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ORIGINAL ARTICLE

Regulation of cation transporter genes by the arbuscular mycorrhizal symbiosis in rice plants subjected to salinity suggests improved salt tolerance due to reduced Na^+ root-to-shoot distribution

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Abstract Rice is a salt-sensitive crop whose productivity is strongly reduced by salinity around the world. Plants growing in saline soils are subjected to the toxicity of specific ions such as sodium, which damage cell organelles and disrupt metabolism. Plants have evolved biochemical and molecular mechanisms to cope with the negative effects of salinity. These include the regulation of genes with a role in the uptake, transport or compartmentation of Na^+ and/or K^+. Studies have shown that the arbuscular mycorrhizal (AM) symbiosis alleviates salt stress in several host plant species. However, despite the abundant literature showing mitigation of ionic imbalance by the AM symbiosis, the molecular mechanisms involved are barely explored. The objective of this study was to elucidate the effects of the AM symbiosis on the expression of several well-known rice transporters involved in Na^+/K^+ homeostasis and measure Na^+ and K^+ contents and their ratios in different plant tissues. Results showed that *OsNHX3*, *OsSOS1*, *OsHKT2;1* and *OsHKT1;5* genes were considerably upregulated in AM plants under saline conditions as compared to non-AM plants. Results suggest that the AM symbiosis favours Na^+ extrusion from the cytoplasm, its sequestration into the vacuole, the unloading of Na^+ from the xylem and its recirculation from photosynthetic organs to roots. As a result, there is a decrease of Na^+ root-to-shoot distribution and an increase of Na^+ accumulation in rice roots which seems to enhance the plant tolerance to salinity and allows AM rice plants to maintain their growing processes under salt conditions.

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Keywords Arbuscular mycorrhizal symbiosis · Ion homeostasis · Plant tolerance · Salinity

Introduction

Rice (*Oryza sativa* L.) is a species native to tropical regions which has been consumed by humans for nearly 9000 years (Callaway 2014). Nowadays, it is considered the most important source of food for more than half of the world population (Kumar et al. 2013). According to FAO (2005), the world population continues to grow and world agriculture should produce 70 % more food by the middle of twenty-first century. However, rice is a salt-sensitive crop and its productivity is strongly reduced by salinity around the world (Kumar et al. 2013). Therefore, investigating different strategies to make rice plants more tolerant and to improve its productivity under salinity is an important challenge for researchers in order to cope with reduced food production due to soil salinization (Ruiz-Lozano et al. 2012, Augé et al. 2014).

Plants growing in saline soils are subjected to the toxic effects of specific ions such as sodium and chloride, which disrupt the structure of enzymes and other macromolecules, damage cell organelles and disrupt photosynthesis and respiration. Soil salinity also induces a physiological drought in the plant and produces nutrient imbalance due to decreased nutrient uptake and/or transport to the shoot (Munns and Tester 2008, Ruiz-Lozano et al. 2012). Thus, accumulation of Na^+ and impairment of K^+ nutrition are a typical characteristic of plant subjected to salt stress, the mechanisms of which are only partially understood (Chen et al. 2007). Na^+ is a major cation present in saline soils, but it is not an essential mineral nutrient for most plants, while many cytosolic enzymes are activated by K^+ and inhibited by Na^+ (Shi et al. 2002). Indeed, under normal physiological conditions, higher plants contain

between 1 and 10 mM of Na^+ in cytosol and between 60 and 100 mM of K^+ (Bassil et al. 2012). When there is an abnormally high cytosolic Na^+/K^+ ratio, inhibition of protein synthesis and cytosolic enzymatic activities is triggered (Shabala and Cuin 2008). Hence, plants have evolved biochemical and molecular mechanisms in order to cope with the negative effects of salinity. These include the regulation of genes with a role in the uptake, transport or compartmentation of Na^+ and/or K^+, which are responsible of the adequate ionic homeostasis in the plant.

Salt-sensitive plants, also known as glycophytes, have several mechanisms to prevent excessive accumulation of Na^+ in the cytosol. The first one is restricting Na^+ entry to plant cells by selective ion uptake. The second one is maximizing the efflux of Na^+ back to the growth medium or to apoplastic spaces. Finally, plants can also restrict the transfer of Na^+ to the shoot by sequestering the internalized Na^+ into vacuoles (Cuin et al. 2011), contributing at the same time to cellular osmotic adjustment (Shi et al. 2002, Zhu 2003). In any case, the two last mechanisms seem to be more important to control Na^+ accumulation in plants (Cuin et al. 2011, Cabot et al. 2014). Na^+ efflux from cytosol to the growth medium or to apoplastic spaces is catalysed by a plasma membrane Na^+/H^+ antiporter SOS1 in several plants, including rice (Kumar et al. 2013). However, the preferential expression of *SOS1* in the cells surrounding xylem vessels suggests also a role of this transporter in the redistribution of Na^+ between roots and shoots and has been related with the ability of plants to prevent Na^+ from reaching the photosynthetic tissues (Shi et al. 2002, Olías et al. 2009). The sequestration of Na^+ into vacuoles is catalysed by vacuolar $(Na^+, K^+)/H^+$ antiporters NHXs (Cuin et al. 2011), of which four genes (*OsNHX1–4*) have been reported in rice (Fukuda et al. 2011, Kumar et al. 2013). Moreover, recirculation of Na^+ from photosynthetic organs to roots and unloading of Na^+ from the xylem have also been described as strategies for salt tolerance (Davenport et al. 2007), with high-affinity HKT transporters being involved in these processes in several plants, including rice (Garciadeblás et al. 2003, Ren et al. 2005).

Apart from intrinsic protective systems, plants can overcome salinity effects by interacting with several beneficial soil microorganisms such as arbuscular mycorrhizal fungi (AMF). Several studies have shown that the AM symbiosis can alleviate salt stress (For reviews see Evelin et al. 2009, Ruiz-Lozano et al. 2012, Porcel et al. 2012, Augé et al. 2014). The improved salt tolerance of AM plants has been attributed to a more efficient uptake of nutrients (Talaat and Shawky 2011, Beltrano et al. 2013), protection of enzyme activities (Rabie and Almadini 2005, Talaat and Shawky 2011), increase in photosynthesis ability (Sheng et al. 2008, Hajiboland et al. 2010, Estrada et al. 2013a), facilitation of water uptake by plants (Aroca et al. 2007, Sheng et al. 2008) and mitigation of ionic imbalance (Giri et al. 2007, Evelin et al. 2012, Estrada et al. 2013b).

Mycorrhizal colonization has been shown to enhance K^+ absorption under saline conditions while preventing Na^+ translocation to shoot tissues (Giri et al. 2007, Sharifi et al. 2007, Talaat and Shawky 2011). Thus, mycorrhizal plants often have a higher K^+/Na^+ ratio under salinity and a lower shoot Na^+ concentration than non-mycorrhizal plants (Rabie and Almadini 2005, Sannazzaro et al. 2006, Estrada et al. 2013a), preventing the disruption of various enzymatic processes and inhibition of protein synthesis. It has been also proposed that AM fungi may act as a first barrier for ion selection during the fungal uptake of nutrients from the soil or during their transfer to the host plant. Hammer et al. (2011) reported that *Rhizophagus intraradices* can selectively take up elements such as K^+, Mg^{2+} and Ca^{2+} while avoiding Na^+ uptake and that the concentration of Na^+ increased in AM plants with increasing salinity levels up to a certain level, and subsequently decreased at higher salinity. This would suggest that AM fungi can induce a buffering effect on the uptake of Na^+ when the content of Na^+ is within a permissible limit (Evelin et al. 2009, Hammer et al. 2011).

Despite the abundant literature showing mitigation of ionic imbalance by the AM symbiosis, the molecular mechanisms involved in such an effect are almost completely unknown. Indeed, a recent review on physiological and molecular perspectives in salt stress alleviation by AMF encouraged analysing the regulation by AMF of plant transporters involved in ion homeostasis, such as SOS1, NHXs, HKT or AKT (Ruiz-Lozano et al. 2012). To our knowledge, Ouziad et al. (2006) analysed the expression of two Na^+/H^+ antiporter genes in mycorrhizal tomato plants. Results showed that, under the conditions assayed, the AM symbiosis did not alter the expression of *LeNHX1* and *LeNHX2* genes. More recently, Estrada et al. (2013b) showed that maize plants inoculated with three native AM fungi from a Mediterranean saline area showed significant increase of K^+ and reduced Na^+ accumulation as compared to non-mycorrhizal plants, concomitantly with higher K^+/Na^+ ratios in their tissues, and these effects correlated with the regulation of *ZmAKT2*, *ZmSOS1* and *ZmSKOR* genes in roots of maize colonized by these native AMF. This was the first report of the regulation of plant transporter genes by the AM symbiosis under saline conditions.

The study by Estrada et al. (2013b) proposed that the protective effect of AM fungi on maize plants under salinity was mediated by improved K^+ retention in the plant tissues due to upregulation of *ZmAKT2* and *ZmSKOR*. The present study extends previous observations and intends to elucidate the effects of the AM symbiosis on the expression of several rice transporters involved in Na^+/K^+ homeostasis and to measure the Na^+ and K^+ contents and their ratios in the different plant tissues. These data should allow understanding if the AM symbiosis affects Na^+ and K^+ uptake, distribution and compartmentation within the

plant tissues and should shed further light on the mechanisms involved in the enhanced tolerance of AM plants to salt stress.

Materials and methods

Experimental design

The experiment consisted of a randomized complete block design with two inoculation treatments: (1) non-mycorrhizal control plants and (2) plants inoculated with the AM fungus *Claroideoglomus etunicatum* (isolate EEZ 163). There were 30 replicates of each inoculation treatment, totalling 60 pots (three plants per pot), so that ten pots of each inoculation treatment were grown under non-saline conditions throughout the entire experiment, while ten pots per treatment were subjected to 75 mM of NaCl for 4 weeks and the remaining ten pots per treatment were subjected to 150 mM of NaCl for 4 weeks.

Soil and biological materials

Loamy soil was collected from Granada province (Spain, 36° 59′ 34″ N; 3° 34′ 47″ W), sieved (5 mm), diluted with quartz-sand (<2 mm) and with vermiculite (1:1:1, soil:sand:vermiculite, *v*/*v*/*v*) and sterilized by steaming (100 °C for 1 h on three consecutive days). The original soil had a pH of 8.2 [measured in water 1:5 (*w*/*v*)], 1.5 % organic matter and nutrient concentrations (g kg^{-1}) as follows: N, 1.9; P, 1 ($NaHCO_3$-extractable P); K, 6.9. The electrical conductivity of the original soil was 0.2 dS m^{-1}.

Four indica rice (*O. sativa* L. cv Puntal) seedlings, previously germinated on sand, were sown in pots containing 900 g of the same soil/sand/vermiculite mixture as described above and thinned to three seedlings per pot after 3 days. The cultivar Puntal has been characterized as sensitive to salinity (http://www.federaciondearroceros.es).

Inoculation treatments

Mycorrhizal inoculum was bulked in an open-pot culture of *Zea mays* L. and consisted of soil, spores, mycelia and infected root fragments. The AM fungus used in this study had been previously isolated from Cabo de Gata Natural Park (Almería, Spain, 36° 45′ 24″ N 02° 13′ 17″ W), which is an area with serious problems of salinity and affected by desertification. The AMF species was *C. etunicatum* (isolate EEZ 163), previously characterized as an efficient AM fungus under salinity (Estrada et al. 2013a,b). Appropriate amounts of the inoculum containing about 700 infective propagules (according to the most probable number test) were added to the corresponding pots at sowing time just below rice seedlings. Non-mycorrhizal control plants received the same amount of autoclaved mycorrhizal inocula together with a 10-ml aliquot of a filtrate (<20 μm) of the AM inocula in order to provide a general microbial population free of AM propagules.

Growth conditions

The experiment was carried out under glasshouse conditions with temperatures ranging from 19 to 25 °C, 16/8 light/dark period and a relative humidity of 50–60 %. Average daily photosynthetic photon flux density of 800 μmol m^{-2} s^{-1} was measured with a light meter (LICOR, Lincoln, NE, USA, model LI-188B). Water was supplied daily to the entire period of plant growth to avoid any drought effect. Plants were established for 32 days prior to salinization to allow adequate plant and symbiotic establishment. After that time, a group of plants were kept under non-saline conditions, by irrigating four times per week with water (50 ml/pot) until the end of the experiment (0 mM NaCl), while two groups of each inoculation treatments were watered four times per week with an aqueous solution (50 ml/pot) containing 75 or 150 mM NaCl, respectively. Plants were maintained under these conditions for additional 4 weeks. During this period, plants received each week 10 ml per pot of Hoagland nutrient solution containing only 25 % P concentration to avoid inhibition of AM root colonization. At the end of the experiment, the electrical conductivities in the soil:sand:vermiculite mixture used as growing substrate were 0.5, 6.3 and 9.4 dS m^{-1} for the salt levels of 0, 75 and 150 mM NaCl, respectively.

Parameters measured

Biomass production

At harvest (60 days after planting), the shoot and root system were separated and the shoot dry weight (SDW) and root dry weight (RDW) was measured after drying in a forced hot-air oven at 70 °C for 2 days.

Symbiotic development

The percentage of mycorrhizal root infection in rice plants was estimated by visual observation of fungal colonization after clearing washed roots in 10 % KOH and staining with 0.05 % trypan blue in lactic acid (*v*/*v*), as described by Phillips and Hayman (1970). The extent of mycorrhizal colonization was calculated according to the gridline intersect method (Giovannetti and Mosse 1980).

Stomatal conductance

Stomatal conductance was measured 2 h after the onset of photoperiod with a porometer system (Porometer AP4, Delta-T Devices Ltd, Cambridge, UK) following the user

manual instructions. Stomatal conductance measurements were taken in the second fully developed leaf from six different plants from each treatment.

Efficiency of photosystem II

The efficiency of photosystem II was measured with FluorPen FP100 (Photon Systems Instruments, Brno, Czech Republic), which allows a non-invasive assessment of plant photosynthetic performance by measuring chlorophyll a fluorescence. The quantum yield of photosystem II is calculated as the ratio between the actual fluorescence yield in the light-adapted state (FV′) and the maximum fluorescence yield in the light-adapted state (FM′), according to Oxborough and Baker (1997). Measurements were taken in the second fully developed leaf of six different plants of each treatment.

Determination of mineral nutrients

P, K^+ and Na^+ ions were extracted from 0.05 g of ground leaf and root dry material after acid digestion. For that, samples were mixed with 4 ml HNO_3 + 1 ml H_2O_2, heated to 220 °C for 20 min and cooled at room temperature for at least 4 h. After that, samples were diluted with Milli-Q water and injected into an ICP plasma analyser (IRIS Intrepid II XDL, Thermo Electron Corporation) for the analysis. Extractions were made from five different plants of each treatment. Mineral analyses were carried out by the Analytical Service of the Centro de Edafología y Biología Aplicada del Segura, CSIC, Murcia, Spain.

RNA extraction, synthesis of cDNA and gene expression analyses

RNA was extracted from rice root and leaf samples by a phenol/chloroform extraction method followed by precipitation with LiCl (Kay et al. 1987) and stored at −80 °C. The RNA was subjected to DNase treatment and reverse-transcription using the QuantiTect Reverse Transcription Kit (Qiagen), following the instructions provided by manufacturer. To rule out the possibility of a genomic DNA contamination, all the complementary DNA (cDNA) sets were checked by running control PCR reactions with aliquots of the same RNA that have been subjected to the DNase treatment but not to the reverse transcription step.

Gene expression analyses were carried out by quantitative reverse transcription (qRT)-PCR using an iCycler iQ apparatus (BioRad, Hercules, CA, USA). The primer sets used to amplify each analysed gene in the synthesized cDNAs were designed with Primer3 tool at NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are shown in Table 1. Individual real-time RT-PCR reactions were assembled with oligonucleotide primers (0.15 μM each), 10.5 μl of 2× iQSYBR Green Supermix (Bio-Rad; containing 100 mM KCl, 40 mM Tris-HCl pH 8.4, 0.4 mM dNTPs, 50 U/μl iTaq DNA polymerase, 6 mM $MgCl_2$, 20 nM SYBR Green I, 20 nM fluorescein) plus 1 μl of a 1:10 dilution of each corresponding cDNA in a final volume of 21 μl.

The PCR program consisted in a 3-min incubation at 95 °C to activate the hot-start recombinant *Taq* DNA polymerase, followed by 32 cycles of 30 s at 95 °C and 30 s at 56 °C, and 40 s at 72 °C, where the fluorescence signal was measured. The specificity of the PCR amplification procedure was checked with a heat dissociation protocol (from 60 to 100 °C) after the final cycle of the PCR. Standardization was carried out based on the expression of the rice ubiquitin gene (accession AK061988) in each sample. The relative abundance of transcripts was calculated by using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen 2001). Experiments were repeated three times, with the threshold cycle (CT) determined in triplicate, using cDNAs that originated from three RNAs extracted from three different biological samples. Negative controls without cDNA were used in all PCR reactions.

Statistical analysis

Statistical analysis was performed using SPSS 19.0 statistical program (SPSS Inc., Chicago, IL, USA). The data were processed by the two-way analysis of variance (ANOVA) with inoculation treatment, salt levels and their interaction as sources of variation. Percentage values were arcsine transformed before statistical analysis. Post hoc comparisons with the Duncan's test were used to find out differences between groups with $P \leq 0.05$ as the significance cut-off.

Results

Plant biomass production and symbiotic development

Rice plants inoculated with *C. etunicatum* grew more than non-AM control plants at whatever salt level assayed (Tables 2, 3). The increase in shoot dry weight due to AM colonization ranged from 40 % under non-saline conditions to 62 % under 75 mM NaCl. A 51 % of increase was observed at 150 mM NaCl. In AM plants, salinity decreased the shoot dry weight by 10 % only at 150 mM NaCl. In contrast, in non-AM plants, salinity decreased shoot dry weight by 17 % at 75 mM NaCl. However, no further decrease was observed at 150 mM NaCl.

Rice plants cultivated under non-saline conditions only reached a 21 % of mycorrhizal root length. The AM root colonization increased to 36 or 43 % of mycorrhizal root length when the plants were subjected to 75 and 150 mM NaCl, respectively, with no significant differences among these two salt levels (Tables 2 and 3). Uninoculated plants did not show mycorrhizal root colonization.

Table 1 Accession numbers of genes used in this study, primer sequences and gene function

Gene name	Accession no.	Primer sequence	Annealing Temp. (°C)	Gene function
OsUBQ5Forw OsUBQ5Rev	AK061988	5′- ACCACTTCGACCGCCACTACT-3′ 5′- ACGCCTAAGCCTGCTGGTT-3′	56	Regulatory protein. Used as constitutive gene to normalize expression data
OsNHX1Forw OsNHX1Rev	AB021878	5′- CATTGATCAGGCTGCTGCTA-3′ 5′- CTTGCATGCTTGTCAGGAGA-3′	56	Vacuolar (Na^+, K^+)/H^+ antiporter. Na^+ sequestration into vacuoles
OsNHX3Forw OsNHX3Rev	AB531433	5′- ACCGGTGGGTCAATGAATCC-3′ 5′- CCACCACTGACGAGCAGAAT-3′	56	The same
OsNHX5Forw OsNHX5Rev	AB531434	5′- TCACTGCCCTTGACAGGAAC-3′ 5′- GTCAGGTGGCAACTCATCCA-3′	56	The same
OsSOS1Forw OsSOS1 Rev	AY785147	5′- CTGGGCCTTGCTTTTGGAAT-3′ 5′- ATTCCCAGTGTCATGACGGT-3′	56	Plasma membrane Na^+/H^+ antiporter. Na^+ efflux to the growth medium or apoplastic spaces. Na^+ redistribution between roots and shoots
OsHKT1;5Forw OsHKT1;5Rev	EF373553	5′- CCTGCCACCTTACACCACTT-3′ 5′- AGCTTCTGCCATATGCTGCT-3′	56	High-affinity Na^+, K^+ transporter. Na^+ recirculation from shoot to root and Na^+ unloading from xylem
OsHKT2;1Forw OsHKT2;1Rev	AF500082	5′- CTTTCCAGCCTCATCACCAT-3′ 5′- TGACCTTGTCCCCTGAAAAC-3′	56	The same
OsHKT2;2Forw OsHKT2;2Rev	EF373552	5′- ACACTGCTGATGCTTGTTGG-3′ 5′- ACAGTTCTTGTCGGCTCGAT-3′	56	The same

Stomatal conductance and efficiency of photosystem II

The stomatal conductance was reduced by increasing salinity (Tables 2 and 3). In non-AM plants, such reduction was only significant (by 20 %) when the plants were exposed to 150 mM NaCl. In AM plants, the reduction was by 30 % at 75 mM NaCl and by 44 % at 150 mM NaCl. However, AM plants exhibited higher stomatal conductance than the non-AM counterparts at whatever salt level. Thus, under non-saline conditions, AM plants had 88 % more stomatal conductance than non-AM plants and this percentage was 43 and 32 % at 75 and 150 mM NaCl, respectively.

The efficiency of photosystem II was also higher in AM plants at whatever salt level applied (Tables 2 and 3). The increase in AM plants ranged from 7 % under non-saline conditions to 21 % at 150 mM NaCl. The salt levels applied reduced the efficiency of photosystem II in non-AM plants up to 23 % at 150 mM NaCl. In AM plants, the decrease was lower and it was significant (by 13 %) only at 150 mM NaCl.

Accumulation of mineral ions in shoots and roots, ratio K^+/Na^+ and ratio shoot Na^+/root Na^+

Phosphorus

The P concentration in shoots and roots was maintained higher in AM plants at whatever salt level assayed (Fig. 1, Table 3). In roots, AM plants increased the P concentration by 175 % on average. In shoots, the increase ranged from 460 % under non-saline conditions to 190 % at the highest salt level applied. Indeed, the shoot P concentration decreased in AM plants due to salinity applied, although these plants always maintained higher shoot P concentration than non-AM plants.

Potassium

The K^+ accumulation in shoots decreased steadily with increasing salinity (Fig. 2a, Table 3). The decrease was similar in AM and in non-AM plants. However, under non-saline conditions, AM plants accumulated slightly more K^+ in shoots than non-AM plants, while under the two saline levels, AM plants accumulated slightly less K^+ in shoots than non-AM plants. In the case of roots, the concentration of K^+ was similar in AM and non-AM plants at the different salt levels, except at 150 mM NaCl, where AM plants had less K^+ than non-AM ones (Fig. 2b, Table 3).

Sodium

Contrarily to K^+, the accumulation of Na^+ in shoots and roots increased steadily with increasing salinity (Fig. 2c, d, Table 3). In shoots, the Na^+ concentration was similar in AM and non-AM plants at whatever salt level. In roots, the concentration of Na^+ was higher in AM plants than in non-AM plants (by 51 % on average) at the two salt levels assayed.

K^+/Na^+ ratios

The ratio K^+/Na^+ in shoots and roots was considerably affected by salinity application (Fig. 3a, b, Table 3). Thus, under non-saline conditions, it was considerably high, with AM plants having enhanced K^+/Na^+ ratio in shoots and reduced

Table 2 Shoot dry weight (SDW), percentage of AM root colonization (AM), stomatal conductance (Gs) and efficiency of photosystem II (PS II) in rice plants. Plants were inoculated with the AM fungus *Claroideoglomus etunicatum* or remained as uninoculated controls. Plants were cultivated in the absence of salinity for the entire experiment (0 mM NaCl) or were subjected to two levels of salinity (75 and 150 mM NaCl) for 4 weeks

Treatment	SDW (g plant^{-1})	AM (%)	Gs (mmol H_2O m^{-2} s^{-1})	Efficiency PS II (r.u.)
0 mM NaCl				
Control	0.42c	0c	64.8de	0.56bc
C. etunicatum	0.59a	21b	122.4a	0.60a
75 mM NaCl				
Control	0.35d	0c	60.0e	0.51d
C. etunicatum	0.57ab	36a	85.9b	0.58ab
150 mM NaCl				
Control	0.35d	0c	51.9f	0.43e
C. etunicatum	0.53b	43a	68.8 cd	0.52 cd

Within each column, means followed by the same letter do not differ significantly at $P < 0.05$ by Duncan's test

r.u. relative units

ratio in roots, as compared to non-AM plants. Under the two salt levels assayed, this ratio decreased substantially, showing no significant differences between AM and non-AM plants.

Table 3 Two-way analysis of variance (ANOVA)

	Significance of sources of variation		
Parameter measured	Mycorrhiza (*M*)	Salt stress (*S*)	M×S
Shoot DW	**	*	*
AM colonization	***	*	*
Stomatal conductance	**	*	ns
PS-II efficiency	***	**	**
Shoot P concentration	***	*	*
Root P concentration	***	ns	ns
Shoot K^+ concentration	ns	***	***
Root K^+ concentration	ns	**	***
Shoot Na^+ concentration	ns	***	ns
Root Na^+ concentration	**	***	*
Shoot K^+/Na^+ ratio	***	***	***
Root K^+/Na^+ ratio	***	***	***
Shoot Na^+/root Na^+	***	***	*
OsNHX3 shoot	***	ns	ns
OsNHX3 root	ns	ns	***
OsSOS1 shoot	***	*	*
OsSOS1 root	ns	ns	*
OsHKT2;1 shoot	**	*	**
OsHKT2;1 root	**	ns	**
OsHKT1;5 shoot	*	ns	***
OsHKT1;5 root	ns	*	ns

The sources of variation are mycorrhizal inoculation (M), salt treatment (S) and their interaction (M×S)

ns not significant

*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$

Ratio shoot Na^+/root Na^+

The ratio shoot Na^+ to root Na^+ is indicative of Na^+ translocation to shoots. This ratio tended to increase by the presence of salinity in both AM and non-AM plants (Fig. 4, Table 3). However, it was maintained always lower in AM plants. Thus, under non-saline conditions, the ratio was 80 % lower in AM plants. Under 75 and 150 mM NaCl, it was 47 and 43 % lower in AM plants, respectively.

Expression of genes encoding for ion transporters

The expression of genes *OsNHX1*, *OsNHX3*, *OsNHX5* (as representatives of NHX antiporters type I and type II, Fukuda et al. 2011), *OsSOS1* (as a plasma membrane Na^+/H^+ antiporter, Kumar et al. 2013), *OsHKT1;5*, *OsHKT2;1* and *OsHKT2;2* (as representatives of HKT transporters from group 1 and group 2, Platten et al. 2006) was analysed in both shoot and root tissues (see gene accessions in Table 1). Only the expression of those genes showing regulation by the AM symbiosis was presented in Fig. 5. The genes *OsNHX1*, *OsNHX5* and *OsHKT2;2* resulted unaltered by the mycorrhizal presence.

OsNHX3

In shoots, the expression of this gene remained constant in non-AM plants, regardless of the salt level applied. In contrast, in AM plants, *OsNHX3* was considerably upregulated under both saline levels as compared to non-AM plants. In root tissues, the expression of *OsNHX3* gene was upregulated by AM fungal presence only at 75 mM NaCl as compared to non-AM plants.

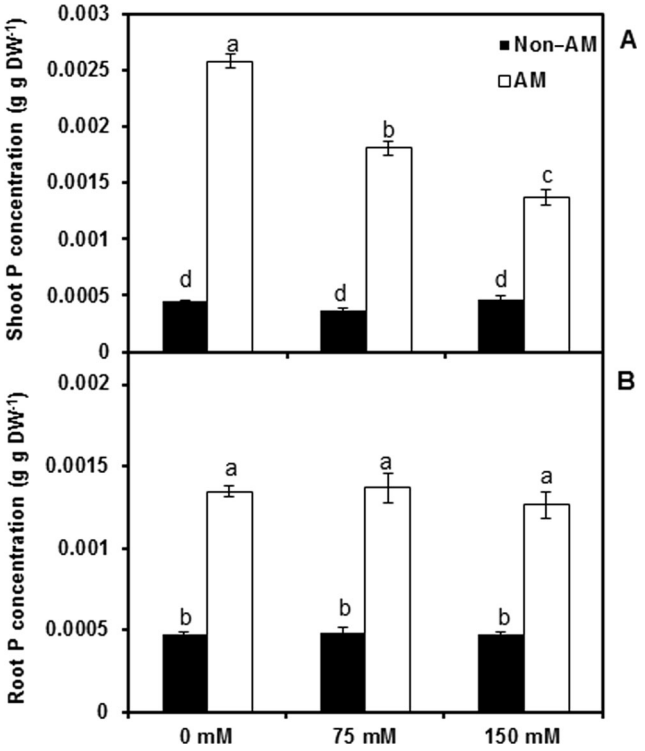

Fig. 1 Shoot (**a**) and root (**b**) P concentrations. Rice plants were inoculated with *Claroideoglomus etunicatum* (AM) or remained as uninoculated controls (Non-AM). Plants were cultivated in the absence of salinity for the entire experiment (0 mM NaCl) or were subjected to two levels of salinity (75 and 150 mM NaCl) for 4 weeks. *Bars* represent the means of five replicates ± standard error. Bars topped by the same letter do not differ significantly at $P \leq 0.05$ by Duncan's test

OsSOS1

In shoots, *OsSOS1* gene expression was consistently upregulated by AM fungal presence at both saline levels, especially at 150 mM NaCl. In contrast, the expression remained constant in shoots of non-AM plants. In roots, the expression of this gene was upregulated in non-AM plants at 150 mM NaCl, while it was downregulated in AM plants at this salt level.

OsHKT2;1

The expression of this gene followed a similar trend than SOS1 since it remained constant in shoots of non-AM plants regardless of the salt level, while the expression of *OsHKT2;1* was considerably upregulated by AM fungal presence at both saline levels, especially at 150 mM NaCl. In roots, the application of 75 mM NaCl decreased *OsHKT2;1* gene expression in non-AM plants while upregulated it in AM plants. However, at 150 mM NaCl, the expression of this gene was reduced in AM plants reaching similar values as under non-saline conditions.

OsHKT1;5

In shoots, the expression of this gene was transiently inhibited in non-AM plants after application of 75 mM NaCl, while it was upregulated sevenfold in AM plants. However, at increasing salinity (150 mM NaCl), the expression of the gene was

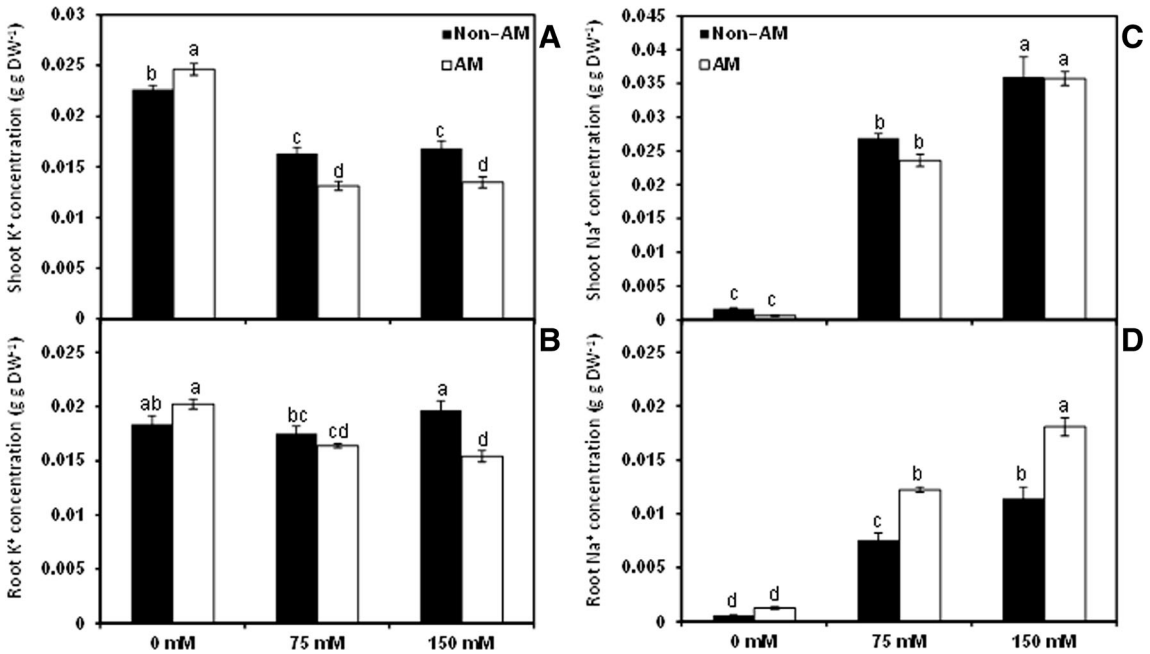

Fig. 2 Shoot (**a**, **c**) and root (**b**, **d**) K^+ and Na^+ concentrations. Rice plants were inoculated with *Claroideoglomus etunicatum* (AM) or remained as uninoculated controls (Non-AM). Plants were cultivated in the absence of salinity for the entire experiment (0 mM NaCl) or were subjected to two levels of salinity (75 and 150 mM NaCl) for 4 weeks. *Bars* represent the means of five replicates ± standard error. Bars topped by the same letter do not differ significantly at $P \leq 0.05$ by Duncan's test

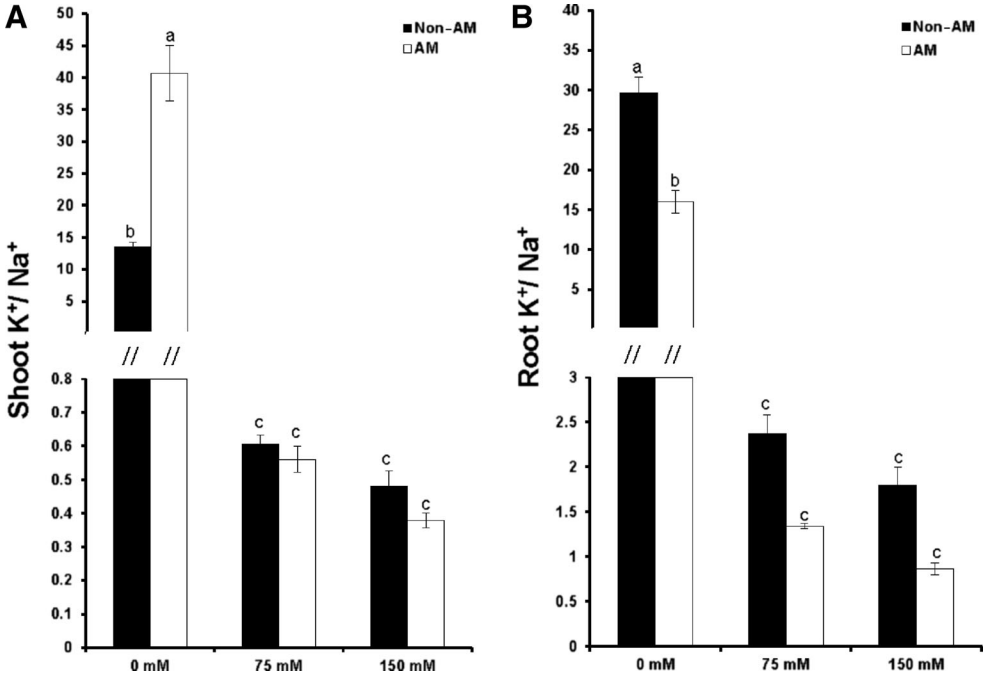

Fig. 3 Ratios K^+/Na^+ in shoots (**a**) and roots (**b**) of rice plants. Rice plants were inoculated with *Claroideoglomus etunicatum* (AM) or remained as uninoculated controls (Non-AM). Plants were cultivated in the absence of salinity for the entire experiment (0 mM NaCl) or were subjected to two levels of salinity (75 and 150 mM NaCl) for 4 weeks. *Bars* represent the means of five replicates ± standard error. Bars topped by the same letter do not differ significantly at $P \leq 0.05$ by Duncan's test

similar for AM and non-AM plants and comparable to non-saline conditions. There were no significant differences in *OsHKT1;5* gene expression in roots.

Discussion

In our experiment, rice plants inoculated with *C. etunicatum* had highest shoot dry biomass at all salinity levels demonstrating the ability of this AM fungal strain native from saline soils (Estrada et al. 2013a,b) to stimulate plant growth under salinity. In non-AM plants, salinity decreased shoot dry weight by 17 % at both saline levels. Plant biomass production is an integrative measurement of plant performance under many types of abiotic stress conditions, and the symbiotic efficiency of AM fungi has been measured in terms of plant growth improvement (see reviews by Evelin et al. 2009, Ruiz-Lozano et al. 2012). Furthermore, the root AM colonization increased to 36 or 43 % when the plants were subjected to 75 and 150 mM NaCl, respectively, suggesting that the lower P availability under salinity may have stimulated fungal root colonization via strigolactone production, as evidenced in another study (Aroca et al., 2013).

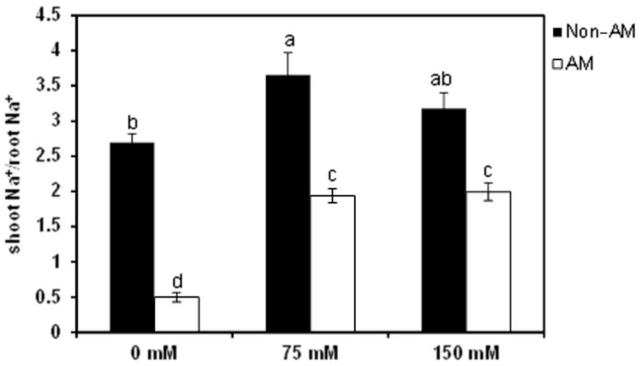

Fig. 4 Shoot Na^+/root Na^+ ratio in rice plants. Rice plants were inoculated with *Claroideoglomus etunicatum* (AM) or remained as uninoculated controls (Non-AM). Plants were cultivated in the absence of salinity for the entire experiment (0 mM NaCl) or were subjected to two levels of salinity (75 and 150 mM NaCl) for 4 weeks. *Bars* represent the means of five replicates ± standard error. Bars topped by the same letter do not differ significantly at $P \leq 0.05$ by Duncan's test

The shoot P concentration decreased with salinity in AM plants, probably because salinity interfered with fungal P uptake. Nevertheless, the accumulation of P in rice plants was maintained always higher in AM plants, both in root and in shoot tissues. This effect has been largely described in AM plants (Smith and Read 2008), including salt stress conditions (Ruiz-Lozano and Azcón 2000, Evelin et al. 2012), and has been correlated with the improved plant growth of AM plants under salinity. Phosphorus is a plant macronutrient which is an integral component of several key plant structural compounds such as phospholipids at the cell membranes. Thus, improved P uptake in AM plants grown under salinity may contribute to keep cell membrane integrity and to reduce electrolyte leakage, as well as to the maintenance of vacuolar membrane stability and facilitating the compartmentalization of Na^+ ions within vacuoles

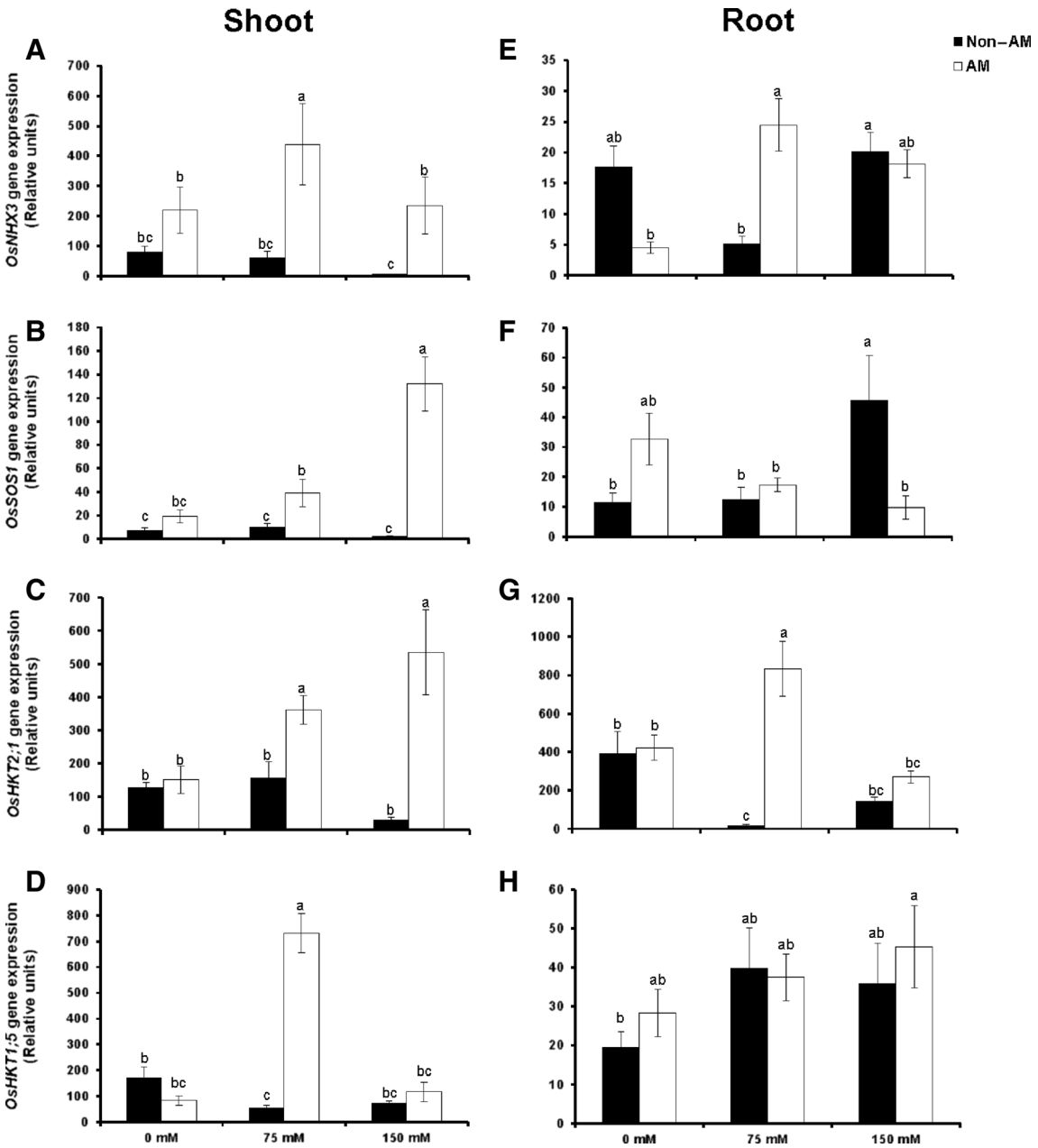

Fig. 5 Expression of genes *OsNHX3*, *OsSOS1*, *OsHKT2;1* and *OsHKT1;5* in shoots (**a**–**d**) and roots (**e**–**h**) of rice plants. Plants were inoculated with *Claroideoglomus etunicatum* (AM) or remained as uninoculated controls. Plants were cultivated in the absence of salinity for the entire experiment (0 mM NaCl) or were subjected to two levels of salinity (75 and 150 mM NaCl) for 4 weeks. *Bars* represent the means of five replicates ± standard error. Bars topped by the same letter do not differ significantly at $P \leq 0.05$ by Duncan's test

(Evelin et al. 2012, 2013). This, in turn, avoids the toxic effects of Na^+ ions on metabolic pathways, thereby reducing the negative impacts of salinity on growth (Evelin et al. 2012).

When plants are subjected to salinity, they accumulate Na^+ ions, which compete with cellular K^+. Hence, Na^+ uptake and distribution within the plant are major determinants for the sensitivity of a plant to salinity. Prevention of Na^+ entry into the root, transport to and allocation within the leaf and sequestration into the vacuole are strategies by which plants cope with excessive soil salinity (Ruiz-Lozano et al. 2012).

In studies on mitigation of salt stress damage by the AM symbiosis, increased plant salt tolerance has been attributed to selective uptake of nutrients by AM fungal hyphae and reduction of toxic ions in the plant tissues (Hammer et al. 2011, Evelin et al. 2012, Ruiz-Lozano et al. 2012). Several authors have reported an increase in K^+ and a decrease in Na^+ concentrations in AM-inoculated plants (Garg and Manchanda 2009, Talaat and Shawky 2011, Evelin et al. 2012). However, our results show a decrease in shoot K^+ concentration while Na^+ accumulation was increased in roots of AMF-inoculated plants.

This would explain why the K^+/Na^+ ratio was not improved by the AM symbiosis under salinity. In any case, this study also shows that the ratio shoot Na^+ to root Na^+ was consistently lower in AM plants than in non-AM plants, suggesting that the translocation of Na^+ ions from roots to shoots was restricted in AM plants as a strategy to limit the accumulation of this toxic ion in photosynthetic tissues (Zhu et al. 2016). It has been proposed that in AM-inoculated plants, Na^+ might be kept inside root cell vacuoles, in vesicles or in intraradical fungal hyphae to prevent the allocation of Na^+ to the shoots (Hammer et al. 2011, Evelin et al. 2013). In a previous study, Evelin et al. (2012) also observed that while there was a continuous increase in the Na^+ shoot to root ratio due to salinity in non-AM fenugreek plants, in AM plants, the increase in Na^+ shoot to root ratio was considerably lower. These findings indicate that AM fungi induce a regulatory effect on the translocation of Na^+ to the aerial parts (Evelin et al. 2012). However, the molecular bases of these protective mechanisms are unknown so far.

Plants have evolved several transport systems that allow them the sequestration of Na^+ into the vacuole or to limit Na^+ entrance into the root and its transport and distribution to the leaves. In the present study, AM plants accumulated more Na^+ in root tissues than non-AM plants and had a lower shoot Na^+ to root Na^+ ratio. Thus, we studied if the AM symbiosis regulated the gene expression of well-known transporters involved in ion homeostasis. These include the following: (1) transporter type NHX, a Na^+/H^+ antiporter system localized in the vacuole. It is expressed in roots and leaves and sequesters Na^+ into the vacuole (Munns 2005); (2) transporter type SOS1, a plasma membrane Na^+/H^+ antiporter involved in Na^+ efflux from cytosol to the growth medium or to apoplastic spaces. It may also participate in the redistribution of Na^+ between roots and shoots, being related with the ability of plants to prevent Na^+ from reaching the photosynthetic tissues (Olias et al. 2009), and in Na^+ extrusion from the root to the external medium (Munns 2005); and (3) transporter type HKT, which allows the transport of Na^+ and K^+ and is involved in recirculation of Na^+ from photosynthetic organs to roots and unloading of Na^+ from the xylem (Davenport et al. 2007). Evidences accumulated so far indicate that cation transporters such as NHXs, SOS1 and HKT are candidate genes for salt tolerance by regulating internal concentrations of Na^+ in various tissues and also indirectly for K^+ homeostasis (Asins et al. 2013).

The expression of *OsNHX3* gene remained constant in shoots of non-AM plants, regardless of the salt level applied. In contrast, in AM plants, *OsNHX3* was considerably upregulated under both saline levels as compared to non-AM plants. The shoot Na^+ concentration was similar in AM and non-AM plants at both saline levels. However, at the molecular level, the upregulation of this gene suggests that in AM plants, part of the Na^+ translocated to shoots may be excluded from cytosol by sequestering it into the vacuole. In this way, AM rice plants would maintain a high K^+/Na^+ ratio within the metabolically active cytosol, which is crucial for plant tolerance to salinity (Cuin et al. 2011). This would additionally contribute to improve osmotic adjustment due to the stored Na^+ (Shi et al. 2002). Contrarily, in the non-AM plants, the low expression of this gene would mean that the Na^+ remains in the cytosol where it may damage cell metabolism. Our data show no significant differences between AM and non-AM plants in their K^+/Na^+ ratios, but we must remark that we measured total K^+ and Na^+ content in plant tissues and we could not assess the fraction of Na^+ which is indeed stored into vacuoles.

The shoot expression of *OsSOS1* and *OsHKT2;1* genes was consistently upregulated by AM fungal presence at both saline levels, especially at 150 mM NaCl. In contrast, the expression was low and remained constant in non-AM plants. This suggests that in AM plants, the enhanced expression of these two genes may contribute to a Na^+ efflux from cytosol to apoplastic spaces (via SOS1), where Na^+ is less toxic, as well as to an increased Na^+ unloading from the xylem and recirculation from photosynthetic organs to roots (via HKT). Indeed, it has been suggested that the transport functions of SOS1 and HKT systems may be coordinated to achieve Na^+ homeostasis and partitioning between plant organs (Pardo et al. 2006, Olias et al. 2009). Besides, it has been recently shown in wheat that *HKT1;4* and *HKT1;5* genes affect the activity and expression levels of the SOS1 Na^+/H^+ exchanger in both root cortical and stelar tissues (Zhu et al. 2016). In this study, both genes seem to be coordinated since they showed a similar expression pattern in shoots. Indeed, at the highest salt level (150 mM NaCl), the expression of *OsSOS1* gene in roots resulted considerably lower in AM plants than in non-AM plants. This may be important in order to restrict the transport of Na^+ to shoots at this high salt level since SOS1 also has a role controlling the long distance Na^+ transport from the root to the shoot via xylem (Olías et al. 2009). Moreover, in wheat, it has been proposed that HKT1;4 and HKT1;5 confer two complementary mechanisms for reducing the xylem Na^+ content. One enhances the Na^+ retrieval from the xylem via its direct action, while the other reduces the rate of Na^+ loading into the xylem via SOS1 (Zhu et al. 2016). All these effects would also explain the higher Na^+ concentration in roots of AM plants.

In shoots, the expression of another HKT gene, *OsHKT1;5* was upregulated by sevenfold in AM plants at 75 mM NaCl. However, at increasing salinity (150 mM NaCl), the expression of the gene was similar for AM and non-AM plants and comparable to non-saline conditions. Different HKT members may have different transport properties. The rice OsHKT1;5 has been shown to transport Na^+, and it was hypothesized to control shoot Na^+ recirculation by withdrawing Na^+ from the xylem stream into the xylem parenchyma cells (Ren et al. 2005). This mechanism may be operating in AM plants at low salt levels (75 mM NaCl) but avoided at higher salt levels due to the prevalence of the other homeostasis mechanisms provided

by OsNXH3, OsSOS1 and OsHKT2;1, which remained upregulated in AM plants at the highest salt level.

Curiously, the effects of the AM symbiosis on the expression of these transporter genes are more evident in shoot tissues than in root tissues. Indeed, in root tissues, only a transient upregulation of *OsNHX3* and *OsHKT2;1* genes by AM fungal presence at 75 mM NaCl was observed. At the highest salt level (150 mM NaCl), the expression of these genes was similar to that in non-AM plants. These data suggest that the effect of the AM symbiosis seems to be directed preferentially to protect the photosynthetic tissues from the detrimental effects of Na^+ rather than the root tissues. The ability of a plant to exclude Na^+ from photosynthetic tissues is considered to be a crucial feature of salinity tolerance in glycophytes (Colmer et al. 2005, Munns and Tester 2008, Cuin et al. 2011). Moreover, it has been proposed that the regulation of the rate of Na^+ transport to the shoot over time is critical for plant salinity tolerance (Maathuis 2014).

Wu et al. (2013) showed that the ability of a plant to maintain a high K^+/Na^+ ratio resides in photosynthetically active tissues and this determines its photosynthetic capacity (and hence growth and yield) under saline conditions. The improved plant growth in AM treatments in this study seems to be related to the preferential protection of photosynthetic tissues by the AM symbiosis. Indeed, a better performance of photosynthesis-related processes was measured. AM plants exhibited higher stomatal conductance and efficiency of photosystem II than the non-AM counterparts at whatever salt level, most probably enhancing CO_2 fixation under salt stress. Several studies have shown a correlation between tolerance to abiotic stresses and maintenance of efficiency of photosystem II (Loggini et al. 1999, Sheng et al. 2008, Redondo-Gómez et al. 2010). A recent study has shown that AM rice plants had a higher photochemical efficiency for CO_2 fixation and solar energy utilization and this increases plant salt tolerance by preventing the injury to the photosystem reaction centres and by allowing a better utilization of light energy in photochemical processes. All these processes translated into higher photosynthetic and rubisco activities in AM rice plants and improved plant biomass production under salinity (Porcel et al. 2015).

In summary, the present results show that the AM symbiosis regulates the expression of several genes encoding plant transporters involved in ion homeostasis. Most of these changes concern shoot tissues rather than root tissues. Thus, we propose that in aerial plant tissues, the AM symbiosis may favour Na^+ extrusion from cytoplasm, its sequestration into the vacuole, the unloading of Na^+ from the xylem and its recirculation from photosynthetic organs to roots through regulation of *OsSOS1*, *OsNHX3*, *OsHKT2;1* and *OsHKT1;5*. As a result, there is a decrease of Na^+ root-to-shoot distribution and an increase of Na^+ accumulation in rice roots which seems to enhance the plant tolerance to salinity and allows AM rice plants to maintain their growing processes under salt stress conditions.

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