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Arbuscular mycorrhizal fungi native from a Mediterranean saline area enhance maize tolerance to salinity through improved ion homeostasis

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ABSTRACT

Soil salinity restricts plant growth and productivity. Na^+ represents the major ion causing toxicity because it competes with K^+ for binding sites at the plasma membrane. Inoculation with arbuscular mycorrhizal fungi (AMF) can alleviate salt stress in the host plant through several mechanisms. These may include ion selection during the fungal uptake of nutrients from the soil or during transfer to the host plant. AM benefits could be enhanced when native AMF isolates are used. Thus, we investigated whether native AMF isolated from an area with problems of salinity and desertification can help maize plants to overcome the negative effects of salinity stress better than non-AM plants or plants inoculated with non-native AMF. Results showed that plants inoculated with two out the three native AMF had the highest shoot dry biomass at all salinity levels. Plants inoculated with the three native AMF showed significant increase of K^+ and reduced Na^+ accumulation as compared to non-mycorrhizal plants, concomitantly with higher K^+/Na^+ ratios in their tissues. For the first time, these effects have been correlated with regulation of *ZmAKT2*, *ZmSOS1* and *ZmSKOR* genes expression in the roots of maize, contributing to K^+ and Na^+ homeostasis in plants colonized by native AMF.

Key-words: adaptation; native arbuscular mycorrhizal fungi.

INTRODUCTION

Salinity is a major and increasing problem which restricts plant growth and productivity. More than 800 million hectares of land throughout the world are salt affected (including both saline and sodic soils) (FAO 2005). This is over 6% of the total land area of the world. High amounts of salts in soils are responsible for yield reduction in one-third of the global arable land (Lambers 2003). This is particularly the case in regions with high rates of evaporation, like arid and semi-arid areas (Hammer *et al*. 2011). Most crops are glycophytic and tolerate salinity to a threshold level. Above this level, yield decreases (Khan *et al*. 2006), since excess of salt inhibits photosynthetic ability and induces physiological drought in plants (Pitman & Läuchli 2002). Maize (*Zea mays* L.) is classified as a salt-sensitive plant (Maas & Hoffman 1977). Although maize is originally from Mesoamerica, nowadays, it is the third most important cereal crop and ranks first in countries with developing economies (Mejía 2003).

Most glycophytes tolerate salinity by restricting the uptake of Na^+ and Cl^- while maintaining uptake of macronutrients such as K^+ or N (Teakle & Tyerman 2010). Although Cl^- is considered an essential micronutrient for higher plants involved in the regulation of important cellular functions such as enzyme activity, maintenance of membrane potentials, and as a co-factor in photosynthesis and pH gradients (White & Broadley 2001), it can be toxic to plants at high concentrations (Xu *et al*. 2000). However, for maize, it has been shown that Na^+ (and not Cl^-) represents the major ion causing toxicity related to salinity (Fortmeier & Schubert 1995) because it can compete with K^+ for binding sites at the plasma membrane. The K^+ ion is essential for protein synthesis, activation of many enzymes and photosynthesis and it plays a central role in osmotic adjustment, turgor maintenance and in the control of stomata opening (Maathuis & Amtmann 1999). It has been shown that chloroplast function is impaired when K^+ is displaced by Na^+, leading to uncontrolled water losses (Slabu *et al*. 2009). Furthermore, adequate K^+ is very important to maintain cytosolic ion homeostasis in Na^+-stressed plants (Zhu 2003), a function which is disrupted by excessive Na^+ entry (Demidchik & Maathuis 2007). Accumulation of Na^+ and impairment of K^+ nutrition is a major characteristic of salt-stressed plants, the mechanisms of which are only partially understood. However, salt stress often causes reduction in plant tissue K^+ content, and the K^+/Na^+ ratio is considered a useful parameter to assess salt tolerance (Maathuis & Amtmann 1999; Chen *et al*. 2007). Another important response of glycophytes to salinity stress, associated with osmoregulation adjustment, is the accumulation of osmotically active organic solutes such as proline and glycine-betaine (Munns 2005). Proline maintains the osmotic balance and protects enzymes in presence of high cytoplasmic electrolyte concentrations (Greenway & Munns 1980; Hajlaoui *et al*. 2010). However, the significance of proline accumulation in osmotic adjustment is still debated and varies according to the species (Lutts, Kinet & Bouharmont 1996; Rodriguez *et al*. 1997).

Plants can overcome salinity effects by interacting with several beneficial soil microorganisms. Soil microbiota, such

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as arbuscular mycorrhizal fungi (AMF) live symbiotically associated with the roots of 80% of terrestrial plants (Smith & Read 2008) and are able to increase plant growth and crop productivity under different environmental stresses (Barea *et al*. 2013). Several studies have shown that inoculation with AMF can alleviate salt stress (Sannazzaro *et al*. 2006; Jahromi *et al*. 2008; Estrada *et al*. 2013). Improved salt tolerance following mycorrhizal colonization may be the result of a more efficient nutrient uptake (Cantrell & Linderman 2001), ion balance (Giri, Kapoor & Mukerji 2007), protection of enzyme activities (Rabie & Almadini 2005), increase in photosynthesis ability (Sheng *et al*. 2008) and facilitation of water uptake in plants (Aroca, Porcel & Ruiz-Lozano 2007).

Mycorrhizal colonization has also been shown to enhance K^+ absorption under saline conditions while preventing Na^+ translocation to shoot tissues (Giri *et al*. 2007; Sharifi, Ghorbanli & Ebrahimzadeh 2007; Talaat & Shawky 2011). Thus, mycorrhizal plants grown under saline conditions often have a higher K^+/Na^+ ratio (Rabie & Almadini 2005; Sannazzaro *et al*. 2006) and a lower shoot Na^+ concentration (Al-Karaki & Hammad 2001) than non-mycorrhizal plants, preventing the disruption of various enzymatic processes and inhibition of protein synthesis. Mycorrhizal fungi may also act as a first barrier for ion selection during the fungal uptake of nutrients from the soil or during transfer to the plant host. It has been indicated that AMF can selectively take up elements such as K^+ and Ca^{2+}, which act as osmotic equivalents while they avoid uptake of toxic Na^+ (Hammer *et al*. 2011; Evelin, Giri & Kapoor 2012). This suggests that AMF induce a buffering effect on the uptake of Na^+ when the content of Na^+ is within the permissible limit (Evelin, Kapoor & Giri 2009; Hammer *et al*. 2011). Indeed, analysing the regulation by AMF of plant genes involved in ion homeostasis has been encouraged in a recent review on physiological and molecular perspectives in studies of salt stress alleviation by AMF (Ruiz-Lozano *et al*. 2012). In this sense, the plasma membrane localized Na^+/H^+ antiporter SOS1 has been shown to fulfil two important roles in plants, restriction of net Na^+ uptake by roots and control of xylem loading for long-distance transport of Na^+ (Shi *et al*. 2002). Transport of K^+ to the shoot depends on xylem delivery, a process largely controlled by SKOR (Gaymard *et al*. 1998) and on phloem K^+ recycling (Maathuis 2007). The molecular mechanism for the latter is unclear but likely to involve the phloem expressed K^+ channel AKT2 (Marten *et al*. 1999). Thus, these three genes were studied in this work.

AMF can be found under severe saline conditions in nature, both in saline inlands and coasts (Aliasgharzadeh *et al*. 2001; Yamato, Ikeda & Iwase 2008) and in salt marshes (Carvalho, Correia & Martins-Louçao 2004; Wilde *et al*. 2009). Moreover, the use of AMF adapted to salinity could be a critical issue for success in recovering saline areas either in natural environments or in agricultural lands affected by salinity. Several studies, describing inoculation strategies used in revegetation of degraded ecosystems, showed a higher benefit of native AMF, which appear to be physiologically and genetically adapted to the stress conditions of the target environment, than non-native isolates (Ferrol *et al*. 2004; Oliveira *et al*. 2005; Querejeta *et al*. 2006). This can be extrapolated to salt-stressed soil, thus the use of salinity-adapted AMF ecotypes should be rewarding.

The objectives of this work were: (1) to investigate whether native AMF isolated from a saline environment (Cabo de Gata Natural Park, Almería, Spain, an area with serious problems of salinity and affected by desertification) can help maize plants to overcome the negative effects of salinity stress better than non-AM plants or plants inoculated with non-native AMF; and (2) to analyse the regulation by these AMF of key plant ion transporters expected to be affected by salinity. Indeed, the molecular mechanisms involved in the better performance of AM plants under salinity stress are almost completely unknown, and there is little information on the effects of the AM symbiosis on plant ion transporters (Ruiz-Lozano *et al*. 2012).

MATERIALS AND METHODS

Identification of the mycorrhizal strains isolated from Cabo de Gata Natural Park

AM fungal spores were separated from the soil samples by a wet sieving process (Sieverding 1991). The morphological spore characteristics and their subcellular structures were described from a specimen mounted in: polyvinyl alcohol-lactic acid-glycerine (PVLG; Koske & Tessier 1983); a mixture of PVLG and Melzer's reagent (Brundrett, Melville & Peterson 1994); a mixture of lactic acid to water at 1:1; Melzer's reagent; and water (Spain 1990). For identification of the AMF species, spores were then examined using a compound microscope at up to 400-fold magnification as described for glomeromycotean classification by Oehl *et al*. (2011). The species were identified based on spore morphology as a *Rhizophagus intraradices* (Schenck & Smith 1982), *Claroideoglomus etunicatum* (Becker & Gerdemann 1977) and *Septoglomus constrictum* (Trappe 1977).

In addition to the morphological identification, a molecular identification was also carried out. For that, spores isolated from the bait cultures of each fungal strain were surface-sterilized with chloramine T (2%) and streptomycin (0.02%) and crushed with a sterile disposable micropestle in 40 μL milli-Q water (Ferrol *et al*. 2004). A two-step polymerase chain reaction (PCR) was conducted to amplify the AM fungal DNA from the spores. The first PCR step was performed with the universal eukaryote primers NS1 and NS4 region of the small subunit ribosomal gene and the second with the specific AM fungal primers AML1 and AML2 (Lee, Lee & Young 2008). The amplified DNA was purified using the Ilustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, UK). DNA fragments were sequenced on an automated DNA sequencer (ABI Prism 373; Perkin-Elmer, Wellesley, MA, USA). Sequence data were compared to gene libraries (EMBL and GenBank) using BLAST program (Altschul *et al*. 1990).

The BLAST analysis unambiguously placed *R. intraradices* as the closest relative of our *R. intraradices* CdG strain, with sequence accession number FR750209 (Krüger *et al*. 2012)

having a 99% identity. *S. constrictum* was the closest relative to our *S. constrictum* CdG strain, with sequence accession number FR750212 (Krüger *et al*. 2012) having a 99% identity. Finally, *C. etunicatum* was the closest relative of our *C. etunicatum* CdG strain, with sequence accession number FR750216.1 (Krüger *et al*. 2012) having also a 99% identity. The AM fungal strains have been incorporated to the collection of Zaidin Experimental Station, Granada, Spain, under accession numbers EEZ 195, EEZ 196 and EEZ 163, respectively.

Experimental design

The experiment consisted of a randomized complete block design with five inoculation treatments: (1) non-mycorrhizal control plants, (2) plants inoculated with the model AM fungus *R. intraradices* (Ri collect), reproduced at collection of the Zaidin Experimental Station (isolate EEZ 58), (3) plants inoculated with the AM fungal strain *R. intraradices* isolated from Cabo de Gata Natural Park (Ri CdG), (4) plants inoculated with the AM fungal strain *S. constrictum* isolated from CdG (Sc CdG) and (5) plants inoculated with the AM fungal strain *C. etunicatum* isolated from CdG (Ce CdG). There were 30 replicates of each inoculation treatment, totalling 150 pots (one plant per pot), so that 10 of each microbial treatment were grown under non-saline conditions throughout the entire experiment, while 10 pots per treatment were subjected to 66 mM of NaCl and the remaining 10 pots per treatment were subjected to 100 mM of NaCl.

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) (1:1, soil:sand, v/v) and sterilized by steaming (100 °C for 1 h on 3 consecutive days). The original soil had a pH of 8.2 [measured in water 1:5 (w/v)]; 1.5% organic matter, nutrient concentrations (g kg^{-1}): N, 1.9; P, 1 ($NaHCO_3$-extractable P); K, 6.9. The electrical conductivity of the original soil was 0.5 dS m^{-1}.

Three seeds of maize (*Z. mays*. L) were sown in pots containing 900 g of the same soil/sand mixture as described above and thinned to one seedling per pot after emergence.

Inoculation treatments

Mycorrhizal inoculum was bulked in an open-pot culture of *Z. mays* L and consisted of soil, spores, mycelia and infected root fragments. The AM species used were three strains 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 native AMF strains were isolated in a salt marsh from the rhizosphere of *Astericus maritimus*, so that plants were collected with their intact root systems up to 40 cm soil depth. The electrical conductivity in such rhizospheric soil varied with soil depth, ranging from 3.95 dS m^{-1} at the surface to 7 dS m^{-1} at the deeper rhizospheric soil layer. The AMF isolates were *R. intraradices* (previously named *Glomus intraradices*), *S. constrictum* and *C. etunicatum*. We also used a *R. intraradices* strain from our culture collection (Ri collect, isolate EEZ 58) which came from the Biosystematic Research Center, Ottawa, Canada, and was originally collected in Pont Rouge, Quebec, Canada. It is the model fungus used in many studies dealing with different topics, including genome sequencing (Tisserant *et al*. 2012). The multiplication of Ri collect (isolate EEZ 58) at our culture collection, always followed standard procedures and the fungus has never been subjected to salt stress. Thus, as the fungus was isolated from non-saline soil and it has never been subjected to salt stress during its multiplication, we assume that it is not adapted to salinity. Appropriate amounts of each inoculum containing about 700 infective propagules (according to the most probable number test), were added to the corresponding pots at sowing time just below maize seeds. 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%. A 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 45 d prior to salinization to allow adequate plant growth and symbiotic establishment. Three concentrations (0, 66 and 100 mM NaCl) of saline solution were added to the soil substrate by adding pre-determined amounts of NaCl from a stock 2 M saline solution, according to the amount of substrate in the pots. The concentration of NaCl in the soil was increased gradually on alternative days to avoid an osmotic shock. It took 8 d, to reach the desired 66 and 100 mM NaCl levels. The electrical conductivities after salt addition in the soil:sand mixture used as growing substrate were 0.2, 5.1 and 7.4 dS m^{-1} for the salt levels of 0, 66 and 100 mM NaCl, respectively. Plants were maintained under these conditions for additional 30 d.

Symbiotic development

The percentage of mycorrhizal root infection in maize 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 & Hayman (1970). The extent of mycorrhizal colonization was calculated according to the gridline intersect method (Giovannetti & Mosse 1980).

Biomass production

At harvest (83 d 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 d.

Proline content

Free proline was extracted from 0.5 g of fresh leaves and roots (Bligh & Dyer 1959). The methanolic phase was used for quantification of proline content. Proline was estimated by spectrophotometric analysis at 530 nm of the ninhydrin reaction according to Bates, Waldren & Teare (1973).

Determination of mineral nutrients

Na^+ and K^+ 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 mili-Q water and injected into an ICP plasma analyser (IRIS Intrepid II XDL, Thermo Electron Corporation, Marietta, OH, USA) 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.

Chloride anions were determined from an aqueous extraction from 0.2 g of dry plant material with 10 mL of deionized water. The extract was shaken for 2 h and then filtered through whatman n°2 filter paper. The quantification was made from five different plants of each treatment as described by Diatloff & Rengel (2001).

RNA extraction and synthesis of cDNA for gene expression analysis

RNA was extracted using the RNeasy plant mini kit (Qiagen, Valencia, CA, USA) from maize roots samples stored at –80 °C. Single-strand cDNA was primed by random hexamers using 100–1000 ng of DNase-treated RNA. RNA samples were denatured at 65 °C for 5 min and then reverse transcribed at 25 °C for 10 min and 42 °C for 50 min in a final volume of 20 μL containing 10 μL of total RNA, 10 μM random primers (Invitrogen, Carlsbad, CA, USA), 0.5 mM dNTPs, 10 U RNase inhibitor, 4 μL of 5x buffer, 2 μL 0.1 M dithiothreitol (DTT) and 1 μL of Superscript II Reverse Transcriptase (Invitrogen). The samples were precipitated with 1 v/v isopropanol and suspended in 20 μL of water.

Quantitative PCR

Gene expression analyses were carried out by quantitative reverse transcription (qRT)-PCR using an iCycler iQ apparatus (Bio-Rad, Hercules, CA, USA). The cDNA samples were standardized to four reference genes: alpha tubulin (gi:450292), elongation factor 1-alpha (EF1-α) (gi:2282583), polyubiquitin (gi:248338) and glyceraldehyde phosphate dehydrogenase (GADPH) (gi:22237). The same reactions were performed with specific primers designed for each of the analysed genes: *ZmAKT2*, For (5′-CCTCAAGCATCAG GTCGAGA-3′) and Rev (5′-CTCTGTAATCTTCCTGGA CG-3′), *ZmSKOR*, For (5′-TCAGATCCAAGATGTCCC AG-3′) and Rev (5′-TTCGTATCCTCTTAACGCAG-3′) *ZmSOS1*, For (5′-GCTTGTCACATACTTCACAG-3′) and Rev (5′-ACTTGTCCACTTCACTACAC-3′).

Individual real-time RT-PCR reactions were assembled with oligonucleotide primers (0.15 μM each), 10.5 μL of 2x iQSYBR Green Supermix (Bio-Rad; containing 100 mM KCl, 40 mM Tris-HCl pH 8.4, 0.4 mM dNTPs, 50 U μL^{-1} iTaq DNA polymerase, 6 mM MgCl2, 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. 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.

The relative levels of transcript were calculated using the Normalization Factor (NF) based on the expression levels of the three best-performing housekeeping genes, in our case polyubiquitin, GADPH and EF1-α. NF was measured using a Visual Basic application for excel (GeNorm) that calculates the gene stability as described by Vandesompele *et al*. (2002). The calculation was done for each cDNA used in the Q-PCR quantification. Expression levels were transformed from Cq values using the PCR efficiencies (Ramakers *et al*. 2003).

Statistical analysis

Statistical analysis was performed using SPSS 19.0 statistical program (SPSS Inc., Chicago, IL, USA) performing first a one-way ANOVA followed by the Tukey test with $P < 0.05$ as the significance cut-off. Two independent statistical analyses were carried out: the first to analyse data from the different AMF treatments within each saline level and the second one to analyse data from each fungal species at increasing salinity.

RESULTS

Symbiotic development

Increasing salinity application enhanced the percentage of AM root colonization in all cases, except in plants colonized by Ri CdG, which exhibited similar colonization rates at all salinity levels (Fig. 1). Within each salinity level, the highest rate of AM root colonization was achieved in plants inoculated with Ri collect (up to 88%). High levels of root colonization were also found in plants inoculated with Ce CdG and Sc CdG (Fig. 1). In contrast, the lowest root colonization was always found in plants colonized by Ri CdG (about 20%).

Plant biomass production

The increase of salt application affected negatively the shoot biomass production in all treatments (Fig. 2a). Such decrease was more evident at the highest salt level applied (100 mM NaCl). In contrast, the root biomass production only decreased with salinity in the non-mycorrhizal plants

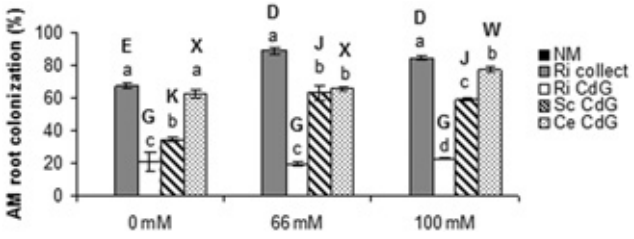

Figure 1. Percentage of mycorrhizal root length in maize plants. Grey bars represent plants inoculated with the collection *Rhizophagus intraradices* strain (Ri collect); white bars, plants inoculated with the native *R. intraradices* CdG strain (Ri CdG); lined bars, plants inoculated with the native *Septoglomus claroideum* CdG strain (Sc CdG) and dotted bars, plants inoculated with the native *Claroideoglomus etunicatum* CdG strain (Ce CdG). Plants were subjected to 0, 66 or 100 mM NaCl. Different letters indicate significant differences ($P < 0.05$) among fungal treatments at each salt level (a, b, c, d) or among salt levels for each arbuscular myocorrhizal fungi (AMF) treatment: Ri collect (D, E, F), Ri CdG (G, H, I), Sc CdG (J, K, L) or Ce CdG (W, X, Y).

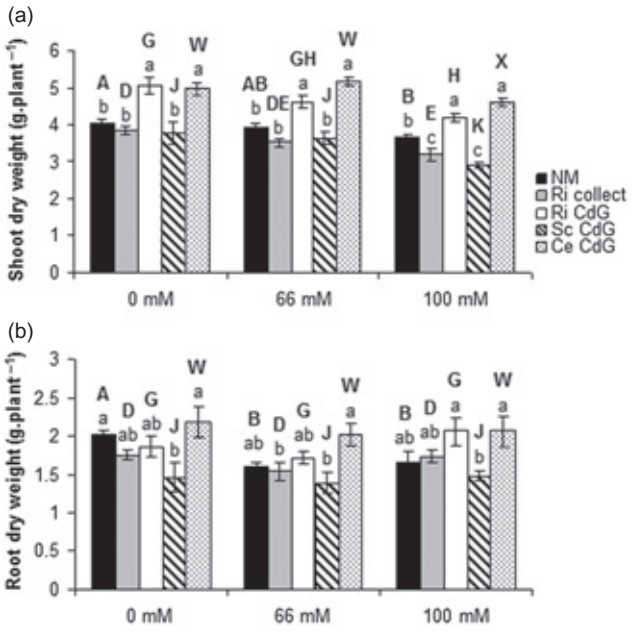

Figure 2. Shoot (a) and root (b) dry weights (g plant^{-1}) in maize plants. Black bars represent non-mycorrhizal control plants (NM); grey bars, plants inoculated with the collection *Rhizophagus intraradices* strain (Ri collect); white bars, plants inoculated with the native *R. intraradices* CdG strain (Ri CdG); lined bars, plants inoculated with the native *Septoglomus claroideum* CdG strain (Sc CdG) and dotted bars, plants inoculated with the native *Claroideoglomus etunicatum* CdG strain (Ce CdG). Plants were subjected to 0, 66 or 100 mM NaCl. Different letters indicate significant differences ($P < 0.05$) among fungal treatments at each salt level (a, b, c, d) or among salt levels for each AMF treatment: NM plants (A, B, C), Ri collect (D, E, F), Ri CdG (G, H, I), Sc CdG (J, K, L) or Ce CdG (W, X, Y).

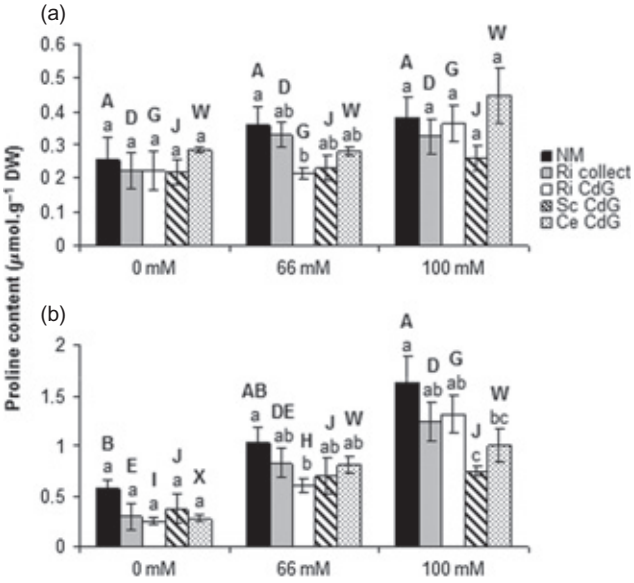

Figure 3. Shoot (a) and root (b) proline accumulation in maize plants. See legend for Fig. 2.

(Fig. 2b). When results were analysed for the three salt treatments, it was clear that Ri CdG and Ce CdG, both enhanced maize shoot biomass as compared to the non-mycorrhizal plants, while Ri collect and Sc CdG did not (Fig. 2a).

Accumulation of proline

The accumulation of proline was more pronounced in root than in shoot tissues (Fig. 3a,b) and it increased in the roots with increasing salinity in the growth medium, except for plants inoculated with Sc CdG, where the differences were not significant (Fig. 3b). The highest accumulation of proline occurred in the roots of non-mycorrhizal plants at 100 mM NaCl. It also increased with salinity in roots of plants inoculated with Ri CdG and Ce CdG. In shoots, no significant differences were found either as a consequence of increasing salinity or by the AM fungus inoculated.

Accumulation of mineral ions in shoots and roots and K^+/Na^+ ratios

Potassium

The increase of salinity in the growing medium decreased the accumulation of K^+ in the root tissues in all treatments, except in plants inoculated with Ri collect, which had similar K^+ levels at 0 mM NaCl and at 100 mM NaCl (Fig. 4b). In contrast, in shoot tissues, maize accumulated more K^+ at increasing salinity levels (Fig. 4a). This was especially evident in plants inoculated with Sc CdG. At 100 mM NaCl, all the mycorrhizal treatments accumulated more K^+ in roots than the non-mycorrhizal plants (Fig. 4b). At this salt level, no significant differences in K^+ accumulation were observed in roots between AM and non-AM treatments. In shoots, at all salinity levels, the non-mycorrhizal plants and the plants

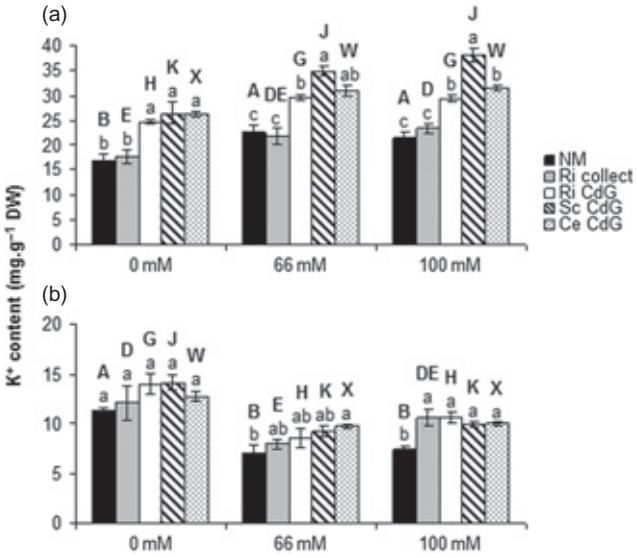

Figure 4. Shoot (a) and root (b) potassium concentration in maize plants. See legend for Fig. 2.

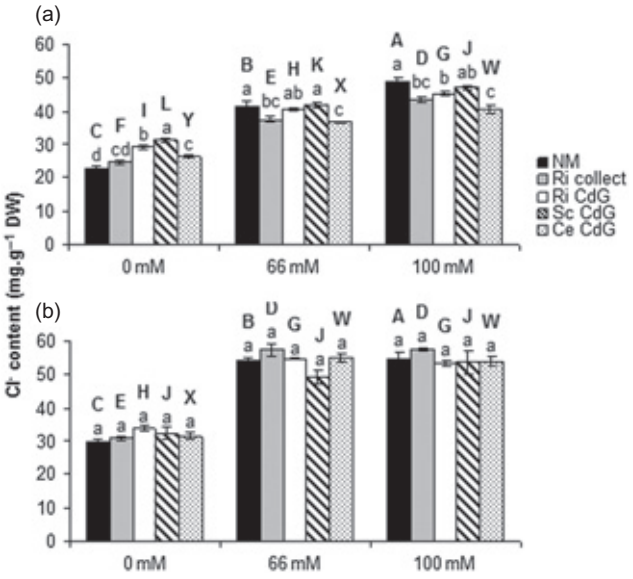

Figure 6. Shoot (a) and root (b) chloride concentration in maize plants. See legend for Fig. 2.

inoculated with Ri collect exhibited always a lower K^+ accumulation than plants inoculated with either of the three native AM fungal strains (Ri CdG, Sc CdG or Ce CdG) (Fig. 4a).

Sodium

The accumulation of Na^+ in maize plants increased considerably both in shoot and in root tissues when the plants were cultivated under salinity (Fig. 5a,b). When data were analysed within each salt level, it was observed that at 0 mM NaCl the three native AMF enhanced the accumulation of Na^+ in root tissues as compared to the non-mycorrhizal plants or those inoculated with Ri collect (Fig. 5b). However, at 66 mM NaCl and 100 mM NaCl no significant differences in Na accumulation in roots were observed among treatments. Only plants inoculated with Ce CdG, had significantly lower Na^+ levels at 100 mM NaCl than the rest of treatments. In the shoot tissues, it was observed that at 0 mM NaCl the levels of Na^+ were very low in all treatments (Fig. 5a). The accumulation of Na^+ was enhanced at 66 and 100 mM NaCl for all treatments, with non-mycorrhizal plants exhibiting the highest Na^+ accumulation and mycorrhizal plants the lowest, especially those inoculated with Ce CdG (Fig. 5a).

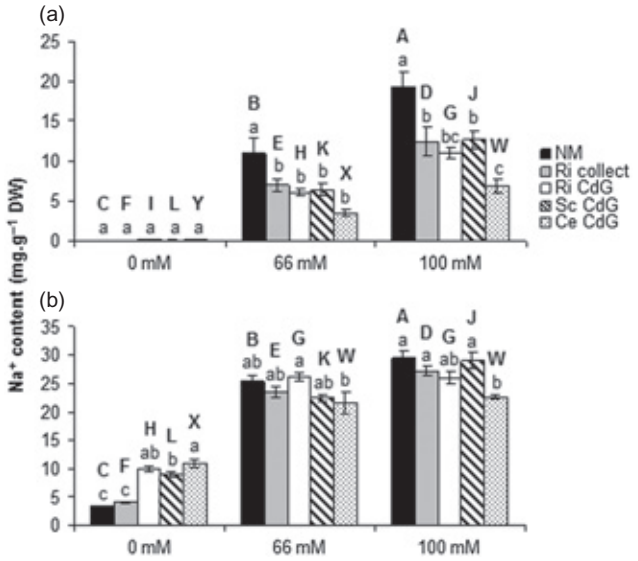

Figure 5. Shoot (a) and root (b) sodium concentration in maize plants. See legend for Fig. 2.

Chloride

The accumulation of Cl^- increased in both shoot and root tissues with increasing salinity in the growth medium (Fig. 6a,b). This was more evident in the shoot tissues (Fig. 6a). When data were analysed within each salt level, it was observed that in roots no remarkable differences in Cl^- accumulation were found among treatments. In shoots, at 0 mM NaCl, plants inoculated with the three native AMF (Ri CdG, Sc CdG and Ce CdG) accumulated more Cl^- than non-mycorrhizal plants or those inoculated with the Ri collect fungus (Fig. 6a). In contrast, when salt was applied to the growth medium, non-mycorrhizal plants always exhibited the highest Cl^- accumulation and no important differences in Cl^- accumulation were observed among fungal treatments. At both saline levels, plants inoculated with Ce CdG showed the lowest accumulation of Cl^-.

K^+/Na^+ ratios

The K^+/Na^+ ratio was negatively affected by salinity in both shoots and roots (Fig. 7a,b). However, the effect was more evident in shoot tissues, where the differences between the

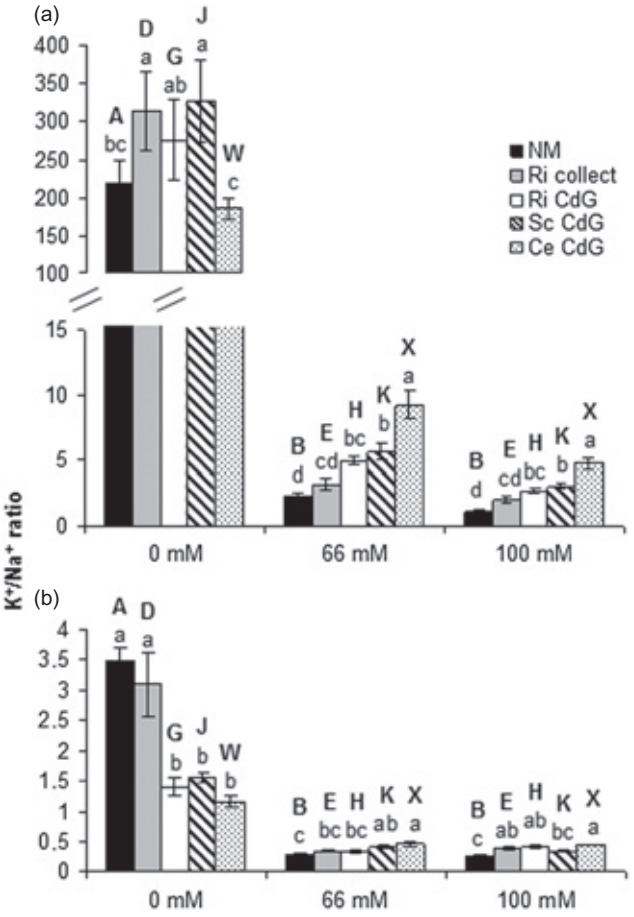

Figure 7. Shoot (a) and root (b) K^+/Na^+ ratio in maize plants. See legend for Fig. 2.

non-saline treatment and either of the two saline treatments were of two orders of magnitude (Fig. 7a). In roots, the K^+/Na^+ ratio at 0 mM NaCl was lower in plants inoculated with either of the three native AMF as compared to non-mycorrhizal plants or plants inoculated with Ri collect (Fig. 7b). In contrast, when salt was applied, non-mycorrhizal plants showed the lowest K^+/Na^+ ratio, especially if compared to roots of plants inoculated with Ce CdG. In the shoots, at both saline levels, the lowest K^+/Na^+ ratios were also found in non-mycorrhizal plants and in plants colonized with the Ri collect strain (Fig. 7a). The three native AMF (Ri CdG, Sc CdG and Ce CdG) showed significantly enhanced K^+/Na^+ ratios in shoots as compared to the non-mycorrhizal plants, especially those colonized by Ce CdG.

Expression of genes encoding for ion transporters

Ion analyses suggest that AMF affect tissue K^+ and Na^+. We therefore tested whether membrane transporters involved in shoot K^+ and Na^+ deposition were affected at the transcript level by AMF colonization.

The expression of the *ZmAKT2* gene was differently affected by increasing salinity in the different fungal treatments (Fig. 8a). In fact, in roots of non-mycorrhizal plants or plants colonized by the Ri collect strain, it decreased its expression at 66 and 100 mM NaCl as compared to 0 mM NaCl. In contrast, the expression of this gene increased steadily with increasing salinity in roots of plants colonized by Sc CdG and Ce CdG. When data were analysed within each salt level, it was observed that in the absence of salt in the growth medium the expression of *ZmAKT2* was notably higher in roots of non-mycorrhizal plants and plants colonized by the Ri collect strain than in roots of plants colonized by either of the three native AM fungal strains. At 66 mM NaCl, few differences among treatments were observed. Finally, at the highest salt level (100 mM NaCl), the expression of this gene increased notably in roots of plants colonized by Sc CdG and Ce CdG, as compared to the other treatments.

The expression of the *ZmSOS1* gene was negatively affected by the highest salinity level in non-mycorrhizal plants or plants inoculated with Ri collect and Ri CdG (Fig. 8b). In contrast, the application of 100 mM NaCl enhanced considerably the expression of the *ZmSOS1* gene in roots of plants colonized by Ce CdG as compared to

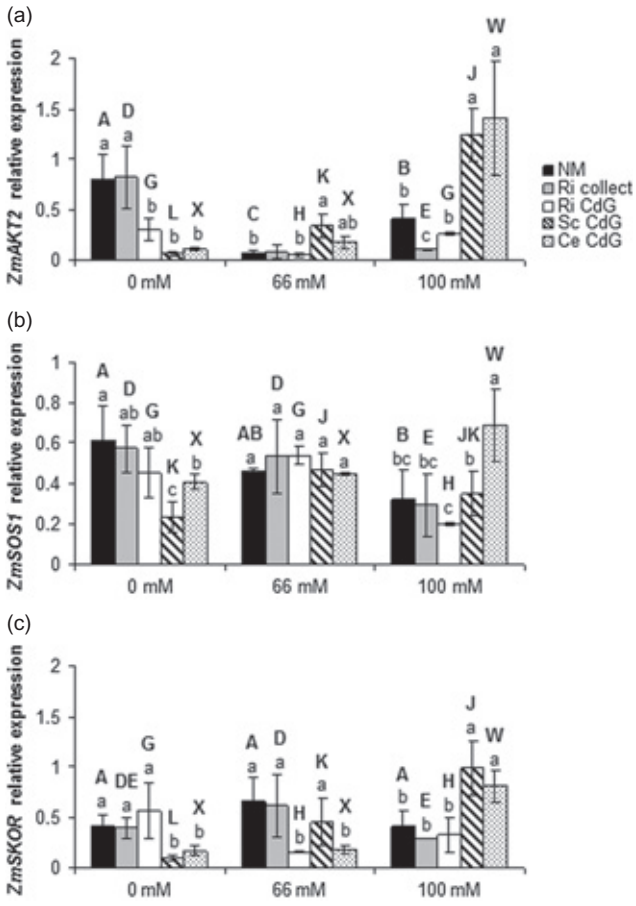

Figure 8. Analysis of *ZmAKT2* (a), *ZmSOS1* (b) and *ZmSKOR* (c) genes expression by real-time quantitative-polymerase chain reaction in roots of maize plants from the different treatments. See legend for Fig. 2.

66 mM NaCl or to the non-saline level. When data were analysed within each salt level, it was observed that in the absence of salinity plants inoculated with Sc CdG or with Ce CdG had a significantly lower expression of *ZmSOS1* than the non-mycorrhizal plants. No significant differences among treatments were observed at 66 mM NaCl, while at the highest salt level (100 mM NaCl) plants inoculated with Ce CdG exhibited the highest expression of this gene in their roots.

The expression of the *ZmSKOR* gene in roots was little affected by the increasing salinity in non-mycorrhizal plants or in plants inoculated with the Ri collect and Ri CdG strains (Fig. 8c). In contrast, in plants colonized by Sc CdG and Ce CdG, the expression of this gene increased steadily with increasing salinity. When data were analysed within each salt level, it was observed that in the absence of salinity plants inoculated with Sc CdG or with Ce CdG had a significantly lower expression of *ZmSKOR* than all other treatments. This lower expression was maintained at 66 mM NaCl in roots of plants colonized by Ce CdG, but again, at the highest salt level (100 mM NaCl) plants inoculated with Ce CdG and also plants inoculated with Sc CdG exhibited the highest expression of this gene.

DISCUSSION

Previous studies have demonstrated that maize plants inoculated with AMF grow better than non-mycorrhizal plants under salt stress conditions (Feng *et al*. 2002; Sheng *et al*. 2008, 2011). However, these experiments were based on the inoculation of a sole AM fungus, *G. mosseae*. Although mycorrhizal symbiosis is usually considered non-specific, Klironomos (2003) found that there was great variation in growth performance with the same fungal isolate. Therefore, AM colonization would depend on the compatibility of both AMF and host plants. In the present work, we assessed the performance of three AM fungal species isolated from a saline area and compared their effectiveness with a *G. intraradices* (= *R. intraradices*) strain belonging to the Zaidin Experimental Station collection, which came from non-saline soil and had never been exposed to salinity during its multiplication at our culture collection. Each species exhibited different mycorrhizal development and symbiotic efficiency under the salt levels assayed.

The results of the present study showed that colonization rates varied among fungal species. Ri collect had the higher rate of root colonization, followed by Ce CdG, Sc CdG and Ri CdG. While most studies reported a decrease in mycorrhizal colonization at increasing salinity levels (Sharifi *et al*. 2007; Evelin *et al*. 2012), our study reveals a significant increase in root colonization in three of the AMF strains, Ri collect, Sc CdG and Ce CdG, while Ri CdG had the same colonization rate at all salinity levels. Yamato *et al*. (2008) also found that colonization rates were not reduced in any of the AMF present in coastal vegetation on Okinawa Island. Similar results were reported by Wu, Zou & He (2010) in citrus colonized by *Paraglomus occultum* isolated from a saline habitat. These results may be explained in terms of salt tolerance of the AMF isolates: they can maintain or even increase colonization capacity under saline conditions. On the other hand, Ri collect has been previously described to have a very high rate of colonization (Graham, Drouillard & Hodge 1996; Ruiz-Lozano *et al*. 2001), thus it seems not surprising that it maintained or even increased the colonization rate. Nevertheless, under saline conditions the native AMF strains isolated from saline areas maintained a higher symbiotic efficiency with maize plants than the collection strain.

Plant biomass production is an integrative measurement of plant performance under many types of abiotic stress conditions and the symbiotic efficiency of AMF has been measured in terms of plant growth improvement (see reviews by Evelin *et al*. 2009; Ruiz-Lozano *et al*. 2012). In our experiment, maize plants inoculated with Ri CdG and Ce CdG had the highest shoot dry biomass at all salinity levels, demonstrating the higher symbiotic efficiency of these native AMF (Oliveira *et al*. 2005; Querejeta *et al*. 2006). The growth of maize inoculated with Ri collect was similar to the non-mycorrhizal plants, except at 100 mM NaCl, where it was lower. The latter can be explained due to the high percentage of root colonization by this fungal strain that could demand excessive carbohydrates from the plant. In fact, plant growth responses to AMF inoculation can range from parasitic to mutualistic (Klironomos 2003). Sc CdG had a similar tendency; previous studies have reported that *G. constrictum* (= *S. constrictu*m) increased plant dry weight less than other AMF tested (Blaszkowski 1993; Yu *et al*. 2010), suggesting a different symbiotic strategy to cope with abiotic stresses rather than a parasitic behaviour. In any case, the positive effect of AM fungal mycorrhization on growth was lower in root tissues than in shoot tissues, which is in agreement with Hajiboland *et al*. (2010).

It has been proposed that mycorrhizal colonization enhances plant salt tolerance by improving photosynthetic ability, water and nutrient uptake, ion balance and osmolite concentration among others (Garg & Manchanda 2009; Ruiz-Lozano *et al*. 2012; Estrada *et al*. 2013). Salt injury can be avoided by maintaining proper osmotic adjustment and ionic homeostasis. Salinity stress induces physiological drought in plants, thus maintaining the water homeostasis is essential to alleviate the impact of salinity on plant growth and crop yield (Dodd & Pérez-Alfocea 2012). Indeed, the extensive hyphal network contributes to water and nutrient uptake because the AM fungus can explore a larger soil volume (Evelin *et al*. 2012). Another method to maintain a favourable gradient for water flow from soil into the roots is to decrease the plant osmotic potential by active accumulation of inorganic ions or organic solutes (Ruiz-Lozano *et al*. 2012). Proline is a major osmoprotectant osmolyte and in plants colonized by AMF, it has been found to increase more than in non-AM plants at different salinity levels (Sharifi *et al*. 2007; Talaat & Shawky 2011). However, reports on the effect of AM symbiosis on proline accumulation are somewhat contradictory and some authors reported that non-AM plants accumulated more proline than AM plants (Rabie & Almadini 2005; Jahromi *et al*. 2008; Sheng *et al*. 2011). Our results did not show significant differences in shoot proline

concentration among fungal treatments. In the root, proline content was several times higher than in the shoot and significantly increased with the salinity levels in all treatments, except in Sc CdG. Higher levels of proline in roots could be beneficial as these are the primary sites for water absorption and must maintain osmotic balance between water absorbing root cells and external media (Sharifi *et al*. 2007). In all, our results suggest that the enhanced salt tolerance in AM maize plants was not due to differences in proline accumulation.

Under salt stress, plants not only accumulate organic solutes like proline, but also inorganic ions such as potassium to maintain osmotic adjustment (Yang *et al*. 2009). In salinity conditions, plants increasingly accumulate Na^+ ions which compete with cellular K^+ (Ruiz-Lozano *et al*. 2012). K^+ functions cannot be replaced by Na^+ ions, thus it is very important to maintain a proper ion homeostasis in terms of K^+/Na^+ ratio (Giri *et al*. 2007; Shabala & Cuin 2008). Our results show a significant increase of K^+ in the leaves of maize plants inoculated with the three native AMF as compared to non-mycorrhizal plants or plants inoculated with the collection fungus. Although in all the treatments Na^+ accumulation increased with salinity, a higher K^+/Na^+ ratio was observed in the plants inoculated with the three native AMF. Several authors have reported a decrease in Na^+ and an increase in K^+ concentrations in AM-inoculated plants (Garg & Manchanda 2009; Talaat & Shawky 2011; Evelin *et al*. 2012). Results are also consistent with Giri *et al*. (2007), who showed higher accumulation of K^+ by mycorrhizal plants in saline soils, thus maintaining a high K^+/Na^+ ratio which influences the ionic balance of the cytoplasm or Na^+ efflux from plants. Recently, Hammer *et al*. (2011) demonstrated that *R. intraradices* can selectively take up elements such as K^+, Mg^{2+} and Ca^{2+} while avoiding Na^+ uptake. Moreover, as a significant proportion of elemental nutrient uptake in plants occurs via mycorrhizal fungi, they help to alleviate the effects of the excess of salts in the soil. Our results confirm that *S. constrictum* also induced a higher K^+/Na^+ ratio compared to non-mycorrhizal plants. In the roots, levels of Na^+ were always higher than in the leaves. It has been proposed that in AM-inoculated plants, Na^+ might be kept inside root cell vacuoles and intraradical fungal hyphae to prevent the allocation of Na^+ to the shoots (Cantrell & Linderman 2001). Plants inoculated with Ce CdG had the lowest Na^+ concentration at 66 and 100 mM of NaCl, being the most efficient fungus in terms of avoiding Na^+ uptake. Hammer *et al*. (2011) found different concentrations and distributions of Na^+ and Cl^- within the fungal tissue and they hypothesized that AMF exclude Na^+ but include Cl^-. Our results showed that all treatments enhanced Cl^- concentration as salinity in the growing medium increased. Mardukhi *et al*. (2011) proposed that mycorrhizal plants had no control on plant Cl^- uptake. Thus, the alleviating effect of AMF on plant growth under salinity stress is more related to Na^+ than Cl^- uptake. Moreover, Na^+ causes higher ion toxicity in maize than Cl^- (Fortmeier & Schubert 1995).

The previous results prompted us to hypothesize that AMF may have regulated the expression of plant genes encoding for ion transporters. It is well documented that overexpression of Na^+/H^+ and K^+/H^+ antiporters improve salt tolerance in plants (Zhang *et al*. 2001; Rodriguez-Rosales *et al*. 2008). However, scarce information is available on the possible regulation by the AM symbiosis of plant genes involved in ion homeostasis. Until now, only Ouziad *et al*. (2006) have studied the effect of AM symbiosis on the expression of two Na^+/H^+ antiporters in tomato under salt stress conditions, showing no regulation of these genes by the AM symbiosis. Nevertheless, we studied the expression of three genes involved in Na^+ and K^+ transport in order to get some clues on molecular mechanisms involved in the enhanced tolerance of mycorrhizal plants to salinity stress.

The SOS signalling pathway has a major role maintaining ion homeostasis by regulating Na^+ and K^+ transport at both the plasma membrane and tonoplast. At the root tissues, SOS1 has been shown to be involved in Na^+ extrusion to the soil solution (Zhu 2002, 2003). The AKT family contributes to a major potassium acquisition by plants. The function of AKT2 has been attributed to phloem loading and/or unloading through bi-directional K^+ transport (Shabala & Cuin 2008). The SKOR channel is involved in K^+ release into the xylem (Munns 2005). Based on that, we analysed the expression of these maize genes in the roots of the different treatments. The most important differences among treatments were observed at 100 mM NaCl for the three genes, where plants inoculated with Se CdG or with Ce CdG exhibited enhanced relative expression. In contrast, under non-saline conditions, these plants always showed reduced expression as compared to non-mycorrhizal plants. These results correlate with the higher K^+ and lower Na^+ concentrations found in shoot tissues of maize plants. The optimal cytosolic K^+/Na^+ ratio can be maintained by either restricting Na^+ accumulation of by preventing K^+ loss from the cell. However, the ability of plants to retain K^+ in their tissues may be crucial in achieving salt tolerance (Shabala & Cuin 2008). Moreover, in barley, it was found that its ability to maintain high K^+/Na^+ ratios was mainly achieved by K^+ retention rather than Na^+ exclusion (Chen *et al*. 2007). In our study, maize plants colonized by Sc CdG and Ce CdG considerably up-regulated the expression of *ZmAKT2* and *ZmSKOR* genes when exposed to 100 mM NaCl. This up-regulation of both genes may have contributed to K^+ retention in the plant tissues and accounted for the enhanced K^+/Na^+ ratios in these plants as compared to the rest of treatments. Indeed, previous reports have shown that AKT2/3 and SKOR channels were up-regulated by salinity in shoots and roots, respectively (Marten *et al*. 1999; Maathuis 2006). Such up-regulation resulted in increased rates of K^+ circulation through the vascular tissue, suggesting a long-distance redistribution of K^+ between the roots and shoots. The same may have occurred in plants colonized by Sc CdG and Ce CdG.

In the case of plants colonized by Ce CdG, enhanced Na^+ exclusion may also have accounted for their high K^+/Na^+ ratios, since these plants exhibited an important up-regulation of *ZmSOS1* gene at 100 mM NaCl and the lowest Na^+ content in their tissues. In any case, it is curious that the *ZmSOS1* gene was down-regulated by increasing salinity in non-AM plants and in those colonized by Ri

collect and Ri CdG. This may be related to post-transcriptional and/or post-translational regulation of this transporter (Zhu 2003).

Thus, we suggest that AMF can affect plant ion transport via modification of gene expression. Moreover, different species of AMF differ in the gene transport efficiency and native AMF have better regulation of these genes, thus enhancing plant salt tolerance.

In summary, the tolerance of maize to salt stress was enhanced by the three native AMF more than by the collection one. Based on our results and on existing literature, a major point for salt tolerance in mycorrhizal maize plants is the improvement of plant nutrition and maintenance of ionic homeostasis. We showed selective regulation by AMF of plant ion uptake and accumulation with subsequent effects on the K^+/Na^+ ratio, correlating with plant transporters gene expression. The more effective AMF were *C. etunicatum* CdG and *S. constrictum* CdG. However, the native *R. intraradices* also had a better ability to alleviate the inhibitory effect of salt stress than the collection *R. intraradices* strain. The characterization of ion transporters of these salt tolerant fungi should be the next step in understanding the molecular mechanisms of salt tolerance acquired by AM symbiosis. The results obtained open important possibilities for sustainable agricultural practices in salinized soils in order to increase crop performance and yield production worldwide.

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