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Differential expression of the TFIIIA regulatory pathway in response to salt stress between *Medicago truncatula* genotypes

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SUMMARY

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 homologue of the abiotic-stress related *CorA1* (Cold-Regulated A1) gene and a TFIIIA-related transcription factor, 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.

INTRODUCTION

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 affect 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 salinisation of arable land is expected to have devastating global effects, resulting in 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 colonise 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 *M. 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 recognised 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 hybridisations (SSH) 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 (Seki et al., 2002; Ozturk et al., 2002; Bartels and Sunkar, 2005). However, few reports analysed 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 homologue of the *CorA1* (Cold-Regulated A1, *MtCorA1*; Laberge et al., 1993) gene, which is a putative target of MtZpt2 transcription factors (Merchan et al., 2007). Accordingly, these transcription factors 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 days 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 mM to 150 mM, as compared with plants grown without salt (Kruskal & 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 & 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 & Wallis test, $p < 0.01$, $n = 20$; Fig. 1C, left panel). 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 post-germination stage (Fig. 1C, middle panel). The ratio root dry weight / leaf dry weight (as percentage of control) reveals the relative effects of increasing NaCl concentration for each genotype (Fig. 1C, right panel). As expected, ratio values for *M. truncatula* 108-R were significantly reduced with increased salt treatments (60-150 mM NaCl; Kruskal & 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 to 150 mM).

Root growth performance was also assessed on a low-nitrogen medium (“i”; Blondon et al., 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 a 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 to monitor the effects of salt treatments on plant growth, and further revealed differential root growth responses between 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 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). In order 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 *S. meliloti* strain 2011 (able to grow up to 300 mM NaCl, Rübberg et al., 2003). Three weeks after inoculation, these plants were assessed in order 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 & 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 at 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 leghaemoglobin 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, being 108-R significantly more affected than Jemalong A17. Perturbations 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 which 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 days of salt stress (150 mM NaCl on “i” medium referred 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: 108-R plants untreated (R0), 108-R plants treated with 150 mM NaCl for 4 days (R4), Jemalong A17 plants untreated (J0), and Jemalong A17 plants treated with 150 mM NaCl for 4 days (J4). To identify statistically significant differentially expressed genes between genotypes during salt treatments, we used a Student *t*-test based on two biological replicates and 4 technical replicates (eg. quadruplicated probes on macroarray), and retained genes with *p* values < 0.01.

Most of the genes were similarly regulated in these genotypes since approximately half of the spots on the “dedicated” macroarray (e.g. about 150 spots) showed similar detectable signals for 108-R and Jemalong A17 hybridisation, 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 normalisation (Merchan et al., 2007). In Jemalong A17, expression of 14 genes was altered after a salt stress: 4 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 vs 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 vs R4; Fig. 3B). Surprisingly, no gene was induced in common between 108-R and Jemalong A17 after a salt stress but 3 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 vs R0), revealed 38 genes up-regulated in Jemalong A17 roots relative to 108-R roots (Supplemental Table S1). In contrast, only 5 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 6 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 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 vs R4). Direct comparison of gene induction/repression between the two varieties after 4 days 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 (6 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 characterised. Sorting of the genes up-regulated at 4n in Jemalong A17 into functional categories (according to Jourmet et al., 2002), revealed that four categories were overrepresented: signal transduction and post-translational 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 RT-PCR on independent biological samples (Supplemental Table S2, genes indicated in yellow). One codes for a homologue 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 histidine-containing phosphotransfer protein potentially related to cytokinin signalling (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 homologue of the CORA (Cold-Regulated A1; Laberge et al., 1993) gene could be a target of TFIIIA-like C₂H₂ zinc finger transcription factor (TF), MtZpt2-1 and MtZpt2-2 (Merchan et al., 2007, Supplemental Fig. S1). Indeed, the other potential target gene *Fpfl* (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 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 *A. rhizogenes*-transformed roots overexpressing MtZpt2-1 (referred as composite plants, Boisson-Dernier et al., 2001). In order to analyse 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 2x35S CaMV promoter in several independent transgenic roots of 108-R or Jemalong A17 genotypes to assay their salt responses. After three weeks of growth in control medium, the composite plants were transferred to a salt-containing (100 mM NaCl) or a control medium and root length was measured 8 and 6 days after transfer in 108-R and Jemalong A17 plants, respectively (Fig. 5). In non-saline 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 *t*-test, $p < 0.01$ in two independent biological experiments; $n > 25$) in primary root growth compared to control roots one 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 absence or presence of salt stress (Fig. 5C 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 three 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 days

post-inoculation. 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. S3A 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. S3A and B)

Hence, even though the MtZpt pathway could not be related to the different nodulation capacity of the genotypes either in presence or in 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 to salinity (Richards, 1996). Salt tolerance is a complex trait involving the function of many genes (Foolad, 2004; Flowers, 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 Wang, 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 N-fixation efficiency are particularly affected (Rai, 1992; Cardovilla et al., 1994). In this work, we analyse 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 transcription factor 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, 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, 1988). Similarly, continued root growth under salt stress may provide additional surfaces for sequestration of toxic ions, leading to lower salt concentration, and enable reaching soil areas with lower salt concentrations. For example, salt tolerance of barley 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 Fahræus 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 Schroeder, 2004). Nevertheless, in *Arabidopsis*, most of the genes were induced by salt stress only in roots, and 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 days 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* (Velagaleti et al., 1990; 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 towards 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 sucrose 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 characterise 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 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 *A. thaliana*, it was shown by comparative transcriptome analysis that the orthologues of several well-

known *A. thaliana* stress-response genes are highly expressed even under control conditions (Taji et al., 2004); similarly, the metal-tolerant *A. halleri* was found to express a number of metal-homeostasis genes at much higher levels than *A. thaliana*, 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 non-stressed conditions. Jemalong A17 does not induce major changes at 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 (post-transcriptional 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 signalling pathways in the control of root growth under adverse conditions (Malamy et al., 2005; Merchan et al., 2007). In addition, among those showing the largest differences, we detected a dehydrin-related gene and a homologue 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 et al., 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 transcription factors (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 which 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 level 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 sulphuric acid for 8 min and rinsed three times with distilled water. The scarified seeds were surface-sterilised for 20 min in bleach (12% [v/v] sodium hypochlorite). After washing with sterilised water, seeds were sown on 1% water-agar plates and stored for 2 days 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 Fahræus medium), and the position of primary root tips was marked on the Petri dish every 24 h. Root length was measured the 5th day 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 hr photoperiod, and 70% of relative humidity.

For measuring the effect of salt concentration on nodulation of *M. truncatula* genotypes, 2-day-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 day after germination (5 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, 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 days after inoculation (30 d.a.g.). This experiment was conducted in a greenhouse minimising evaporation to keep the total water volume of the pots constant. Two biological replicates per salinity treatment were performed (20 plants/genotype).

Fifteen and thirty days 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 non-stress 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 non-stress treatment) was determined for individual plants of each genotype.

RNA Extraction and DNA Array Construction

Roots were collected after 4 days 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, Hoerd, France). Macroarray hybridisations were performed as described in the “EMBO *Medicago 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 labelling, hybridisation of macroarrays, and quantification of hybridisation signals were performed as described in (<http://www.isv.cnrs-gif.fr/embo01/manuels/pdf/module5.pdf> and Merchan et al., 2007). Experiments were normalised relative to expression of 6 “house-keeping” 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 normalise all signals obtained from macroarray hybridisations (Merchan et al., 2007).

For real-time RT-PCR, after DNase treatment (RQ1 RNase-free DNase, Promega, France), 1.5 µg of total RNA were retrotranscribed for 1 h at 42 °C using the “SUPERSCRIPT® II first-strand synthesis system” (Invitrogen, Cergy Pontoise, France) and subsequently denature 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, Meylan, France) 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, Tm: 60°C). The following gene-specific primers were used, forward and reverse, respectively: 5’-GGTTGTTTGCGAAGAAGGAG-3’ and 5’-GTACCCACGGTTTCAACAT-3’ for TC67888 (*MtADPr*); 5’-GGTGTCGTCGGAGTCACAG-3’ and 5’-TGGCCTTGAGAAGCTTAGGA-3’ for TC85295 (*MtDor*); 5’-TGTCTCAGCAGATGGCACAG-3’ and 5’-CGAGGAGGAAGTTGATGGAG-3’ for TC76699 (*MtDehyd* for 108-R); 5’-GAGCGAGGAGGAAGTTGATGG-3’ and 5’-TGGTGCTGGTGGAGTTGTTA-3’ for TC106659 (*MtDehyd* for Jemalong A17); 5’-GGCGGAGGTGGTTACAATGG-3’ and 5’-GGCAACAGATTCAGCAGCAC-3’ for TC68024 (*MtCorA1*); 5’-ATAGATGCGTGCCGAGG TG-3’ and 5’-GCATCTCTACAGATCCACTC-3’ for TC74018 (*MtHP2*); 5’-AAGTCCGAAAAGCCGGGAGG-3’ and 5’-

GCACTTAACTCACCCACCACTGC-3' for *MtZpt2-1*; 5'-GGCAACGGACTTTCTACCTC-3' and 5'-CTCCTCCATCAGCCACCGTG-3' for *MtZpt2-2*. Parallel reactions to amplify *MtActin11* were used to normalise the amount of template cDNA. Synthesis of three independent cDNA preparations from the same RNA sample (technical duplicates) allowed 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 & 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 *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 analysed the significance of differences in root growth lengths or nodulation capacities using a Student *t*-test ($p < 0.01$).

A. rhizogenes Root Transformation

A. 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, USA) 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 days 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 realised ($n > 25$).

For nodulation assays, composite plants were transferred to Fahræus medium without nitrogen containing the brown filter paper, and five days later were inoculated with 10 mL of *S. meliloti* strain 2011 suspension ($D.O_{600nm}=0.05$) per plate for 1 h *in vitro*. Nodulation efficiency was first evaluated by counting nodules at 10 days post-inoculation (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 days) 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 supplemental material is available in the online version of this article.

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

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

Supplemental Figure S3. Evaluation of nodulation capacity in *A. rhizogenes*-transformed *M. truncatula* roots over-expressing *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 vs 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 vs R4).

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LITERATURE CITED

- Apse MP, Aharon GS, Snedden WA, and Blumwald E** (1999) Salt tolerance conferred by overexpression of a vacuolar Na⁺/H⁺ antiport in *Arabidopsis*. *Science* **285**: 1256-1258
- Arrese-Igor C, González E, Gordon A, Minchin F, Gálvez L, Royuela M, Cabrerizo P, and Aparicio-Tejo P** (1999) Sucrose synthase and nodule nitrogen fixation under drought and environmental stresses. *Symbiosis* **27**: 189-212
- Assmann S, and Wang X** (2001) From milliseconds to millions of years: guard cells and environmental responses. *Plant Biol* **4**: 421-428
- Austin RB** (1993) Augmenting yield-based selection. p. 391-405. In: Hayward MD, and Romagosa I (eds.). *Plant breeding principles and prospects*. Chapman y Hall, London
- Bartels D, and Sunkar R** (2005) Drought and salt tolerance in plants. *Crit Rev Plant Sciences* **24**: 23-58
- Barker DG, Bianchi S, Blondon F, Dattée Y, Duc G, Essad S, Flament P, Gallusci P, Génier G, Guy P, Muel X, Tourneur J, Dénarié J, and Huguet T** (1990) *Medicago truncatula*, a model plant for studying the molecular genetics of the *Rhizobium*-legume symbiosis. *Plant Mol Biol Rep* **8**: 40-49
- Becher M, Talke IN, Krall L, and Krämer U** (2004) Cross-species microarray transcript profiling reveals high constitutive expression of metal homeostasis genes in shoots of the zinc hyperaccumulator *Arabidopsis halleri*. *Plant J* **37**: 251-268
- Bekki A, Trinchant JC, and Rigaud J** (1987) Nitrogen fixation (C₂H₂ reduction) by *Medicago* nodules and bacteroids under sodium chloride stress. *Physiol Plant* **71**: 61-67

- Bell CJ, Dixon RA, Farmer AD, Flores R, Inman J, Gonzales RA, Harrison MJ, Paiva NL, Scott AD, Weller JW, and May GD** (2001) The Medicago genome initiative: a model legume database. *Nucleic Acids Res* **29**: 114-117
- Bernstein L** (1962) Salt-affected soils and plants. The problem of the arid zones. Proc UNESCO Symp UNESCO, Paris, France. pp. 139-174
- Blondon F** (1964) Contribution à l'étude du développement de graminées fourragères: ray-grass et dactyle. *Rev Gen Bot* **71**: 293-381
- Boisson-Dernier A, Chabaud M, García F, Becard G, Rosenberg C, and Barker DG** (2001) *Agrobacterium rhizogenes*-transformed roots of *Medicago truncatula* for the study of nitrogen-fixing and endomycorrhizal symbiotic associations. *Mol Plant Microbe Interact* **14**: 695-700
- Bouhmouch I, Souad-Mouhsine B, Brhada F, and Aurag J** (2005) Influence of host cultivars and Rhizobium species on the growth and symbiotic performance of *Phaseolus vulgaris* under salt stress. *J Plant Physiol* **162**: 1103-1113
- Cardovilla MP, Ligerio F, and Lluch C** (1994) The effect of salinity on N fixation and assimilation in *Vicia faba*. *J Exp Bot* **45**: 1483-1488
- Chinnusamy V, Jagendorf A, and Zhu JK** (2005) Understanding and improving salt tolerance in plants. *Crop Sci* **45**: 437-448
- Cook DR** (1999) *Medicago truncatula*, a model in the making! *Curr Opin Plant Biol* **2**: 301-304
- Crespi M, and Gálvez S** (2000) Molecular mechanisms in root nodule development. *J Plant Growth Regul* **19**: 155-166

- Dana MM, Pintor-Toro JA, and Cubero B** (2006) Transgenic tobacco plants overexpressing chitinases of fangal origin show enhanced resistance to biotic and abiotic stress agent. *Plant Physiol* **142**: 722-730
- Davletova S, Schlauch K, Coutu J, and Mittler R** (2005) The zinc-finger protein Zat12 plays a central role in reactive oxygen and abiotic stress signaling in Arabidopsis. *Plant Physiol* **139**: 847-856
- Delgado MJ, Garrido JM, and Lluch C** (1994) Effects of salt stress on growth and nitrogen fixation by pea, faba-bean, common bean and soybean plants. *Soil Biol Biochem* **26**: 371-376
- Duzan HM, Zhou X, Souleimanov A, and Smith DL** (2004) Perception of *Bradyrhizobium japonicum* Nod factor by soybean [*Glycine max* (L.) Merr.] root hairs under abiotic stress conditions. *J Exp Bot* **55**: 2641-2646
- Foolad MR** (2004) Recent advances in genetics of salt tolerance in tomato. *Plant Cell Tissue Organ Cult* **76**: 101-119
- Flowers TJ** (2004) Improving crop salt tolerance. *J Exp Bot* **55**: 307-319
- Garg BK, and Gupta IC** (2000) Nodulation and symbiotic nitrogen fixation under salt stress. *Curr Agric* **24**: 23-35
- Georgin P, and Gouet M** (2000) Statistiques avec excell. Eyrolles, París, France. p. 338
- González EM, Gálvez L, Royurla M, Aparicio-Tejo PM, and Arresse-Igor C** (2001) Insights into the regulation of nitrogen fixation in pea nodules: lessons from drought, abscisic acid and increased photoassimilate availability. *Agronomie* **21**: 607-613

- González-Rizzo S, Crespi M, and Frugier F** (2006) The *Medicago truncatula* CRE1 cytokinin receptor regulates lateral root development and early symbiotic interaction with *Sinorhizobium meliloti*. *Plant Cell* **18**: 2680-2693
- Gordon AJ, Minchin FR, Skot L, and James CL** (1997) Stress induced declines in soybean N₂ fixation are related to nodule sucrose synthase activity. *Plant Physiol* **114**: 937-946
- Graham PH, and Vance CP** (2003) Legumes: importance and constraints to greater use. *Plant Physiol* **131**: 872-877
- Guo ZJ, Chen XJ, Wu XL, Ling JQ, and Xu P** (2004) Overexpression of the AP2/EREBP transcription factor OPBP1 enhances disease resistance and salt tolerance in tobacco. *Plant Mol Biol* **55**: 607-618
- Hasegawa PM, Bressan RA, Zhu JK, and Bohnert HJ** (2000) Plant cellular and molecular responses to high salinity. *Annu Rev Plant Physiol Plant Mol Biol* **51**: 463-499
- Hoffmann B, Trinh TH, Leung J, Kondorosi A, and Kondorosi E** (1997) A new *Medicago truncatula* line with superior in vitro regeneration, transformation, and symbiotic properties isolated through cell culture selection. *Mol Plant Microbe Interact* **10**: 307-315
- Hong JK, and Hwang BK** (2006) Promoter activation of pepper class II basic chitinase gene, *CAChi2*, and enhanced bacterial disease resistance and osmotic stress tolerance in the *CAChi2*-overexpressing Arabidopsis. *Planta* **223**: 433-448
- Horié T, and Schroeder J** (2004) Sodium transporters in plants. Diverse genes and physiological functions. *Plant Physiol* **136**: 2457-2462
- Jain RK, and Selvaraj G** (1997) Molecular genetic improvement of salt tolerance in plants. *Biotech Annu Rev* **3**: 245-267

- Journet EP, Van Tuinen D, Gouzy J, Crespeau H, Carreau V, Farmer MJ, Niebel A, Schiex T, Jaillon O, Chatagnier O, Godiard L, Micheli F, Kahn D, Gianinazzi-Pearson V, and Gamas P** (2002) Exploring root symbiotic programs in the model legume *Medicago truncatula* using EST analysis. *Nucleic Acids Res* **30**: 5579-5592
- Kania T, Russenberger D, Peng S, Apel K, and Melzer S** (1997) FPF1 promotes flowering in Arabidopsis. *Plant Cell* **9**: 1327-1338
- Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, and Shinozaki K** (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nature Biotech* **17**: 287-291
- Kawasaki S, Borchert C, Deyholos M, Wang H, Brazille S, Kawai K, Galbraith D, and Bohnert H** (2001) Gene expression profiles during the initial phase of salt stress in rice. *Plant Cell* **13**: 889-905
- Kim S, Kang JY, Cho DI, Park JH, and Kim SY** (2004) ABF2, an ABRE-binding bZIP factor, is an essential component of glucose signaling and its overexpression affects multiple stress tolerance. *Plant J* **40**: 75-87
- Kreps JA, Wu Y, Chang H-S, Zhu T, Wang X, and Harper JF** (2002) Transcriptome changes for Arabidopsis in responses to salt, osmotic, and cold stress. *Plant Physiol* **130**: 2129-2141
- Laberge S, Castonguay Y, and Vezina LP** (1993) New cold- and drought-regulated gene from *Medicago sativa*. *Plant Physiol* **101**: 1411-1412
- Limpens E, and Bisseling T** (2003) Signaling in symbiosis. *Curr Opin Plant Biol* **6**: 343-350
- Malamy JE** (2005) Intrinsic and environmental response pathways that regulate root system architecture. *Plant Cell Environ* **28**: 67-77

- Merchan F, Breda C, Hormaeche JP, Sousa C, Kondorosi A, Aguilar OM, Megías M, and Crespi M** (2003) A krüppel-like transcription factor gene is involved in salt stress responses in *Medicago* spp. *Plant Soil* **257**: 1-9
- Merchan, F, de Lorenzo L, González-Rizzo S, Niebel A, Megías M, Frugier F, Sousa C, and Crespi M** (2007) Analysis of regulatory pathways involved in the reacquisition of root growth after salt stress in *Medicago truncatula*. *Plant J* **51**: 1-17
- Moore S, Payton P, Wright M, Tanksley S, and Giovannoni J** (2005) Utilization of tomato microarrays for comparative gene expression analysis in the Solanaceae. *J Exp Bot* **56**: 2885-2895
- Morón B, Soria-Díaz ME, Ault J, Verroios G, Noreen S, Rodríguez-Navarro DN, Gil-Serrano A, Thomas-Oates J, Megías M, and Sousa C** (2005). Low pH changes the profile of nodulation factors produced by *Rhizobium tropici* CIAT899. *Chem Biol* **12**: 1029-1040
- Mukhopadhyay A, Vij S, and Tyagi AK** (2004) Overexpression of a zinc-finger protein gene from rice confers tolerance to cold, dehydration, and salt stress in transgenic tobacco. *Proc Natl Acad Sci USA* **101**: 6309-6314
- Munns R, Hare RA, James RA, and Rebetzke GJ** (2000) Genetic variation for improving the salt tolerance of durum wheat. *Aust J Agric Res* **51**: 69-74
- Ozturk ZN, Talame V, Deyholos M, Michalowski CB, Galbraith DW, Gozukirmizi N, Tuberosa R, and Bohnert HJ** (2002) Monitoring large-scale changes in transcript abundance in drought- and salt-stressed barley. *Plant Mol Biol* **48**: 551-573
- Park JM, Park CJ, Lee SB, Ham BK, Shin R, and Paek KH** (2001) Overexpression of the tobacco *Tsi1* gene encoding an EREBP/AP2-type

transcription factor enhances resistance against pathogen attack and osmotic stress in tobacco. *Plant Cell* **13**: 1035-1046

Rai R (1992) Effect of nitrogen levels and *Rhizobium* strains on symbiotic N₂ fixation and grain yield of *Phaseolus vulgaris* L. genotypes in normal and saline-sodic soils. *Biol Fertil Soils* **14**: 293-299

Richards RA (1996) Defining selection criteria to improve yield under drought. *Plant Growth Regul* **20**: 157-166

Rüberg S, Tian Z-X, Krol E, Linke B, Meyer F, Wang Y, Pühler A, Weidner S, and Becker A (2003) Construction and validation of a *Sinorhizobium meliloti* whole genome DNA microarray: genome-wide profiling of osmoadaptive gene expression. *J Biotechnol* **106**: 255-268

Seki M, Narusaka M, Ishida J, Nanjo T, Fujita M, Oono Y, Kamiya A, Nakajima M, Enju A, Sakurai T, Satou M, Akiyama K, Taji T, Yamaguchi-Shinozaki K, Carninci P, Kawai J, Hayashizaki Y, and Shinozaki K (2002) Monitoring the expression profiles of 7000 Arabidopsis genes under drought, cold, and high-salinity stresses using a full-length cDNA microarray. *Plant J* **31**: 279-292

Sharp RE, Hsiao TC, and Silk WK (1988) Growth of the maize primary root at low water potentials. I. Spatial distribution of expansive growth. *Plant Physiol* **87**: 50-57

Shisong M, Gong Q, and Bohnert HJ (2006) Dissecting salt stress pathways. *J Exp Bot* **57**: 1097-1107

Steudle E (2000) Water uptake by roots: effects of water deficit. *J Exp Bot* **51**: 1531-1542

- Spollen WG, Sharp RE, Saab IN, and Wu Y** (1993) Regulation of cell expansion in roots and shoots at low water potentials. In: Water Deficits: Plant Responses from Cell to Community. Smith JAC, and Griffiths H (eds). BIOS Scientific Publishers, Oxford, UK. pp. 37-52
- Taji T, Seki M, Satou M, Sakurai T, Kobayashi M, Ishiyama K, Narusaka Y, Narusaka M, Zhu JK, and Shinozaki K** (2004) Comparative genomics in salt tolerance between *Arabidopsis* and *Arabidopsis*-related halophyte salt cress using *Arabidopsis* microarray. *Plant Physiol* **135**: 1697-1709
- Takahashi S, Seki M, Ishida J, Satou M, Sakurai T, Narusaka M, Kamiya A, Nakajima M, Enju A, Akiyama K, Yamaguchi-Shinozaki K, and Shinozaki K** (2004) Monitoring the expression profiles of genes induced by hyperosmotic, high salinity, and oxidative stress and abscisic acid treatment in *Arabidopsis* cell culture using a full-length cDNA microarray. *Plant Mol Biol* **56**: 29-55
- Takatsuji H** (1999) Zinc-finger proteins: the classical zinc finger emerges in contemporary plant science. *Plant Mol Biol* **39**: 1073-1078
- Tester M, and Davenport RJ** (2003) Na⁺ transport and Na⁺ tolerance in higher plants. *Ann Bot* **91**: 503-527
- Truchet G, Débelle F, Vasse J, Terzaghi B, Garnerone AM, Rosenberg C, Batut J, Maillet F, and Dénarié J** (1985) Identification of a *Rhizobium meliloti* pSym2011 region controlling the host specificity of root hair curling and nodulation. *J Bacteriol* **164**: 1200-1210
- Velagaleti RR, Marsh S, and Kramer D** (1990) Genotypic differences in growth and nitrogen fixation among soybean [*Glycine max* (L.) Merr.] cultivars grown under salt stress. *Trop Agric* **67**: 169-177
- Volkov V, Wang B, Dominy PJ, Fricke W, and Amtmann A** (2004) *Thellungiella halophila*, a salt-tolerant relative of *Arabidopsis thaliana*, possesses effective

mechanisms to discriminate between potassium and sodium. *Plant Cell Environ* **27**: 1-14

Walia H, Wilson C, Condamine P, Liu X, Ismail AM, Zeng L, Wanamaker S, Mandal J, Xu J, Cui X, and Close TJ (2005) Comparative transcriptional profiling of two contrasting rice genotypes under salinity stress during the vegetative growth stage. *Plant Physiol* **139**: 822-835

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

Weber M, Harada E, Vess C, von Roepenack-Lahaye E, and Clemens S (2004) Comparative microarray analysis of *Arabidopsis thaliana* and *Arabidopsis halleri* roots identifies nicotianamine synthase, a ZIP transporter and other genes as potential metal hyperaccumulation factors. *Plant J* **37**: 269-281

Weber M, Trampczynska A, and Clemens S (2006) Comparative transcriptome analysis of toxic metal responses in *Arabidopsis thaliana* and the Cd²⁺-hypertolerant facultative metallophyte *Arabidopsis halleri*. *Plant Cell Environ* **29**: 950-963

Westgate ME, and Boyer JS (1985) Osmotic adjustment and the inhibition of leaf, root, stem and silk growth at low water potentials in maize. *Planta* **164**: 540-549

Winicov I, and Bastola DR (1999) Transgenic overexpression of the transcription factor Alfin1 enhances expression of the endogenous *MsPRP2* gene in alfalfa and improves salinity tolerance of the plants. *Plant Physiol* **120**: 473-480

Winicov I (2000) Alfin1 transcription factor overexpression enhances plant root growth under normal and saline conditions and improves salt tolerance in alfalfa. *Planta* **210**: 416-422

- Xiong L, and Yang Y** (2003) Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid-inducible mitogen-activated protein kinase. *Plant Cell* **15**: 745-759
- Yeo AR** (1998) Molecular biology of salt tolerance in the context of whole-plant physiology. *J Exp Bot* **49**: 915-929
- Young ND, Cannon SB, Sato S, Kim DJ, Cook DR, Town CD, Roe BA, and Tabata S** (2005) Sequencing the genespaces of *Medicago truncatula* and *Lotus japonicus*. *Plant Physiol* **137**: 1174-1181
- Zahran HH** (1999) Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiol Mol Biol Rev* **63**: 968-989
- Zahran HH, and Sprent JI** (1986) Effects of sodium chloride and polyethylene glycol on root-hair infection and nodulation of *Vicia faba* L. plants by *Rhizobium leguminosarum*. *Planta* **167**: 303-309
- Zhang HX, and Blumwald E** (2001) Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. *Nat Biotech* **19**: 765-768
- Zhu JK** (2001) Cell signaling under salt, water and cold stresses. *Curr Opin Plant Biol* **4**: 401-406

FIGURE LEGENDS

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 Fahræus or “i” medium plates for 5 days, in the presence of different NaCl concentration (0, 30, 60, 90, 120, and 150 mM).

A. Relative root length of each variety at 5 days after germination (d.a.g.) in *in vitro* conditions with Fahræus medium is shown as percentage of control root growth without salt.

B. Representative pictures taken 5 days after transfer of the seedlings to 0 mM, 30 mM, and 120 mM NaCl on Fahræus medium.

C. Relative dry weights of roots and leaves from *M. truncatula* 108-R and Jemalong A17 after 15 days of growth in Fahræus medium submitted to different salt-stress treatments. Results are shown as percentage of control without salt. Ratio (right panel) 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 non-stressed and salt-stressed conditions in “i” medium for 5 days.

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 (α : 0.01). *: Columns labelled with asterisk are significantly different ($p < 0.01$) within a given salinity level. In both cases, the Kruskal & 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 concentration (0, 30, 60, 90, 120, and 150 mM). The inoculation with *S. meliloti* strain 2011 was made 9 days

after germination (d.a.g.). Nodule number, root and leaf dry weights were determined at 30 d.a.g. (21 days post-infection, 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 (α : 0.05). *: Columns labelled with asterisk are significant differences between genotypes within a given salinity level, with $p < 0.05$. Statistical comparisons were performed using the Kruskal & Wallis test. A representative example out of two biological experiments is shown.

Figure 3. Gene expression profiles of *M. truncatula* 108-R and Jemalong A17.

A. Experimental design is based on series of pairwise comparisons. Four days after germination, seedlings from different genotypes (R for 108-R and J for Jemalong A17) were grown for 4 days without (0 mM; R0 and J0) or with 150 mM NaCl (R4 and J4). 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 on the left side, and Jemalong A17 on the right side).

D. Distribution of differentially expressed genes into functional categories according to BLASTN hits (based on Journet et al., 2002 and Merchan et al., 2007). Percentages were calculated from the total number of differentially expressed genes from Jemalong A17 (112 genes).

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 condition (4 days at 150 mM NaCl). Induction ratios were

calculated between the salt-treated and non-treated samples. A representative example out of two biological experiments is shown, and error bars represent standard deviation of three technical replicates. Numbers on the X-axis indicate the fold-induction of gene expression in relation to the non-salt stress condition.

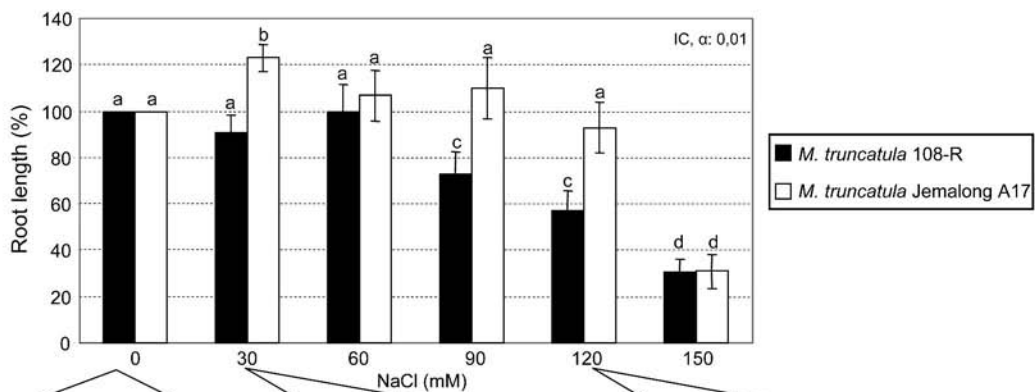
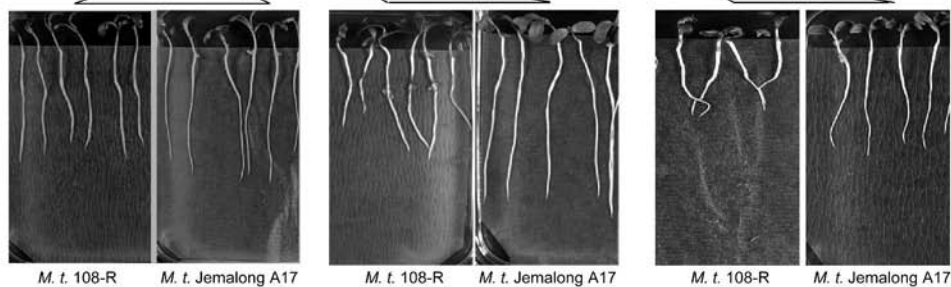
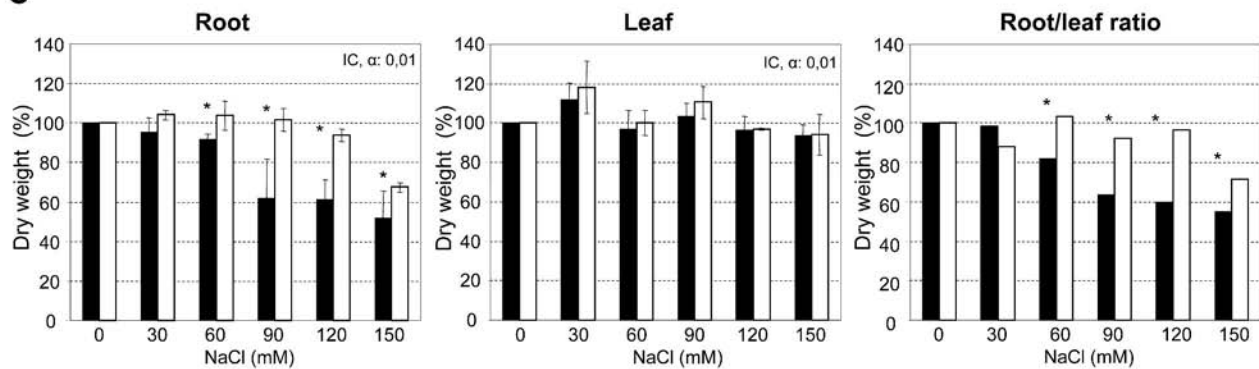
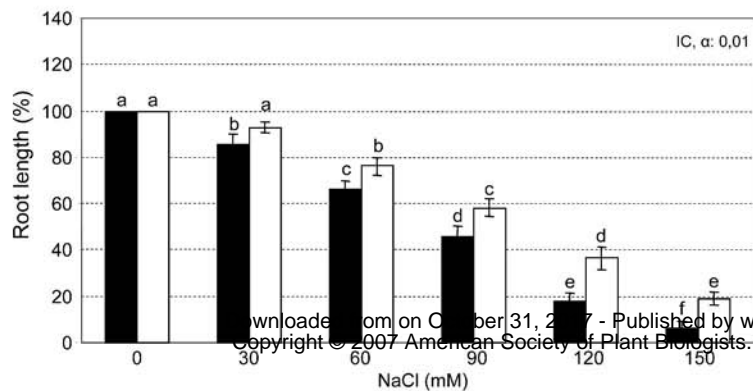
A. Real-time RT-PCR of 5 randomly selected differentially expressed genes between genotypes: *MtHP2*, a histidine-containing phosphotransfer protein homologue gene (a gene involved in cytokinin signal transduction); *MtCorA1*, a cold-and drought-regulated CORA protein homologue gene; *MtADPr*, a gene encoding an ADP-ribosylation factor homologue protein; *MtDor*, a gene coding for a dormancy-associated protein; *MtDehyd*, a gene encoding a dehydrin-related protein.

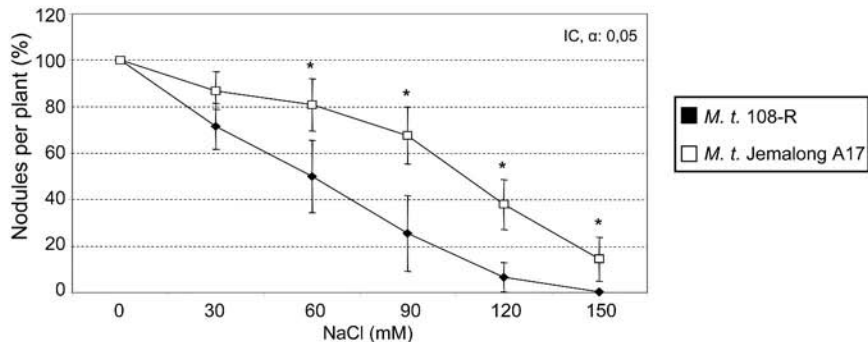
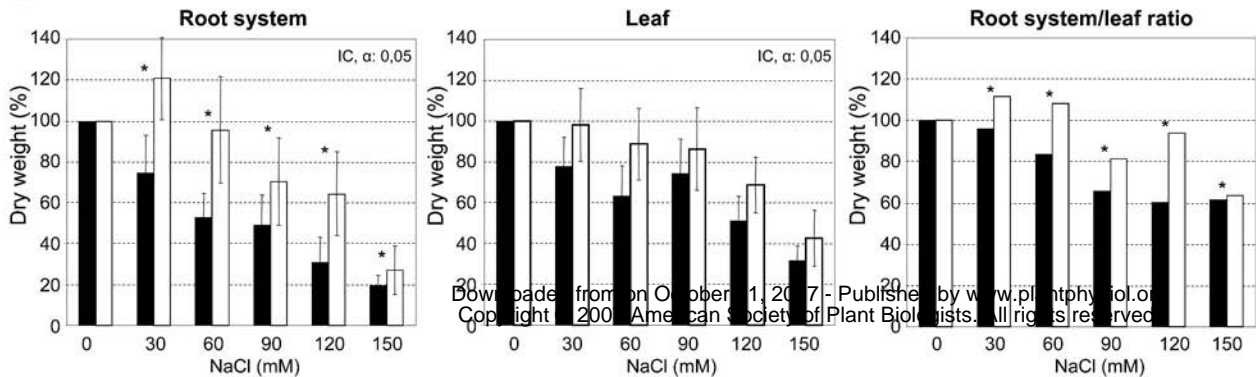
B. Real time RT-PCR analysis of 2 genes that encode putative TFIIIA-type transcription factors (*MtZpt2-1* and *MtZpt2-2*)

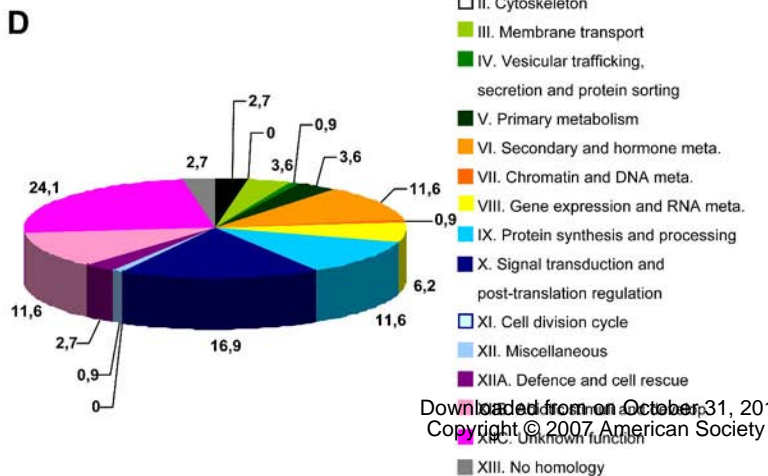
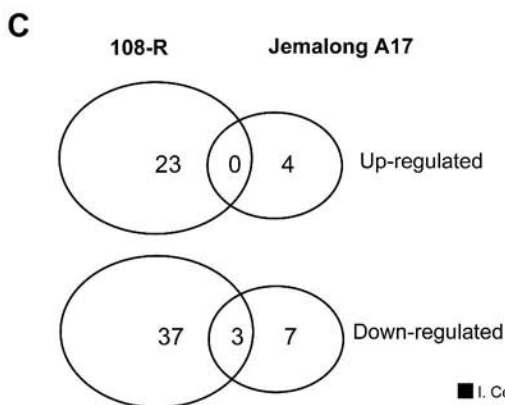
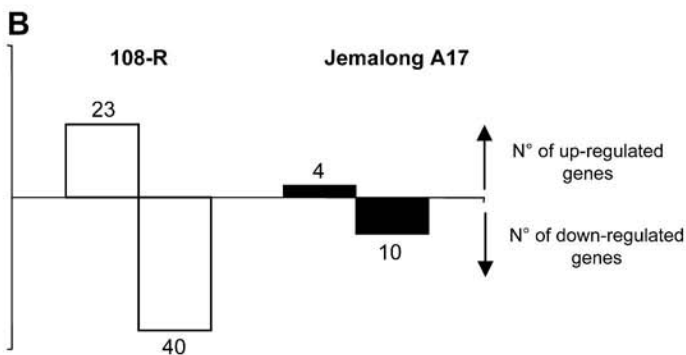
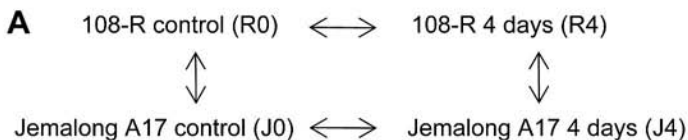
Figure 5. Evaluation of root growth in *A. rhizogenes*-transformed *M. truncatula* roots over-expressing *MtZpt2-1* or *MtZpt2-2*. Composite plants were prepared as described in Boisson-Dernier et al. (2001) using control empty vector and *MtZpt2-1* or *MtZpt2-2* overexpressing constructs. After kanamycin 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. Asterisk indicates statistically significant differences ($p < 0.01$, $n > 25$).

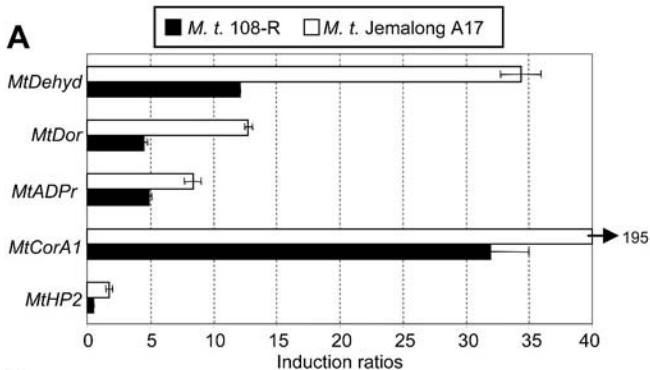
A and B. Growth of transgenic roots over-expressing either *MtZpt2-1* or *MtZpt2-2* in *M. truncatula* 108-R was monitored one week after transfer into control (**A**) or a salt-containing medium (100 mM NaCl; **B**). The initial position of the root apex after transfer was monitored to determine the 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**).

A**B****C****D**

A**B**



A**B**