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ORIGINAL ARTICLE

Regulation of plasma membrane aquaporins by inoculation with a *Bacillus megaterium* strain in maize (*Zea mays* L.) plants under unstressed and salt-stressed conditions

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Abstract It is documented that some plant-growth-promoting rhizobacteria (PGPR) enhance plant salt tolerance. However, as to how PGPR may influence two crucial components of plant salt tolerance such as, root hydraulic characteristics and aquaporin regulation has been almost unexplored. Here, maize (*Zea mays* L.) plants were inoculated with a *Bacillus megaterium* strain previously isolated from a degraded soil and characterized as PGPR. Inoculated plants were found to exhibit higher root hydraulic conductance (*L*) values under both unstressed and salt-stressed conditions. These higher *L* values in inoculated plants correlated with higher plasma membrane type two (PIP2) aquaporin amount in their roots under salt-stressed conditions. Also, ZmPIP1;1 protein amount under salt-stressed conditions was higher in inoculated leaves than in non-inoculated ones. Hence, the different regulation of *PIP* aquaporin expression and abundance by the inoculation with the *B. megaterium* strain could be one of the causes of the different salt response in terms of root growth, necrotic leaf area, leaf relative water content and *L* by the inoculation treatment.

Keywords Aquaporins · Maize · PGPR · Root hydraulic conductance · Salt stress

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Abbreviations

L	Root hydraulic conductance
PGPR	Plant-growth-promoting rhizobacteria
PIP	Plasma membrane aquaporins

Introduction

Plants in nature interact with several beneficial soil microorganisms, which improve plant stress tolerance (Aroca and Ruiz-Lozano 2009a; Ryan et al. 2009). Among such microorganisms, plant-growth-promoting rhizobacteria (PGPR) are one of the most studied (Dimkpa et al. 2009; Lugtenberg and Kamilova 2009). PGPR can be classified as extracellular bacteria (existing in the rhizosphere, on the rhizoplane, or in spaces between cells) and intracellular bacteria (mainly N_2 fixing bacteria) (Gray and Smith 2005). The action mechanisms of PGPR can be divided also into direct and indirect ones. Direct mechanisms include N_2 fixation, soil mineral solubilization, production of plant-growth-promoting substances (auxins, cytokinins or gibberellins), and reduction of ethylene levels, among others. Indirect mechanisms include favoring colonization by other beneficial soil microorganisms, as mycorrhizal fungi, and repressing the growth of plant pathogenic microorganisms (Vessey 2003; Lugtenberg and Kamilova 2009).

It is well documented that inoculation with PGPR induces plant tolerance to abiotic stresses with an osmotic component like drought or salinity (Kohler et al. 2009; Liddycoat et al. 2009). The beneficial effects of PGPR inoculation under osmotic stress conditions are not only recorded as a biomass growth increment, but also as an improvement in water status (Nadeem et al. 2007; Kohler et al. 2009). In order to keep tissue water status under osmotic stress conditions, plants need to reach a balance between water lost by leaf transpiration

and water gained by root uptake. The effect of PGPR inoculation on leaf transpiration has been largely studied with contrasting results (Rincon et al. 2008; Alguacil et al. 2009; Bashan et al. 2009). However, as to how PGPR inoculation influenced root water uptake capacity remains almost unexplored. Thus, there is only one report describing an increase in root hydraulic conductance (*L*) in sorghum plants by inoculation with *Azospirillum brasilense* under control and osmotic stress conditions (Sarig et al. 1992).

Root water uptake capacity depends on *L* value, which could be determined ultimately by aquaporin activity (Siefritz et al. 2002; Javot et al. 2003; Postaire et al. 2010). Higher plant aquaporins belong to a large gene family of major intrinsic proteins, which is subdivided into five subfamilies based on amino acid sequence similarity: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin-26-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs), and the recently discovered uncharacterized intrinsic proteins (XIPs), each group being also divided into several groups (Chaumont et al. 2001; Maurel et al. 2008; Gupta and Sankararamakrishnan 2009). Among plant aquaporins, PIPs seem to be the more determinant in regulating *L* and root water uptake (Siefritz et al. 2002; Javot et al. 2003; Postaire et al. 2010). However, only Alguacil et al. (2009) have analyzed how inoculation with one PGPR (*Pseudomonas mendocina*) modulates expression of a *PIP2* gene in lettuce plants, finding an up-regulation under unstressed conditions, but no effect under drought conditions. Unfortunately, measurements of *L* or root water uptake rate were not undertaken in this study.

Recently, a PGPR from degraded soil of Southern Spain has been isolated by our group and identified as *Bacillus megaterium* (Marulanda-Aguirre et al. 2008). This *B. megaterium* strain grows in both the rhizosphere and into the roots. This bacterial strain promotes clover growth under drought conditions. Furthermore, this *B. megaterium* strain increases its own levels of proline and indol acetic acid (an auxin) when it was grown in vitro under osmotic stress conditions without affecting its own growth (Marulanda et al. 2009). Here, it was intended to elucidate how this *B. megaterium* strain could modify *L* and PIP aquaporin gene expression and protein abundance under salt-stressed conditions in maize (*Zea mays* L.) plants. Maize was chosen based on our experience in measuring *L* and PIP aquaporin regulation in this species (Aroca et al. 2005; Ruiz-Lozano et al. 2009).

Materials and methods

Biological material and experimental design

Maize (*Zea mays* L. cv. Gerona, Abonos Bolivar, Granada, Spain) seeds were surface-disinfected by incubating them for 10 min in 0.1% (w/v) NaClO and rinsed three times in distilled water. No data about the salt tolerance of this particular maize cultivar are available in the literature. Seeds were sown in 650 ml pots (10 cm height, 9 cm diameter) freely draining filled with a mixture of sterilized sand and soil (1:1). Soil was collected from Estación Experimental del Zaidín (Granada, Spain), sieved (2 mm) and sterilized by steaming (1 h at 100°C for three consecutive days). The soil had a pH of 8.1 (water); 1.81% organic matter, nutrient concentrations (mg kg^{-1}): N, 2.5; P, 6.2 ($NaHCO_3$-extractable P); K, 132.0. The soil texture was made up of 35.8% sand, 43.6% silt and 20.5% clay. This soil was previously used in growing maize plants (Vázquez et al. 2000; Ruiz-Lozano et al. 2009). Plants were grown in a glass house with temperature ranging from 20 to 25°C, RH from 50 to 60%, photoperiod of 16:8 (light:dark) supplemented with fluorescent lamps to achieve a medium photosynthetic photon flux density of 800 μE m^{-2} s^{-1}.

After sowing, 5 and 10 days later, pots were inoculated with a *Bacillus megaterium* strain, which was isolated earlier (Marulanda-Aguirre et al. 2008; Marulanda et al. 2009). *B. megaterium* culture grown in nutrient broth medium for 48 h at 28°C was centrifuged at 4,500*g* for 5 min. The pellet was suspended in sterilized water. One milliliter of the suspension containing 10^8 cfu ml^{-1} was added to each pot. After 3 weeks from the last inoculation, pots were watered four times on alternate days with 10 ml of 150 mM NaCl solution or with distilled water. Plants were harvested 3 days after the last application. Alongside the experiment, plants were irrigated with 10 ml nutrient solution (Marulanda-Aguirre et al. 2008) each 5 days and supplied with water as needed to keep soil water content at field capacity measured with a ML2 ThetaProbe (AT Delta-T Devices Ltd., Cambridge, UK) as reported earlier Ruiz-Lozano et al. (2009). The electrical conductivity of the soil at the end of the experiment was 0.84 and 2.59 ds m^{-1} for untreated and salt-treated soils, respectively.

Thereby, the experiment was composed of four treatments: plants not inoculated with the *B. megaterium* strain and without salt added, plants inoculated with the *B. megaterium* strain and without salt added, plants not inoculated with the *B. megaterium* strain and with salt added, and plants inoculated with the *B. megaterium* strain and with salt added. Each treatment was composed of 10 pots.

Biomass production and necrotic leaf area

At harvest, plants were carefully removed from pots under tap water and roots were thoroughly cleaned. Each plant was divided into roots and shoots and dried in an oven (75°C) for 2 days. After that, the dry weight (DW) was measured. Before introducing shoots in the oven, the whole leaves of each plant were divided into green and yellow

ones and scanned. Hence the percentage of necrotic leaf area was calculated as: [YL/(YL + GL)] × 100, where YL is the area of the yellow leaves and GL is the area of the green leaves of each plant. Five plants per treatment were used for theses analyses. For further analyses only green tissues were employed.

Leaf relative water content (RWC)

Leaf samples of around 1 cm^2 were weighed [fresh weight (FW)] immediately after harvesting, then placed in a water saturated vial at 5°C for 48 h and weighed [turgid weight (TW)]. The samples were dried in an oven at 75°C for a period of 48 h and their dry weights (DW) were obtained. Then RWC was calculated using the following equation: [(FW − DW)/(TW − DW)] × 100. Leaves from eight different plants of each treatment were used.

Leaf transpiration rate

Leaf transpiration rate was measured by a gravimetric method (Aroca et al. 2006, 2007). Briefly, the surfaces of five pots of each treatment were covered with aluminum foil. The pot–plant system was weighed and is referred to as W0. The pot–plant system was weighed again after 2 h and is referred to as Wf. The leaf transpiration rate was calculated as: (W0 − Wf)/t × A, where t is the time in hours, and A is the leaf area in m^2. Leaf area was calculated as follows: leaves of a whole plant were detached and scanned (HP Scanjet 5550c, Hewlett Packard, Palo Alto, CA, USA). The corresponding images were analyzed with Adobe Photoshop CS (Adobe Systems Incorporated, San Jose, CA, USA). Measurements started 2 h after the lights were turned on.

Root hydraulic conductance (L)

Root hydraulic conductance (L) was measured in plants exuding under atmospheric pressure (Aroca et al. 2006; Ruiz-Lozano et al. 2009). Briefly, five pots of each treatment were immersed in aerated nutrient solution. Plants were cut 1 cm above the root insertion and a pipette tip with a silicone tube was attached to the stem. The liquid exuded in the first 15 min was discarded. The exudate of the following 2 h was collected with a syringe and weighed. The osmolarities of the exuded sap and the nutrient solution were determined using a cryoscopic osmometer (Osmomat 030, Gonotec Gmbh, Berlin, Germany). L was calculated as Jv/ΔΨ, where Jv is the exuded sap flow rate expressed in a root dry weight basis and ΔΨ the osmotic potential difference between the exuded sap and nutrient solution. Although it is more appropriate to express L in root area basis, it is also well accepted the expression of L in root dry weight basis. For details about the significance of expressing L in root area or dry weight basis, see Tyree et al. (1998).

Plant mineral analyses

Ions were extracted from 0.05 g of ground leaf dry material with 10 ml of deionized water. This extract was diluted, and for analysis, an ICP plasma analyzer (IRIS Intrepid II XDL, Thermo Electron Corporation) was used. Extractions were made from four 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 extracted from 0.05 g of ground dry material with 10 ml of deionized water. This extract was diluted and injected into a Dionex-D-100 (Sunnyvale, CA, USA) ion chromatograph, using an Ionpac AS12A (Sunnyvale, CA, USA) 4 mm (10–32) column and guard column. The chloride anions were detected with a conductivity detector and quantified by comparing peak areas with those of known standards (Silva et al. 2008).

PIP aquaporin expression

Expression analyses of the three *ZmPIP1s* and the three *ZmPIP2s* genes most expressed in maize roots were carried out by quantitative real-time PCR (Ruiz-Lozano et al. 2009). The selected PIP genes were *ZmPIP1;1*, *ZmPIP1;2*, *ZmPIP1;5*, *ZmPIP2;1*, *ZmPIP2;5*, and *ZmPIP2;6* based on their high-expression levels reported by Hachez et al. (2006). Total RNA was isolated from three independent samples of roots and leaves of each treatment by a phenol/chloroform extraction method followed by precipitation with LiCl (Kay et al. 1987). cDNAs were synthesized as described Ruiz-Lozano et al. (2009) and the primer sets for each gene expression analysis were selected as described Hachez et al. (2006). The *Zea mays α-tubulin* gene was used for standardization of the results (Hachez et al. 2006; Ruiz-Lozano et al. 2009). Q-RT-PCR reaction mixture consisted in 1 ml of a dilution 1:10 of the cDNA, 200 nM dNTPs, 400 nM each primer, 3 mM $MgCl_2$, 2.5 µl of 1× SyBR Green (Molecular Probes, Eugene, OR, USA), and 0.5 U Platinum Taq DNA polymerase (Invitrogen) in 1× PCR buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl), in a total volume of 25 µl. The PCR program was as described Ruiz-Lozano et al. (2009). The relative levels of transcription were calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

PIP protein abundance

Microsomes from two different root and leaves samples of each treatment were isolated as described by Hachez

et al. (2006) with slight modifications. About 0.5 g of fresh plant tissues were ground in 8 ml of a mixture of 250 mM sorbitol, 50 mM Tris–HCl (pH 8), 2 mM EDTA, and protease inhibitors [1 mM phenylmethylsulfonyl fluoride, 1 μg ml^{-1} each of leupeptin, aprotinin, antipain, chymostatin, and pepstatin (Sigma, St. Louis, MO, USA)]. The homogenate was filtered through a nylon mesh and centrifuged at 4,400*g* for 5 min, and the resulting supernatant was then centrifuged at 130,000*g* for 2 h. The resulting pellet was resuspended in 40 μl of 5 mM KH_2PO_4, 330 mM saccharose, and 3 mM KCl with a final pH of 7.8.

Thereafter, Western blot analyses were carried out as described Hachez et al. (2006) and Ruiz-Lozano et al. (2009). Ten micrograms of crude microsomal fractions were solubilized for 15 min at 60°C in 27 mM Tris–HCl, 0.7% SDS, 3.3% glycerol, 0.0016% bromophenol blue, and 1% DTT. The proteins were separated by SDS-PAGE on 12% polyacrylamide gel. Proteins were transferred to PVDF membrane at 100 mA for 1 h. The membranes were blocked during 2 h at room temperature with 5% (w/v) non-fat milk in Tris-buffered saline (TBS) with 0.05% Tween 20. After that, membranes were incubated overnight at 4°C with 1:1,000 dilution of each primary antibody. A goat anti-rabbit Ig or a goat anti-rat Ig coupled to horseradish peroxidase (Sigma) was used as secondary antibody at a 1:10,000 dilution. The signal was developed using a chemiluminescent substrate (West-Pico, Super Signal, Pierce, Rockford, IL, USA).

The primary antibodies used here were anti-ZmPIP2;1(2;2), and anti-ZmPIP1;2, previously described by Hachez et al. (2006). Furthermore, we used antibodies which recognize several PIP2 or PIP1 proteins of several plant species because they were designed against the most conservative regions of these aquaporin subgroups. More precisely, for detecting PIP1 proteins the 26 first aa of the N-terminal part of *Phaseolus vulgaris* PvPIP1;3 protein (Acc. No. DQ855475; Aroca et al. 2007) were used as a peptide to immunize rats. For detecting PIP2 proteins, the last 12 aa of the C-terminal part of *P. vulgaris* PvPIP2;1 protein (Acc. No. AY995195; Aroca et al. 2006) were used as a peptide to immunize rabbits.

Results

Physiological parameters

Inoculation with *B. megaterium* had no effect on plant biomass under control conditions (Fig. 1). However, salt treatment only decreased root and total DW in non-inoculated plants and it had no effect in shoot DW in both inoculated and non-inoculated plants (Fig. 1).

The percentage of necrotic leaf area was used to quantify salt injury (Cassaniti et al. 2009). Only non-inoculated

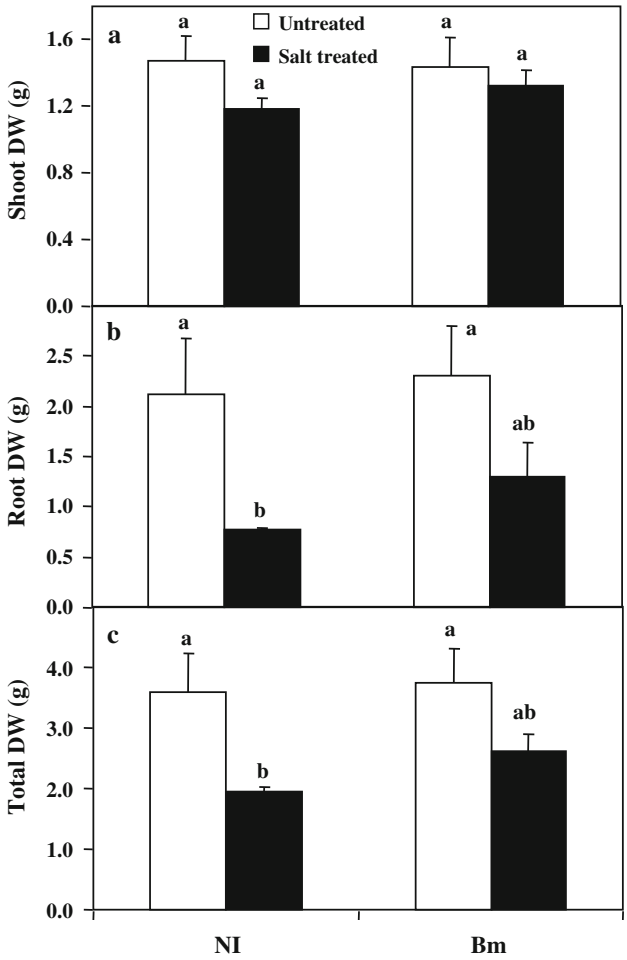

Fig. 1 Shoot (**a**), root (**b**) and total (**c**) dry weights of maize plants inoculated (*Bm*) or non-inoculated (*NI*) with a *B. megaterium* strain. Plants were untreated (*white columns*) or salt treated (*black columns*) as described in "Materials and methods" section. *Columns* represent mean ± SE ($n = 5$). *Different letters* mean significant differences ($p < 0.05$) among treatments after ANOVA and Fisher LSD tests

plants increased significantly ($p < 0.05$) such percentage (about five times) when compared to untreated plants (Fig. 2a). At the same time, salt treatment only caused a diminution on leaf RWC in non-inoculated plants (Fig. 2b). These two parameters demonstrated that inoculation with the *B. megaterium* strain used here diminished the deleterious effects caused by salt treatment in maize plants.

In the shoots, salt treatment increased K^+ concentration in non-inoculated plants, Cl^- concentration in both kinds of plants (inoculated or not), and Mg^{2+} only in inoculated plants (Table 1). Similar ion concentration changes caused by salt were observed in the roots, but here an elevation of Na^+ concentration was also observed in inoculated and non-inoculated plants (Table 1).

Inoculated plants under both unstressed and salt-added conditions had higher *L* than non-inoculated plants (Fig. 3). However, salt treatment diminished *L* only in inoculated

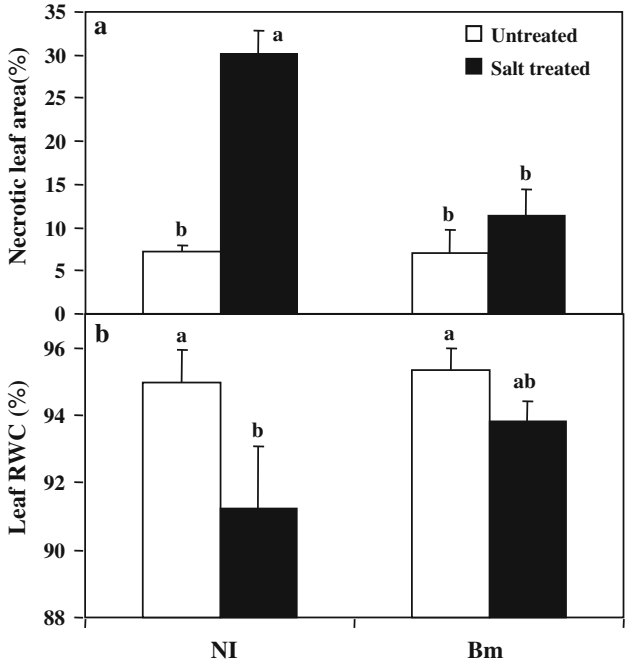

Fig. 2 Necrotic leaf area (**a**) and leaf relative water content (**b**) of maize plants inoculated (*Bm*) or non-inoculated (*NI*) with a *B. megaterium* strain. Plants were untreated (*white columns*) or salt treated (*black columns*) as described in "Materials and methods" section. *Columns* represent mean ± SE ($n = 8$). *Different letters* mean significant differences ($p < 0.05$) among treatments after ANOVA and Fisher LSD tests

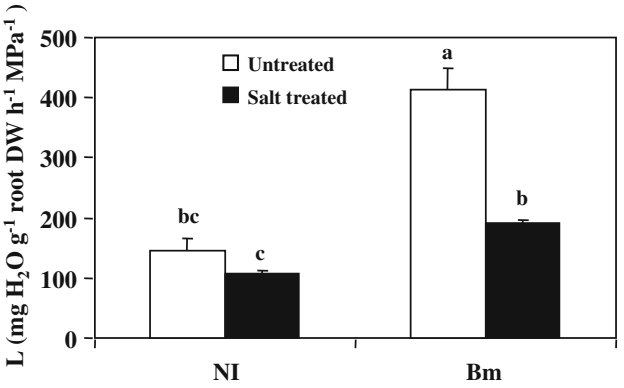

Fig. 3 Root hydraulic conductance (*L*) of maize plants inoculated (*Bm*) or non-inoculated (*NI*) with a *B. megaterium* strain. Plants were untreated (*white columns*) or salt treated (*black columns*) as described in "Materials and methods" section. *Columns* represent mean ± SE ($n = 5$). *Different letters* mean significant differences ($p < 0.05$) among treatments after ANOVA and Fisher LSD tests

plants, although it kept higher values than non-inoculated plants (Fig. 3). No significant differences in leaf transpiration rate were observed among treatments after ANOVA analysis with a p value of 0.52. The values ranged from 75 ± 13 g H_2O m^{-2} h^{-1} in salt-treated non-inoculated plants to 103 ± 19 g H_2O m^{-2} h^{-1} in non-stressed inoculated plants.

PIP aquaporin expression

Regulation in roots of the three most abundant *ZmPIP1* and *ZmPIP2* genes by salt treatment and *B. megaterium* inoculation was analyzed by q-RT-PCR (Hachez et al. 2006; Ruiz-Lozano et al. 2009). Under unstressed conditions, *B. megaterium* inoculation caused an up-regulation of the three *ZmPIP2* genes analyzed (*ZmPIP2;1*, *ZmPIP2;5*, and *ZmPIP2;6*) in root tissues (Fig. 4). In untreated plants, no more differences in *ZmPIP* expression were observed between inoculated and non-inoculated plants in either root or leaf tissues (Figs. 4, 5).

The expression of *ZmPIP1;1* and *ZmPIP1;5* was up-regulated by salt treatment in roots of both inoculated

Table 1 Mineral contents of plant tissues

Treatment	K^+	Na^+	Cl^-	Ca^{2+}	Mg^{2+}
Leaves					
NI	27.6 ± 1.3b	0.4 ± 0.1a	5.7 ± 1.0b	5.8 ± 0.4a	2.6 ± 0.2b
NIS	35.1 ± 2.2a	0.3 ± 0.1a	18.7 ± 2.2a	5.1 ± 0.5a	2.8 ± 0.2b
Bm	31.1 ± 1.5ab	0.5 ± 0.1a	8.9 ± 0.5b	5.5 ± 0.2a	2.4 ± 0.1b
BmS	34.8 ± 0.4a	0.5 ± 0.1a	16.0 ± 1.1a	6.8 ± 1.0a	3.5 ± 0.3a
Root					
NI	7.4 ± 1.0c	2.8 ± 0.4b	1.0 ± 0.1b	9.0 ± 1.2a	5.5 ± 0.6ab
NIS	9.6 ± 0.6b	12.6 ± 1.7a	15.2 ± 1.0a	7.8 ± 0.6a	4.8 ± 0.1b
Bm	12.1 ± 0.7a	3.8 ± 0.3b	1.9 ± 0.3b	7.8 ± 0.6a	5.4 ± 0.2ab
BmS	10.5 ± 0.7ab	12.8 ± 0.5a	16.7 ± 1.1a	10.2 ± 0.7a	6.9 ± 0.7a

Mineral contents are expressed as (mg g^{-1} DW) in leaves and roots of non-inoculated and without salt treatment (NI), non-inoculated and salt treated (NIS), inoculated and without salt treatment (Bm), and inoculated and salt treated (BmS)

Different letters indicates significant differences ($p < 0.05$) among treatments, in leaves and roots independently, after ANOVA and Fisher LSD tests. Data are mean ± SE ($n = 4$)

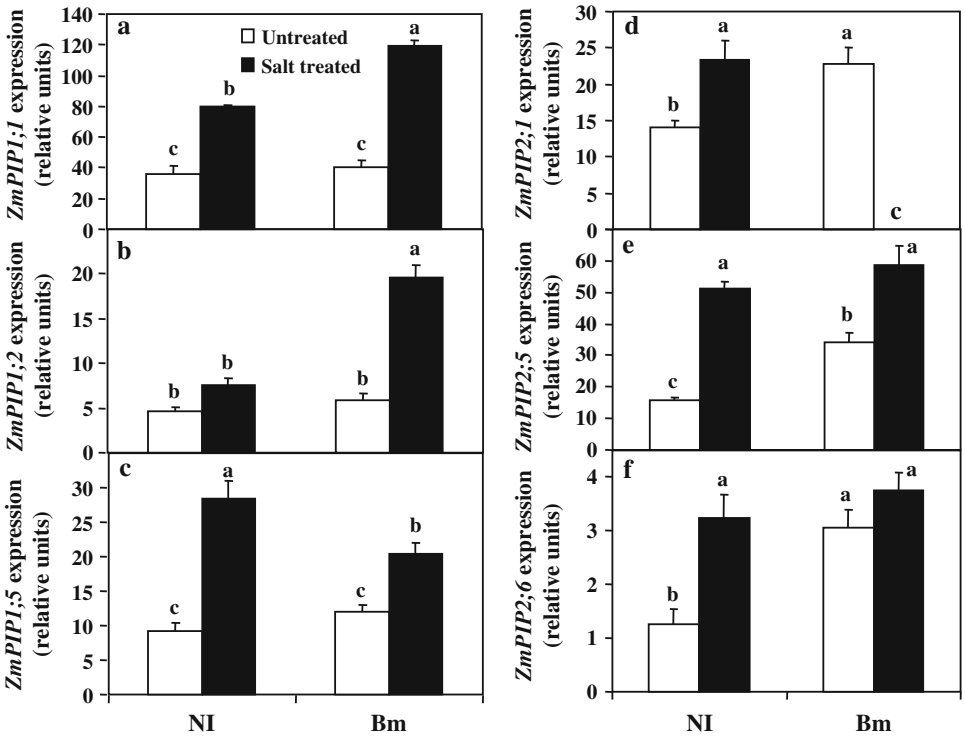

Fig. 4 Relative root *mRNA* expression of *ZmPIP1;1* (**a**), *ZmPIP1;2* (**b**), *ZmPIP1;5* (**c**), *ZmPIP2;1* (**d**), *ZmPIP2;5* (**e**) and *ZmPIP2;6* (**f**) genes of maize plants inoculated (*Bm*) or non-inoculated (*NI*) with a *B. megaterium* strain. Plants were untreated (*white columns*) or salt treated (*black columns*) as described in "Materials and methods" section. *Columns* represent mean ± SE ($n = 3$) of three different RNA samples. *Different letters* mean significant differences ($p < 0.05$) among treatments after ANOVA and Fisher LSD tests

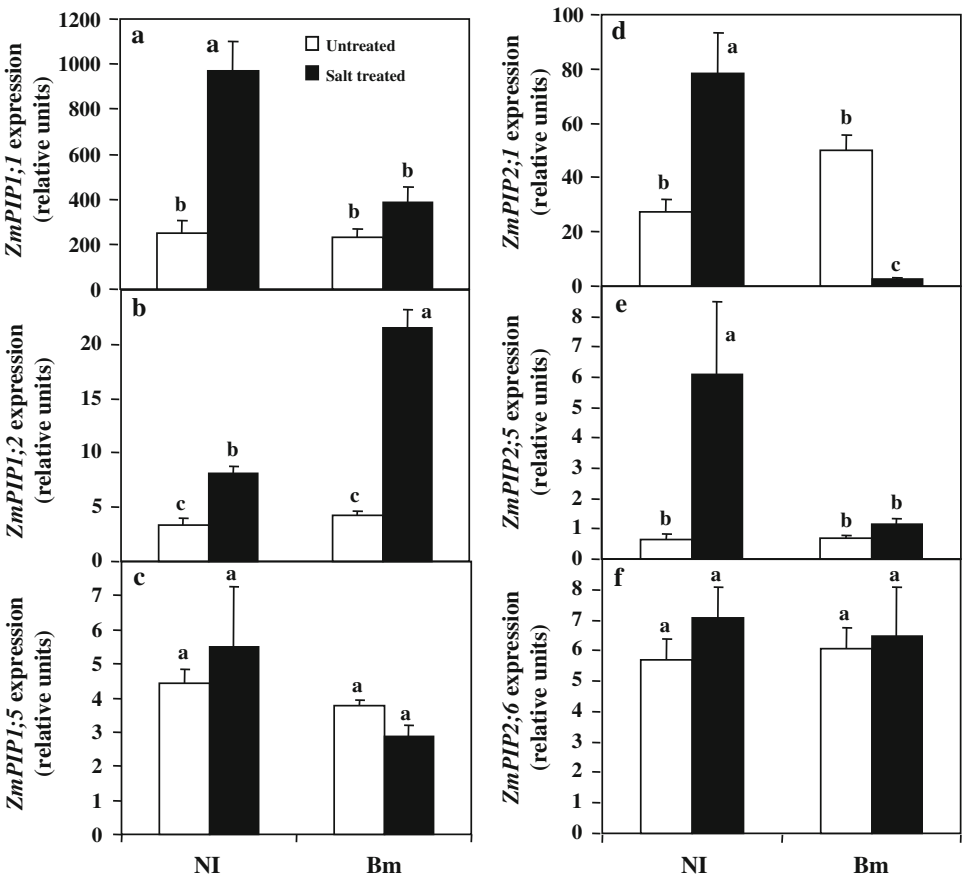

Fig. 5 Relative leaf *mRNA* expression of *ZmPIP1;1* (**a**), *ZmPIP1;2* (**b**), *ZmPIP1;5* (**c**), *ZmPIP2;1* (**d**), *ZmPIP2;5* (**e**) and *ZmPIP2;6* (**f**) genes of maize plants inoculated (*Bm*) or non-inoculated (*NI*) with a *B. megaterium* strain. Plants were untreated (*white columns*) or salt treated (*black columns*) as described in "Materials and methods" section. *Columns* represent mean ± SE ($n = 3$) of three different RNA samples. *Different letters* mean significant differences ($p < 0.05$) among treatments after ANOVA and Fisher LSD tests

and non-inoculated plants (Fig. 4a, c). However, *ZmPIP1;1* was up-regulated to a greater extent in inoculated roots and *ZmPIP1;5* increased its expression more in non-inoculated roots (Fig. 4a, c). *ZmPIP1;2* expression was up-regulated by salt treatment only in inoculated roots, while *ZmPIP2;6* expression was up-regulated only in non-inoculated ones (Fig. 4b, f). *ZmPIP2;5* expression was up-regulated by salt treatment up to the same levels in inoculated and non-inoculated roots (Fig. 4e). Finally, *ZmPIP2;1* expression was conversely regulated in inoculated and non-inoculated roots by salt treatment, thus it was up-regulated in non-inoculated roots, and showed an extreme down-regulation in inoculated ones (Fig. 4d).

In the leaves, *ZmPIP2;1* followed the same expression pattern as in the roots, with an up-regulation in non-inoculated plants, and with a strong down-regulation in the inoculated ones (Fig. 5d). *ZmPIP1;5* and *ZmPIP2;6* expression was unaffected by salt treatment in leaf tissues in both inoculated and non-inoculated plants (Fig. 5c, f). On the contrary, *ZmPIP1;2* expression was up-regulated by salt treatment in both inoculated and non-inoculated plants in leaf tissues, although to a greater extent in inoculated ones (Fig. 5b). Finally, *ZmPIP1;1* and *ZmPIP2;5* expression was up-regulated by salt in leaf tissues but only in non-inoculated plants (Fig. 5a, e).

PIP protein abundance

ZmPIP protein abundance was analyzed by using two specific antibodies raised against ZmPIP1;2 and ZmPIP2;1(2;2) proteins, respectively (Hachez et al. 2006), and by using two antibodies raised against conserved regions of PIP1 and PIP2 proteins, respectively (Fig. 6). Hence, most probably the antibody against PIP1 proteins recognized all ZmPIP proteins except ZmPIP1;6. Furthermore, PIP2 antibodies should recognize ZmPIP2;1, ZmPIP2;2 and ZmPIP2;7 proteins; about ZmPIP2;3, ZmPIP2;4 and ZmPIP2;6 proteins it is not sure if the antibody could recognize them, but absolutely, it does not recognize ZmPIP2;5 protein (Fig. 6). In fact, similar PIP1 and PIP2 antibodies have been previously used in maize plants (Aroca et al. 2005; Hachez et al. 2006). Interestingly, all the Western blots showed an increase in PIP protein amount by salt treatment in both kinds of plants (inoculated or not) and in both tissues (roots and leaves), except for PIP1 proteins in leaves of both kinds of plants, and for ZmPIP1;2 protein in leaves of non-inoculated plants, for which a descent was observed (Fig. 7).

The *B. megaterium* inoculation had no significant effects on PIP protein abundance in root tissues, but decreased PIP2s, PIP1s and ZmPIP1;2, and increased ZmPIP2;1(2;2) amounts in leaf tissues (Fig. 7). Furthermore, owing to the inoculation treatment, the accumulation of PIP2 and

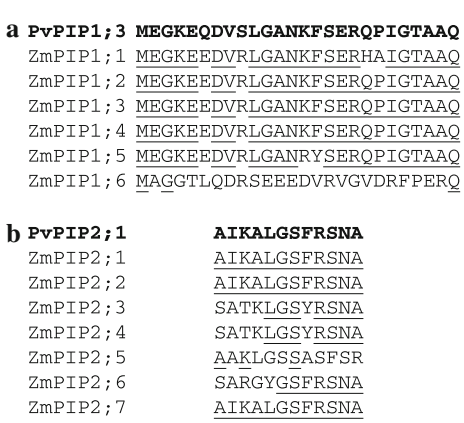

Fig. 6 Multiple alignment of N-terminal (**a**) and C-terminal (**b**) regions of ZmPIP1 (**a**) and ZmPIP2 (**b**) proteins, respectively, with *Phaseolus vulgaris* PIP1;3 (**a**) and PIP2;1 (**b**), respectively. The consensus amino acids are *underlined*. The PvPIP1;3 and PvPIP2;1 sequences correspond to the peptide used to make the respective antibody

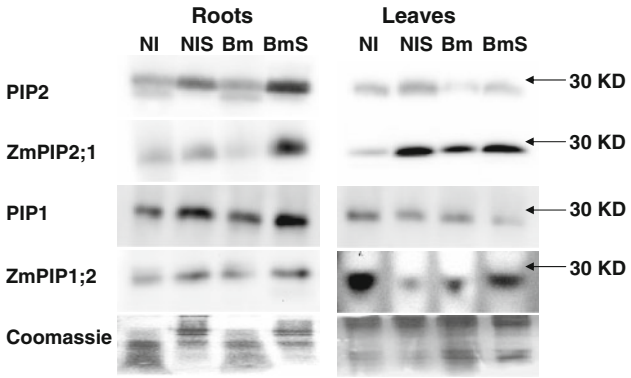

Fig. 7 Western blots of microsomal extracts of roots (*left panels*) or leaves (*right panels*) of maize plants non-inoculated and without salt treatment (*NI*), non-inoculated and salt treated (*NIS*), inoculated and without salt treatment (*Bm*), and inoculated and salt treated (*BmS*) plants. The antibodies used are indicated on the right, PIP2s means an antibody raised against several PIP2 proteins, ZmPIP2;1 means an antibody raised against only ZmPIP2;1 and ZmPIP2;2 proteins, PIP1s means an antibody raised against several PIP1 proteins, and ZmPIP1;2 means an antibody raised against only ZmPIP1;2 protein

ZmPIP2;1(2;2) proteins induced by salt treatment in root tissues was even higher (Fig. 7).

Discussion

Bacillus megaterium induced different salt response

In earlier studies the bacterial strain—used here—was shown to improve nutritional deficiency tolerance in lettuce plants (Marulanda-Aguirre et al. 2008) and drought tolerance in clover plants (Marulanda et al. 2009). Here, *B. megaterium* inoculation caused a different salt response

in maize plants in terms of root growth, necrotic leaf area, leaf relative water content and *L*. Thus, it is confirmed that a particular *B. megaterium* strain is able to modify plant response to several abiotic stresses and in different plant species. Less inhibition of root growth is considered a salt tolerance trait (Ferdose et al. 2009), since roots support shoot growth. It was documented that the *B. megaterium* strain—used here—is able to produce indol acetic acid (an auxin) (Marulanda et al. 2009), which is involved in keeping root growth and development under saline conditions (Wang et al. 2009). Hence, auxin production by this bacterial strain could be implicated in the reduced growth inhibition caused by the salt treatment.

Leaf necrosis under saline conditions has been attributed to both Cl^- toxicity and leaf dehydration (Cui et al. 1995). Here, inoculated and non-inoculated plants presented the same concentration of Cl^- anions in their leaves. We calculated the Cl^- concentration in leaf tissues in mM, being 91 and 103 mM to salt-treated non-inoculated and inoculated plants, respectively, without significant differences between them. Benes et al. (1996a, b) found that Cl^- leaf concentration of 80 mM caused growth inhibition in maize plants at harvest time. Therefore, Cl^- leaf concentration should not be the main cause of leaf yellowing, but different subcellular Cl^- redistribution could be taking place (Fricke et al. 1996; Dabuxilatu and Ikeda 2005), that is, more Cl^- accumulation in the vacuole of inoculated plants. Also, non-inoculated plants suffered from a water deficit whereas inoculated plants kept their RWC values similar to non-stressed plants. This leaf dehydration observed in non-inoculated plants could cause, in part, the leaf necrosis observed (Cui et al. 1995; Quian et al. 2001). Therefore, both leaf dehydration and Cl^- accumulation in the cytosol of non-inoculated salt-treated plants could cause leaf necrosis. However, although the precise cause of leaf necrosis cannot be fully elucidated, leaf necrosis data clearly support the fact that non-inoculated plants suffer more damage owing to salt treatment than do inoculated ones.

Higher leaf dehydration caused by salt treatment in non-inoculated plants could be caused by higher leaf transpiration rates and/or lower root water uptake capacity. Since no significant differences in leaf transpiration rate were observed among treatments, it is possible that such higher leaf dehydration observed in non-inoculated plants was caused by a lower root water uptake capacity under saline conditions. The lack of response of transpiration rate to salt treatment in this particular maize cultivar is not strange since stomatal closure under abiotic stresses is genotype-dependent. Hence, under cold conditions, only maize-tolerant genotypes are able to close their stomata in order to avoid leaf dehydration (Aroca et al. 2003). On the other hand, it has been proposed that root water uptake capacity can be crucial to alleviate salt-stress symptoms (Martínez-Ballesta et al. 2006). Furthermore, it is known that root water uptake is determined by root hydraulic properties and aquaporin activity (Boursiac et al. 2005; Martínez-Ballesta et al. 2006). Here, under both non-stressed and stressed conditions, inoculated plants had higher *L* than non-inoculated plants. Hence, such higher *L* values could be one of the causes of the better water status of inoculated plants under salt stress.

PIP aquaporin regulation

Since significant differences in *L* values were observed between inoculated and non-inoculated plants, the expression and abundance of the most expressed PIP genes in maize roots (Hachez et al. 2006) were evaluated. The fact that the expression of the three *ZmPIP2* genes analyzed were up-regulated in roots by the inoculation treatment under non-stressed conditions could explain the higher *L* values of these plants, since PIP2 proteins usually had more water transport capacity than PIP1 (Fetter et al. 2004). However, the four antibodies used revealed non-significant differences in PIP root protein abundance between inoculated and non-inoculated plants under unstressed conditions. According to Fig. 6, it is possible that the greater *L* values of inoculated plants under unstressed conditions could be determined by ZmPIP2;5 activity, since this protein is not recognized by the antibodies used. In fact, ZmPIP2;5 protein has high water transport capacity (Fetter et al. 2004) and it is accumulated in mature zones of the roots and with a polar localization in the external periclinal side of epidermal cells, indicating an important role in root water transport (Hachez et al. 2006). The other explanation, for the apparent disagreement between *L* values and PIP protein abundance under unstressed conditions, could be a different PIP protein localization along the root axis (Benabdellah et al. 2009) or among cellular membranes (Boursiac et al. 2005; Zelazny et al. 2007). Furthermore, different phosphorylation states of PIP proteins could be causing the greater *L* values observed in inoculated plants (Johansson et al. 1998; Van Wilder et al. 2008).

Under saline conditions a general up-regulation of *ZmPIPs* genes was observed in roots, with small differences between inoculated and non-inoculated plants. These data corroborate those observed when salt treatment was imposed for a week or so (Kawasaki et al. 2001; Aroca et al. 2007), but contrary to what was observed when saline treatment was imposed for short periods less than 2 days (Martínez-Ballesta et al. 2003; Boursiac et al. 2005). These two phases in the response of *PIPs* expression to salt stress could be interpreted as follows: first, an inhibition of expression could be related to a mechanism for avoiding cell dehydration, and second, an increase of expression could be related to a gain of cell turgor after cell solute

accumulation takes place (Aroca and Ruiz-Lozano 2009b). Moreover, PIP protein abundance also increased in root tissues by saline treatment, although the rise was greater in inoculated roots for those aquaporin proteins recognized by the PIP2 and ZmPIP2;1 antibodies used.

The *B. megaterium* strain—used here—also produces the auxin idol acetic acid (Marulanda et al. 2009), which can up- or down-regulate plant aquaporin expression (Werner et al. 2001; Lin et al. 2007; Mut et al. 2008). Anyway, the higher amount of PIP2 proteins in inoculated roots under salt stress could be the cause of the higher *L* of those plants (Sade et al. 2010).

Curiously, *ZmPIP2;1* expression in root and leaf of inoculated plants decreased drastically by salt treatment, but protein accumulation took place. This opposite behavior between *PIP* gene expression and PIP protein abundance was observed before (Aroca et al. 2005). It is possible that *mRNA* synthesis was inhibited by the accumulation of the corresponding encoded protein. In fact, Staiger et al. (2003) found that accumulation of the clock-regulated RNA-binding protein AtGRP7 reduced the expression of the mature *AtGRP7 mRNA* by a splicing mechanism. How a similar mechanism could act in plant aquaporin expression should be the matter of future research. Although ZmPIP2;1 antibody also recognizes *ZmPIP2;2* protein, the expression of *ZmPIP2;2* gene is between 10 and 100 lower than that of *ZmPIP2;1* (Hachez et al. 2006, 2008; Ruiz-Lozano et al. 2009). In fact, in the present study no significant changes in *ZmPIP2;2* expression were detected by any of the treatments (salt or inoculation; data not shown).

Recently, Sade et al. (2010) found that overexpression of tobacco *aquaporin1* (a PIP1 member) in tomato increased water use efficiency and salt tolerance of transformed plants. Also, it is known that the same tobacco aquaporin protein may transport CO_2 (Uehlein et al. 2003) and it is partly localized in the inner chloroplast membrane (Uehlein et al. 2008). Under salt stress, *ZmPIP1;1*, *ZmPIP2;1* and *ZmPIP2;5* expressions were higher in non-inoculated leaves than in inoculated ones, while the contrary happened for *ZmPIP1;2* expression. However, as commented above, not always *PIP* gene expression correlates with PIP protein abundance. Thus, the most significant difference between inoculated and non-inoculated leaves was the opposite response of ZmPIP1;2 protein amount to salt treatment. While in inoculated leaves a strong accumulation was observed, in non-inoculated leaves a diminution took place. The higher accumulation of ZmPIP1;2 protein in inoculated leaves under salt stress could increased their water use efficiency (Sade et al. 2010). However, as it was mentioned above, which signal mechanisms (hormonal or not) are behind this different aquaporin regulation between inoculated and non-inoculated plants will be the matter of future studies.

Conclusion

Here it was confirmed that the *B. megaterium* strain used was able modify salt response in maize plants in terms of root growth, necrotic leaf area, leaf relative water content and *L*. Also, it was found that inoculated plants had higher *L* values under both control and salt-stressed conditions than the one measured in non-inoculated plants, correlating with the PIP2 protein amount of their roots only under saline conditions. Thus, the modification of PIP aquaporin expression and abundance by inoculation with *B. megaterium* could be one of the causes of the different plant salt response. However, which mechanisms are behind this different PIP aquaporin regulation should be investigated in future studies. At the same time, since the plant response to PGPR could vary among different maize genotypes (Pan et al. 1999), this study should be repeated with an ample range of maize genotypes in order to extrapolate the present results to field studies.

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