K^+ (Yamaguchi *et al.,* 2005) implies that AtNHX1 mediates the uptake of K^+ from cytosol into vacuoles under regular growth conditions and Na^+ sequestration into vacuoles will also take place because of the rising concentration of this ion in the cytosol under salinity stress. Overexpression of *AtNHX1* in tomato has conferred salt tolerance, although the leaves accumulated high Na^+ concentrations (Zhang and Blumwald, 2001). This observation, together

with the fact that the Arabidopsis *nhx1* mutant exhibited Na^+ sensitivity and significantly less vacuolar Na^+/H^+ antiport activity (Apse *et al.,* 2003), strongly supported the role of NHXs in Na^+ compartmentalization under salinity stress. However, Leidi *et al*., (2010) showed that the overexpression of the *AtNHX1* in tomato resulted in enhanced accumulation of K^+, but not Na^+. Moreover, Arabidopsis *nhx1 nhx2* double mutants displayed reduced K^+ concentration in vacuoles, supporting the role of AtNHX1 and AtNHX2 in mediating H^+ efflux coupled to K^+ uptake (Bassil *et al.,* 2011). Fukuda et al. (2004) showed that rice *OsNHX1* encodes a vacuolar Na^+, K^+/H^+ antiporter, and suggested that OsNHX1 plays important roles in the compartmentalization of excess cytosolic Na^+ and K^+ into the vacuoles. In the present study, under salt stress, *OsNHX1* expression was induced in the leaf tissues of Sakha 102 (Figure 2.8E), which had high Na^+ (Figure 2.4A) and K^+ (Figure 2.5A) contents. This increased accumulation of Na^+ and K^+ is likely to be a consequence of the activity of OsNHX1, as the antiporter is supposed to facilitate K^+ as well as Na^+ uptake into vacuoles in exchange for H^+ into the cytoplasm. However, *OsNHX1* was repressed in Egyptian Yasmine leaves, which had lower Na^+ content but high K^+ levels. This result indicates that the induced expression of *OsNHX1* would be mainly in response to elevated Na^+ levels but not K^+ in Sakha 102.

2.4.4 K^+ acquisition under salt stress

OsAKT1 is mapped to chromosome 1 as one of the quantitative trait loci (QTLs) controlling K^+ concentration and the Na^+/K^+ ratio in salt-stressed plants (Koyama *et al.,* 2001). Our results showed that salt stress induced the expression of *OsAKT1* in both cultivars leaves (Figure 2.9A), which might enable the maintenance of higher K^+ levels in the leaves of both cultivars. Egyptian Yasmine had abundant *OsAKT1* transcripts under salinity stress conditions, and that would explain the increased K^+ accumulation in the leaves and roots as compared to Sakha 102.

Although high affinity K^+ transporters (HAK) are inhibited by Na^+, some may also transport this ion, and this was shown in barley HvHAK1 and reed plants PhaHAK5 (Santa-Maria *et al.,* 1997 and Takahashi *et al.,* 2007). In this study, *OsHAK7* was induced in the roots and leaves of both cultivars under stress conditions (Figure 2.9B). There were higher transcript levels of *OsHAK7* in the roots and leaves of Sakha 102 and lower transcript amounts in Egyptian Yasmine, which may have resulted in the excessive accumulation of Na^+ in Sakha 102 leaves and lower Na^+ levels in Egyptian Yasmine leaves (Figure 2.4A). These results are consistent with the results of Senadheera et al., (2009), where *OsHAK7* was up-regulated in the salt sensitive rice cultivar IR29 and down-regulated in the tolerant line FL478, which may prevent the overall Na^+ load in tolerant plants.

In summary a hypothesized model depicting the afore-mentioned Na^+ /K^+ uptake and transport mechanisms in the studied rice plants can be seen in Figure 2.10.

2.5 Conclusion

In this study, it was demonstrated that Egyptian Yasmine is a relatively salt-tolerant cultivar compared to Sakha 102, which is due to its ability to restrict Na^+ accumulation in leaves under salt stress. Differences in the mechanisms of salinity tolerance between the two cultivars may be partly explained by the distinct regulation of gene expression of Na^+/K^+ transport proteins. This is evident in the inducible expression of *OsHKT1;5* (Na^+ retrieval), *OsLti6b* (restriction of Na^+ entry), and *OsAKT1* (K^+ uptake), as well as the repressed expression of *OsHKT2;1* (Na^+ influx). Divergent regulation of Na^+ and K^+ transporters may be involved in the maintenance of lower Na^+/K^+ ratios in Egyptian Yasmine under salt stress.

Chapter 3

Identification of a type 3 metallothionein-like gene (*OsMT-3a*) from rice through functional screening analysis in *Escherichia coli*, confers tolerance against salinity and heavy-metal stresses

3.1 Introduction

Salinity, drought, temperature, and heavy metals are major factors that reduce plant productivity and are proving to be an increasing threat to agriculture (Sreenivasulu *et al.,* 2007). Such stresses have similar consequences: causing ionic imbalance and generating reactive oxygen species (ROS). Overproduction of ROS damages cell membranes, nucleic acids, and photosynthetic pigments (Zhang *et al.,* 2007), and therefore, living organisms have developed cellular ROS detoxifying systems. ROS are scavenged by enzymes such as superoxide dismutase, catalase, and peroxidase (Apel and Hirt, 2004; Jang *et al.,* 2012), and also by non-enzymatic components that include low molecular weight antioxidants, such as ascorbate, glutathione, carotenoids, and metallothioneins (MTs) (Gechev *et al.,* 2006).

MTs are a group of polypeptides that can bind with heavy metals through their thiol group via chelation. Because of this chelating activity, MTs are involved in the homeostasis of essential metals (Cu^{2+} and Zn^{2+}) and cellular detoxification of nonessential metals (Cd^{2+} and Hg^{2+}) (Hamer, 1986; Huang and Wang, 2010). A structural characteristic of MTs is the cysteine (Cys) residue, which is the basis of its classification. On the basis of the arrangement of Cys residues, MTs are divided in two classes (Cobbett and Goldsbrough, 2002). Plant MTs belong to Class II and are further classified into four types (1-4) based on the position of Cys residues (Cobbett and Goldsbrough, 2002). Through characterization of biochemical properties of some plant MTs in heterologous expression system, its potential contribution to abiotic stress tolerance has been discussed (Chaturvedi *et al*. , 2014; Dundar *et al*. , 2015; Turchi *et al*. , 2012; Xue *et al*. , 2009; Yang *et al*. , 2009).

Studies in animals as well as plants showed that MTs are not only involved in maintaining homeostasis of essential metals and metal detoxification (Cobbett and Goldsbrough, 2002) but are also implicated in a range of physiological processes, including scavenging ROS (Akashi *et al.,* 2004; Wong *et al.,* 2004; Kumar *et al.,* 2012; Chaturvedi *et al.,* 2014). The antioxidant function of MTs has been attributed to the presence of a large number of Cys residues, which besides metal binding are also capable of scavenging ROS (Hassinen *et al.,* 2011).

MT gene expression is regulated by abiotic stress, including heavy-metal stress, and plays an important role in metal detoxification and homeostasis (Huang and Wang, 2010; Kim *et al.,* 2014). Earlier studies of plant MTs focused on their role in maintaining intracellular metal homeostasis and mediating responses to metal toxicity (Cobbett and Goldsbrough, 2002). However, recent studies have shown additional roles of plant MTs in development, fruit ripening, senescence, and defense against oxidative stress; Zhigang *et al.,* (2006) showed that expression of the *Brassica juncea MT2* in Arabidopsis enhanced tolerance to Cu and Cd, but inhibited root elongation. Two *MT*-like genes were upregulated during natural leaf senescence in sweet potato (Chen *et al.,* 2003). Cotton *MT3* scavenged ROS and enhanced plant tolerance to oxidative stresses caused by NaCl, polyethylene glycol, and low temperature (Xue *et al.,* 2009). In rice, most studies of MT isoforms have focused on the gene expression patterns under different stress conditions (Jin *et al.,* 2006; Yang *et al.,* 2009) and metal-binding ability of different MT proteins (Nezhad *et al.,* 2013). It is necessary to investigate the role of rice MTs in response to salinity stress, which could be another adaptation strategy of rice plants to such stress conditions.

In the previous study, the adaptation mechanisms of the two Egyptian rice cultivars, Egyptian Yasmine and Sakha 102 to salinity stress has been characterized, and found that the difference in tolerance between the two lies in the ability to exclude Na^+ to the leaf blade. This exclusion

mechanism was shown to be under the control of transporters such as the HKT1;5, which functions in restricting the transport of Na^+ to the leaf and hence in stress tolerance. However, it is unclear whether the tolerance of Egyptian Yasmine is limited only to Na^+ exclusion or regulation of Na^+ transport protein coding genes. Therefore, the present study was designed to isolate salinity-inducible genes from Egyptian Yasmine and examine their functional roles which could be involved in other adaptation processes under salinity stress conditions, in an attempt to further elucidate the molecular mechanism of salinity stress tolerance in this cultivar.

3.2 Materials and Methods

3.2.1 Plant material, growth conditions, salt treatment, and sample collection

Seeds of the rice cultivars Egyptian Yasmine and Sakha 102 were surface-sterilized via immersion in a 5% NaClO solution for 30 min, and then thoroughly rinsed with distilled water. Seeds were subsequently soaked in tap water for 24 h at 28 °C. After germination, the seeds were transferred to a nylon mesh and allowed to float on 20 L of tap water for two days. Water was then replaced with half-strength Kimura B solution. Twenty one-day-old seedlings were transferred to either Kimura B nutrient solution (control) or Kimura B nutrient solution supplemented with 50 mM NaCl (salinity) for 10 days. The solutions were replaced every two days and the pH was daily adjusted to 5.0–5.5. Seedlings were grown in a growth chamber under the following controlled environmental conditions: 70% relative humidity, 24 ± 2 °C, and a 16 h photoperiod at a photosynthetic photon flux density of 250 - 350 μmol m^{-2} s^{-1}. The root and leaf tissues from experimental and control plants were separated and frozen in liquid nitrogen, prior to being stored at -80 °C.

3.2.2 Total RNA isolation, cDNA synthesis, and construction of cDNA expression library

Total RNA was extracted from the leaves and roots of the control and stressed plants of the rice cultivar, Egyptian Yasmine, by using TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA synthesis was carried out by a PCR-based method using the In-Fusion® SMARTer™ Directional cDNA library construction kit (Clontech, Takara, Japan) as described by the manufacturer. The double stranded cDNA was purified using CHROMA SPIN™ + TE-1000 size exclusion column chromatography (Clontech, Takara, Japan). Three microliters of each fraction was electrophoresed on a 1.1% agarose/ethidium bromide gel to determine the peak fractions by visualizing the intensity of the bands under UV light. Fractions containing large, medium, and small-sized cDNA were pooled, which were then ligated into the pSMART2IFD vector (Clontech Laboratories).

3.2.3 *E. coli* functional screening

The resulting ligation reactions were transformed into electrocompetent *E. coli* (KNabc) cells. The transformed *E. coli* cells were then selected and inoculated into sterile 96-well microtiter plates containing L-medium (1% Bacto tryptone, 0.5% yeast extract, and 0.05% NaCl) supplemented with 0.2 M of NaCl, then incubated at 37 °C for 24 h with gentle shaking (30 rpm). Subsequently, the individual bacterial transformants were printed on quadruplet selection plates (L-medium, 100 µg/mL ampicillin, 1.0 mM isopropyl β-D-1-thiogalactopyranoside [IPTG], 1.5% agar) containing different concentrations of NaCl (0, 0.5, 1.0, 1.5, and 2 M NaCl), and incubated. The successfully transformed *E. coli* cells grown on selection plates containing the highest NaCl concentrations were then isolated and grown in a liquid culture medium supplemented with 100, 200, 500, and 1,000 mM NaCl for 24 h. Growth was verified by measuring the optical density (OD) at 600 nm (OD_{600}). Then, the inserts of these clones were confirmed by colony PCR using

the forward, 5′-TCACACAGGAAACAGCTATGA-3′ and reverse, 5′-CCTCTTCGCTATTACGCCAGC-3′ screening primers (Clontech Laboratories).

3.2.4 Bacterial strains and salt stress experiment

The bacterial strains *E. coli* TG1 and its derivative KNabc (Δ*nhaA*, Δ*nhaB*, Δ*chaA*), [a triple mutant of *E. coli*, this mutant was disabled in the function of three Na^+/H^+ antiporters which exclude Na^+ from the cell in the wild-type (TG1)] were used (Nozaki et al., 1996). For bacterial salinity stress experiment, *E. coli* mutant (KNabc) expressing a rice cDNA, *E. coli* KNabc (negative control carrying the empty vector pUC19) and *E. coli* WT (TG1/pUC19) strains were used. A preparatory culture was grown under selective conditions in L-medium plus 100 µg/ml ampicillin at 37°C under aerobic conditions until a defined cell density was achieved. Subsequently, equal cell densities were used to inoculate 20 ml of L-medium plus 100 µg/ml ampicillin. Gene expression was induced by adding IPTG at 1 mM. After a 3 h induction phase, 100 mM of NaCl was added. These bacterial cells were incubated at 37°C with shaking (200 rpm). Bacterial growth was measured photometrically at OD_{600} at 2 h intervals for 24 h.

3.2.5 Colony PCR, plasmid DNA extraction and digestion with restriction enzymes

The conditions for colony PCR analysis were as follows: an initial incubation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 68 °C for 30 s. *E. coli* transformants (representing the plasmid expression vector containing rice cDNA) were then cultured, and plasmid DNA was extracted as described in the HiYield Plasmid Mini Kit (RBC Bioscience). Plasmid extraction was followed by digestion with the *Eco*RI and *Bam*HI to confirm the size of the insert.

3.2.6 Gene sequencing and identification

Sequencing of the rice cDNA conferring salinity tolerance was performed using BigDye Sequencing Kit (ABI, USA) according to the manufacturer's instructions, and analyzed using Genetic Analyzer (ABI 3100, USA). The sequencing was carried out using vector specific primers (forward and reverse screening primers). Computational search against sequence databases at NCBI was performed to identify the gene.

3.2.7 Quantification of H_2O_2 levels in *OsMT-3a* transformed *E. coli* under NaCl stress conditions

The *OsMT3a*-transformed *E. coli* cells and those with empty vector were cultured overnight in L-medium (OD_{600} = 5.0–6.0). Equal cell densities were used to inoculate 10 mL of L-medium plus 100 µg/mL ampicillin. Gene expression was induced by adding 1 mM IPTG. After a 3-h induction phase, 200 mM NaCl was added and incubated with shaking for 24 h at 37 °C. Then, 100 µL of each culture was added to 1 mL of reaction buffer (0.25 mM $FeSO_4$, 0.25 mM $(NH_4)_2SO_4$, 25 mM H_2SO_4, 1.25 mM xylenol orange, and 1 mM sorbitol) and allowed to stand for 1 h at room temperature. The H_2O_2 levels were quantified photometrically at 560 nm, and calculated by reference to standards (Suharsono *et al.,* 2002).

3.2.8 Tolerance of *E. coli* to H_2O_2 stress

Overnight cultures of the cells KNabc harboring pSMART2IFD-*OsMT-3a*, *E. coli* KNabc/ pUC19 and TG1/pUC19 strains were used. 100 µl from the overnight cultures were added to 10 mL L-medium plus ampicillin, and cultured at 37 °C. When the cells reached OD_{600} = 0.6, 1 mM IPTG was added, and culturing was continued for another 12 h. Equal densities of cells (OD_{600}=

0.1) were then cultured in the presence of either 0, 1, 3 or 4 mM H_2O_2 at 37 °C, and the growth was monitored (OD_{600}) at 2-h intervals for 12 h.

3.2.9 Measurement of growth and heavy metal accumulation in *E. coli* overexpressing the *OsMT-3a* gene

Initially, we run the experiments with the control strain, KNabc/pUC19 under varied concentrations of $CdCl_2$, $CuSO_4$, or $ZnSO_4$ and detected the used experimental concentration of each (100 µM of $CdCl_2$, 1.5 mM of $CuSO_4$, 250 µM of $ZnSO_4$) depending on the substantial effect on growth rate (toxic level). We added 100 µl from the overnight cultures to 10 mL L-medium plus ampicillin, and cultured at 37 °C. When the cells reached OD_{600} = 0.6, 1 mM IPTG was added, and culturing was continued for another 12 h. Equal densities of cells (OD_{600}= 0.05) were then cultured in the presence of either 1.5 mM $CuSO_4$, 100 µM $CdCl_2$, or 250 µM $ZnSO_4$ at 37 °C, and the growth was monitored (OD_{600}) at 12-h intervals for 60 h.

To measure the accumulation of heavy metals, *E. coli* cells were grown in liquid medium containing 100 µM $CdCl_2$, 250 µM $ZnSO_4$, or 1.5 mM of $CuSO_4$ at 37 °C. When cells reached OD_{600} = 0.6, 1 mM IPTG was added and then incubated for another 24 h at 37 °C. After incubation, the cells were harvested and washed twice with L-medium. Dry weight was determined after 3 days at 70 °C. The cells were then digested using 5 mL concentrated HNO_3, incubated at 95 °C for 2 h. Prior to the measurement, the lysate was diluted using 0.1N HNO_3. The Cu, Zn, and Cd concentrations were determined by using inductively coupled plasma-atomic emission spectrometry (ICP-AES) (iCAP 6300, Thermo Fisher Scientific Inc., UK).

3.2.10 Expression analysis of the *OsMT-3a* in the salinity-tolerant and salinity-sensitive rice cultivars

Total RNA was extracted from the leaves and roots of control and stressed plants using TRIzol reagent (Invitrogen, Carlsbad, CA). After digestion with DNaseI, total RNA (1 µg) was reverse-transcribed to cDNA using a ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). Quantitative polymerase chain reaction (qPCR) was conducted as previously described (Ueda et al., 2013), using a Thunderbird SYBR qPCR Mix (Toyobo) and an ABI Step One Plus system (Applied Biosystems). RT-qPCR was performed as described previously. The sequences of the primers used are as follows: *OsMT-3a* forward primer, 5′-TGGTTTGTGTGTGTGCTGATT-3′ and reverse primer, 5′-TAGCACGGGTACATCACATCA-3′.

3.2.11 *In vivo* localization of O_2^- in rice plants

Histochemical staining was performed for the *in vivo* detection of O_2^- using nitro-blue tetrazolium (NBT) (Shi et al., 2010). For detecting O_2^-, the apical 3 cm of the third leaves from the rice plants (Egyptian Yasmine and Sakha 102) exposed to either NaCl (50 mM) and/or $CdCl_2$ (100 µM) for 10 days were used. The presence of O_2^- was detected by immersing the leaf samples in NBT solution (1 mg ml^{-1} in 10 mM phosphate buffer; pH 7.8) and vacuum infiltrated for 30 min, kept at room temperature for 2 h and then illuminated for 12 h in light until blue spots appeared. Rice leaves were then decolorized in a bleaching solution (ethanol: acetic acid: glycerol [3:1:1]), and the tubes with the leaves were placed in 100 °C water bath for about 30 min until the chlorophyll bleached out, and then were stored in a glycerol-ethanol (1:4) (v/v) solution. The O_2^- staining was repeated three times.

3.2.12 Quantification of H_2O_2 in rice leaves under NaCl and $CdCl_2$ stress conditions

The leaf tissues of 10-days treated plants (50 mM NaCl or 100 µM $CdCl_2$) from Egyptian Yasmine and Sakha 102 were ground in liquid nitrogen, and homogenized with 4 ml cold acetone. The homogenate was centrifuged at 8,000 *g* for 15 min at 4 °C. Then, 100 µl of each was added to 1 ml of reaction buffer (0.25 mM $FeSO_4$, 0.25 mM $(NH_4)_2SO_4$, 25 mM H_2SO_4, 125 µM xylenol orange, and 10 mM sorbitol) and allowed to stand for 1 h at room temperature. The H_2O_2 levels were quantified photometrically at 560 nm, and calculated by reference to standards (Suharsono, 2002).

3.2.13 Statistical analysis

The collected data were subjected to one-way analysis of variance (ANOVA), using the SPSS statistics package, version 21 (IBM Inc., USA), and the means ($n = 4$) were separated using the Duncan's multiple range test at $p < 0.05$.

3.3 Results

3.3.1 *E. coli* functional screening

To further elucidate the molecular mechanisms of salinity tolerance in the rice cultivar Egyptian Yasmine, we planned to isolate and characterize the salinity-inducible genes from this plant through their functional validation analysis in *E. coli* expression system. To achieve that, we screened a cDNA library that was prepared from salinity-stressed seedlings of Egyptian Yasmine in the Na^+-sensitive *E. coli* mutant cells KNabc (Δ*nhaA*, Δ*nhaB*, Δ*chaA*), that lack the three Na^+/H^+ antiporters which exclude Na^+ from the cell. Due to deactivation of the major Na^+ efflux systems, the KNabc mutant could not grow in the presence of 0.2 M NaCl. Such genetic backgrounds in the *E. coli* mutant would be useful for identification of determinant genes for salinity tolerance in rice through functional screening.

Successfully transformed *E. coli* cells were grown on selection plates containing different concentrations of salt up to 2 M NaCl. We obtained few colonies that were able to grow at the highest NaCl concentration (Figure 3.1). These colonies were picked out and inoculated in a liquid culture medium supplemented with different concentrations of salt up to 1 M NaCl to confirm their ability to grow under more severe salt stress conditions (salt stress is more severe in liquid media). We identified one such clone, which showed the best growth both at 2 M NaCl on plates (on solid medium) and at 200 mM NaCl in the liquid medium, for further characterization. The presence of the insert in that clone was confirmed using colony PCR analysis.

3.3.2 Sequence analysis and multiple alignment of *OsMT-3a*

From the library screening, one isolated gene shared significant sequence homology with that of plant type-3 metallothionein (*MT-3a*). The full-length coding sequence of *OsMT-3a* is 189 bp

long, and encodes a 62-amino acid protein (Figure 3.2A). Similar to other plant type-3 MT isoforms, this amino acid sequence contains 10 Cys residues arranged in the forms CXXCXCXXXXC and CXCXXXCXCXXCXC at its N and C termini, respectively (Figure 3.2B). The genetic similarity of *OsMT-3a* with other plant MTs was determined using Blastx and CLUSTAL W. Multiple alignments indicated that *OsMT-3a* shared high homology with many MT3-like genes from other plant species (Figure 3.2B).

3.3.3 *OsMT-3a* confers salinity-stress tolerance to transformed *E. coli* through the scavenging of ROS

To assess the role of *OsMT-3a* in response to salinity stress, *E. coli* KNabc cells were transformed with either the empty vector pUC19 or with pSMART2IFD containing *OsMT-3a* cDNA. After transformation of the parental wild-type TG1 strain with pUC19 (TG1/pUC19), we monitored the growth of these three sets of transformants in response to salinity stress (using 100 mM NaCl) (Figure 3.3A). *OsMT-3a*-transformed *E. coli* cells had a moderate growth rate between TG1/pUC19 cells and KNabc/pUC19 cells, and grew more efficiently compared to KNabc/pUC19 cells in media containing 100 mM of NaCl. These results demonstrated that the expression of *OsMT-3a* enabled *E. coli* cells to become more tolerant to NaCl stress. However, this gene did not completely complement the Na^+-sensitivity defect in this bacterial mutant KNabc, as the growth did not reach the TG1/pUC19 growth rate as seen in Figure 3.3A. This finding implied that OsMT-3a improved salinity tolerance of KNabc cells through any other mechanisms rather than Na^+ exclusion from the cells. To study mechanisms of the potential contribution by OsMT-3a in salinity tolerance, Na^+ concentration was examined in these three cells (Figure 3.3B). In comparison to Na^+ accumulation under the control condition (1.23 μmol/10^6 cells in TG1/pUC19, 3.51 μmol/10^6 cells in KNabc/pUC19, and 5.8 μmol/10^6 cells in KNabc/OsMT-3a), KNabc/OsMT-3a cells

accumulated 180 times higher Na^+ than TG1/pUC19 cells (2.06 μmol/10^6 cells in TG1/pUC19, 1300 μmol/10^6 cells in KNabc/pUC19, and 363.5 μmol/10^6 cells in KNabc/OsMT-3a), suggesting that OsMT-3a did not directly participate in Na^+ exclusion from the cells (Figure 3.3B).

To investigate whether the increased tolerance to NaCl stress in *OsMT-3a*-transformed *E. coli* cells might be due to the scavenging of ROS by OsMT-3a, we compared the H_2O_2 concentrations in KNabc/OsMT-3a, KNabc/pUC19, and TG1/pUC19 under salinity stress. In comparison to control conditions, H_2O_2 concentrations were significantly increased in both KNabc/OsMT-3a and KNabc/pUC19 in the presence of 200 mM NaCl (Figure 3.3C). However, H_2O_2 concentration in KNabc/OsMT-3a cells was less than one third of that in KNabc/pUC19 cells (Figure 3.3C),

3.3.4 Tolerance of *OsMT-3a*-transformed *E. coli* cells to H_2O_2 stress

Initial experiments with the KNabc/pUC19 cells revealed that the concentration of H_2O_2 up to 0.75 mM had no substantial effect on the bacterial growth rate. Therefore, the concentrations of 1, 3, and 4 mM of H_2O_2 were considered to be used to investigate the effect of *OsMT-3a* on the tolerance of the transformed *E. coli* cells. *E. coli* cells expressing *OsMT-3a* showed increased tolerance to H_2O_2 stress, and had a similar or even better growth rate as the WT TG1/pUC19 cells under exposure to high concentrations of H_2O_2 (Figure 3.4A). The final cell density of KNabc/OsMT-3a was 33%, 99%, and 98% higher than KNabc/pUC19 in a culture medium supplemented with 1, 3 and 4 mM H_2O_2, respectively (Figure 3.4B, C, D), as the higher concentrations of H_2O_2 were lethal for KNabc/pUC19 cells. These data suggest that the heterologous expression of *OsMT-3a* gave the *E.coli* cells more tolerance to H_2O_2, probably through a scavenging activity.

3.3.5 *OsMT-3a* confers heavy-metal tolerance to transformed *E. coli*

Previous reports have discussed expressing other classes of plant MTs, such as *Brassica juncea Bj*MT2 (Zhigang *et al.,* 2006), *Salicornia brachiata SbMT-2* (Chaturvedi *et al.,* 2012), sweet potato *IbMT1* and *IbMT3* (Kim *et al.,* 2014), and olive *OeMT2* (Dundar *et al.,* 2015), in *E. coli* to assess if they confer tolerance to heavy metals. Here, we examined the functions of *OsMT-3a* using *E. coli* transfected with a pSMART2IFD-*OsMT-3a* vector, and assessed cell growth in the presence of heavy metals (Figure 3.5). Initially, we run the experiments with the control strain, KNabc/pUC19 under varied concentrations of $CdCl_2$, $CuSO_4$, or $ZnSO_4$ and detected the used experimental concentration of each (100 μM of $CdCl_2$, 1.5 mM of $CuSO_4$, 250 μM of $ZnSO_4$) depending on the substantial effect on growth rate (toxic level). In the absence of heavy metals, all bacterial strains exhibited a normal growth rate; TG1/pUC19 exhibited a higher growth rate compared to both KNabc/pUC19 and KNabc/OsMT-3a with almost similar growth rate (Figure 3.5A). However, exposure to supplemental $CdCl_2$, $CuSO_4$, or $ZnSO_4$ differentially affected cell growth. Cell growth of the KNabc/pUC19 was affected by toxic concentrations of heavy metals, but *OsMT-3a* overexpression significantly improved growth kinetics, especially under $CdCl_2$ stress (Figure 3.5B) at which the growth rate of KNabc/OsMT-3a cells until 24 h exposure was significantly higher compared to that of the TG1/pUC19 and KNabc/pUC19. With respect to $CuSO_4$, only with 12-h exposure, *E. coli* cells expressing *OsMT-3a* grew better than did the controls, TG1/pUC19 and KNabc/pUC19 (Figure 3.5C). Under $ZnSO_4$ stress conditions, no significant difference in the growth appeared between the *OsMT-3a*-transformed cells and the controls until 12 h exposure, after that time, a decline in the growth rate of the cells, KNabc/pUC19, was observed (Figure 3.5D).

To determine whether the tolerance to heavy metal in *E. coli* cells induced by *OsMT-3a* is associated with accumulation of heavy metals, *E. coli* cells overexpressing *OsMT-3a* were cultured in media containing $CdCl_2$, $ZnSO_4$, or $CuSO_4$, and their metal ion content was measured (Figure 3.6). Under $CdCl_2$ stress, Cd^{2+} levels were increased in the cells of all the strains, TG1/pUC19, KNabc/OsMT-3a and KNabc/pUC19. However, *E. coli* cells overexpressing *OsMT-3a* contained a significantly higher concentration of Cd^{2+} than did those transformed with the empty vector, KNabc/pUC19 (Figure 3.6A). Under both $ZnSO_4$ and $CuSO_4$ stresses, a similar increase in the concentrations of Zn^{2+} and Cu^{2+} in the cells of all the strains examined, but with no significant difference in Zn^{2+} and Cu^{2+} concentrations between the transformed KNabc/OsMT-3a and KNabc/pUC19 cells (Figure 3.6B, C), indicating that OsMT-3a may binds heavy metals selectively.

3.3.6 Tissue-specific expression of *OsMT-3a* in rice plants

To examine the tissue-specific expression patterns of *OsMT-3a* in rice, qRT-PCR analysis was performed using total RNAs extracted from the leaf and root tissues of the rice cultivars Egyptian Yasmine and Sakha 102. In the previous study, we demonstrated that Egyptian Yasmine has higher salinity tolerance than does Sakha 102, which is due to its ability to restrict Na^+ accumulation in leaves when under salt stress, mainly through the inducible expression of *OsHKT1;5*, which functions in Na^+ retrieval (Ren *et al.,* 2005). In the current study, gene expression analysis revealed that *OsMT-3a* was highly induced by salinity stress in the leaves of the salinity-tolerant cultivar Egyptian Yasmine but not in those of the salinity-sensitive, Sakha 102. However, the gene expression in the roots of both cultivars was repressed under salinity stress conditions, suggesting that *OsMT-3a* might function predominantly in the leaves, but not in the roots, under salinity stress (Figure 3.7).

3.3.7 *In vivo* localization of O_2^- in rice plants and H_2O_2 concentration analysis

Under NaCl and $CdCl_2$ stress conditions, O_2^- production was visualized by a histochemical method using NBT staining (Figure 3.8). It was observed that leaves of Sakha 102 plants showed more accumulation of O_2^- compared to those of Egyptian Yasmine as these leaves showed intense blue colored polymers accumulation with NBT staining under NaCl and $CdCl_2$ stress condition (Figure 3.8). Moreover, by analyzing the H_2O_2 concentrations in the leaves of the two cultivars under both NaCl and $CdCl_2$ stress conditions, Egyptian Yasmine showed significantly lower H_2O_2 concentrations under stress conditions compared to those of Sakha 102 (Figure 3.9). Lower concentrations of H_2O_2 implied the better ROS scavenging system in Egyptian Yasmine compared to Sakha 102.

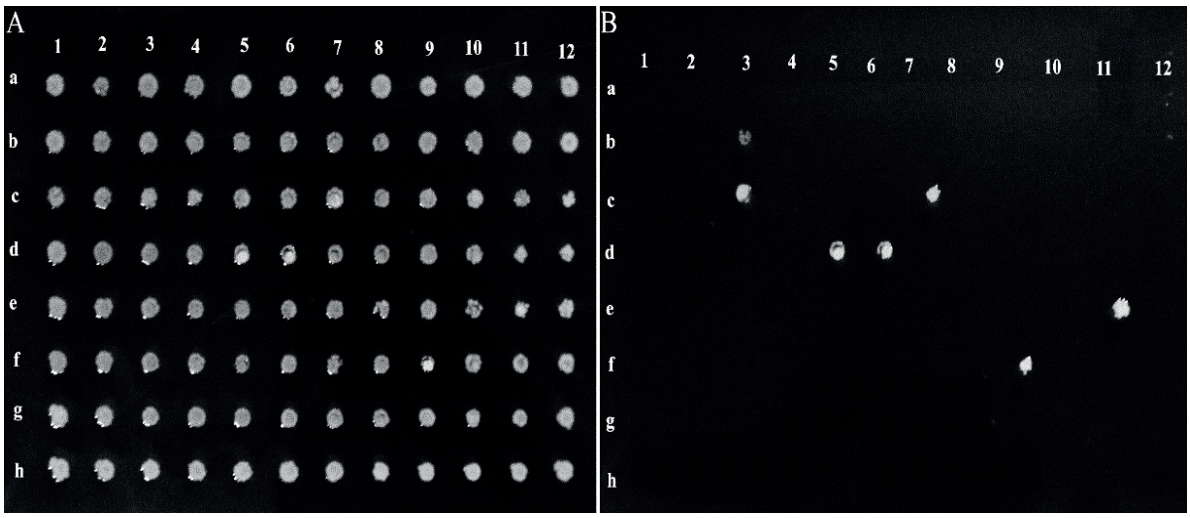


Figure 3.1. Rice leaf cDNA library screening in *E. coli* cells KNabc (*ΔnhaA, ΔnhaB, ΔchaA*). The individual bacterial transformants were printed on quadruplet selection plates (L-medium, 100 μg/mL ampicillin, 1.0 mM IPTG, 1.5% agar) as a control (A) or supplemented with 2 M NaCl (B) and incubated at 37 °C for 24 h.

A

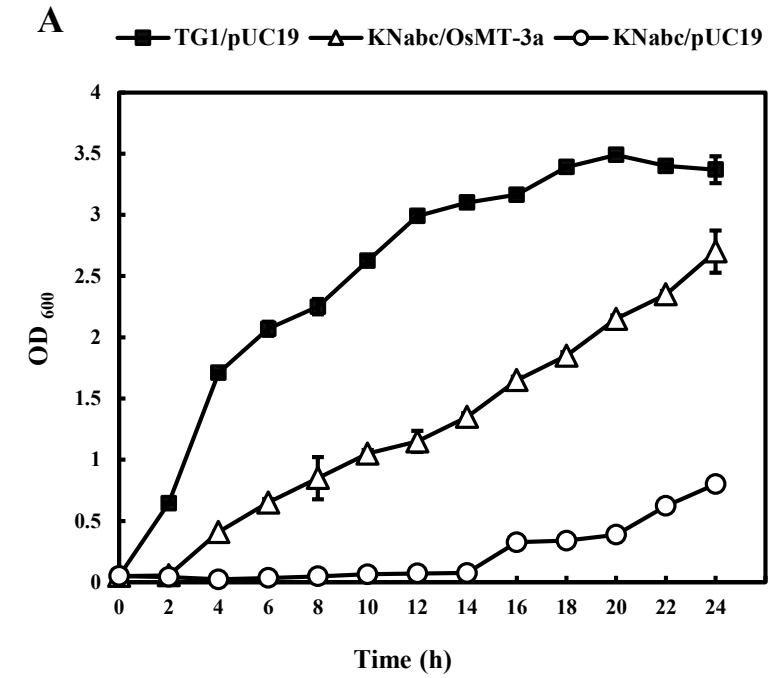
```
ATG TCG GAC AAG TGC GGC AAC TGC GAC TGT GCT GAC AAG AGC CAG
M   S   D   K   C   G   N   C   D   C   A   D   K   S   Q
TGC GTG AAG AAA GGT ACC AGC TAT GGC GTC GTC ATA GTT GAA GCC
C   V   K   K   G   T   S   Y   G   V   V   I   V   E   A
GAG AAG AGC CAC TTC GAG GAG GTC GCC GCC GGC GAG GAG AAC GGC
E   K   S   H   F   E   E   V   A   A   G   E   E   N   G
GGC TGC AAG TGC GGC ACC AGC TGC TCC TGC ACC GAC TGC AAG TGC
G   C   K   C   G   T   S   C   S   C   T   D   C   K   C
GGC AAG TGA
G   K   *
```

B

```
OsMT3     1 MSDKCGNCDCADKSQCVKKGTSYGVVIVEAEKSHFEEVAAGEENG------GCKCGTSCS
HvMT3     1 MADKCGNCDCADKTQCVKKGDSYGIVMVDTEKSHLEVQETAENDD------KCKCGTSCT
ThMT3     1 MSGKCGNCSCADKSQCVQKRNQYGFDLIETQTYAESTVVMDDPPTAAENGGQCKCGDRCA
GhMT3     1 MADKCGNCDCADKSQCVKKGN---SLVIETEESYISTVVVEP---LAENDGKCKCGTSCS
AtMT3     1 MSSNCGSCDCADKTQCVKKGTSYTFDIVETQESYKEAMIMDVGAEENNANCKCKCGSSCS
BjMT3     1 MSDKCGSCDCADKTQCVKKGTSYTFDIVETQESYKEAMFMDVGAEEN--GCQCKCGSTCS
                C  C C     C                                    C C   C

OsMT3    55 CTDCKCGK-
HvMT3    55 CTNCTCGH-
ThMT3    61 CVNCTCGSH
GhMT3    55 CTNCTCGSH
AtMT3    61 CVNCTCCPN
BjMT3    59 CVNCTCCPN
            C  C C
```

Figure 3.2. (A) Nucleotide and deduced amino acid sequences of *OsMT-3a* cDNA, (B) Comparison of the deduced amino acid sequences of *Oryza sativa* MT-3a with its homologues from other plant species. Conserved Cys residues are indicated by the letter C. Identical or conserved amino acid residues are shaded in dark or grey colors, respectively. The accession numbers in GenBank of the other plant type 3 MTs are as follows: *Hordeum vulgare* subsp. *vulgare* (HvMT3), CAD88266; *Tamarix hispida* (ThMT3), EH057039; *Gossypium hirsutum* (GhMT3), AY857933; *Arabidopsis thaliana* (AtMT3), NP_566509; and *Brassica juncea* (BjMT3), AB057415.


A
TG1/pUC19
KNabc/OsMT-3a
KNabc/pUC19
OD 600
Time (h)
0
0.5
1
1.5
2
2.5
3
3.5
4
0
2
4
6
8
10
12
14
16
18
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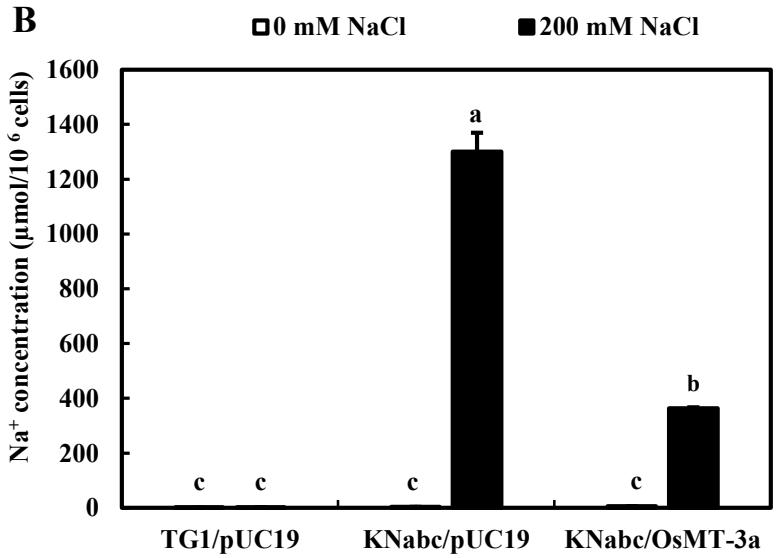
B
0 mM NaCl
200 mM NaCl
Na+ concentration (µmol/10 6 cells)
0
200
400
600
800
1000
1200
1400
1600
a
b
c
c
c
c
c
TG1/pUC19
KNabc/pUC19
KNabc/OsMT-3a

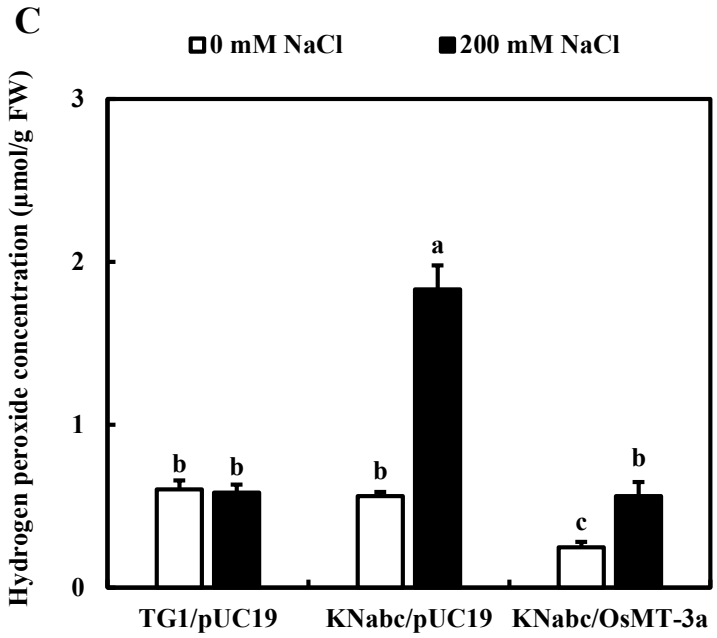

Figure 3.3. (A) Increased tolerance of *OsMT-3a*-expressing *E. coli* cells to NaCl stress. At the beginning of the assay (OD_{600} = 0.05), 1.0 mM IPTG was added to all three cultures. Bacterial cells were cultivated in L-medium supplemented with 100 mM NaCl. (B) Na^+ concentration in *OsMT-3a*-transformed *E. coli* cells grown in the presence of 200 mM NaCl compared with the concentration in control cells. (C) H_2O_2 concentrations in *OsMT-3a*-transformed *E. coli* cells grown in the presence of 200 mM NaCl compared with that in control cells. Data are represented as the means of three independent experiments ± SE. The same letters indicate no significant differences ($P < 0.05$).

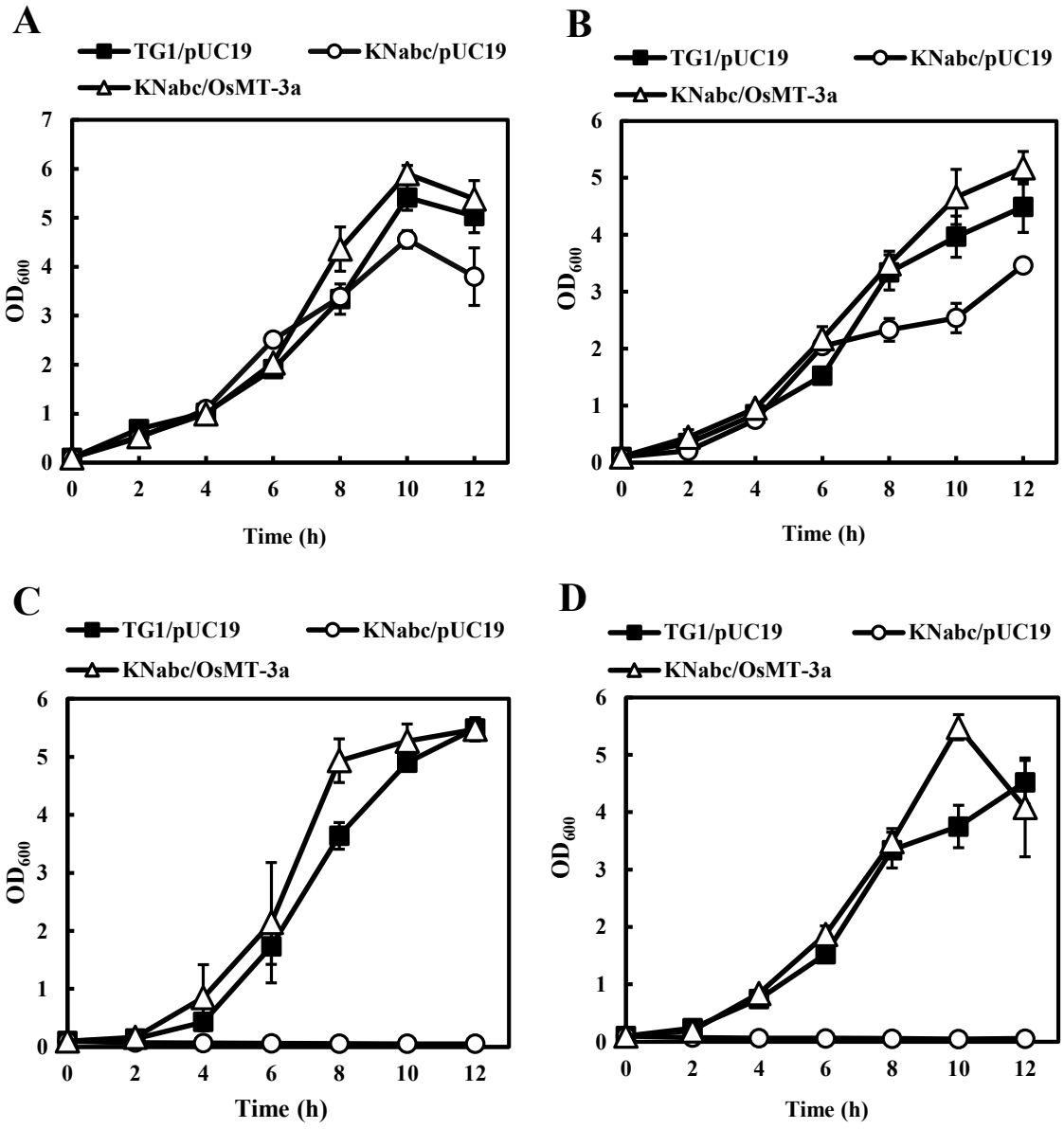

Figure 3.4. Growth curve of the strains, TG1/pUC19, KNabc/pUC19 and KNabc/OsMT-3a under H_2O_2 stress. The medium supplemented with (A) 0 mM, (B) 1 mM, (C) 3 mM, or (D) 4 mM H_2O_2. Data are represented as the means of three independent experiments ± SE.

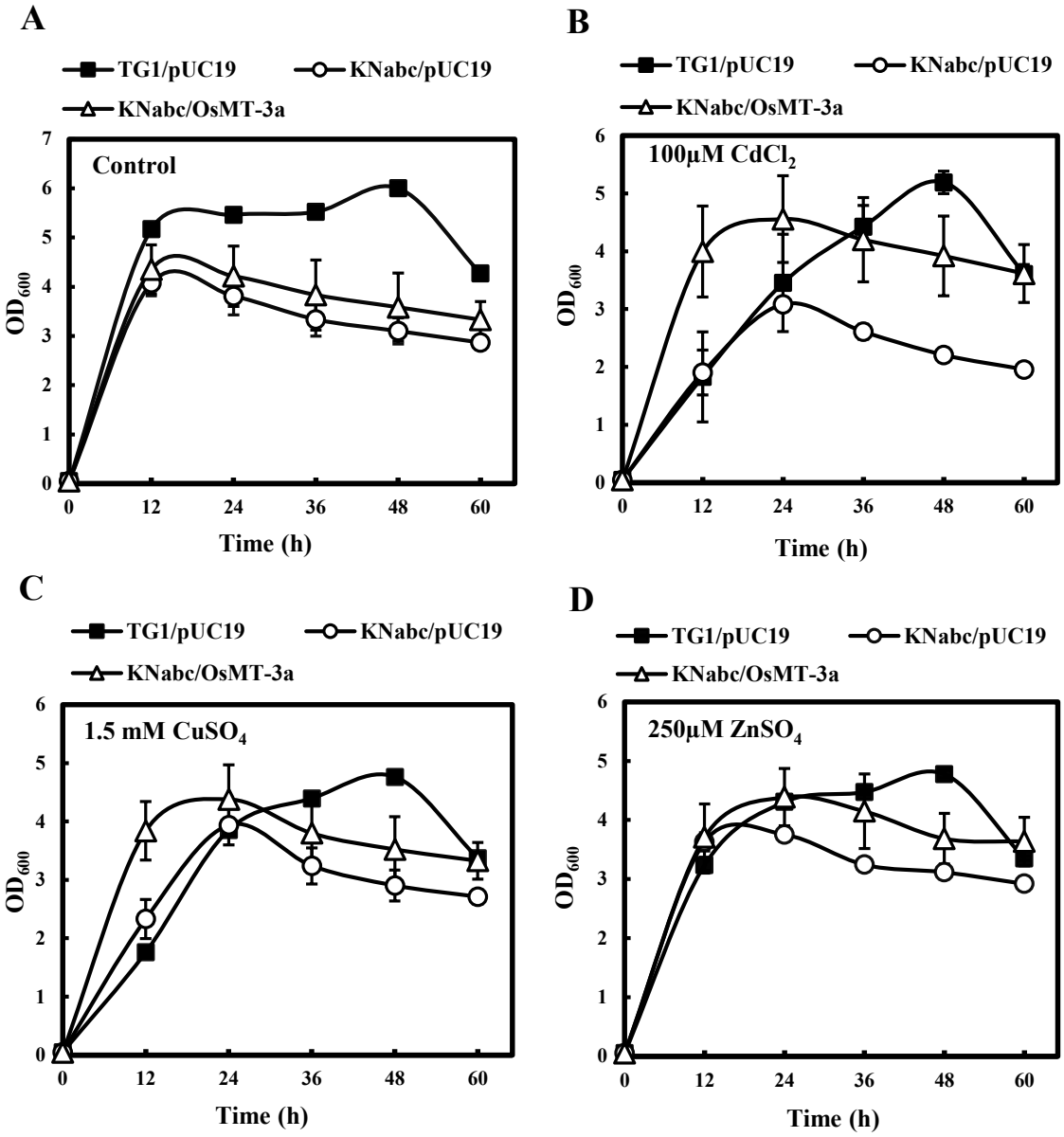

Figure 3.5. Increased tolerance of *OsMT-3a*-expressing *E. coli* cells to Cd^{2+}, Cu^{2+}, and Zn^{2+} stresses. At the beginning of the assay (OD_{600} = 0.05), 1.0 mM IPTG was added to all three cultures. Bacterial cells were cultivated either (A) in L-medium only as control or (B) supplemented with 100 μM $CdCl_2$, (C) 1.5 mM $CuSO_4$, (D) 250 μM $ZnSO_4$. Data are represented as the means of three independent experiments ± SE.

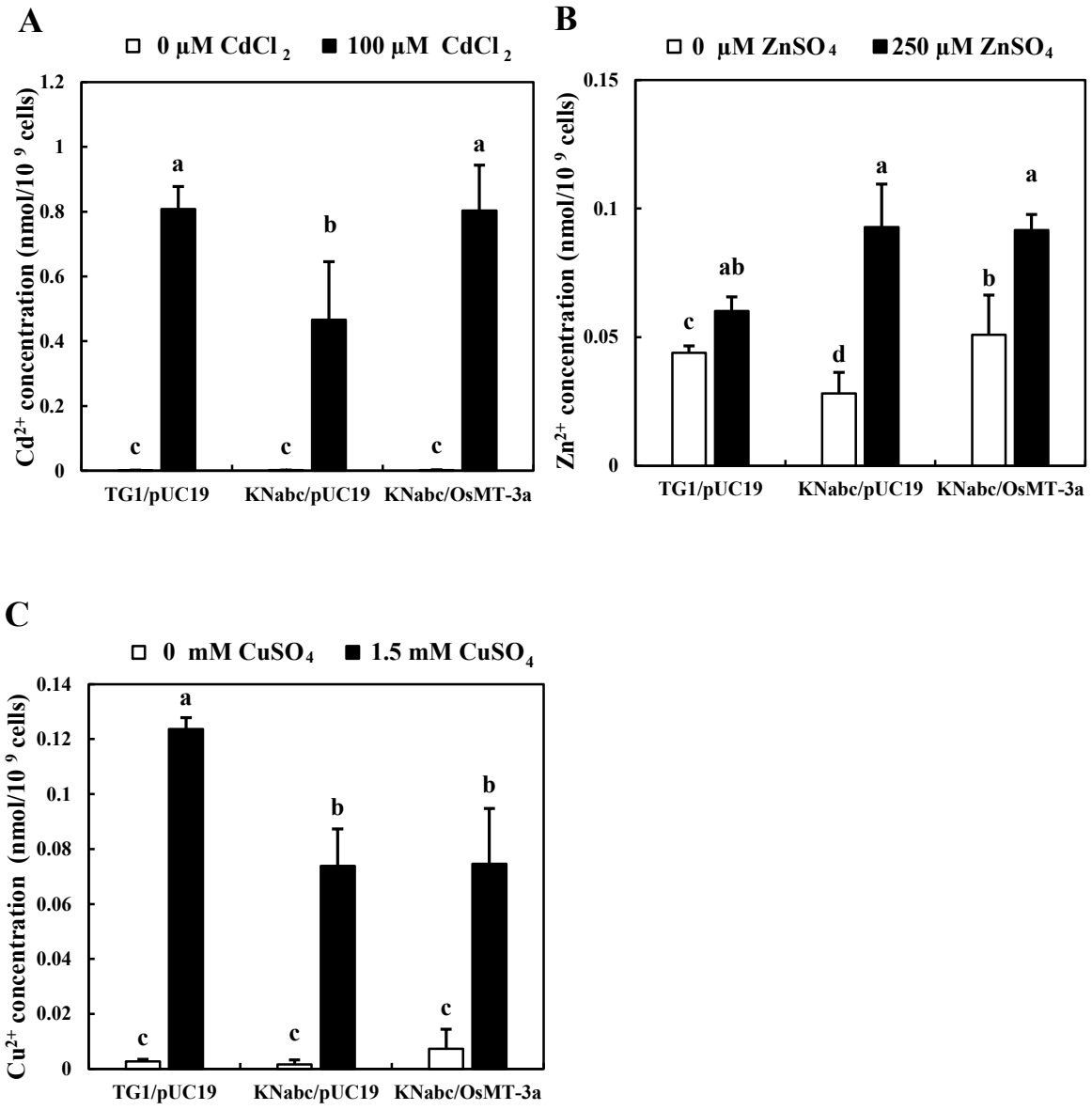

Figure 3.6. Metal ions concentration (Cd^{2+}, Zn^{2+} and Cu^{2+}) in *OsMT-3a*-expressing *E. coli* cells in the presence of (A) 100 μM $CdCl_2$, (B) 250 μM $ZnSO_4$, (C) 1.5 mM $CuSO_4$ compared with that in control cells. At the beginning of the assay (OD_{600} = 0.05), 1.0 mM IPTG was added to all three cultures and they were then grown for 24 h. Data are represented as the means of three independent experiments ± SE. The same letters indicate no significant differences ($P < 0.05$).

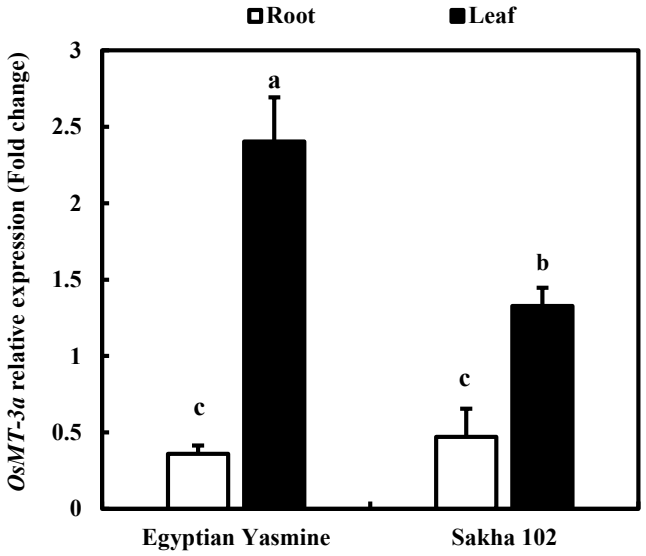

Figure 3.7. Relative expression of *OsMT-3a* in the roots and leaves of rice seedlings of the cultivars Egyptian Yasmine and Sakha 102 grown under salinity stress conditions (50 mM NaCl) for 10 days. Data are represented as the means of three independent experiments ± SE. The same letters indicate no significant differences ($P < 0.05$).

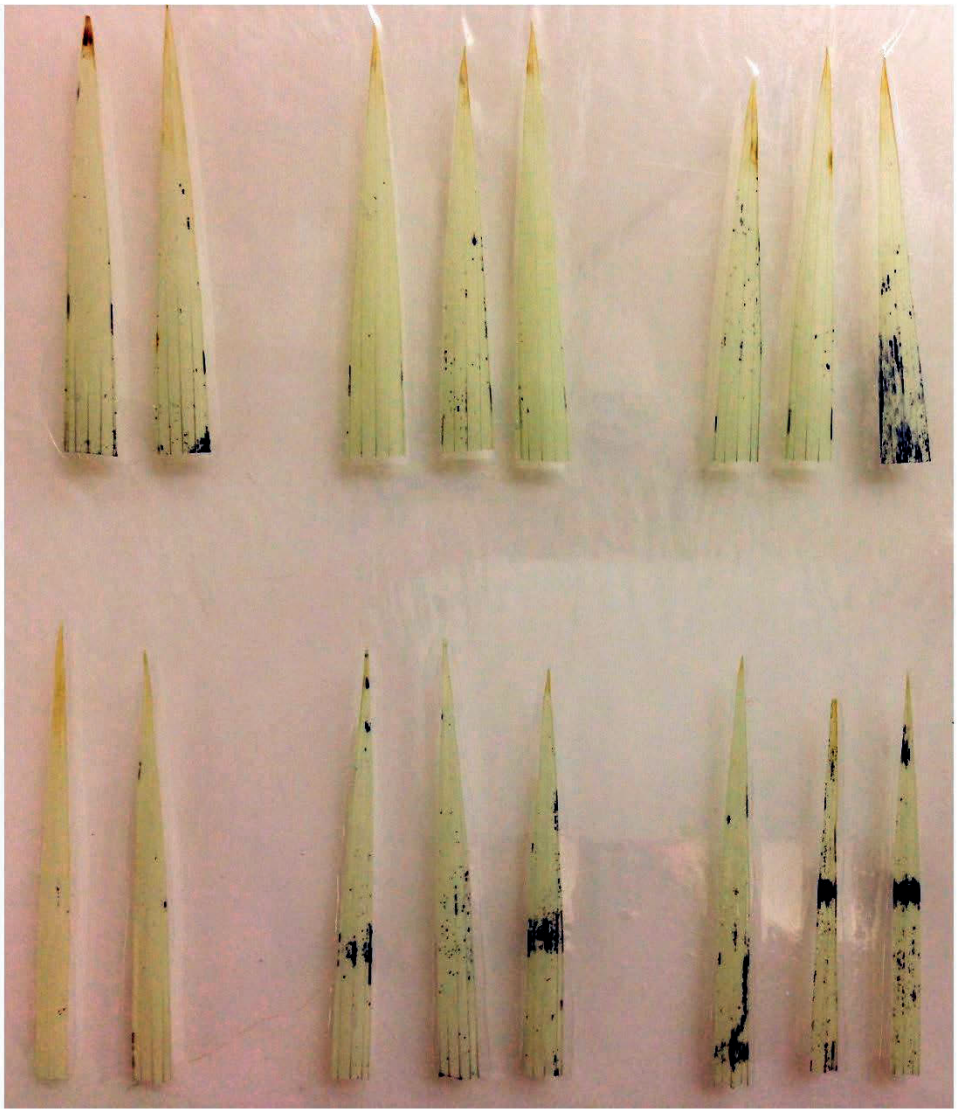

Figure 3.8. Histochemical detection of O_2^- with NBT in the leaves of rice plants grown under different stress condition. Egyptian Yasmine and Sakha 102 cultivars were grown in hydroponic (control) or treated with NaCl (50 mM) and/or $CdCl_2$ (100 µM) for 10 days.

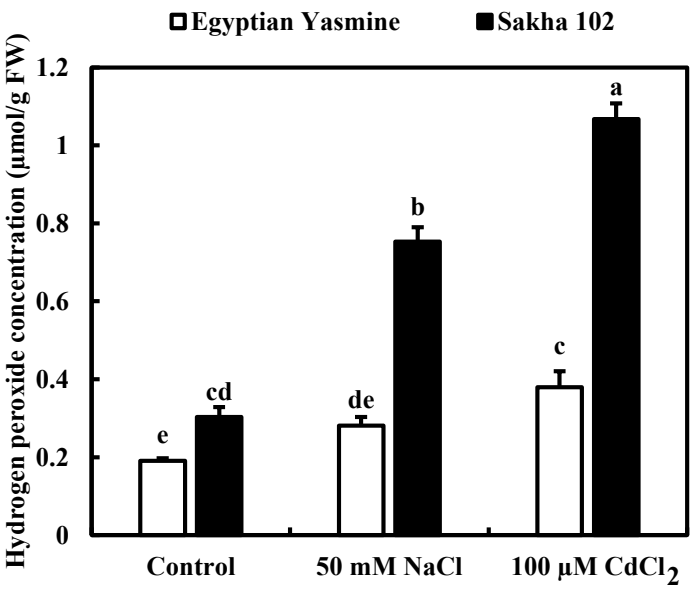

Figure 3.9. Comparison of H_2O_2 concentrations in the leaves of the rice cultivars, Egyptian Yasmine and Sakha 102 grown under control conditions and in the presence of 50 mM NaCl and/or 100 µM $CdCl_2$. Data are represented as the means of four replicates ± SE. The same letters indicate no significant differences ($P < 0.05$).

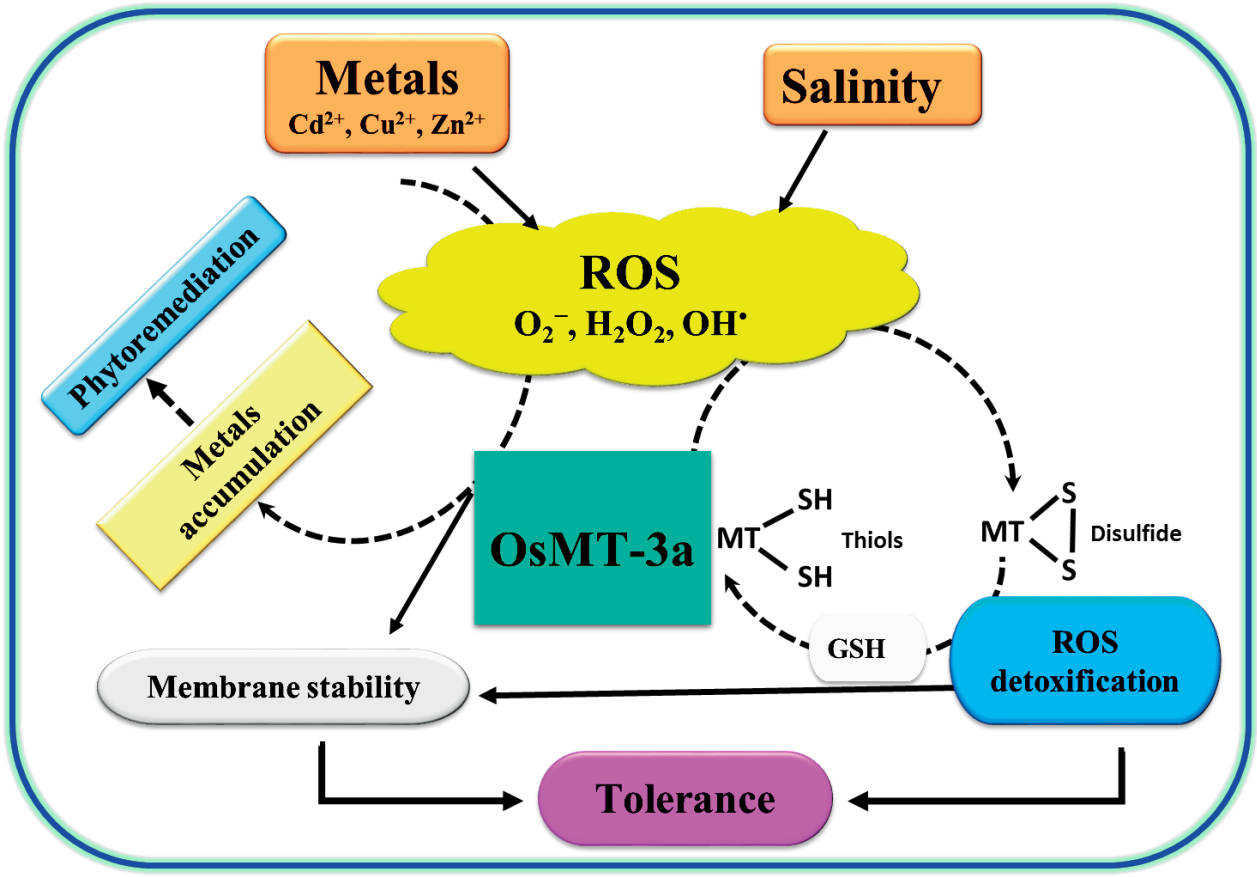

Figure 3.10. A hypothetical model for the role of OsMT-3a in ROS scavenging and phytoremediation under abiotic stress.

3.4 Discussion

Oxidative stress is a secondary consequence when organisms are challenged with abiotic stresses, such as high salinity. Oxidative stress results from the disturbance in balance between the production and scavenging of ROS such as hydrogen peroxide, superoxide anions, and hydroxyl radicals, which damage cells by destroying lipids, nucleic acids, and proteins (Apel and Hirt, 2004). Living organisms have developed a variety of adaptive mechanisms to cope with various internal and external stresses. MTs are efficient scavengers of produced ROS in plants (Grennan, 2011; Hassinen *et al.,* 2011).

In the current study, in an effort to further elucidate the molecular mechanisms of salinity stress tolerance in the rice cultivar Egyptian Yasmine, we set out to isolate salinity-inducible genes and examine their functional roles which could be involved in other adaptation mechanisms under salinity stress conditions. To achieve this, we screened a cDNA library that is prepared from salinity-stressed seedlings of the salinity-tolerant rice cultivar, Egyptian Yasmine, in a Na^+-sensitive *E. coli* cells KNabc [(Δ*nhaA*, Δ*nhaB*, Δ*chaA*), a triple mutant of *E. coli*, these mutants are disabled in the function of three Na^+/H^+ antiporters which exclude Na^+ from the cell in the wild-type (TG1)] under different salt concentrations of up to 2 M NaCl. From the library screening, one isolated gene shared significant sequence homology with that of plant type-3 metallothionein (MT-3a). Quantitative RT-PCR analysis indicated that the expression of *OsMT-3a* in rice seedlings was highly induced by salinity stress. Functional screening analysis of the gene in *E. coli* cells, and *in vivo* localization of O_2^- and quantification of H_2O_2 in rice plants demonstrated that the *OsMT-3a* possibly contribute to the tolerance of rice plants to oxidative stress brought about by salinity and heavy metal stresses, through its ability to scavenge ROS.

There is substantial experimental evidence of the involvement of plant MTs in various environmental stress resistance through its ability to scavenge ROS, which relieves the oxidative stress produced by such stresses. Akashi *et al*. (2004) have demonstrated the strong hydroxyl radical-scavenging activity of the drought-induced CLMT2 that was isolated from wild watermelon, and that the hydroxyl radical-catalyzed degradation of genomic DNA was effectively suppressed by CLMT2 *in vitro*. Also Wong *et al*. (2004) showed that the rice recombinant protein OsMT2b possessed superoxide and hydroxyl radicals scavenging activities *in vitro*. Xue *et al*. (2009) reported that H_2O_2 levels in transgenic tobacco plants overexpressing *GhMT3a*, a type 3 MT isolated from cotton (*Gossypium hirsutum*), were only half of those in wild-type plants when placed under salinity, drought, and low temperature stresses, suggesting that the higher tolerance to stress might be due to changes in ROS signaling. In the current study, we found that under 200 mM NaCl stress conditions, H_2O_2 concentrations in transformed *E. coli* cells, KNabc/*OsMT-3a* were less than one third those in KNabc/pUC19 (Figure 3.3C), indicating a correlation between the expression of *OsMT-3a* and the imbalance of ROS production in *E. coli* cells. Thus, OsMT-3a may act as an antioxidant to minimize ROS toxicity.

Moreover, a different class of rice MT (rgMT) was shown to improve the salinity tolerance of *E. coli* (Jin *et al.,* 2006). In the current study, *E. coli* cells expressing *OsMT-3a* were able to tolerate high salinity levels, and grew more efficiently when compared to KNabc/pUC19, in media containing a high concentration of NaCl (Figure 3.3A), indicating a potential role of the gene in enhancing the growth performance of the bacterial cells under such severe salinity stress conditions. However, *OsMT-3a* did not completely recover the growth of the bacterial mutants KNabc when compared to TG1/pUC19 growth rate under salinity stress (Figure 3.3A), and the KNabc/OsMT-3a accumulated much more Na^+ compared to TG1/pUC19 under such conditions

(Figure 3.3B), implying that OsMT-3a improved salinity tolerance of KNabc cells through another mechanism rather than Na^+ exclusion from the cells.

The roles of plant MT isoforms in modulation of ROS are well documented (Akashi *et al.,* 2004; Wong *et al.,* 2004; Yang *et al.,* 2011). Here, we demonstrated that the heterologous expression of *OsMT-3a* increased the tolerance of *E. coli* cells to H_2O_2 stress (Figures 3.4), in terms of growth enhancement under higher concentration of H_2O_2 that was lethal to KNabc/pUC19 cells, suggesting that *OsMT-3a* is involved in cell protection against an excess of ROS. This result supports a previous study showing that in transgenic rice plants overexpressing *OsMTI-1a*, another rice MT type 1 isoform, the activity of catalase, peroxidase, and ascorbate peroxidase antioxidant enzymes was significantly increased under H_2O_2 treatment (Yang *et al.,* 2009). However, further investigation is needed to study the relationship between MT isoforms and the activities of antioxidant enzymes.

Many reports have assigned the expression of plant *MT* genes in *E. coli* to assess their ability to confer tolerance to heavy-metal stress (Zhigang *et al.,* 2006; Chaturvedi *et al.,* 2012; Kim *et al.,* 2014; Dundar *et al.,* 2015). In the current study, we have investigated the growth of the different bacterial strains in the presence of heavy metals (Figure 3.5). *E. coli* expressing *OsMT-3a* grew better in media containing high concentrations of $CdCl_2$, $CuSO_4$, and $ZnSO_4$, respectively, than did those without *OsMT-3a* (Figure 3.5), indicating that *OsMT-3a* conferred tolerance to heavy-metal stress.

Several types of plant MTs have been identified, and it was proposed that each MT may have higher affinity for a specific heavy metal and lower affinity for other metals (Foley *et al.,* 1997). The results reported by Nezhad *et al.,* (2013) showed that, *in vitro* analysis of metal-binding characteristics and spectroscopic behaviors of recombinant OsMT proteins revealed that the two

isoforms OsMTI-1b and OsMTII-1a have different ability to bind Cd^{2+} ions, and that the heterologous expression of OsMTI-1b, but not OsMTII-1a, augments Cd^{2+}, Ni^{2+} and Zn^{2+} tolerance of *E. coli* cells by sequestering more metal ions. Type 2 MT from *Avicennia marina* (AmMT2) when expressed in *E. coli*, increased its tolerance to Zn^{2+}, Cu^{2+}, Pb^{2+}, and Cd^{2+} by binding to these metals, with the highest affinity for Cd^{2+} (Huang and Wang, 2010). Our results showed that, under $CdCl_2$, $CuSO_4$ and $ZnSO_4$ stress conditions, both KNabc/pUC19 and KNabc/OsMT-3a cells accumulated metal ions (Figure 3.6); however, *OsMT-3a*-expressing cells accumulated a significantly higher levels of Cd^{2+}, than did KNabc/pUC19 (Figure 3.6A), while no significant difference in the concentrations of Zn^{2+} and Cu^{2+} between KNabc/OsMT-3a and KNabc/pUC19. These results indicate that, OsMT-3a has greater affinity to bind Cd^{2+} (about two times higher than that of control cells) (Figure 3.6A) than for Zn^{2+} and Cu^{2+}, suggesting that OsMT-3a may protect *E. coli* cells against the toxic effect of Cd^{2+} and play a role in detoxification by sequestering more metal ions. Similarly, in a previous study, Arabidopsis MT3 was shown to enhance Cd^{2+} tolerance when expressed in *Vicia faba* guard cells (Lee *et al.,* 2004) and also, the *Tamarix hispida* MT-like ThMT3 protein was proved to enhance tolerance to Cd^{2+}, Zn^{2+}, Cu^{2+}, and NaCl stress when expressed in yeast cells (Yang *et al.,* 2011).

MT expression is under spatial and temporal control in plants, and plant MTs display tissue-specific expression. Plant type 1 MTs are more abundantly expressed in roots than in leaves, whereas type 2 MTs are expressed primarily in leaves, and at a lower level in roots (Cobbett and Goldsbrough, 2002). Type 3 MTs are expressed in leaves or in ripening fruit (Ledger and Gardner, 1994), whereas expression of type 4 MTs appear to be restricted to developing seeds (Kawashima *et al.,* 1992). Similarly, the current study found that *OsMT-3a* was expressed more abundantly in the leaves than in the roots of both assessed cultivars (Figure 3.7). Previous studies reported that

MT expression was induced by heavy metals and abiotic stresses in various plants (Lee *et al.,* 2004; Zhigang *et al.,* 2006; Xue *et al.,* 2009; Kim *et al.,* 2014). In particular, the rice *rgMT* expression has been shown to be induced by several abiotic stresses, including salt (NaCl) and alkali ($NaHCO_3$) stresses, drought (polyethylene glycol), and metal ions ($CuCl_2$, $ZnCl_2$, and $CdCl_2$) (Jin *et al.,* 2006). Moreover, expression of the rice type 1 *OsMT1a* gene was induced by dehydration and Zn^{2+} treatment (Yang *et al.,* 2009), and the expression of the Pokkali rice *OsMT1e-P* gene was induced in response to salinity stress (Kumar *et al.,* 2012). In the current study, we found that *OsMT-3a* exhibited expression patterns similar to those of these two *MT* genes and was strongly induced in response to salinity stress (Figure 3.7). However, the expression level of *OsMT-3a* was higher in the salinity-tolerant Egyptian Yasmine than in the salinity-sensitive Sakha 102. These results clearly show that *OsMT-3a* is of importance in the tolerance mechanism of Egyptian Yasmine under salinity stress conditions.

Because heterologous expression of *OsMT-3a* improved the salinity tolerance of *E. coli* cells probably through scavenging ROS, we examined that proposed function of this gene in rice plants by determining the ROS levels in the two contrasting rice genotypes. For this purpose, leaves of the rice cultivars Egyptian Yasmine and Sakha 102, subjected to NaCl and $CdCl_2$ stresses for 10 days were stained with NBT to reveal the accumulation of O_2^-. Histochemical staining showed that under both salinity and heavy-metal stresses, notable increase in ROS levels in Sakha 102 as these leaves showed intense blue colored polymers accumulation (Figure 3.8). However under the same condition, the leaves of Egyptian Yasmine showed remarkably less accumulation of blue colored polymers implying less ROS production. As ROS levels during stress greatly relies on the homeostasis between ROS generation and removal (Miller et al., 2010), accumulation of less ROS in Egyptian Yasmine seems to indicate that ROS scavenging systems in this cultivar might work

more effectively as compared to Sakha 102. MTs can protect plants by participating in signaling (early response) or adaptation (late response). Increased production of the antioxidant thiol protein, MT during late phases of oxidative stress, helps in mitigating stress by scavenging the ROS probably through the oxidation of cysteine thiols (Chubatsu and Meneghini, 1993). Based on this analysis, we propose that OsMT-3a may enhance plant stress tolerance through the reduction of ROS. A hypothetical model summarizing the role of *Os*MT-3a in modulating ROS scavenging/detoxification during stress conditions is shown in Figure 3.10.

Although the functions of plant MTs in ROS removal or signaling and the interplay between the metal ions and ROS scavengers remain unclear, it is likely that plant MTs provide an efficient antioxidant system that enables plants to cope with oxidative stress (Yang *et al.,* 2009). Because salinity and metal stress can both induce the production of ROS and consequently damage (Li and Trush, 1993), it is possible that the ROS scavenging activity of OsMT-3a enhanced the tolerance of the transformed *E. coli* to salt and heavy metals.

3.5 Conclusion

In conclusion, we cloned a MT gene, classified as a type 3 MT, from rice leaves (*OsMT-3a*). *OsMT-3a* expression was highly induced in the leaves of the salinity-tolerant rice cultivar Egyptian Yasmine, and expression was strongly induced by NaCl stress. Heterologous expression of *OsMT-3a* in *E. coli* reduced the deleterious effects of H_2O_2 stress and conferred tolerance to NaCl and heavy metals in *E. coli* cells, suggesting that OsMT-3a is important not only for metal homoeostasis/detoxification but also for imparting tolerance to oxidative stress, and that OsMT-3a can potentially contribute to the tolerance of rice plants to oxidative stress brought about by salinity stress, through its ability to scavenge ROS. Therefore, this gene (*OsMT-3a*) was shown to

be of great importance for the adaptation to salinity stress and might be the key gene for salinity tolerance in the cultivar Egyptian Yasmine.

Chapter 4

General discussion

This study was conducted to: (1) investigate the physiological responses and mode of adaptation to salinity stress in the Egyptian rice cultivars, Sakha 102 and Egyptian Yasmine by comparing the growth criteria, Na^+ and K^+ accumulation patterns and other physiological parameters under salinity stress conditions; (2) elucidate differences in the mechanisms of salinity tolerance and the molecular basis for Na^+ accumulation patterns in the salinity-tolerant, Egyptian Yasmine and the salinity-sensitive, Sakha 102 cultivars through the analysis of gene expression profiles of some genes encoding Na^+ and K^+ transport protein; and (3) further elucidate the molecular mechanisms of salinity stress tolerance in the salinity-tolerant rice cultivar, through the isolation and characterization of candidate genes that may function in adaptation to other stress conditions such as osmotic or oxidative stresses, which could reveal other strategies of adaptation of this cultivar in response to environmental stresses.

4.1 Growth, physiological adaptation and gene expression analysis of two Egyptian rice cultivars under salt stress

To explore the mode of adaptation under salinity stress, the physiological parameters of two local Egyptian rice (*Oryza sativa* L.) cultivars, Sakha 102 and Egyptian Yasmine were grown under 50 mM NaCl stress for 14 days. The results indicated that Egyptian Yasmine was relatively salt tolerant compared Sakha 102, and this was evident in its higher dry mass production, lower leaf Na^+ levels, and enhanced water conservation under salt stress conditions. Reduction in plant growth under salinity stress is often associated with salt-induced osmotic effect, nutrient deficiency or ion toxicity (Munns, 2002). Moreover, Egyptian Yasmine exhibited lower Na^+/K^+ ratios in all tissues examined under salinity stress. In contrast, Sakha 102 appeared salt sensitive and accumulated much higher Na^+ in the leaves, with reduced growth and higher Na^+/K^+ ratio

especially in the leaves. Low cytosolic Na^+ contents and high K^+/Na^+ ratios aid in maintaining an osmotic and biochemical equilibrium in the cells (Tester and Davenport, 2003). Therefore, the reduced Na^+ accumulation in the leaves of Egyptian Yasmine might be due to a mechanism which excludes Na^+ from shoot, and this mechanism may not be operating in Sakha 102. Such an exclusion mechanism could be operating through, efflux of excess Na^+ out of cells, retrieval of excess Na^+ from the xylem stream or compartmentalization of excess Na^+ into the cell vacuoles (Munns, 2002). These processes are under the control of a number of transporters that have been implicated in leaf Na^+ exclusion including, the plasma membrane Na^+/H^+ antiporter SOS1, the high-affinity K^+ transporter (HKT 1), and the tonoplast Na^+/H^+ antiporter (NHX1) (Shi *et al.,* 2002; Ren *et al.,* 2005; Horie *et al.,* 2009; Venema *et al.,* 2002). However, this mechanism of Na^+ exclusion needs to be elucidated, in order to understand the basis for the differences in Na^+ accumulation between the two cultivars. We analyzed the transcript levels of genes encoding Na^+ and K^+ transport proteins in different tissues of Egyptian Yasmine and Sakha 102. In response to salinity stress, Egyptian Yasmine showed induction of expression of some membrane transporter/channel genes that may contribute to Na^+ exclusion from the shoots including *OsHKT1;5*, which mediates Na^+ retrieval from the xylem into xylem parenchyma cells before it is transported in the transpiration stream to the shoot (Ren *et al.,* 2005). Thus, it is possible that in the present study, the improved growth of Egyptian Yasmine under salt stress may be due to induced expression of *OsHKT1*;5, whereas, the sensitivity of Sakha 102 might be due to the repression of *OsHKT1*;5 expression, leading to unregulated Na^+ transport to the leaves and hence growth impairment. Also, PMP3 proteins can potentially contribute to Na^+ exclusion from cells (Navarre and Goffeau, 2000). The marked induction of *OsLti6b* (the rice PMP3 ortholog) expression in the roots of Egyptian Yasmine indicated a role in limiting excess Na^+ entry into the

roots of this cultivar, whereas such a function would be unlikely in the Sakha 102 cultivar. Furthermore, Egyptian Yasmine had abundant *OsAKT1* transcripts under salinity stress conditions, and that would explain the increased K^+ accumulation in the leaves and roots as compared to Sakha 102. In contrast to Na^+ exclusion, OsHKT2;1 is known to mediate Na^+ influx in yeast and plants (Garciadeblás *et al.,* 2003). Repressed expression of *OsHKT2;1* in both cultivars indicated that the OsHKT2;1 transporter is important for the restriction of toxic accumulation of Na^+ in both cultivars. Therefore, the active regulation of genes related to Na^+ transport at the transcription level may be involved in salt tolerance mechanisms of Egyptian Yasmine.

4.2 Identification of a type 3 metallothionein-like gene (*OsMT-3a*) from rice through functional screening analysis in Escherichia coli, confers tolerance against salinity and heavy-metal stresses

Screening using microorganisms like *Escherichia coli* and yeast, which can efficiently express heterologous cDNA clones of a library, is a powerful tool for identifying genes with specific functions (Serrano and Gaxiola, 1994; Kumari *et al.,* 2009). *E. coli* or yeast cells expressing plant cDNA clones have been previously screened to identify genes involved in enhanced stress tolerance (Rausell *et al.,* 2003; Yamada *et al.,* 2003; Ezawa and Tada, 2009; Joshi *et al.,* 2009).

In the previous study, the adaptation mechanisms of two Egyptian rice cultivars to salinity stress have been characterized. The difference in tolerance between the two cultivars lies in the ability to exclude Na^+ to the leaf blade. This exclusion mechanism was shown to be under the control of transporters such as the HKT1;5, which functions in restriction the transport of Na^+ to the leaf and hence in stress tolerance. However, it is unclear whether the tolerance of Egyptian Yasmine is limited only to Na^+ exclusion or expression of Na^+ transport genes. Therefore, the

present study was designed to isolate and characterize some genes from Egyptian Yasmine that could be involved in other adaptation processes, for example against osmotic or oxidative stresses under salt stress conditions, which could reveal other adaptation strategies of this cultivar to environmental stresses.

A metallothionein-like type 3 (*OsMT-3a*) gene was identified from a rice (*Oryza sativa* L.) leaf cDNA library prepared from plants grown under NaCl stress, through functional screening in *Escherichia coli* cells. Expression analysis of *OsMT-3a* in rice seedlings revealed that *OsMT-3a* was highly upregulated by high salinity in the leaves of the salinity-tolerant cultivar Egyptian Yasmine, but not in those of the salinity-sensitive one, Sakha 102. Previous studies have reported that *MT* expression was induced by heavy metals and abiotic stresses in various plants (Lee *et al.,* 2004; Zhigang *et al.,* 2006; Xue *et al.,* 2009; Kim *et al.,* 2014). In particular, the rice *rgMT* expression has been shown to be induced by several abiotic stresses, including salt (NaCl) and alkali ($NaHCO_3$) stresses, drought (polyethylene glycol), and metal ions ($CuCl_2$, $ZnCl_2$, and $CdCl_2$) (Jin *et al.,* 2006). *E. coli* cells expressing *OsMT-3a* showed higher tolerance to NaCl, H_2O_2 and heavy metal (Cd^{2+}) stresses than did control cells. Transformed *E. coli* cells also accumulated higher amounts of Cd^{2+} than other metal ions. The main action mechanism of MTs is in ROS scavenging, which relieves the oxidative stress produced by high salt concentrations (Nishiuchi *et al.,* 2007; Grennan, 2011; Akashi et al., 2004; Hassinen *et al.,* 2011). In this study, H_2O_2 levels under NaCl stress conditions in transformed *E. coli* were less than one third those in control cells. *In vivo* localization of O_2^- and quantification of H_2O_2 in rice plants demonstrated that the *OsMT-3a* possibly contribute to the tolerance of rice plants to oxidative stress brought about by salinity and heavy metal stresses, through its ability to scavenge ROS. Also, the reduced ELR observed for Egyptian Yasmine in chapter 2 can be attributed to the activity of this MT in protecting

biological membranes against oxidative damage. Together, these results provided evidence that the rice *OsMT-3a* could function as a reactive oxygen species scavenger that could improve salinity tolerance.

4.3 Conclusion

This study aimed at characterizing the physiological responses of the Egyptian rice cultivars, Sakha 102 and Egyptian Yasmine to salinity stress and to elucidate differences in the mechanisms of salinity tolerance between them. By comparing the growth parameters, Na^+ and K^+ accumulation and the analysis of gene expression of Na^+ and K^+ transport protein coding genes, it was demonstrated that Egyptian Yasmine is relatively salt tolerant compared to Sakha 102, and this was evident in its higher dry mass production, lower leaf Na^+ levels, and enhanced water conservation under salt stress conditions. Moreover, Egyptian Yasmine exhibited lower Na^+/K^+ ratios in all tissues examined under salinity stress. Furthermore, its ability to restrict Na^+ accumulation in leaves under salt stress is believed to be one of its main adaptation strategies, which may be partly explained by the distinct regulation of gene expression of Na^+/K^+ transport proteins. This was evident in the inducible expression of *OsHKT1;5* (Na^+ retrieval), *OsLti6b* (restriction of Na^+ entry), and *OsAKT1* (K^+ uptake), as well as the repressed expression of *OsHKT2;1* (Na^+ influx). Divergent regulation of Na^+ and K^+ transporters may be involved in the maintenance of lower Na^+/K^+ ratios in Egyptian Yasmine under salt stress. Further evaluation of salinity stress-induced genes in Egyptian Yasmine yielded the *OsMT-3a* gene, whose overexpression in *E. coli* showed that it is important in mediating ROS scavenging. Overall, these studies have shown that the tolerance of Egyptian Yasmine to salt stress lies in its ability to exclude Na^+ in the leaf and to scavenge ROS.

Summary

Soil salinity is one of the most severe problems in agriculture. Absorption of excessive salt inhibits both root and shoot growth, reduces reproductive activity and affects viability of plants. To counter salinity stress, plant cells have several adaptive mechanisms. However, the molecular mechanisms regulating biochemical and physiological changes in response to salinity stress are not well understood. Rice, *Oryza sativa* L., is one of the most important crop species and the major food crop for much of the world's population. Since it is a relatively salt-sensitive crop species, it is important to understand the mode of adaptation of the plant to salinity stress in order to produce new salinity tolerant rice varieties with increased productivity. In Egypt, soil salinization is becoming increasingly challenging for agriculture, and the response of many rice cultivars to salinity stress has not been clearly established. Thus, our study was conducted to investigate the physiological responses of two important local rice cultivars to salinity stress and to elucidate differences in the mechanisms of salinity tolerance between them by comparing the growth parameters, Na^+ and K^+ accumulation and the expression profiles of some genes encoding Na^+ and K^+ transport protein. Also, to elucidate the further molecular mechanisms of stress tolerance in the salinity-tolerant cultivar, through the isolation and characterization of genes which could be involved in other tolerance pathways that might reveal other adaptation strategies of this cultivar in response to salinity stress.

1. Growth, physiological adaptation and gene expression analysis of two Egyptian rice cultivars under salt stress

To investigate the mode of adaptation under salinity stress, the physiological parameters of two local Egyptian rice cultivars, Sakha 102 and Egyptian Yasmine were examined under 50 mM NaCl stress for 14 days. The results indicated that Egyptian Yasmine was relatively salt tolerant compared Sakha 102, and this was evident in its higher dry mass production, lower leaf Na^+ levels, and enhanced water conservation under salt stress conditions. Moreover, Egyptian Yasmine exhibited lower Na^+/K^+ ratios in all tissues under salinity stress. In contrast, Sakha 102 appeared salt sensitive and accumulated much higher Na^+ in the leaves, with reduced growth and higher Na^+/K^+ ratio especially in the leaves. Therefore, the adaptation of Egyptian Yasmine to salt stress involves reduced Na^+ accumulation in the leaves, which might be due to a mechanism which excludes Na^+ from shoot, and that this mechanism is not operating in Sakha 102. However, this mechanism of Na^+ exclusion needs to be elucidated, in order to understand the basis for the differences in Na^+ accumulation between the two cultivars. Na^+/K^+ transport proteins have been shown to control the transport of Na^+ and/or K^+ across membranes and regulate ion homeostasis in cells. We analyzed the transcript levels of some key genes encoding Na^+ and K^+ transport proteins in different tissues of Egyptian Yasmine and Sakha 102. Moreover, in response to salinity stress, Egyptian Yasmine showed induction of expression of some membrane transporter/channel genes that may contribute to Na^+ exclusion from the shoots (*OsHKT1;5*), limiting excess Na^+ entry into the roots (*OsLti6b*), K^+ uptake (*OsAKT1*), and reduced expression of a Na^+ transporter gene (*OsHKT2;1*). Therefore, the active regulation of genes related to Na^+ transport at the transcription level might be involved in salt tolerance mechanisms of Egyptian Yasmine. Differences in the mechanisms of salinity tolerance between the two cultivars may be partly explained by the distinct

regulation of gene expression of Na^+ and K^+ transport proteins, and these mechanisms offer the promise of improved salinity stress tolerance in local Egyptian rice genotypes.

2. Identification of a type 3 metallothionein-like gene (*OsMT-3a*) from rice through functional screening analysis in Escherichia coli, confers tolerance against salinity and heavy-metal stresses

To further elucidate the molecular mechanism of stress tolerance in Egyptian Yasmine, we set out to isolate and characterize the salinity-inducible genes which could be involved in other adaptation processes under salinity stress conditions. A metallothionein-like type 3 (*OsMT-3a*) gene was identified from rice plants (cv. Egyptian Yasmine) grown under NaCl stress, through cDNA library screening in *Escherichia coli* cells. Heterologous expression of *OsMT-3a* in *E. coli* cells has induced their tolerance to NaCl and heavy metals, Cd^{2+}, Zn^{2+}, and Cu^{2+} (mostly Cd^{2+}) than did control cells, in terms of growth performance. Under high concentrations of H_2O_2, *OsMT-3a*-overexpressing *E. coli* cells showed enhanced growth, while the growth of control cells was completely inhibited. Hydrogen peroxide levels under NaCl stress conditions in *OsMT-3a*-transformed cells were less than one third those in control cells. Quantitative real-time PCR analysis revealed that expression of *OsMT-3a* was highly induced by salinity stress in the leaves of the salinity-tolerant cultivar Egyptian Yasmine, but not in those of the salinity-sensitive one, Sakha 102. *In vivo* localization of oxygen superoxide (O_2^-) and quantification of H_2O_2 in the leaves of the rice plants grown under NaCl and $CdCl_2$ stress conditions showed that, Egyptian Yasmine maintained lower levels of O_2^- and H_2O_2 concentrations compared to those in Sakha 102. Taken together, these results suggested that, heterologous expression of *OsMT-3a* has conferred tolerance to NaCl and heavy metals and reduced the deleterious effects of H_2O_2 stress in *E. coli* cells, and that OsMT-3a can potentially contribute to the tolerance of rice plants to oxidative stress brought about by salinity and heavy metal stresses, through its ability to scavenge ROS.

In conclusion, the divergent regulation of Na^+ and K^+ transporters may be involved in the maintenance of lower Na^+/K^+ ratios in Egyptian Yasmine under salt stress. Moreover, further evaluation of salinity stress-induced genes in Egyptian Yasmine yielded the *OsMT-3a* gene, whose overexpression in *E. coli* showed that it is important in mediating ROS scavenging. Overall, these studies have shown that the tolerance of Egyptian Yasmine to salinity stress lies in its ability to exclude Na^+ in the leaf and to scavenge ROS.

References

Abd Allah, A.A., Amar, M.H., Badawi, A.T. 2010. Screening rice genotypes for drought resistance in Egypt. J. Plant Breed. Crop Sci., 7, 205–215.

Abebe, T., Guenzi, A. C., B, M., Cushman, J. C. 2003. Tolerance of mannitol accumulating transgenic wheat to water stress and salinity. Plant Physiol., 131, 1748–1755.

Akashi, K., Nishimura, N., Ishida, Y., Yokota, A. 2004. Potent hydroxyl radical-scavenging activity of drought-induced type-2 metallothionein in wild watermelon. Biochem. Biophys. Res. Commun., 323, 72–78.

Apel, K., Hirt, H. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol., 55, 373–399.

Apse, M. P., Aharon, G. S., Snedden, W. A., Blumwald, E. 1999. Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ anitport in Arabidopsis. Science, 285, 1256–1258.

Apse, M.P., Sottosanto, J.B., Blumwald, E. 2003. Vacuolar cation/H^+ exchange, ion homeostasis, and leaf development are altered in a T-DNA insertional mutant of *AtNHX1*, the Arabidopsis vacuolar Na^+/H^+ antiporter. Plant J., 36, 229–239.

Arafat, S., Afify, A., Aboelghar, M., Belal, A. 2010. Rice crop monitoring in Egyptian Nile delta using EGYPTSAT-1 data. Joint U.S.-Egypt workshop for space technology and geo-information for sustainable development, NARSS-Cairo.

Arzani, A. 2008. Improving salinity tolerence in crop plants: a biotechology view. In Vitro Cell. Dev. Biol., Plant, 44, 373–383.

Assaha, D.V.M., Ueda, A., Saneoka, H., 2013. Comparison of growth and mineral accumulation of two solanaceous species, *Solanumm scabrum* Mill. (huckleberry) and *S. melongena* L. (eggplant), under salt stress. Soil Sci. Plant Nutr., 59, 912–920.

Badawi, A.T. 2005. Sustainability of rice productivity in Egypt. Egypt J. Agirc. Res., 83 (5A).

Bassil, E., Tajima, H., Liang, Y.C., Ohto, M.A., Ushijima, K., Nakano, R., Esumi, T., Coku, A., Belmonte, M., Blumwald, E. 2011. The Arabidopsis Na^+/H^+ antiporters NHX1 and NHX2 control vacuolar pH and K^+ homeostasis to regulate growth, flower development, and reproduction. Plant Cell, 23, 3482–3497.

Bates, L.S., Waldren, R.P., Teare, I.D. 1973. Rapid determination of free proline for water stress studies. Plant Soil, 39, 205–207.

Berthomieu, P., Conejero, G., Nublat, A., Brackenbury, W.J., Lambert, C., Savio, C., Uozumi, N., Oiki, S., Yamada, K., Cellier, F., Gosti, F., Simonneau, T., Essah, P.A., Tester, M., Very, A.A., Sentenac, H., Casse, F. 2003. Functional analysis of *AtHKT1* in Arabidopsis shows that Na^+ recirculation by the phloem is crucial for salt tolerance. EMBO J., 22, 2004–2014.

Blumwald, E. 2000. Sodium transport and salt tolerance in plants. Curr. Opin. Cell Biol., 12, 431–434.

Carden, D.E., Walker, D.J., Flowers, T.J., Miller, A. J. 2003. Single-cell measurements of the contributions of cytosolic Na^+ and K^+ to salt tolerance. Plant Physiol., 131, 676–683.

Chaturvedi, A.K., Mishra, A., Tiwari, V., Jha, B. 2012. Cloning and transcript analysis of type 2 metallothionein gene (*SbMT-2*) from extreme halophyte *Salicornia brachiata* and its heterologous expression in *E. coli*. Gene, 499, 280–287.

Chaturvedi, A.K., Patel, M.K., Mishra, A., Tiwari, V., Jha, B. 2014. The *SbMT-2* gene from a halophyte confers abiotic stress tolerance and modulates ROS scavenging in transgenic tobacco. PLoS One, 9, e111379.

Chen, H.J., Hou, W.C., Yang, C.Y., Huang, D.J., Liu, J.S., Lin, Y.H. 2003. Molecular cloning of two metallothionein-like protein genes with differential expression patterns from sweet potato (*Ipomoea batatas*) leaves. J. Plant Physiol., 160, 547–555.

Chubatsu, L.S., Meneghini, R. 1993. Metallothionein protects DNA from oxidative damage. Biochem. J., 291, 193–198.

Cobbett, C., Goldsbrough, P. 2002. Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. Annu. Rev. Plant Biol., 53, 159–182.

Cotsaftis, O., Plett, D., Shirley, N., Tester, M., Hrmova, M. 2012. A two-staged model of Na^+ exclusion in rice explained by 3D modeling of HKT transporters and alternative splicing. PLoS One, 7, e39865.

Cuartero, J., Bolarin, M.C., Asins, M.J., Moreno, V. 2006. Increasing salt tolerance in the tomato. J. Exp. Bot., 57, 1045–1058.

Cuin, T.A., Miller, A.J., Laurie, S.A., Leigh, R.A. 2003. Potassium activities in cell compartments of salt-grown barley leaves. J. Exp. Bot., 54, 657–661.

Darwish, E., Testerink, C., Khalil, M., El-Shihy, O., Munnik, T. 2009. Phospholipid signaling responses in salt-stressed rice leaves. Plant Cell Physiol., 50, 986–997.

Davenport, R.J., Tester, M. 2000. A weakly voltage-dependent, nonselective cation channel mediates toxic sodium influx in wheat. Plant Physiol., 122, 823–834.

Ding, L., Zhu, J.K. 1997. Reduced Na^+ uptake in the NaCl hypersensitive *sos1* mutant of *Arabidopsis thaliana*. Plant Physiol., 113, 795–799.

Dundar, E., Sonmez, G.D., Unver, T. 2015. Isolation, molecular characterization and functional analysis of OeMT2, an olive metallothionein with a bioremediation potential. Mol. Genet. Genom., 290, 187–99.

Ezawa, S., Tada, Y. 2009. Identification of salt tolerance genes from the mangrove plant *Bruguiera gymnorrhiza* using Agrobacterium functional screening. Plant Sci., 176, 272–278.

FAO. 2008. FAO Land and plant nutrition management service. http://www.fao.org/ag/agl/agll/spush.

Flowers, T.J. 1999. Salinization and horticultural production. Sci. Hortic. 78, 1–4.

Flowers, T.J. 2004. Improving crop salt tolerance. J. Exp. Bot., 55, 307–319.

Flowers, T.J., Troke, P.F., Yeo, A.R. 1977. The mechanism of salt tolerance in halophytes. Annu. Rev. Plant Physiol., 28, 89–121.

Foley, R.C., Liang, Z.M., Karam, B.S. 1997. Analysis of type 1 metallothionein cDNAs in *Vicia faba*. Plant Mol. Biol., 33, 583–591.

Foyer, C.H., Lelandais, M., Kunert, K.J. 1994. Photooxidative stress in plants. Physiol. Plant., 92, 696–717.

Fukuda, A., Nakamura, A., Tagiri, A., Tanaka, H., Miyao, A., Hirochika, H., Tanaka, Y. 2004. Function, intracellular localization and the importance in salt tolerance of a vacuolar Na^+/H^+ antiporter from rice. Plant Cell Physiol., 45, 146–159.

Fukuda, A., Nakamura, A., Tanaka, Y. 1999. Molecular cloning and expression of the Na^+/H^+ exchanger gene in *Oryza sativa*. Biochim. Biophys. Acta., 1446, 149–155.

Fukuda, A., Yazaki, Y., Ishikawa, T., Koike, S., Tanaka, Y. 1998. Na^+/H^+ antiporter in tonoplast vesicles from rice roots. Plant Cell Physiol., 39, 196–201.

Gao, X.H., Ren, Z.H., Zhao, Y.X. and Zhang, H. 2003. Overexpression of SOD2 increases salt tolerance of Arabidopsis. Plant Physiol., 133, 1873–1881.

Garciadeblás, B., Senn, M.E., Bañuelos, M.A., Rodríguez-Navarro, A. 2003. Sodium transport and HKT transporters: the rice model. Plant J., 34, 788–801.

Gechev, T.S., Van Breusegem, F., Stone, J.M., Denev, I., Laloi, C. 2006. Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. Bioessays, 28, 1091–1101.

Golldack, D., Quigley, F., Michalowski, C.B., Kamasani, U.R., Bohnert, H.J. 2003. Salinity stress-tolerant and -sensitive rice (*Oryza sativa* L.) regulates AKT1-type potassium channel transcripts differently. Plant Mol. Biol., 51, 71–81.

Golldack, D., Su, H., Quigley, F., Kamasani, U.R., Munoz-Garay, C., Balderas, E., Popova, O.V., Bennett, J., Bohnert, H.J., Pantoja, O. 2002. Characterization of a HKT-type transporter in rice as a general alkali cation transporter. Plant J., 31, 529–542.

Grennan, A.K. 2011. Metallothioneins, a diverse protein family. Plant Physiol., 155, 1750–1751.

Hamer, D.H. 1986. Metallothionein. Annu. Rev. Biochem., 55, 913–951.

Hassinen, V.H., Tervahauta, A.I., Schat, H., Karenlampi, S.O. 2011. Plant metallothioneins- metal chelators with ROS scavenging activity? Plant Biol., 13, 225–232.

Hong, Z., Lakkineni, K., Zhang, Z., Verma, D. P. S. 2000. Removal of feedback inhibition of Δ^1-pyrroline-5-carboxylate systhetase results in increased proline accumulation and protection of plants from osmotic stress. Plant Physiol., 122, 1119–1136.

Horie, T., Costa, A., Kim, T.H., Han, M.J., Horie, R., Leung, H.Y., Miyao, A., Hirochika, H., Schroeder, J.I. 2007. Rice OsHKT2;1 transporter mediates large Na^+ influx component into K^+-starved roots for growth. EMBO J., 26, 3003–3014.

Horie, T., Hauser, F., Schroeder, J.I. 2009. HKT transporter-mediated salinity resistance mechanisms in Arabidopsis and monocot crop plants. Trends Plant Sci., 14, 660–668.

Horie, T., Yoshida, K., Nakayama, H., Yamada, K., Oiki, S., Shinmyo, A. 2001. Two types of HKT transporters with different properties of Na^+ and K^+ transport in *Oryza sativa*. Plant J., 27, 129–138.

Huang, G.Y., Wang, Y.S. 2010. Expression and characterization analysis of type 2 metallothionein from grey mangrove species (*Avicennia marina*) in response to metal stress. Aquat. Toxicol., 99, 86–92.

Inada, M., Ueda, A., Shi, W., Takabe, T. 2005. A stress-inducible plasma membrane protein 3 (AcPMP3) in a monocotyledonous halophyte, *Aneurolepidium chinense*, regulates cellular Na^+ and K^+ accumulation under salt stress. Planta, 220, 395–402.

Jain, M., Nijhawan, A., Tyagi, A.K., Khurana, J.P. 2006. Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. Biochem. Biophys. Res. Commun., 345, 646–651.

Jang, S.J., Wi, S.J., Choi, Y.J., An, G., Park, K.Y. 2012. Increased polyamine biosynthesis enhances stress tolerance by preventing the accumulation of reactive oxygen species: T-DNA mutational analysis of *Oryza sativa* lysine decarboxylase-like protein 1. Mol. Cells, 34, 251–262.

Jin, S., Cheng, Y., Guan, Q., Liu, D., Takano, T., Liu, S. 2006. A metallothionein-like protein of rice (rgMT) functions in *E. coli* and its gene expression is induced by abiotic stresses. Biotechnol. Lett., 28, 1749–1753.

Joshi, A., Dang, H.Q., Vaid, N., Tuteja, N. 2009. Isolation of high salinity stress tolerant genes from *Pisum sativum* by random overexpression in *Escherichia coli* and their functional validation. Plant Signal Behav., 4, 400–412.

Kaiser, W.M. 1979. Reversible inhibition of the Calvin cycle and activation of oxidative pentose phosphate cycle in isolated intact chloroplast by hydrogen peroxide. Planta 145, 377–382.

Kandil, A.A., Sharief, A.E., Nassar, E.S. E. 2012. Response of some rice (*Oryza sativa* L.) cultivars to germination under salinity stress. Int. J. Agric. Sci., 4, 272–277.

Karahara, I., Ikeda, A., Kondo, T., Uetake, Y. 2004. Development of the Casparian strip in primary roots of maize under salt stress. Planta, 219, 41–47.

Kawashima, I., Kennedy, T.D., Chino, M., Lane, B.G. 1992. Wheat Ec metallothionein genes. Like mammalian Zn^{2+} metallothionein genes, wheat Zn^{2+} metallothionein genes are conspicuously expressed during embryogenesis. Eur. J. Biochem., 209, 971–976.

Kim, S.H., Jeong, J.C., Ahn, Y.O., Lee, H.S., Kwak, S.S. 2014. Differential responses of three sweet potato metallothionein genes to abiotic stress and heavy metals. Mol. Biol. Rep., 41, 6957–66.

Koyama, M.L., Levesley, A., Koebner, R.M.D., Flowers, T.J., Yeo, A.R. 2001. Quantitative trait loci for component physiological traits determining salt tolerance in rice. Plant Physiol., 125, 406–422.

Kumar, G., Kushwaha, H.R., Panjabi-Sabharwal, V., Kumari, S., Joshi, R., Karan, R., Mittal, S., Pareek, S.L., Pareek, A. 2012. Clustered metallothionein genes are co-regulated in rice and ectopic expression of *OsMT1e-P* confers multiple abiotic stress tolerance in tobacco via ROS scavenging. BMC Plant Biol., 12, 107.

Kumari, S., Singh, P., Singla-Pareek, S.L., Pareek, A. 2009. Heterologous expression of a salinity and developmentally regulated rice cyclophilin gene (*OsCyp2*) in *E. coli* and *S. cerevisiae* confers tolerance towards multiple abiotic stresses. Mol. Biotechnol., 42, 195–204.

Kushizaki, M. 1968. An extraction procedure of plant materials for the rapid determination of Mn, Cu, Zn, and Mg by the atomic absorption analysis. J. Sci. Soil Manure, 39, 489–490.

Ledger, S.E., Gardner, R.C. 1994. Cloning and characterization of five cDNAs for genes differentially expressed during fruit development of kiwifruit (*Actinidia deliciosa* var. *deliciosa*). Plant Mol. Biol., 25, 877–886.

Lee, J., Shim, D., Song, W.Y., Hwang, I., Lee, Y. 2004. Arabidopsis metallothioneins 2a and 3 enhance resistance to cadmium when expressed in *Vicia faba* guard cells. Plant Mol. Biol., 54, 805–815.

Lefévre, I., Gratia, E., Lutts, S. 2001. Discrimination between the ionic and osmotic components of salt stress in relation to free polyamine level in rice (*Oryza sativa*). Plant Sci., 161, 943–952.

Leidi, E. O., Barragán, V., Rubio, L., El-Hamdaoui, A., Ruiz, M. T., Cubero, B., Fernández, J. A., Bressan, R. A., Hasegawa, P. M., Quintero, F. J., Pardo, J. M. 2010. The AtNHX1 exchanger mediates potassium compartmentation in vacuoles of transgenic tomato. Plant J., 61, 495–506.

Li, Y., Trush, M.A. 1993. DNA damage resulting from the oxidation of hydroquinone by copper: role for a Cu (II)/Cu (I) redox cycle and reactive oxygen generation. Carcinogenesis. 7, 1303–1311.

Livak, K.J., Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods, 25, 402–408.

Maathuis, F. J. M., Sanders, D. 2001. Sodium uptake in *Arabidopsis thaliana* is regulated by cyclic nucleotides. Plant Physiol., 127, 1617–1625.

Mandhania, S., Madan, S., Sawhney, V. 2006. Antioxidant defense mechanism under salt stress in wheat seedling. Biol. Plant., 227, 227–231.

Martínez-Atienza, J., Jiang, X., Garciadeblás, B., Mendoza, I., Zhu, J. K., Pardo, J. M., Quintero, F. J. 2007. Conservation of the salt overly sensitive pathway in rice. Plant Physiol., 143, 1001–1012.

Miller, G., Suzuki, N., Ciftci-Yilmaz, S., Mittler, R. 2010. Reactive oxygen species homeostasis and signaling during drought and salinity stresses. Plant Cell Environ., 33, 453–467.

Mittler, R. 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci., 7, 405–410.

Mohanty, A., Kathuria, H., Ferjani, A., Sakamoto, A., Mohanty, P., Murata, N., Tyagi, A. 2002. Transgenics of an elite *indica* rice variety Pusa Basmati 1 harboring the *codA* gene are highly tolerant to salt stress. Theor. Appl. Genet., 106, 51–57.

Munns, R. 2002. Comparative physiology of salt and water stress. Plant Cell Environ., 25, 239–250.

Munns, R., Tester, M. 2008. Mechanisms of salinity tolerance. Annu. Rev. Plant Biol., 59, 651–681.

Munns, R., James, A. J., Läuchli, A. 2006. Approaches to increasing the salt tolerance of wheat and other cereals. J. Exp. Bot., 57, 1025–1043.

Navarre, C., Goffeau, A. 2000. Membrane hyperpolarization and salt sensitivity induced by deletion of PMP3, a highly conserved small protein of yeast plasma membrane. EMBO J., 19, 2515–2524.

Nezhad, R. M., Shahpiri, A., Mirlohi, A. 2013. Heterologous expression and metal-binding characterization of a type 1 metallothionein isoform (*Os*MTI-1b) from rice (*Oryza sativa*). Protein J., 32, 131–137.

Nishiuchi, S., Liu, S., Takano, T. 2007. Isolation and characterization of a metallothionein-1 protein in *Chloris virgata* Swartz that enhances stress tolerances to oxidative, salinity and carbonate stress in *Saccharomyces cerevisiae*. Biotechnol. Lett., 29, 1301–1305.

Noctor, G., Foyer, C. 1998. Ascorbate and glutathione: keeping active oxygen under control. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 49–279.

Nozaki, K., Inaba, K., Kuroda, T., Tsuda, M., Tsuchiya, T. 1996. Cloning and sequencing of the gene for Na^+/H^+ antiporter of *Vibrio parahaemolyticus*. Biochem. Biophys. Res. Commun., 222, 774–779.

Nylander, M., Heino, P., Helenius, E., Palva, E.T., Ronne, H., Welin, B.V. 2001. The low-temperature and salt-induced *RCI2A* gene of Arabidopsis complements the sodium sensitivity caused by a deletion of the homologous yeast gene *SNA1*. Plant Mol. Biol., 45, 341–352.

Pardo, J. M. 2010. Biotechnology of water and salinity stress tolerance. Curr. Opin. Biotechnol., 21, 185–196.

Polle, A. 2001. Dissecting the superoxide dismutase-ascorbate-glutathione pathway in chloroplasts by metabolic modeling, Computer simulations as a step towards flux analysis. Plant Physiol. 126, 445–462.

Rausell, A., Kanhonou, R., Yenush, L., Serrano, R., Ros, R. 2003. The translation initiation factor eIF1A is an important determinant in the tolerance to NaCl stress in yeast and plants. Plant J., 34, 257–267.

Ren, Z.H., Gao, J.P., Li, L.G., Cai, X.L., Huang, W., Chao, D.Y., Zhu, M.Z., Wang, Z.Y., Luan, S., Lin, H.X. 2005. A rice quantitative trait locus for salt tolerance encodes a sodium transporter. Nature Genet., 37, 1141–1146.

Roberts, S.K., Tester, M., 1997. Patch clamp study of Na^+ transport in maize roots. J. Exp. Bot., 48, 431–440.

Rodríguez-Navarro, A., Rubio, F. 2006. High-affinity potassium and sodium transport systems in plants. J. Exp. Bot., 57, 1149–1160.

Santa-Maria, G.E., Rubio, F., Dubcovsky, J., Rodríguez-Navarroa, A. 1997. The *HAK 1* gene of barley is a member of a large gene family and encodes a high-affinity potassium transporter. Plant Cell, 9, 2281–2289.

Senadheera, P., Singh, R.K., Maathuis, F.J.M. 2009. Differentially expressed membrane transporters in rice roots may contribute to cultivar dependent salt tolerance. J. Exp. Bot., 60, 2553–2563.

Sentenac, H., Bonneaud, N., Minet, M., Lacroute, F., Salmon, J.M., Gaymard, F. Grignon, C. 1992. Cloning and expression in yeast of a plant potassium ion transport system. Science, 256, 663–665.

Serrano, R., Gaxiola, R. 1994. Microbial models and salt stress tolerance in plants. CRC Crit. Rev. Plant Sci., 13, 121–138.

Shehata, S.M., Abd Allah, A.A., Zayed, B.A. 2009. Development of salt tolerant rice lines through mutation breeding. J. Agric. Res. Kafrelsheikh Univ., 35 (4).

Shi, H., Lee, B. H., Wu, S. J., Zhu, J. K. 2003. Overexpression of a plasma membrane Na^+/H^+ antiporter gene improves salt tolerance in *Arabidopsis thaliana*. Nature Biotechnol., 21, 81–85.

Shi, H., Quintero, F.J., Pardo, J.M., Zhu, J.K. 2002. The putative plasma membrane Na^+/H^+ antiporter SOS1 controls long-distance Na^+ transport in plants. Plant Cell, 14, 465–477.

Sivakumar, P., Sharmila, P., Pardha Saradhi, P. 2000. Proline alleviates salt stress induced enhancement in ribulose-1,5-bisphosphate oxygenase activity. Biochem. Biophys. Res. Commun., 279, 512–515.

Sondergaard, T.E., Schulz, A., Palmgren, M.G. 2004. Energization of transport processes in plants. Roles of the plasma membrane H^+-ATPase. Plant Physiol., 136, 2475–2482.

Sreenivasulu, N., Sopory, S. K., Kavi Kishor, P. B. 2007. Deciphering the regulatory mechanisms of abiotic stress tolerance in plants by genomic approaches. Gene, 388, 1–13.

Subbarao, G.V., Ito, O., Berry, W.L., Wheeler, R.M. 2003. Sodium – A functional plant nutrient. Crit. Rev. Plant Sci., 22, 391–416.

Suharsono, U., Fujisawa, Y., Kawasaki, T., Iwasaki, Y., Satoh, H., Shimamoto, K. 2002. The heterotrimeric G protein alpha subunit acts upstream of the small GTPase Rac in disease resistance of rice. Proc. Natl. Acad. Sci. USA, 99, 13307–13312.

Sunarpi, Horie, T., Motoda, J., Kubo, M., Yang, H., Yoda, K., Horie, R., Chan, W.Y., Leung, H.Y., Hattori, K., Konomi, M., Osumi, M., Yamagami, M.,Schroeder, J.I., Uozumi, N. 2005. Enhanced salt tolerance mediated by AtHKT1 transporter-induced Na^+ unloading from xylem vessels to xylem parenchyma cells. Plant J., 44, 928–938.

Takahashi, R., Nishio, T., Ichizen, N., Takano, T. 2007. High-affinity K^+ transporter PhaHAK5 is expressed only in salt-sensitive reed plants and shows Na^+ permeability under NaCl stress. Plant Cell Rep., 26, 1673–9.

Tester, M., Davenport, R.J. 2003. Na^+ tolerance and Na^+ transport in higher plants. Ann. Bot., 91, 503–527.

Turchi, A., Tamantini, I., Camussi, A.M., Racchi, M.L. 2012. Expression of a metallothionein A1 gene of *Pisum sativum* in white poplar enhances tolerance and accumulation of zinc and copper. Plant Sci., 183, 50–56.

Ueda, A., Kanechi, M., Uno, Y., Inagaki, N. 2003. Photosynthetic limitations of a halophyte sea aster (*Aster tripolium* L.) under water stress and NaCl stress. J. Plant Res., 116, 65–70.

Ueda, A., Kathiresan, A., Bennett, J., Takabe, T. 2006. Comparative transcriptome analyses of barley and rice under salt stress. Theor. Appl. Genet., 112, 1286–1294.

Ueda, A., Kathiresan, A., Inada, M., Narita, Y., Nakamura, T., Shi, W., Takabe, T., Bennett, J. 2004. Osmotic stress in barley roots regulates expression of a different set of genes than salt stress does. J. Exp. Bot., 406, 2213–2218.

Ueda, A., Shi, W., Nakamura, T., Takabe, T. 2002. Analysis of salt inducible genes in barley roots by differential display. J. Plant Res., 115, 119–130.

Ueda, A., Yahagi, H., Fujikawa, Y., Nagaoka, T., Esaka, M., Calcaño, M., González, M.M., Martich, J.D.H., Saneoka, H. 2013. Comparative physiological analysis of salinity tolerance in rice. Soil Sci. Plant Nutr., 59, 896–903.

USDA-ARS. 2008. Research Databases. Bibliography on Salt Tolerance. George E. Brown, Jr. Salinity Lab. US Dep. Agric., Agric. Res. Serv. Riverside, CA. http://www. ars.usda.gov/Services/docs.htm?docid=8908

Venema, K., Quintero, F.J., Pardo, J.M., Donaire, J.P. 2002. The Arabidopsis Na^+/H^+ exchanger AtNHX1 catalyzes low affinity Na^+ and K^+ transport in reconstituted liposomes. J. Biol. Chem., 277, 2413–2418.

Walia, H., Wilson, C., Zeng, L., Ismail, A.M., Condamine, P., Close, T.J. 2007. Genome-wide transcriptional analysis of salinity-stressed *japonica* and *indica* rice genotypes during panicle initiation stage. Plant Mol. Biol., 63, 609–623.

Wang, J., Zuo, K., Wu, W., Song, J., Sun, X., Lin, J. 2004. Expression of a novel antiporter gene from *Brassica napus* resulted in enhanced salt tolerance in transgenic tobacco plants. Plant Biol., 48, 509–515.

Wang, W.B., Vinocur, A., Altman, A. 2003. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta, 218, 1–14.

Weatherly, P.E. 1950. Studies in water relations of cotton plants, the field measurement of water deficit in leaves. New Phytol., 49, 81–87.

White, P.J. 2001. The pathways of calcium movement to the xylem. J. Exp. Bot., 52, 891–899.

Wong H. L., Sakamoto T., Kawasaki T., Umemura K., Shimamoto K. 2004. Down-regulation of metallothionein, a reactive oxygen scavenger, by the small GTPase OsRac1 in rice. Plant Physiol., 135, 1447–1456.

Xue, T., Li, X., Zhu, W., Wu, C., Yang, G., Zheng, C. 2009. Cotton metallothionein GhMT3a, a reactive oxygen species scavenger, increased tolerance against abiotic stress in transgenic tobacco and yeast. J. Exp. Bot., 60, 339–349.

Xue, Z. Y., Zhi, D. Y., Xue, G. P., Zhang, H., Zhao, Y. X., Xia, G. M. 2004. Enhanced salt tolerance of transgenic wheat (*Triticum aesativum* L.) expressing a vacuolar Na^+/H^+ antiporter gene with improved grain yields in saline soils in the field and a reduced level of leaf Na^+. Plant Sci., 167, 849–859.

Yamada, A., Tsutsumi, K., Tanimoto, S., Ozeki, Y. 2003. Plant RelA/ SpoT homolog confers salt tolerance in *Escherichia coli* and *Saccharomyces cerevisiae*. Plant Cell Physiol., 44, 3–9.

Yamaguchi, T., Aharon, G.S., Sottosanto, J.B., Blumwald, E. 2005. Vacuolar Na^+/H^+ antiporter cation selectivity is regulated by calmodulin from within the vacuole in Ca^{2+} and pH-dependent manner. Proc. Natl. Acad. Sci. USA, 102, 16107–16112.

Yan, Y. X., Fang, Y. A., Wei, Z. K., Ren, Z. J. 2004. Production and analysis of transgenic maize with improved salt tolerance by the introduction of *AtNHX1* gene. J. Integr. Plant Biol., 46, 854–861.

Yang, J., Wang, Y., Liu, G., Yang, C., Li, C. 2011. *Tamarix hispida* metallothionein-like ThMT3, a reactive oxygen species scavenger, increases tolerance against Cd^{2+}, Zn^{2+}, Cu^{2+}, and NaCl in transgenic yeast. Mol. Biol. Rep., 38, 1567–1574.

Yang, Z., Wu, Y., Li, Y., Ling, H.Q., Chu, C. 2009. OsMT1a, a type 1 metallothionein, plays the pivotal role in zinc homeostasis and drought tolerance in rice. Plant Mol. Biol., 70, 219–229.

Yao, X., Horie, T., Xue S., Leung, H.Y., Katsuhara, M., Brodsky, D.E., Wu, Y., Schroeder, J.I. 2010. Differential sodium and potassium transport selectivities of the rice OsHKT2;1 and OsHKT2;2 transporters in plant cells. Plant Physiol., 152, 341–355.

Zhang, F.Q., Wang, Y.S., Lou, Z.P., Dong, J.D. 2007. Effect of heavy metal stress on antioxidative enzymes and lipid peroxidation in leaves and roots of two mangrove plant seedlings (*Kandelia candel* and *Bruguiera gymnorrhiza*). Chemosphere, 67, 44–50.

Zhang, H., Blumwald, E. 2001. Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. Nature Biotechnol., 19, 765–768.

Zhang, H. X., Hodson, J. N., William, J. P., Blumwald, E. 2001. Engineering salt tolerant Brassica plants: Characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium accumulation. Proc. Natl. Acad. Sci. USA, 98, 12832–12836.

Zhigang, A., Cuijie, L., Yuangang, Z., Yejie, D., Wachter, A., Gromes, R. and Rausch, T. 2006. Expression of *BjMT2*, a metallothionein 2 from Brassica juncea, increases copper and cadmium tolerance in *Escherichia coli* and *Arabidopsis thaliana*, but inhibits root elongation in *Arabidopsis thaliana* seedlings. J. Exp. Bot., 57, 3575–3582.

Zhu J.K., Liu J, Xiong L. 1998. Genetic analysis of salt tolerance in Arabidopsis: evidence for a critical role to potassium nutrition. Plant Cell, 10, 1181–1191.

Zhu, J.K. 2001. Plant salt tolerance. Trends Plant Sci., 6, 66–71.

Zhu, J.K. 2002. Salt and drought stress signal transduction in plants. Annu. Rev. Plant Biol., 53, 247–273.

Zhu, J.K. 2003. Regulation of ion homeostasis under salt stress. Curr. Opin. Plant Biol., 6, 441–445.

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