Differential Expression of the TFIIIA Regulatory Pathway in Response to Salt Stress between *Medicago truncatula* Genotypes[1][W]

Laura de Lorenzo, Francisco Merchan, Sandrine Blanchet, Manuel Megías, Florian Frugier, Martin Crespi*, and Carolina Sousa

Institut des Sciences du Végétal, Centre National de la Recherche Scientifique, F–91198 Gif-sur-Yvette cedex, France (L.d.L., F.M., S.B., F.F., M.C.); and Departamento de Microbiología y Parasitología, Facultad de Farmacia, Universidad de Sevilla, 41012 Seville, Spain (L.d.L., F.M., M.M., C.S.)

Soil salinity is one of the most significant abiotic stresses for crop plants, including legumes. These plants can establish root symbioses with nitrogen-fixing soil bacteria and are able to grow in nitrogen-poor soils. *Medicago truncatula* varieties show diverse adaptive responses to environmental conditions, such as saline soils. We have compared the differential root growth of two genotypes of *M. truncatula* (108-R and Jemalong A17) in response to salt stress. Jemalong A17 is more tolerant to salt stress than 108-R, regarding both root and nodulation responses independently of the nitrogen status of the media. A dedicated macroarray containing 384 genes linked to stress responses was used to compare root gene expression during salt stress in these genotypes. Several genes potentially associated with the contrasting cellular responses of these plants to salt stress were identified as expressed in the more tolerant genotype even in the absence of stress. Among them, a homolog of the abiotic stress-related *COLD-REGULATEDA1* gene and a TFIIIA-related transcription factor (TF), MtZpt2-1, known to regulate the former gene. Two MtZpt2 TFs (MtZpt2-1 and MtZpt2-2) were found in Jemalong A17 plants and showed increased expression in roots when compared to 108-R. Overexpression of these TFs in the sensitive genotype 108-R, but not in Jemalong A17, led to increased root growth under salt stress, suggesting a role for this pathway in the adaptive response to salt stress of these *M. truncatula* genotypes.

Maintaining crop yields under adverse stress environmental conditions is a major challenge in modern agriculture. To meet this goal, it is necessary to understand the contrasting adaptations of plants to growth in stressed conditions. Salinity is one of the major abiotic stresses that affects crop productivity and quality and has been described as one of the most serious threats to agriculture and the natural status of the environment (Chinnusamy et al., 2005). Increased salinization of arable land is expected to have devastating global effects, resulting in a 30% land loss within the next 25 years and up to 50% by the year 2050 (Wang et al., 2003).

Plant responses to salt stress are diverse and include modifications of root system architecture, activation of stress-induced transcriptional programs, and biochemical adaptations, as well as plant growth inhibition. Salinity imposes ionic, osmotic, and secondary stresses, such as nutritional disorders and oxidative stress (Zhu, 2001). Legumes, like most crop plants, are susceptible to salinity (Duzan et al., 2004; Chinnusamy et al., 2005). These plants are widely grown for grain and forage purposes, their world-wide economic importance being second only to grasses (Graham and Vance, 2003). In addition, legumes can establish root symbioses with nitrogen-fixing soil bacteria, enabling the plants to grow in nitrogen-poor soils. This ability to colonize soils where other plants cannot thrive makes the study of legumes and their symbioses important for agriculture. The establishment of successful symbiosis involves an elaborate exchange of molecular signals (Limpens and Bisseling, 2003). In the plant host, root nodule organogenesis is regulated by diverse hormonal, metabolic, and environmental conditions (Crespi and Gálvez, 2000), and this interaction is specifically affected in saline soils (Arrese-Igor et al., 1999; Zahran, 1999).

The problem of salinity has been approached through better management practices and the introduction of salt-tolerant varieties in the affected areas. Unfortunately, these approaches are generally uneconomical and difficult to implement on a large scale. However, major progress could be achieved through genetic improvement (Walia et al., 2005). Various legumes, such as the model legume *Medicago truncatula*, show a large diversity of varieties adapted to varying environmental conditions, including saline soils

(http://www.noble.org/medicago/ecotypes.html). In recent years, *M. truncatula* has been recognized as an excellent legume model in view of its small, diploid genome, self fertility, and short life cycle, as well as availability of various genomic and genetic tools (Barker et al., 1990; Bell et al., 2001; Young et al., 2005). All these traits make *M. truncatula* suitable for identifying genes that could improve agronomic performances such as abiotic stress resistance (Cook, 1999).

Stress responses involve alterations in gene expression (Tester and Davenport, 2003), suppressive subtractive hybridizations, and array technologies using cDNAs or oligonucleotides are increasingly being used to monitor global gene expression changes in various plants in response to abiotic stresses (Ozturk et al., 2002; Seki et al., 2002; Bartels and Sunkar, 2005). However, few reports analyzed differences in gene expression between salt-sensitive and salt-tolerant genotypes in plants (Walia et al., 2005). Although such genes are useful in the dissection of genotype-specific regulatory pathways and mechanisms of salt tolerance, they usually represent only a fraction of all salt-regulated genes, and isolating them is a challenging task. The mechanisms underlying the genotype-dependent difference in expression of such genes are largely unknown.

Two genotypes (108-R and Jemalong A17) were shown to have a different adaptation to salt stress (Merchan et al., 2003). We have developed, in the *M. truncatula* 108-R genotype, a dedicated macroarray containing genes linked to salt stress and recovery responses in roots (Merchan et al., 2007). Here, we aimed to identify genes involved in genotype-specific regulatory pathways and mechanisms of salt acclimation. A comparison of molecular and physiological responses to salt stress in sensitive and tolerant *M. truncatula* genotypes (108-R and Jemalong A17, respectively) was performed. Expression analysis using the dedicated macroarray revealed several genes potentially associated with the contrasting responses to salt stress in these plants. Among them, we identified a homolog of the *COLD-REGULATEDA1* (*CorA1*; *MtCorA1*; Laberge et al., 1993) gene, which is a putative target of MtZpt2 transcription factors (TFs; Merchan et al., 2007). Accordingly, these TFs showed higher expression levels in the tolerant variety. Overexpression of *MtZpt2-1* or *MtZpt2-2* in roots of the salt-sensitive variety allowed significant increase in root growth specifically under salt stress conditions, suggesting a role of these pathways in the differential adaptive response to salt stress of these *M. truncatula* genotypes.

RESULTS

Evaluation of Salt Stress Growth Responses of Two *M. truncatula* Genotypes

We have examined root growth and dry weight biomass in two genotypes of the model legume *M. truncatula* (108-R and Jemalong A17) in response to different salt stress conditions. Root length was measured after 5 d of growth on a rich medium (Fahräeus; Truchet et al., 1985) containing various concentrations of NaCl (Fig. 1). Root length of *M. truncatula* 108-R was significantly reduced after NaCl treatments in the range of 90 to 150 mM, as compared with plants grown without salt (Kruskal and Wallis test, $P < 0.01$; $n = 20$; Fig. 1A). However, root length in Jemalong A17 was negatively affected by salinity only at 150 mM (Kruskal and Wallis test, $P < 0.01$; $n = 20$; Fig. 1A). Altogether, we could identify a differential response to salt stress between the 108-R and Jemalong A17 genotypes. As shown in Figure 1B, *M. truncatula* Jemalong A17 grew well at high salinity levels (120 mM NaCl), while 108-R plants grew more slowly under these salt conditions. Similarly, salt treatment reduced the root dry weight more noticeably for the variety 108-R already after a 60 mM NaCl treatment, whereas it was only significantly affected at 150 mM in Jemalong A17 (Kruskal and Wallis test, $P < 0.01$; $n = 20$; Fig. 1C, left). No significant differences were, however, found for leaf dry weight biomass of each variety and in all treatments, suggesting greater salt sensitivity of the root than of the aerial part at this early postgermination stage (Fig. 1C, middle). The ratio of root dry weight to leaf dry weight (as percentage of control) reveals the relative effects of increasing NaCl concentration for each genotype (Fig. 1C, right). As expected, ratio values for *M. truncatula* 108-R were significantly reduced with increased salt treatments (60–150 mM NaCl; Kruskal and Wallis test, $P < 0.01$; $n = 20$). In contrast, this ratio was constant for the tolerant genotype, at least up to 120 mM NaCl. These results suggest that root dry weight is significantly different between the two genotypes at medium and high salt concentrations (60–150 mM).

Root growth performance was also assessed on a low-nitrogen medium ("i"; Blondon, 1964) to examine the impact of the medium on the range of variability for salinity tolerance between these two genotypes of *M. truncatula*. At the same salinity concentration, root length of 108-R was even more significantly affected by salt treatment when compared with the results obtained in the rich medium. This difference is likely to be a consequence of minimal nutrient capacity of the "i" medium that may potentiate salt stress effects on growth. Statistically significant differences between 108-R and Jemalong A17 genotypes were found for all assayed salinity conditions (Fig. 1D). *M. truncatula* 108-R growth was already reduced by more than 50% at 90 mM of salt, and an NaCl concentration of 150 mM nearly abolished root growth. This negative effect of salt on root growth was less pronounced in Jemalong A17, in agreement with the results obtained on rich (Fahräeus) medium (Fig. 1A).

These various parameters allowed us to monitor the effects of salt treatments on plant growth and further revealed differential root growth responses between
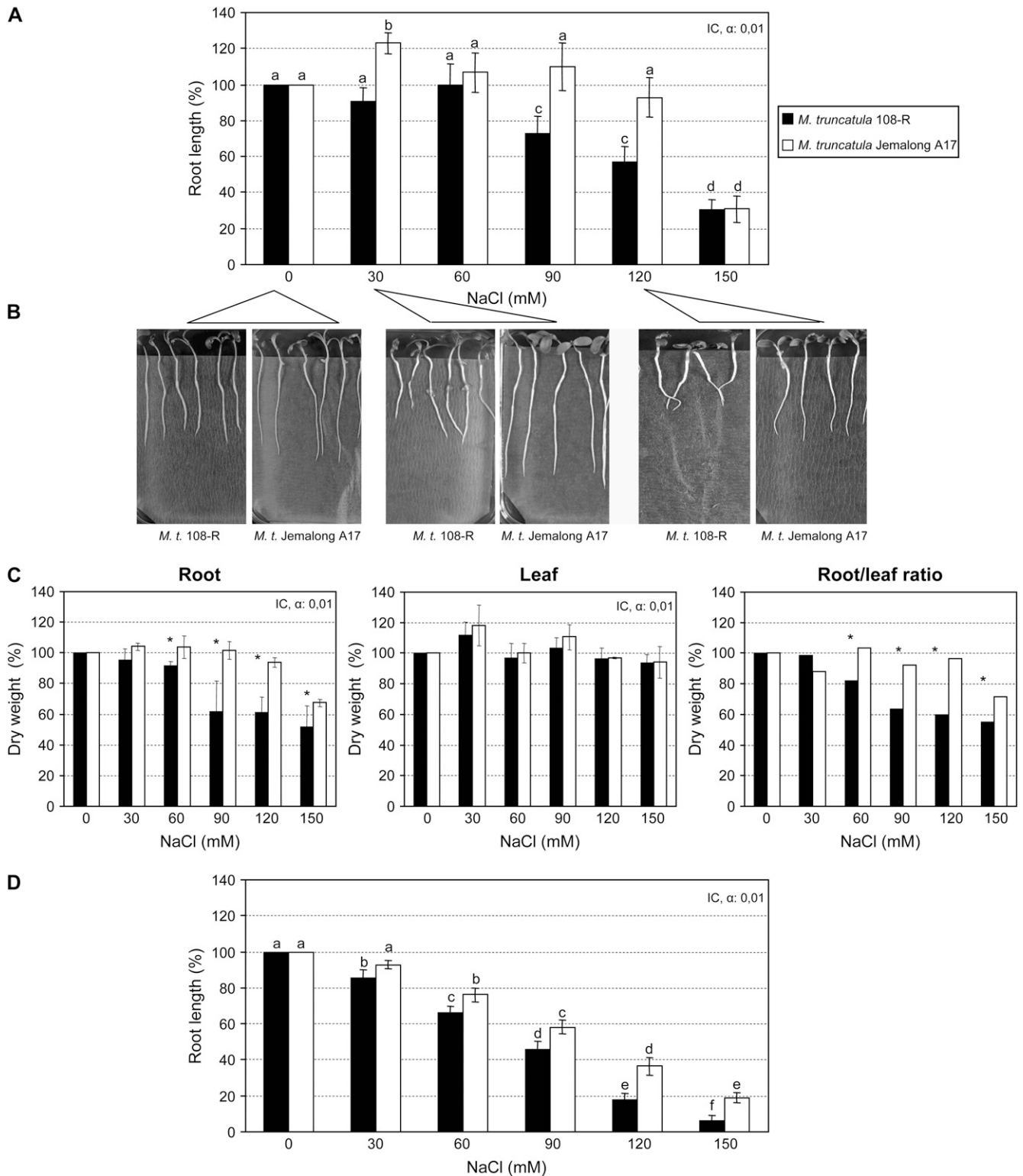
Figure 1. Effect of NaCl on root growth and dry weight of two *M. truncatula* genotypes. Root length and dry weight biomass under different salt stress conditions were evaluated in an in vitro system. Germinated seedlings were grown on vertical Fahräeus or ''i'' medium plates for 5 d in the presence of different NaCl concentrations (0, 30, 60, 90, 120, and 150 mM). A, Relative root length of each variety at 5 d.a.g. in in vitro conditions with Fahräeus medium is shown as percentage of control root growth without salt. B, Representative pictures taken 5 d after transfer of the seedlings to 0, 30, and 120 mM NaCl on Fahräeus medium. C, Relative dry

the two genotypes. Plants grown in low-nitrogen medium were more affected by salinity even though, independently of the growth medium, the Jemalong A17 genotype had a greater ability to tolerate salt stress conditions than 108-R.

Effects of Salt Stress on the Rhizobial Symbiotic Interaction in *M. truncatula* 108-R and Jemalong A17 Genotypes

The formation of nitrogen-fixing nodules results from the symbiotic interaction between legumes and rhizobia, a process sensitive to salinity at several stages (initiation, development, and function of nodules; Garg and Gupta, 2000). To study the effect of salinity on the nodulation capacity in these two *Medicago* genotypes, plants were grown in the presence of different NaCl concentrations and subsequently inoculated with *Sinorhizobium meliloti* strain 2011 (able to grow up to 300 mM NaCl; Rüberg et al., 2003). Three weeks after inoculation, these plants were assessed to determine their nodule number. A negative effect on nodule formation was clearly observed in both varieties at high salt concentration in the medium (Fig. 2A; Kruskal and Wallis test, $P < 0.05$; $n = 20$). Salinity decreased nodule number in the 108-R genotype at 60 mM NaCl by more than 50% versus only 20% in Jemalong A17. In contrast, the latter genotype reaches a similar reduction level only at 120 mM NaCl. These results correlated with the differential root growth sensitivity to salt stress of these genotypes. A drastic reduction in the total number of nodules was observed at 150 mM NaCl although to a lesser extent for Jemalong A17 (Fig. 2A). In addition, salinity reduced nodule size and differentiation in both varieties, as evidenced by the appearance of white nodules without high leghemoglobin content (data not shown). These perturbations of the nodulation process could explain the decrease in plant growth under saline conditions.

The effects of salinity on the dry weight of both 108-R roots and leaves are very significant already at 60 mM NaCl in contrast to what is observed for the Jemalong A17 variety (Fig. 2B). In nodulated plants, a major effect of salt in the aerial part was observed in contrast to recently germinated seedlings. The effect in root and leaf dry weights correlated with the reduction in nodule number under salt stress, with 108-R being significantly more affected than Jemalong A17. Per-

turbations in the nodulation process and in nodule functionality could explain the decrease in plant growth under saline conditions. The contrasting root physiological responses of the two genotypes to salt treatments may be partially responsible for their different ability to interact with *Rhizobium* under stress conditions. Nevertheless, independently of the nitrogen status (either from added combined nitrogen or through symbiotic fixation), the Jemalong A17 genotype is more tolerant to salt stress than 108-R.

Gene Expression Profiles of *M. truncatula* 108-R and Jemalong A17

Transcriptional profiling of selected salt stress-related genes is useful for the assessment and comparison of gene expression on a comprehensive scale across genotypes. To reveal molecular mechanisms that may sustain the contrasting salt tolerance responses in roots of both genotypes, we performed a comparative analysis of expression profiles using a salt stress dedicated macroarray containing 384 genes (Merchan et al., 2007). Gene expression analysis was performed using samples from roots of 108-R and Jemalong A17 after 0 or 4 d of salt stress (150 mM NaCl on "i" medium, referred to as "4i" and "4n," respectively). This concentration and time was previously used for the analysis of recovery responses in *M. truncatula* (Merchan et al., 2003, 2007) and represent a condition that does not permanently affect root growth. Both varieties under these conditions can fully recover their root growth after changing into a normal medium. We directed our experimental design to detect genes differentially regulated between the two genotypes in control and stressed conditions (Fig. 3A). Four different treatments were used: untreated 108-R plants (R0), 108-R plants treated with 150 mM NaCl for 4 d (R4), untreated Jemalong A17 plants (J0), and Jemalong A17 plants treated with 150 mM NaCl for 4 d (J4). To identify statistically significant differentially expressed genes between genotypes during salt treatments, we used a Student's t test based on two biological replicates and four technical replicates (e.g. quadruplicated probes on macroarray) and retained genes with P values < 0.01.

Most of the genes were similarly regulated in these genotypes, because approximately one-half of the spots on the dedicated macroarray (e.g. about 150

Figure 1. (*Continued.*)
weights of roots and leaves from *M. truncatula* 108-R and Jemalong A17 after 15 d of growth in Fahräeus medium submitted to different salt stress treatments. Results are shown as percentage of control without salt. Ratio (right) represents root dry weight/leaf dry weight to evaluate the effect of salinity on dry weight biomass of the plants. D, Relative root growth (%) of the two *M. truncatula* varieties grown under nonstressed and salt-stressed conditions in "i" medium for 5 d. Values indicated with different letters indicate statistically significant differences ($P < 0.01$), whereas those marked with the same letters show statistically similar values. IC, Interval of confidence ($\alpha = 0.01$). Columns labeled with an asterisk are significantly different ($P < 0.01$) within a given salinity level. In both cases, the Kruskal and Wallis test has been used. A representative example out of two biological experiments is shown.
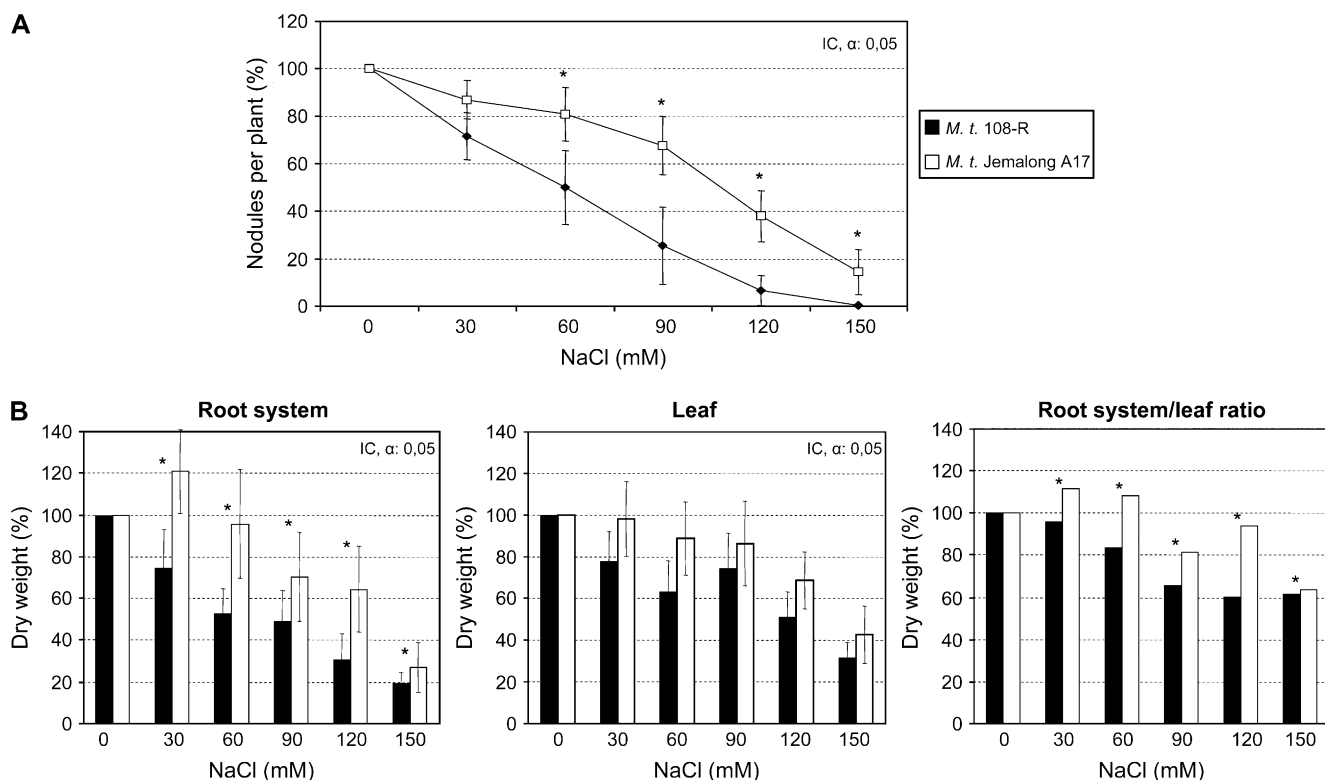
Figure 2. Effect of salt stress on nodulation in *M. truncatula* 108-R and Jemalong A17. Seeds of the two genotypes were germinated on water agar plates, and seedlings were grown in the greenhouse in the presence of different NaCl concentrations (0, 30, 60, 90, 120, and 150 mM). The inoculation with *S. meliloti* strain 2011 was made 9 d.a.g.. Nodule number and root and leaf dry weights were determined at 30 d.a.g. (21 d.p.i.). A, The effect of salt concentration on nodulation of *M. truncatula* 108-R and Jemalong A17 by *S. meliloti* strain 2011 is measured as percentage of the total nodule number 21 d.p.i. observed in control (without salt) conditions. B, Relative dry weights (%) of root system and aerial part of *M. truncatula* 108-R and Jemalong A17 genotypes at 30 d.a.g. under different salt stress conditions. IC, Interval of confidence ($\alpha = 0.05$). Columns labeled with an asterisk are significant differences ($P < 0.05$) between genotypes within a given salinity level. Statistical comparisons were performed using the Kruskal and Wallis test. A representative example out of two biological experiments is shown.

spots) showed similar detectable signals for 108-R and Jemalong A17 hybridization, validating the use of the array for both genotypes. These included internal constitutive controls for gene expression analysis such as tubulin, elongation factor, and actin genes used for array normalization (Merchan et al., 2007). In Jemalong A17, expression of 14 genes was altered after a salt stress: four genes (1.4% of the 280 stress-related genes present in the array; Merchan et al., 2007) induced and 10 (3.6%) down-regulated (J0 versus J4; Fig. 3B). In contrast, in the salt-sensitive genotype, transcript levels of 63 genes were altered by the salt treatment. Among them, 23 genes (8.2%) were up-regulated and 40 (14.3%) down-regulated at 4n (R0 versus R4; Fig. 3B). Surprisingly, no gene was induced in common between 108-R and Jemalong A17 after a salt stress but three genes were down-regulated in both genotypes (Fig. 3C).

We can conclude that the tolerant and sensitive varieties have a different regulation of their transcriptome in response to salt stress.

Differences in Gene Regulation between the Salt-Sensitive and Salt-Tolerant *M. truncatula* Genotypes

The differential expression patterns that may lead to the increased salt tolerance of Jemalong A17 plants in comparison to 108-R were tested in two ways. First, a comparison of expression profiles under normal growth conditions between the salt-sensitive and the salt-tolerant varieties (J0 versus R0) revealed 38 genes up-regulated in Jemalong A17 roots relative to 108-R roots (Supplemental Table S1). In contrast, only five genes were more expressed in the sensitive genotype in the same conditions. This large gene activation observed in Jemalong A17 under control growth conditions may account for its relatively lower levels of gene regulation by salt stress when compared to 108-R. We found six salt-inducible genes in 108-R that were common with those up-regulated in Jemalong A17 in control conditions (Supplemental Table S1, genes marked in yellow), confirming that abiotic stress-related
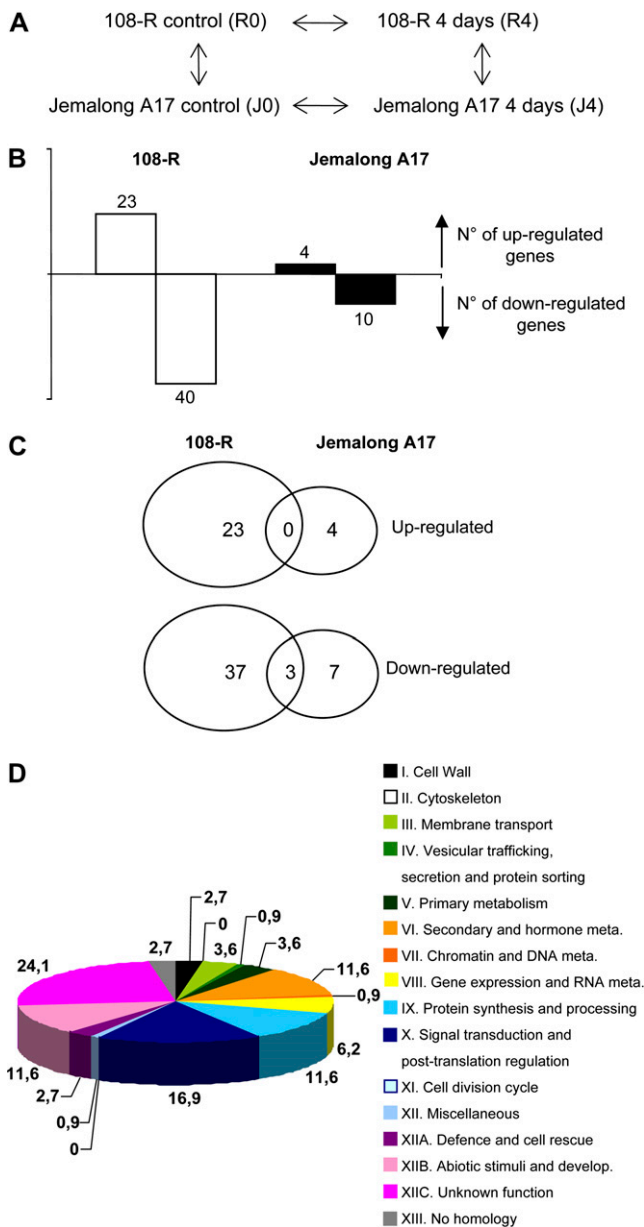
Figure 3. Gene expression profiles of *M. truncatula* 108-R and Jemalong A17. A, Experimental design is based on series of pairwise comparisons. Four d.a.g. seedlings from different genotypes (R for 108-R and J for Jemalong A17) were grown for 4 d without (0 mM; R0 and J0) or with (R4 and J4) 150 mM NaCl. Two biological and four technical replicates were available for each gene, for each physiological condition, and for each genotype. B, The histogram shows the total number of transcripts up- or down-regulated in 108-R (left bars) and Jemalong A17 (right bars) in response to salinity stress at a level of $P < 0.01$. C, Venn diagrams illustrating the number of genes up-regulated or down-regulated under salinity stress in either or both genotypes of *M. truncatula* (108-R, left, and Jemalong A17, right). D, Distribution of differentially expressed genes into functional categories according to BLASTN hits (based on Journet et al., 2002; Merchan et al., 2007). Percentages were calculated from the total number of differentially expressed genes from Jemalong A17 (112 genes).

genes for the 108-R genotype are expressed at higher levels in the tolerant genotype in the absence of stress.

Second, intrinsic differences between the two genotypes after a salt stress were searched (J4 versus R4). Direct comparison of gene induction/repression between the two varieties after 4 d of salt stress detected large differences in expression patterns between the individual genotypes. This may be particularly relevant for genes whose levels of expression in control conditions are not the same for each genotype. This comparison allowed the detection of 118 differentially expressed genes that were classified into two groups: (1) those genes that are up-regulated in 108-R at 4n of salt (six genes); and (2) those genes that are up-regulated in Jemalong A17 at 4n (112 genes; Supplemental Table S2). These latter genes could be of interest in determining the differential tolerance to salt stress of these *M. truncatula* varieties. For this reason, these genes were further characterized. Sorting of the genes up-regulated at 4n in Jemalong A17 into functional categories (according to Journet et al., 2002) revealed that four categories were overrepresented: signal transduction and posttranslational regulation (16.9%), abiotic stimuli and development (11.6%), protein synthesis and processing (11.6%), and hormone and secondary metabolism (11.6%), whereas a significant proportion could not be assigned (unknown function, 24.1%; Fig. 3D). Among those classes underrepresented, we found genes linked to the cell cycle (0%) and to DNA synthesis and chromatin (0.9%) in correlation with the reduction of meristem activity induced by salt stress.

Among the 112 differentially expressed genes up-regulated in Jemalong A17 roots under salt stress, five genes showing different levels of induction in the tolerant variety were randomly selected to validate the changes detected on macroarrays using real-time reverse transcription (RT)-PCR on independent biological samples (Supplemental Table S2, genes indicated in yellow). One codes for a homolog to a ADP-ribosylation factor (*MtADPr*, TC67888); one has homology to a dormancy-associated protein (*MtDor*, TC85295), another codes for a cold-inducible CORA protein (*MtCorA1*, TC68022), a fourth matches the sequence of a dehydrin-related protein (*MtDehyd*, TC76699), and the fifth is *MtHP2*, a His-containing phosphotransfer protein potentially related to cytokinin signaling (González-Rizzo et al., 2006). The expression patterns revealed using real-time RT-PCR followed the same trends seen in macroarrays (Fig. 4A). Interestingly, we previously relate that a homolog of the *CorA1* (Laberge et al., 1993) gene could be a target of TFIIIA-like C_2H_2 zinc finger TF, MtZpt2-1 and MtZpt2-2 (Merchan et al., 2007; Supplemental Fig. S1). Indeed, the other potential target gene, *Fpf1* (Kania et al., 1997), as well as the MtZpt2-1 TF, were found to be up-regulated in the tolerant variety (Supplemental Table S2, genes indicated in light blue). Hence, we tested whether the MtZpt2 TF may be differentially regulated between these genotypes. Real-time RT-PCR showed that both TFs were
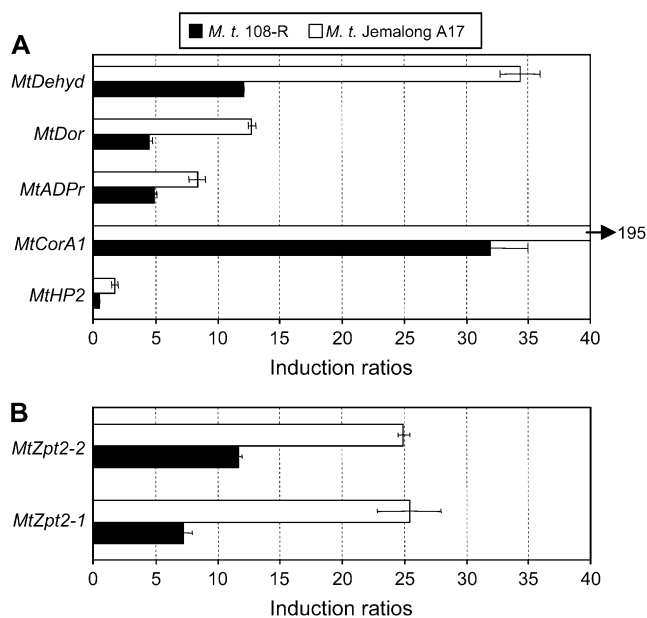
Figure 4. Real-time RT-PCR analysis of selected genes differentially expressed between genotypes in response to salt stress. Specific gene expression in the salt-tolerant (Jemalong A17) versus salt-sensitive (108-R) genotypes were analyzed in control and salt stress conditions (4 d at 150 mM NaCl). Induction ratios were calculated between the salt-treated and nontreated samples. A representative example out of two biological experiments is shown, and error bars represent SD of three technical replicates. Numbers on the x axis indicate the fold-induction of gene expression in relation to the nonsalt stress condition. A, Real-time RT-PCR of five randomly selected differentially expressed genes between genotypes: MtHP2, a His-containing phosphotransfer protein homolog gene (a gene involved in cytokinin signal transduction); MtCorA1, a cold-and drought-regulated CORA protein homolog gene; MtADPr, a gene encoding an ADP-ribosylation factor homolog protein; MtDor, a gene coding for a dormancy-associated protein; and MtDehyd, a gene encoding a dehydrin-related protein. B, Real-time RT-PCR analysis of two genes that encode putative TFIIIA-type TFs (MtZpt2-1 and MtZpt2-2).

more expressed in the salt-tolerant Jemalong A17 genotype than in 108-R (Fig. 4B). These regulatory genes could then be involved in the differential response to salt stress between these *Medicago* genotypes.

Role of the *MtZpt* Pathway in *M. truncatula* 108-R and Jemalong A17 Genotypes

Our previous work (Merchan et al., 2003, 2007) demonstrated a potential role of MtZpt2 TFs in the adaptation of *M. truncatula* 108-R to salt stress, based either on stable transgenic antisense plants or on *Agrobacterium rhizogenes*-transformed roots overexpressing MtZpt2-1 (referred as composite plants; Boisson-Dernier et al., 2001). To analyze a potential physiological role of the *MtZpt2* genes in root adaptation of *M. truncatula* Jemalong A17 to salt stress, we comparatively overexpressed the *MtZpt2-1* or *MtZpt2-2* genes from Jemalong A17 under the control of the constitutive 2× 35S cauliflower mosaic virus promoter

in several independent transgenic roots of 108-R or Jemalong A17 genotypes to assay their salt responses. After 3 weeks of growth in control medium, the composite plants were transferred to a salt-containing (100 mM NaCl) or control medium and root length was measured 8 and 6 d after transfer in 108-R and Jemalong A17 plants, respectively (Fig. 5). In nonsaline conditions, the growth of *MtZpt2-1* or *MtZpt2-2* overexpressing 108-R roots, as well as control roots expressing the GUS reporter gene, were not significantly different (Fig. 5A). However, we detected for both TF-overexpressing 108-R roots a significant increase (Student's *t* test, $P < 0.01$ in two independent biological experiments; $n > 25$) in primary root growth compared to control roots 1 week after transfer on salt medium (Fig. 5B). These results agreed for *MtZpt2-1* with our previous experiments (Merchan et al., 2007). In contrast, Jemalong A17 roots overexpressing *MtZpt2-1* or *MtZpt2-2* were not significantly affected in their growth either in the absence or presence of salt stress (Fig. 5, C and D, respectively), even though these roots were effectively overexpressing the corresponding *MtZpt2* transgene (Supplemental Fig. S2).

We also examined whether the overexpression of MtZpt2-1 or MtZpt2-2 TFs in *M. truncatula* 108-R and Jemalong A17 affected the nodulation capacity of these plants under salt stress conditions. We assayed nodulation capacity in control and salt stress conditions of several independent transgenic roots overexpressing these genes in these two genotypes. After 3 weeks of growth in control medium, composite plants were transferred to a salt-containing medium (100 mM NaCl) and inoculated with *S. meliloti* 2011. We determined the total number of nodules per plant 21 d postinoculation (d.p.i.). Overexpression of either one or the other MtZpt TFs did not alter significantly the nodulation capacity of these two genotypes under salt stress (108-R and Jemalong A17; Supplemental Fig. S3, A and B, respectively). Moreover, no differences in nodulation were observed even in the absence of salt, although the genotype-dependent differential inhibitory effect of salt stress on the symbiotic interaction was detected (Supplemental Fig. S3, A and B).

Hence, even though the MtZpt pathway could not be related to the different nodulation capacity of the genotypes either in the presence or absence of salt stress, their differential root growth response to salt stress may be partially linked to the higher expression levels of the two MtZpt2 TFs in the tolerant variety. These data suggest that the MtZpt2 pathway is activated in Jemalong A17.

DISCUSSION

Soil salinity is one of the major abiotic stresses reducing agricultural productivity. The direct selection of superior salt-tolerant genotypes under field conditions is hindered by the considerable influence that environmental factors have on the response of plants
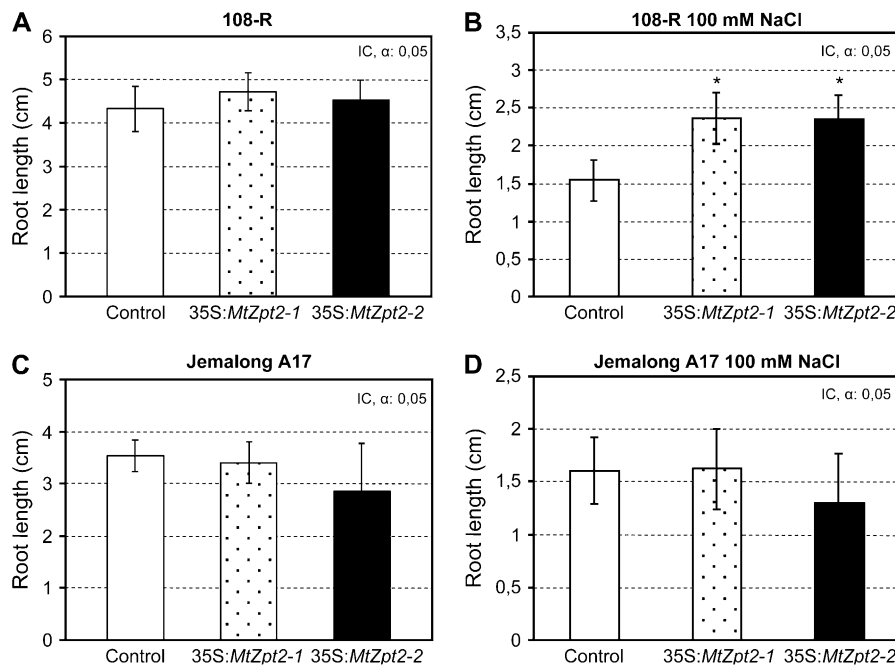
Figure 5. Evaluation of root growth in *A. rhizogenes*-transformed *M. truncatula* roots overexpressing *MtZpt2-1* or *MtZpt2-2*. Composite plants were prepared as described in Boisson-Dernier et al. (2001) using control empty vector and *MtZpt2-1* or *MtZpt2-2* overexpressing constructs. After kanamycin selection of transgenic roots, composite plants were transferred to salt-containing or control media. A representative example out of two biological experiments is shown in all cases. *, Statistically significant differences ($P < 0.01$; $n > 25$). A and B, Growth of transgenic roots overexpressing either *MtZpt2-1* or *MtZpt2-2* in *M. truncatula* 108-R was monitored 1 week after transfer into control (A) or a salt-containing medium (100 mM NaCl; B). The initial position of the root apex after transfer was monitored to determine the degree of root elongation during the week. C and D, Idem as A and B for Jemalong A17 indicating transgenic root growth in control (C) or salt-containing medium (100 mM NaCl; D).

to salinity (Richards, 1996). Salt tolerance is a complex trait involving the function of many genes (Flowers, 2004; Foolad, 2004). In fact, the exploitation of natural genetic variations and the generation of transgenic plants introducing novel genes or altering expression levels of the existing genes are being used to improve salt tolerance (Austin, 1993; Jain and Selvaraj, 1997; Yeo, 1998; Hasegawa et al., 2000; Park et al., 2001; Xiong and Yang, 2003; Guo et al., 2004; Davletova et al., 2005; Dana et al., 2006; Hong and Hwang, 2006). In legumes, salt stress significantly limits productivity because of its adverse effects on the growth of the host plant and its symbiotic interactions. Root growth, nodule development, and nitrogen-fixation efficiency are particularly affected (Rai, 1992; Cardovilla et al., 1994). In this work, we analyze the differential adaptation to salt stress of different *M. truncatula* genotypes (108-R and Jemalong A17) with regard to root growth and nodulation responses. Analysis of gene expression patterns between these genotypes allowed us to propose the implication of the TFIIIA-type TF pathway in the increased tolerance of Jemalong A17 to salt stress.

Arrest of plant growth during stress conditions depends largely on the severity of the stress (Westgate and Boyer, 1985; Sharp et al., 1988; Spollen et al., 1993; Munns et al., 2000; Bartels and Sunkar, 2005). However, continuation of root growth under drought stress is an adaptive mechanism that facilitates water uptake from deeper soil layers (Sharp et al., 1988). Similarly, continued root growth under salt stress may provide additional surfaces for sequestration of toxic ions, leading to lower salt concentration, and enables reaching soil areas with lower salt concentrations. For example, salt tolerance of barley (*Hordeum vulgare*) was correlated with better root growth rates coupled with fast growth and early flowering (Munns et al., 2000; Bartels and Sunkar, 2005). In this study, specific experiments using two *M. truncatula* genotypes (108-R and Jemalong A17) revealed that increased primary root growth after germination may be linked to their different tolerance to salt stress. The magnitude of root growth inhibition was mainly dependent upon the genotype rather than on growth media. The poor "i" medium is probably more similar to the conditions of the rhizosphere than is the relatively rich Fahräeus medium and was chosen for macroarray analysis because good phenotypic variability in response to salinity was observed between these *M. truncatula* varieties.

The aerial organs also contribute to the various mechanisms aimed at postponing adverse saline effects or tolerating salt stress. These include reduction of water loss by increased stomatal resistance and accumulating sizeable amounts of Na^+ in the vacuole (Apse et al., 1999; Steudle, 2000; Assmann and Wang,

2001; Zhang and Blumwald, 2001; Horié and Schroender, 2004). Nevertheless, in Arabidopsis (*Arabidopsis thaliana*), most of the genes were induced by salt stress only in roots, not in leaves (Shisong et al., 2006). The reason for this might be that leaves, compared with roots, have a large sodium storage capacity, or it might be a consequence of the relatively high concentration of sodium ions in roots as they perceive the salt conditions from the soil environment (Kreps et al., 2002; Volkov et al., 2004; Shisong et al., 2006). Under the conditions imposed in this work, 108-R and Jemalong A17 did not differ significantly in leaf dry weight after 15 d of treatment. However, in nodulated plants, the effects of salt on the aerial part were more significant, suggesting a later effect of salinity in this case. In the initial stages of growth, the aerial part may be less sensitive to salt stress than is the root in legumes.

In legume-*Rhizobium* symbiosis, the adverse effects of stresses on nodule functioning vary in intensity, depending on aspects such as plant species, rhizobial strain, and duration and conditions of exposure to the stressful condition (Garg and Gupta, 2000; Bouhmouch et al., 2005; Morón et al., 2005). Furthermore, the number and total weight of nodules decreased in salinity treatments (Delgado et al., 1994; Merchan et al., 2003). We showed that total nodule number in the two *M. truncatula* genotypes was adversely affected by salt stress. The salt present in the growth medium may inhibit the absorption of calcium, reducing the emergence and growth of roots and root hairs and decreasing potential infection sites (Zahran and Sprent, 1986). Nodule growth has previously been reported to be affected by salt stress in *Glycine max* (Delgado et al., 1994; Gordon et al., 1997) and *Phaseolus vulgaris* (Delgado et al., 1994; Bouhmouch et al., 2005). It has been proposed that the inhibition of photosynthesis in plants subjected to salt stress leads to a restriction of photosynthate transport toward nodules and a reduction in their size (Bekki et al., 1987). However, González et al. (2001) suggested that nodule growth reduction is linked to the inhibition of enzymes associated with Suc degradation in nodules. In nodulated plants, both aerial and ground tissues are affected by salt, and complex interactions between them may explain the better nodulation of Jemalong A17 plants.

Array analysis to characterize gene expression profiles for large numbers of transcripts has been used to describe the response to environmental stresses in various species, including Arabidopsis for cold, drought, and salt (Kreps et al., 2002; Seki et al., 2002; Takahashi et al., 2004), *M. truncatula* for recovery from salt stress (Merchan et al., 2007), and rice (*Oryza sativa*) for response to salt stress (Kawasaki et al., 2001). The large number of stress-regulated genes detected in these studies indicates that comparative transcript profiling could be applied between genotypes of the same species (Taji et al., 2004; Moore et al., 2005; Walia et al., 2005; Weber et al., 2006). This comparative analysis might reveal the responses shared across genotypes and also identify differential responses.

Comparison of EST sequences from Jemalong A17 and 108-R revealed a high DNA sequence identity (90%–95%) for the large majority of transcripts, indicating that the macroarray of 108-R genotype (Merchan et al., 2007) can be used for expression profiling of Jemalong A17 genes.

The constitutive high expression of certain stress response genes in plants able to thrive in a particular stress environment emerges as a possibly widespread adaptive mechanism. This would imply that rather than the expression of particular species-specific stress-tolerance genes, it is the altered regulation of conserved genes that enables certain plants to survive in harsh environments. For instance, in the salt cress, *Thellungiella halophila*, a salt-tolerant relative of the glycophyte Arabidopsis, it was shown by comparative transcriptome analysis that the orthologs of several well-known Arabidopsis stress response genes are highly expressed even under control conditions (Taji et al., 2004); similarly, the metal-tolerant *Arabidopsis halleri* was found to express a number of metal-homeostasis genes at much higher levels than Arabidopsis, independently of micronutrient status (Becher et al., 2004; Weber et al., 2004). Our analysis suggests that Jemalong A17 is more tolerant than 108-R to salt stress, because several stress-related genes are expressed under nonstressed conditions. Jemalong A17 does not induce major changes at the transcriptional level among the dedicated macroarray population in response to salinity conditions as compared with 108-R. Additionally, gene repression may be moderated in the tolerant genotype, allowing growth to be achieved in the adverse conditions. In fact, many different functions have been identified as being differentially regulated between the two genotypes in response to salt stress (more than 30% of the tested genes in the macroarray). As expected, a major class is linked to stress-related pathways, together with a large class of regulatory genes (posttranscriptional regulation and signal transduction). Interestingly, several response regulators, a cytokinin receptor, and ethylene-related and auxin-inducible genes were found (Supplemental Table S2). This further reinforces the hypothesis of a cross talk between hormone- and environment-related signaling pathways in the control of root growth under adverse conditions (Malamy, 2005; Merchan et al., 2007). In addition, among those showing the largest differences, we detected a dehydrin-related gene and a homolog of imbibition proteins probably linked to the well-known osmotic stress component of salt stress.

TFs are crucial elements for the regulation of development and adaptation to abiotic stresses in plants, and the overexpression of specific TFs leads to increased tolerance to abiotic stress, such as salt stresses (Kasuga et al., 1999; Winicov and Bastola, 1999; Winicov, 2000; Guo et al., 2004; Kim et al., 2004; Mukhopadhyay et al., 2004; Davletova et al., 2005). We have previously studied the MtZpt2 TFIIIA-related TFs (MtZpt2-1 and MtZpt2-2) in the 108-R genotype (Merchan et al., 2003, 2007). Gene expression patterns in antisense plants

and *A. rhizogenes*-transformed roots overexpressing MtZpt2-1 together with transient assays revealed that MtZpt2-1 may be an activator of the *MtCorA1* genes in 108-R (Merchan et al., 2007). In this work, we found increased expression of the *MtCorA1* gene in the tolerant Jemalong A17 variety that correlated with the differential expression of these TFs. Overexpression of either one or the other MtZpt2 TFs enhanced root growth of 108-R plants but not of Jemalong A17 plants under salt stress conditions. We speculate that activation of the TFIIIA Krüppel-like pathway may be partially involved in the tolerance of Jemalong A17 to salt stress.

In conclusion, our results demonstrate the contrasting salt tolerance at physiological and transcriptional levels between two *M. truncatula* genotypes. This variability in salt sensitivity may be linked to the activation of MtZpt TFs in the tolerant Jemalong A17 genotype and could be a potential genetic resource for improving the salt tolerance of *M. truncatula* and legume crops in selective breeding programs.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Two genotypes of *Medicago truncatula*, 108-R (Hoffmann et al., 1997) and Jemalong A17 (Barker et al., 1990), were used in this work. Seeds of these varieties of *M. truncatula* were scarified in concentrated sulfuric acid for 8 min and rinsed three times with distilled water. The scarified seeds were surface sterilized for 20 min in bleach (12% [v/v] sodium hypochlorite). After washing with sterilized water, seeds were sown on 1% water-agar plates and stored for 2 d at 4°C before incubating overnight at 24°C in the dark to ensure uniform germination. Germinated seedlings were transferred to square plates or pots containing appropriate medium and treatment (see below) and grown vertically in a growth chamber or in a greenhouse, respectively.

In this work, the Fahräeus and "i" media were used. The poor "i" medium (Blondon, 1964) was used for root growth and nodulation assays of seedlings. The Fahräeus medium (Truchet et al., 1985) with added nitrogen (1 mM) was used to measure root growth and to prepare composite plants (see below). For nodulation experiments on composite plants, Fahräeus medium without nitrogen was used for optimal nodulation.

Treatment and Evaluation of Salinity Tolerance

Plants were grown under various salt stress conditions (0, 30, 60, 90, 120, and 150 mM of sodium chloride) to elucidate differences in the root growth, dry weight biomass, and nodulation capacity between the varieties studied. NaCl is a common salt that adversely affects plant growth under natural conditions, although a unique salt is generally not found in soils (Bernstein, 1962). Different levels of NaCl were used in this study to provide a range of root growth and nodulation responses from the control treatment and of selected varieties.

For root growth comparisons, seedlings were placed in petri dishes with the appropriate medium (poor "i" medium or richer Fahräeus medium), and the position of primary root tips was marked on the petri dish every 24 h. Root length was measured the 5th d for around 20 seedlings in petri dishes, and two replications of each treatment (20 plants/genotypes) were performed. The experiment was conducted in a growth chamber with mean temperature 24°C under a 16-/8-h photoperiod, and 70% of relative humidity.

For measuring the effect of salt concentration on nodulation of *M. truncatula* genotypes, 2-d-old seedlings were placed in plastic pots containing perlite:sand (3:1, v/v) as mixed substrate (five seedlings per pot) and irrigated with the appropriate medium ("i" medium; Blondon, 1964) in the greenhouse. Plants were grown in this medium until the 5th d after germination (d.a.g.) when salt stress treatments were initiated. The plant growth media with salt stress was identical to that of controls, except for the addition of NaCl at

different concentrations (30, 60, 90, 120, and 150 mM). After four additional days, plants were infected with a stationary-phase culture of wild-type *Sinorhizobium meliloti* strain 2011. Nodules were counted 21 d after inoculation (30 d.a.g.). This experiment was conducted in a greenhouse, minimizing evaporation to keep the total water volume of the pots constant. Two biological replicates per salinity treatment were performed (20 plants/genotype).

Fifteen and 30 d after the start of salt treatment in both types of experiments (root growth comparisons and determination of nodulation capacity in these two varieties, respectively), individual plants of salt stress and nonstress treatments were harvested. Plants were separated into root system and aerial part. Plant materials were dried at 60°C for 48 h and plant dry weight (under salt stress and as a percentage of dry weight under the nonstress treatment) was determined for individual plants of each genotype.

RNA Extraction and DNA Array Construction

Roots were collected after 4 d of salinity treatment at 150 mM NaCl and immediately frozen in liquid nitrogen for RNA extraction (Merchan et al., 2007). For real-time quantitative RT-PCR experiments, total RNA was extracted using the Total RNA Isolation kit (Macherey-Nagel). Macroarray hybridizations were performed as described in the EMBO *M. truncatula* Practical Course manual (http://www.isv.cnrs-gif.fr/embo01/manuels/pdf/module5.pdf). DNA macroarrays were constructed as described previously in Merchan et al. (2007).

Macroarray Expression Analysis and Real-Time RT-PCR Measurement of Transcript Level of Selected Genes

cDNA probe labeling, hybridization of macroarrays, and quantification of hybridization signals were performed as described in http://www.isv.cnrs-gif.fr/embo01/manuels/pdf/module5.pdf (Merchan et al., 2007). Experiments were normalized relative to expression of six housekeeping genes, whose expression was between 0.5- and 1.5-fold that of the control in all tested conditions and genotypes. These constitutive controls were selected to normalize all signals obtained from macroarray hybridizations (Merchan et al., 2007).

For real-time RT-PCR, after DNase treatment (RQ1 RNase-free DNase, Promega), 1.5 μg of total RNA were retrotranscribed for 1 h at 42°C using the SUPERSCRIPT II first-strand synthesis system (Invitrogen) and subsequently denatured for 10 min at 75°C. One-tenth of the cDNAs were used as a template in 10-μL PCR reactions. PCR was performed with a Light Cycler apparatus and the LC FastStart DNA Master SYBR Green I (Roche Diagnostics) according to the manufacturer's instructions. Gene-specific PCR primers were designed according to the cDNA sequences using the PRIMER3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi; maximum size, 300 bp; melting temperature, 60°C). The following gene-specific primers were used (forward and reverse, respectively): 5′-GGTTGTTTGCGAAGAAG-GAG-3′ and 5′-GTACCCCACGGTTTCAACAT-3′ for TC67888 (*MtADPr*); 5′-GGTGTCGTCGGAGTCACAG-3′ and 5′-TGGCCTTGAGAAGCTTAGGA-3′ for TC85295 (*MtDor*); 5′-TGTCTCAGCAGATGGCACAG-3′ and 5′-CGA-GGAGGAAGTTGATGGAG-3′ for TC76699 (*MtDehyd* for 108-R); 5′-GAG-CGAGGAGGAAGTTGATGG-3′ and 5′-TGGTGCTGGTGGAGTTGTTA-3′ for TC106659 (*MtDehyd* for Jemalong A17); 5′-GGCGGAGGTGGTTACAATGG-3′ and 5′-GGCAACAGATTCAGCAGCAC-3′ for TC68024 (*MtCorA1*); 5′-ATA-GATGCGTGCCGCAGGTG-3′ and 5′-GCATCTCTACAGATCCACTC-3′ for TC74018 (*MtHP2*); 5′-AAGTCCGGAAAAGCCGGGAGG-3′ and 5′-GCACT-TAACTCACCCACCACTGC-3′ for *MtZpt2-1*; 5′-GGCAACGGACTTTCTAC-CTC-3′ and 5′-CTCCTCCATCAGCCACCGTG-3′ for *MtZpt2-2*. Parallel reactions to amplify *MtActin11* were used to normalize the amount of template cDNA. Synthesis of three independent cDNA preparations from the same RNA sample (technical duplicates) allowed us to monitor reproducibility of the assay. A representative example out of the two biological replicates performed is shown for each figure.

Statistical Analysis

Root length, dry weight, and number of nodules in the various treatments were tested for significant differences using a Kruskal and Wallis test (Georgin and Gouet, 2000) because of the low sample size ($n = 20$). Statistical analysis of the macroarray data was based on a moderated Student's t test and is described in detail in Merchan et al. (2007). Genes with adjusted P values < 0.01 were selected as statistically significant. For composite plants, we analyzed the

significance of differences in root growth lengths or nodulation capacities using a Student's t test ($P < 0.01$).

Agrobacterium rhizogenes Root Transformation

Agrobacterium rhizogenes-transformed *M. truncatula* roots were prepared as described in Boisson-Dernier et al. (2001), and the binary plasmid used was described in Merchan et al. (2007). Two weeks after inoculation with *A. rhizogenes*, plants developed transgenic roots and were transferred to the same medium without kanamycin, containing a brown filter paper (recovered from a growth pouch, Mega International) to allow root growth on the surface of the paper (without penetration into the agar), and easy transfer to a new plate with salt-containing medium (100 mM NaCl in "i" medium). Root length was measured 8 and 6 d after transfer in 108-R and Jemalong A17 plants, respectively, from the initial position of the root apex at the time of transfer. Two biological replicates were realized ($n > 25$).

For nodulation assays, composite plants were transferred to Fahräeus medium without nitrogen containing the brown filter paper and 5 d later were inoculated with 10 mL of *S. meliloti* strain 2011 suspension ($OD_{600nm} = 0.05$) per plate for 1 h in vitro. Nodulation efficiency was first evaluated by counting nodules at 10 d.p.i.. Then, infected composite plants (containing the *A. rhizogenes*-transformed roots obtained in vitro) were transferred into the greenhouse support (perlite:sand [3:1, v/v] mixed substrate) in "i" medium. After recovery of the transfer (3–4 d) in high humidity conditions, plants were submitted to different stress media and further inoculated with symbiotic bacteria. Nodule number was determined 21 d.p.i. after this second inoculation.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Homology analysis between *Zpt2* sequences.

Supplemental Figure S2. Expression levels of *MtZpt2* TFs in composite 108-R and Jemalong A17 plants.

Supplemental Figure S3. Evaluation of nodulation capacity in *A. rhizogenes*-transformed *M. truncatula* roots overexpressing *MtZpt2-1* or *MtZpt2-2* in 108-R and Jemalong A17.

Supplemental Table S1. Genes differentially regulated between the salt-tolerant and salt-sensitive genotypes grown under normal conditions (J0 versus R0).

Supplemental Table S2. Genes up-regulated in the salt-tolerant genotype grown under salt stress conditions compared with the sensitive one in the same conditions (J4 versus R4).

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