Identification of regulatory pathways involved in the reacquisition of root growth after salt stress in *Medicago truncatula*

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Summary

Root growth and function are determined by the action of environmental stresses through specific genes that adapt root development to these restrictive conditions. We have defined *in vitro* conditions affecting the growth and recovery of *Medicago truncatula* roots after a salt stress. A dedicated macroarray containing 384 genes, based on a large-scale subtractive hybridization approach, was constructed and used to analyze gene expression during salt stress and recovery of root growth from this stress. Several potential regulatory genes were identified as being linked to this recovery process: a novel RNA-binding protein, a small G-protein homologous to ROP9, a receptor-like kinase, two TF II/IA-like and an AP2-like transcription factors (TF), MtZpt2-1, MtZpt2-2 and MtAp2, and a histidine kinase associated with cytokinin transduction pathways. The two ZPT2-type TFs were also rapidly induced by cold stress in roots. By analyzing transgenic *M. truncatula* plants showing reduced expression levels of both TFs and affected in their capacity to recover root growth after a salt stress, we identified potential target genes that were either activated or repressed in these plants. Overexpression of MtZpt2-1 in roots conferred salt tolerance and affected the expression of three putative targets in the predicted manner: a cold-regulated A (CORA) homolog, a flower-promoting factor (FPF1) homolog and an auxin-induced proline-rich protein (PRP) gene. Hence, regulatory networks depending on TFIIIA-like transcription factors are involved in the control of root adaptation to salt stress.

Keywords: salt stress regulators, TFIIIA-like transcription factors, macroarrays, legume, SSH.

Introduction

Tolerance to abiotic stresses is associated with modifications of morphological and physiological traits; these include changes in plant architecture, variation in leaf cuticle thickness, stomatal regulation, germination, antioxidant capacity, hormonal regulation, membrane and protein stability, maintenance of photosynthesis and root morphology (Edmeades et al., 2001). Soil salinity is one of the most significant abiotic stresses for crop plants, including legumes (Duzan et al., 2004; Zahran, 1999). These latter plants are very important both ecologically and agriculturally because legume roots are able to interact symbiotically with soil microorganisms to form nodules that fix atmospheric nitrogen. Hence, legumes are interesting candidates for improving soil fertility and incorporating salty soils into agriculture. Several legumes, such as the model *Medicago truncatula*, show a large diversity of cultivars adapted to varying environmental conditions, including saline soils (http://www.noble.org/medicago/ecotypes.html). In general, high NaCl concentrations produce water deficit, ion toxicity, nutrient imbalance and oxidative stress (Vinocur and Altman, 2005). These adverse effects cause modifications of root morphology and inhibition of plant growth, and can result in plant death. In *Arabidopsis*, alterations in root morphology caused by external environmental conditions...
are perturbed in several hormone-related mutants, suggesting a connection between perception of soil conditions and modification of endogenous phytohormonal balances to establish a new root architecture (Malamy, 2005; Zhang and Forde, 1998). For example, in winter wheat, changes in root growth and architecture in response to abiotic stresses in a resistant cultivar differ from those observed in a sensitive one (Abdrakhamanova et al., 2003). In legumes, the root lateral organs (nodules and lateral roots) are also regulated by diverse hormonal, metabolic and environmental signals (Crespi and Galvez, 2000). However, little is known about molecular links between morphological adaptations to environmental stresses and endogenous signals (e.g. hormones).

Several genomic analyses have been performed to examine stress responses in plants and to identify signal transduction mechanisms (Price et al., 2004). Suppressive subtractive hybridizations (SSH) and array technologies using cDNAs or oligonucleotides have become powerful and useful tools for identifying and analyzing gene expression profiles of plants that are exposed to abiotic stresses, such as drought, cold and high salinity or to abscisic acid (ABA) treatment. Both types of microarrays have been used to analyze gene expression profiles under these conditions (Kawasaki et al., 2001; Seki et al., 2001, 2002). Arabidopsis transcriptome analysis has also revealed many genes that respond to rehydration after drought stress, suggesting their potential involvement in the process of recovery from an abiotic stress (Oono et al., 2003). Recently, SSH approaches have also been successfully used to isolate differentially expressed genes linked to drought stress (Zheng et al., 2004).

Several regulatory genes, including transcription factors (TFs), have been identified as being involved in stress responses. In certain cases the overexpression of TFs induced by abiotic stresses has suggested potential roles in the induction of higher tolerance to drought and freezing stresses, such as the case of the AP2/EREBP TFs in tobacco, Alfin1 in alfalfa, DREB/CBF in Zea mays L., ZPT2-3 in petunia and ZAT12 in Arabidopsis thaliana (Guo et al., 2004; Qin et al., 2004; Sugano et al., 2003; Vogel et al., 2005; Winicov, 2000). Only in a few cases have target genes for these TFs been identified (e.g. Alfin1 in Winicov and Bastola, 1999; SCOF-1 in Kim et al., 2001; WRKY6 in Robatzek and Somssich, 2002; WRKY53 in Miao et al., 2004; ZAT12 in Vogel et al., 2005).

Our earlier work (Merchan et al., 2003) defined the range of tolerance to NaCl and the conditions for growth recovery from this stress for the M. truncatula cultivar 108R, and identified a TFIIIA-like TF, MtZpt2-1, required for both symbiotic nodule development and root growth recovery after salt stress (Frugier et al., 2000; Merchan et al., 2003). Our present study aimed to identify regulatory genes and their targets involved in salt-stress recovery responses in this model legume species, focusing particularly on root adaptation. Two SSH libraries were made and analyzed in order to construct a dedicated macroarray carrying 384 genes. Subsequently, expression studies in plants exposed to salt stress and allowed to reassume growth revealed novel regulatory genes associated with recovery processes in M. truncatula, including a potential cytokinin receptor. Using transgenic approaches affecting the expression of the MtZpt2-1 and MtZpt2-2 TFs, we could define three putative target genes, two of which could be trans-activated in transient expression assays in M. truncatula. Furthermore, overexpression of MtZpt2-1 in transgenic roots allowed them to grow under salt stress conditions. These results establish a role for a TFIIIA-like TF and its regulated network in the adaptation of legume roots to salt stress.

Results

Identification of 328 genes linked to salt-stress responses and root growth recovery after salt stress in M. truncatula

To identify genes expressed during the reacquisition of root growth after salt stress or recovery in M. truncatula, we used RNA samples from the conditions defined in Merchan et al. (2003). When grown for 4 days on 150 m NaCl (4n), M. truncatula seedlings of the cultivar 108R can rapidly recover root growth, whereas 7 days of treatment (7n) prevents recovery. This salt concentration inhibits the activity of root meristems, an arrest mainly overcome during recovery through the formation of lateral roots, either de novo or because primary and lateral meristems may have different sensitivities to salt stress. This 4n salt treatment induces a significant change in root architecture even though in certain cases both primary and lateral root growth resume. This recovery is very rapid after a 4n treatment whereas plants grown for 7 days under stress require at least 2 weeks before reinitiating root growth (Merchan et al., 2003; Figure S1). By analyzing changes occurring between 4-day-treated (rapidly recoverable) and 7-day-treated (slowly recoverable) roots, we aim to identify genes linked to changes in adaptation of roots to stress. Since roots at both 4n and 7n are morphologically similar and have grown under stress conditions, many of the stress-related genes will be activated in both samples. Hence we may be able to identify novel root genes linked to stress adaptation rather than to general stress responses.

An SSH approach was used to identify recovery-associated genes (subtraction of cDNA from roots of 4n plants as the tester, with cDNA from 7n plants as the driver) and to compare them with genes activated by salt stress (subtraction of cDNA from roots of 4n plants as the tester with cDNA from untreated control roots as the driver). The efficiency of subtraction was analyzed using known markers of recovery and salt-stress responses and control (non-differentially
Expressed clones from the two cDNA libraries were screened with probes derived from the respective driver and tester cDNA preparations to characterize genes giving differential expression in at least one of these conditions. Sequence analysis of all 418 positive clones allowed the identification of 328 different EST clusters. BLASTN and BLASTX results revealed that 31 did not have any match within the TIGR M. truncatula database (release 7.0), validating the SSH approach for identification of novel stress-related genes (Table S1). The highest BLASTN results were considered to assign a potential function to a cloned gene (a score above $1.0 \times 10^{-30}$ was obtained for 90% of the ESTs). More detailed homology searches for specific domains were performed for the remaining sequences having lower scores. The genes were then grouped into different functional categories depending on the homologies found, and this classification was compared with the reference classification of about 25,000 unsubtracted root ESTs grouped into 6000 clusters (Journet et al., 2002) as shown in Figure 1(a). Although such a direct comparison is difficult because the subtraction process enriches for medium- and low-expressed genes, the main clear difference between the two classifications is a large increase in clusters corresponding to categories XIIA (defense and cell rescue; 9% of clones instead of 2%) and XIIIB (abiotic stimuli and development; 11% instead of 2%). This analysis confirmed that our subtractive approach (SSH) successfully enriched the library in stress-related genes. Interestingly, eight ESTs were found only in legume species (using both BLASTN and BLASTX), suggesting the presence of novel pathways linked to environmental stress responses in this plant family (Table S2).

We have thus identified 328 M. truncatula transcripts that are linked to stress responses and recovery of root growth in this model species.

Expression analysis revealed novel genes associated with the recovery process

From the 328 transcripts identified in these two SSH libraries, several belong to closely related gene families. Hence, 280 non-redundant family members were used to generate a ‘dedicated’ macroarray: 105 from the recovery SSH library and 175 from the salt-stress SSH library. These 280 transcripts and diverse control genes (potentially constitutive, tissue-specific markers, genes homologous to known markers of abiotic stress responses in other species), were amplified by PCR to build a macroarray with a total of 384 transcripts (Table S1). This macroarray was hybridized in total with 21 cDNA probes prepared from total RNA (examples are depicted in Figure 1 and all expression data are shown in Table S1). These RNA samples included: (i) RNAs from roots treated with 100 mM NaCl for 1, 2 or 4 days (1d, 2d and 4d; Figure 1c). This salt concentration allows slow rates of root growth but does not arrest primary root meristems and revealed salt-regulated genes with different kinetics and patterns, whereas the first condition aims to identify genes associated with the capacity of roots to resume growth.

To monitor gene expression based on this macroarray dedicated to salt stress, only replicates (belonging to one or two independent biological experiments) with a high determination coefficient ($r^2 > 0.8$) were conserved for further analysis (Beissbarth et al., 2000; Ramirez et al., 2005). Moreover, an intensity threshold equal to 1.5 times the local background was used to avoid false positives showing high ratios but very low absolute intensity signals (Beissbarth et al., 2000).

Signal intensities of eight potential ‘constitutive’ genes were examined in all conditions, and five or six genes whose expression was not significantly affected were selected to normalize hybridization signals from the macroarray (Figure 2a). Genes differentially expressed between two conditions were defined using both Student’s t-test at $P < 0.05$ normalized by expression of the previously defined set of reference genes, and the ‘std separation’ criterion (see Experimental procedures). This statistical analysis revealed that 156 transcripts of the macroarray showed a significant reduction of their expression in 7n as compared to 4n plants. Sixty-four out of 105 genes derived from the ‘recovery’ SSH library show this pattern (60% of SSH recovery transcripts), whereas the 92 remaining regulated genes were identified from the salt-stress SSH library. Among the 156 ‘recovery’ transcripts, 45 were induced in 4n plants (see Table S3 and Figure 2b, left panel) and not at 7n.

Among the 37 genes induced from the earliest time point tested (1 day) in the salt-stress kinetic, 12 were expressed at every time point (1d, 2d and 4d, Figure 2b, right panel) and 10 were also ‘recovery associated’ transcripts (Table S4), suggesting that ‘recovery’ genes do not overlap with early induced ones. Finally, to test the root specificity of the ‘recovery’ genes, RNAs from 4n- and 7ntreated leaves and cotyledons were also hybridized in the macroarray (Figure 1b), and revealed a partial overlap between these genes.

Among the genes showing no significant variation in our macroarray experiments, an actin-encoding gene was consistently giving lower variation in all experiments (Figure 2a), and we therefore chose it as the reference gene for subsequent quantitative RT-PCR studies (qRT-PCR).

To confirm the macroarray results, we selected 19 genes, based on their salt induction pattern, and analyzed their differential expression under various salt treatments using qRT-PCR in two new biological experiments (see Table S5).
Salt recovery-related ESTs

- I. Cell Wall
- II. Cytoskeleton
- III. Membrane transport
- IV. Vesicular trafficking, secretion and protein sorting
- V. Primary metabolism
- VI. Secondary meta. and hormone meta.
- VII. Chromatin and DNA metabolism
- VIII. Gene expression and RNA meta.
- IX. Protein synthesis and processing
- X. Signal transduction and post-translational regulation
- XI. Cell division cycle
- XII. Miscellaneous
- XII.A Defense and cell rescue
- XII.B Abiotic stimuli and development
- XII.C Unknown function
- XIII. No homology

Referred root ESTs (Journet et al., 2002)

(b) (c) (d)

150 mM NaCl

- Control
- As-Mzt2

Potential MtZpt2 target

Down regulated genes
Up regulated genes
Coregulated genes

Recovery related genes

IR 0.5 2

Recovery

Kinetics of Salt response

Late induced genes
Early induced genes
Intensity ratios (IR) were calculated between the salt-treated and untreated samples (control condition). Six representative examples of expression pattern clusters and upregulated genes showing different responses in antisense plants are indicated. Co-regulated genes indicate genes showing a ‘recovery’ expression pattern in corresponding to early induced genes and late-induced genes (in c), recovery-related genes (in b) and potential MtZPT2-1 target genes (in d) are highlighted. Down-regulated genes). (d) Comparison of clustering analysis of gene expression in or 7 days, respectively, with 150 mM NaCl; R, treatment for 4 days with 150 mM NaCl followed by transfer to normal medium for 3 days (recovery condition).

Figure 1. Functional and hierarchical clustering of M. truncatula genes differentially regulated during salt stress.

(a) Distribution of M. truncatula ESTs identified from SSH libraries into functional categories according to their BLASTN best hits. Percentages were calculated from the total number of ESTs identified (418). Upper and lower distributions correspond respectively to our experiments and those reported by Journet et al. (2002). (b–d) The expression patterns of 384 genes on a salt-stress-dedicated macroarray, were analyzed under different salt treatments and control conditions. Hierarchical clustering was carried out using average linkage values. (b) Clustering analysis of expression patterns in M. truncatula roots and leaves treated for 4 and 7 days with 150 mM NaCl (recovery-related genes). (c) Clustering analysis of gene expression patterns in roots of plants treated for 1, 2 or 4 days with 100 mM NaCl (salt-stress-regulated genes). (d) Comparison of clustering analysis of gene expression in MtZpt2-1 antisense and control roots.

Control, M. truncatula 108R roots; As, antisense MtZpt2-1 roots; C, control condition used for normalization (roots of non-treated plants); 4n or 7n, plants stressed for 4 or 7 days, respectively, with 150 mM NaCl; R, roots of plants treated for 4 days with 150 mM NaCl followed by transfer to normal medium for 3 days (recovery condition).

Figure 2. Expression analysis revealed sets of genes induced by the different salt treatments.

(a) Expression summary of constitutive genes present in the dedicated macroarray. The bold line highlights the MtActin gene expression showing the lowest variations under all tested conditions among all other putative constitutive genes. As, roots of As-MtZpt2-1 plants; 108R, roots of M. truncatula 108R; control, roots of untreated plants; 4n or 7n, plants stressed for 4 or 7 days with 150 mM NaCl; R, roots of plants treated for 4 days with 150 mM NaCl followed by transfer to normal medium for 3 days (recovery condition).

(b) Vent diagram displaying gene numbers induced in roots during the salt-recovery (4n and 7n, as defined above; left panel) and salt-treatment kinetic (1, 2 and 4 days with NaCl 100 mM; right panel) experiments.

Fifteen of the genes detected in the macroarray showed the same expression pattern in all tested conditions and the other four in at least 80% of them, demonstrating a very good correlation between the macroarray and qRT-PCR analyses as shown in the different figures described below.

The expression pattern of nine genes linked to ‘recovery’, according to the macroarray studies, was analyzed using qRT-PCR and the same pattern was observed for all (Figure 3a). This included potential regulatory genes such as a new RNA-binding-type protein (MtRbp2) and a homolog of a gene encoding FP1 (MtFpf1, Flowering Promoting Factor 1; Kania et al., 1997). The new RNA-binding protein MtRbp2 is homologous to the At5g04600 gene (E-value 2E-40) of Arabidopsis which is apparently repressed by salt and cold stresses (Genevestigator data, http://www.genevestigator.ethz.ch/). However, MtRbp2 expression is induced after 4 days of salt stress, presents a recovery-type pattern, and leaf expression is minimal compared with root expression (Figure 3a and data not shown).

Another group of 10 genes, certain having homologs encoding proteins linked to abiotic stresses in other organisms, were induced by salt at both 100 mM (kinetic) and 150 mM of salt (4n) in the macroarray studies. For six of them, qRT-PCR results confirmed the macroarray results with even higher induction levels (Figure 3b). In addition, the previously mentioned potential regulatory gene, MtFpf1, showing a ‘recovery’ expression pattern, is also induced from 1 day of salt treatment using qRT-PCR (Figure 3a,b). Interestingly, only 4 of these 10 stress-related genes presented a significant induction in leaves (Figure 3c), further confirming that root tissues displayed different responses to salt stress. Furthermore, 18 transcripts had higher levels of expression at 7n compared with 4n in the macroarray.
studies (Table S6), indicating that 7n plants are still transcriptionally active. Three of them were tested by qRT-PCR and all showed the expected expression pattern (Figure 3b, MtRrp, MtPrp1 and MtRps27).

Hence, we found that different sets of genes are linked to salt-stress and recovery responses in *M. truncatula*, including known genes linked to stress responses in other organisms and new potential regulatory genes.
Identification of *M. truncatula* regulatory genes involved in the recovery response to salt stress

To identify additional regulatory genes derived from our SSH library and linked to recovery, we analyzed by real-time qRT-PCR the expression of several potential regulatory genes giving no detectable induction in macroarray experiments, but selected based on their sequence homology (such as transcription or translation factors, receptor kinases, or G-proteins; see Table S5). A regulatory gene that we previously showed to be involved in recovery responses was the *MtZpt2-1* gene, encoding a TFIIIA-like TF (Merchan et al., 2003). Progress in the sequencing of the *M. truncatula* genome has enabled us to identify a new *MtZpt2-1*-related gene, *MtZpt2-2*. The salt-stress induction patterns of these two TFs in roots were similar, and followed the recovery capacity of roots (Figure 4a). Thus, *MtZpt2-1* has a recovery-related expression pattern and is involved in this process. In contrast, the two *MtZpt2* genes showed different responses to cold stress: a specific, strong, transitory induction of *MtZpt2-1* during the first 6 h of cold treatment, while *MtZpt2-2* was induced later (Figure 4a). This suggests that these TFs may play additional roles in other abiotic stresses.

Another potential regulatory gene identified from the SSH library was a homolog of the Arabidopsis *At3 g28450* gene (E-value 3.4E -53 ) that encodes a receptor-like kinase (http://www.arabidopsis.org), potentially inducible by salt and cold stresses according to Genevestigator data (http://www.genevestigator.ethz.ch/). In *M. truncatula*, this receptor-like kinase gene, referred to as *MtSrlk* (for salt-induced receptor-like kinase) was induced by both salt and cold stresses (Figure 4b,c). A third gene, *MtAp2*, encoding an AP2-domain TF (E-value 1.0E -65 ) sharing a domain found in transcriptional regulators such as ethylene-responsive element-binding protein (EREBP) or C-repeat/dehydration-responsive element-binding factor 1 (CBF1) (Chen et al., 2003) was weakly induced by salt stress in comparison with cold stress (Figure 4b,c).

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**Figure 4.** Expression of potential regulatory genes in response to salt or cold stress in *M. truncatula*.

(a) Induction of *MtZpt2-1* and *MtZpt2-2* genes that encode putative TFs in roots of *M. truncatula* 108R plants treated with 150 mM NaCl (C, control plants; 4n, 7n, plants treated for 4 and 7 days respectively; left panel) and cold-treated plants (4°C for 1 and 6 h; right panel).

(b) Expression patterns of potential regulatory genes in *M. truncatula* roots and aerial parts in response to salt stress (150 mM NaCl). Genes analyzed are described in the text (C, 4n, and 7n as before; Lc, leaf and cotyledon).

(c) Potential regulatory genes induced by cold treatment in *M. truncatula* roots (4°C for 1 and 6 h): *MtAp2* and *MtSrlk*. C, roots of control plants not subjected to cold. Error bars indicate standard deviation.
regulatory gene was MtSui1, a gene encoding for a translation factor SUI1 homolog (around 85% homology to Arabidopsis proteins encoded by the At4 g27130, At1 g54290 and At5 g54760 genes reported to be linked to diverse abiotic stresses; Genevestigator data, http://www.genevestigator.ethz.ch/). The M. truncatula gene is induced by salt but not by cold stress (Figure 4b). We have also identified a Rho protein homolog, annotated as MtRop9, which is slightly induced by salt stress (Figure 4b). Arabidopsis Rop9 is involved in the activation of a stress-related kinase and in ABA responses (Park et al., 2004; Zheng et al., 2002).

We could thus identify several putative M. truncatula regulatory genes, including a receptor-like kinase, transcription and translation factors, an RNA-binding protein, and a small G-protein, showing expression patterns associated with the salt-stress recovery process in roots.

**Regulation of cytokinin signaling genes by salt stress**

Among the kinases identified in the SSH library, we found a histidine kinase, MtHK2 homologous to Arabidopsis genes encoding cytokinin receptors (Kakimoto, 2003). A potential transduction pathway for cytokinin signaling has been defined (Kakimoto, 2003) that involves membrane receptors (AHKs), histidine-containing phosphotransfer proteins (AHPs) probably relaying cytoplasmic-to-nuclear signaling, and transcriptional response regulators (ARRs). We recently identified several members of these gene families in M. truncatula, including a second histidine kinase gene (MtCRE1) and two A-type RR genes (MtRR4 and MtRR5), and functionally linked them to the cytokinin pathway (Gonzalez-Rizzo et al., 2006). Hence, we analyzed the expression of all these cytokinin-signaling genes during salt stress and recovery (Figure 5). Both M. truncatula HK genes were induced by salt stress, following a recovery expression pattern, although reduction in their expression at 7 days was less pronounced than for other recovery genes (Figure 5a). The MtRR genes, however, did not show a recovery expression pattern (data not shown).

To further investigate a possible link between salt-stress responses and cytokinin signaling, we analyzed the expression of these genes in roots treated with 100 mM NaCl for various incubation times. Again, both MtHK genes were induced at 4 days, but their expression started to be significantly higher than controls after 6 h of salt stress. After treatment for 1 h, MtRR4 was already induced, whereas the other MtRR showed an expression that increased later (Figure 5b).

These results suggested that the adaptation induced by recovery from a salt stress may involve cytokinin signaling pathways. The early activation of A-type RRa and cytokinin receptors by salt stress also suggests a possible cross-talk between environmental and cytokinin signaling.

**Deregulated expression of salt-stress-related genes in As-MtZpt2-1 plants**

Insight into the regulatory networks that might affect salt-stress recovery in roots was gained by analyzing gene expression in antisense MtZpt2-1 transgenic plants (As-MtZpt2-1 plants) which are impaired in their recovery response (Merchan et al., 2003). In these plants, salt induction of the two MtZpt2 TFs is reduced at different levels (Figure 6a), indicating that both genes are affected by the MtZpt2-1 antisense construct. Macrorarray analysis using the As-MtZpt2-1 line 1.4.2 (in two independent biological repetitions) enabled us to identify 48 genes deregulated (positively or negatively) in the antisense plants submitted to the same stress conditions (Figure 1d, Table S7). Ten of these deregulated genes were among our collection of 33 genes optimized for qRT-PCR analysis (Table S4). These 10 genes were then further analyzed as potential candidates for regulation by these TFs in two new biological replicates and by comparing their induction in three independent antisense lines (1.4.2, 1.2.3 and 2.4.2) and control plants (Figure 6b,c). Three genes showed similarly modified expression patterns in the three antisense lines, albeit at variable levels: MtCorA1 and MtFpf1 were downregulated, and MtPrp2 (encoding a homolog of a proline-rich protein induced by auxin) was upregulated. The partial reduction of MtZpt2 expression levels was sufficient to induce a very strong expression of MtPrp2 in response to salt stress (Figure 6c).

We have therefore identified three genes either up- or downregulated in independent transgenic lines showing reduced expression of MtZpt2-1 and MtZpt2-2 genes, suggesting that these transcription factors act as both positive and negative regulators in salt-stress responses.
Figure 6. Identification of potential targets of MtZpt2 transcription factors by comparing control and antisense plants.
(a) Expression analysis by qRT-PCR of MtZpt2 genes in roots of control and three independent antisense lines (lines 1.2.3, 1.4.2, 2.4.2).
(b) Expression of putative positively regulated target genes.
(c) Expression of putative negatively regulated target genes.
Induction ratios (IR) were calculated between samples salt-treated for 4 days with 150 mM NaCl and untreated samples (control condition). TIGR TC accession numbers are shown only for genes encoding proteins of unknown function (Up.). C, plants expressing an empty-vector construct. Error bars indicate standard deviation.
Overexpression of MtZpt2-1 induces salt tolerance and deregulation of the three putative target genes in roots

To further explore a possible connection between the action of MtZpt2-1 and the expression of target genes, overexpression of this TF was achieved. Stable transgenic plants overexpressing MtZpt2-1 could, however, not be propagated, since they were sterile (Frugier et al., 2000). We therefore prepared transgenic ‘composite’ M. truncatula plants carrying Agrobacterium rhizogenes-transformed roots selected on kanamycin (according to Boisson-Dernier et al., 2001) overexpressing MtZpt2-1 under the control of the 35S CaMV promoter. Two representative transgenic roots, transformed with the empty vector, and four carrying the overexpressing construct (belonging to independent composite plants), were analyzed for their levels of MtZpt2-1 transcripts (Figure 7a). Overexpression of this gene was found in all lines tested, and no correlative increase was observed for the homologous MtZpt2-2 gene in these lines (Figure 7a, left panel). This suggests that the reduced

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**Figure 7.** Overexpression of MtZpt2-1 affects expression of at least three putative target genes and salt tolerance of roots. Composite plants were prepared as described in Boisson-Dernier et al. (2001) using a control empty vector and a 35S CaMV: MtZpt2-1 construct.

(a) Left panel: roots from two representative independent control lines (C) and four independent transgenic lines overexpressing MtZpt2-1 in sense orientation (S) were analyzed by qRT-PCR. Histograms show relative quantification of MtZpt2-1 transcript levels (left panel) and of three putative targets of MtZPT2-1 (middle panel, positively, and right panel, negatively regulated genes).

(b) Expression of MtZpt2-1 and potential targets in transiently transfected M. truncatula leaves. RNA from leaves transformed with a 35SCaMV: MtZpt2-1 (S) or empty vector (V) construct was used for qRT-PCR after 24 or 48 h of transfection (S24 h, S48 h or V24 h, V48 h, respectively). Histograms show relative quantification of the transgene and the putative targets.

(c) Percentage of GUS-stained cells after bombardment of 35S CaMV: MtZpt2-1 or empty vector (control) constructs. The percentage is calculated in relation to the total number of transformed cells monitored by a 35S CaMV: GFP construct in three independent transient assays. Error bars indicate standard deviation.

(d) Primary root length measured from the point of transfer on the salt-containing medium (100 mM NaCl) or on a normal medium after 1 week. Error bars indicate interval of confidence (IC, $\alpha = 0.05$). Exp. 1 and Exp. 2 are two independent biological experiments. Plants transformed with an empty vector construct were used as controls.
induction of MtZpt2-2 in the antisense lines is due to a homology-dependent effect of the antisense construct, and not a consequence of the suppression of MtZpt2-1.

In these MtZpt2-1-overexpressing roots, the three previously characterized potential target genes of this TF showed patterns of expression correlated to the presence of the transgene in the four different lines: the MtCorA1 and MtFpf1 genes were transcriptionally induced, whereas MtPrp2 was repressed, even in the absence of stress (Figure 7a, central and right panels). However, no strict correlation could be found between transgene and putative target expression levels. Further evidence that MtZpt2-1 may regulate these targets was obtained by transient assays. First, transient expression of MtZpt2-1 in M. truncatula leaves lead to a concomitant increase of the transgene, MtCorA1 and MtFpf1 endogenous genes (Figure 7b). The expression of MtPrp2 is very low in leaf tissues (more than 35 PCR cycles are required to detect this transcript), and may therefore limit the detection of a reliable repression. Second, by cloning the MtCorA1 promoter fused to the uidA reporter gene, we could show that co-expression with MtZpt2-1 yields a large increase in the number of GUS-stained cells in a heterologous system (bombarded onion cells; Figure 7c). A 35SSCaMV::GFP construct was used as an independent control to estimate the efficiency transformation in this transient assay.

To analyze a potential physiological role for this MtZpt2-1 TF, we assayed the salt response of several independent transgenic roots overexpressing this gene. After 3 weeks of growth in normal medium, the composite plants were transferred to a salt-containing medium (100 mM), and root length was measured 7 days after transfer. In normal medium, growth of overexpressing and control roots was not significantly different (Figure 7d). However, we detected a significant (Student’s t-test, \( P < 0.007 \) in two independent biological experiments) increase in primary root growth in the population of MtZpt2-1-overexpressing lines compared with control roots after 1 week on salt medium (Figure 7d).

Hence, overexpression of MtZpt2-1 allows sustained root growth under salt-stress conditions. At least three putative MtZpt2 targets, including the new MtFpf1 and MtPrp2 genes and a known stress-related protein (MtCorA1), could be linked to the action of this regulator in root tissues.

Discussion

In this study, we have identified a group of genes linked to the capacity of the roots to resume growth after salt stress in the model legume M. truncatula using large-scale SSH approaches. Among them, several regulatory genes were characterized and a pathway linking the action of the MtZPT2-1 transcription factor with three target genes has been proposed.

The recent development of transcriptome analysis has enabled the determination of the expression profile of a large number of genes concerned with stress responses in model plants (Arabidopsis and rice; Oono et al., 2003; Seki et al., 2002; Takahashi et al., 2004). The number of stress-regulated genes identified through these analyses can be relatively low (Lan et al., 2004; Seki et al., 2002). Furthermore, the sensitivity of microarray techniques is often not sufficient to study genes expressed at low levels (such as many regulatory genes), especially in comparison with techniques such as RT-PCR (Czechowski et al., 2004). An alternative strategy is to combine the use of SSH and subsequently ‘dedicated’ macroarrays carrying a smaller number of genes but all potentially involved in the studied biological process (Rishi et al., 2004). In legumes, large-scale SSH approaches have been used to study symbiotic interaction (Brechenmacher et al., 2004; Godiard et al., 2006) and germination (Bouton et al., 2005). We constructed SSH libraries to identify genes linked to recovery responses after a salt stress in M. truncatula roots. A large number of novel genes linked to these processes were identified by sequencing clones selected as a result of their differential expression (400 out of 3000 clones). We attempted here not only to identify genes induced by salt stress, but also to refine the screening to select for potential candidates that may be linked to the reacquisition of root growth.

Abiotic stresses strongly affect root growth and architecture, and various responses at the physiological, biochemical and molecular levels have been detected even in different root zones (Sharp et al., 2004). Most of the studies analyzing large-scale changes in gene expression induced by abiotic stresses such as salt stress involved short-term treatments. It is also clearly of interest to address long-term performance and the degree of recovery from stress to assess the stress tolerance of crops (Vinocur and Altman, 2005). Indeed, much less is known about how plants may re-initiate growth once conditions become more favorable. The recovery process in response to different abiotic stresses has been studied for certain plants (rice, Abbasi et al., 2004; Populus euphratica, Gu et al., 2004; alfalfa, Miller et al., 2005; Tortula rurales, Oliver et al., 2004; Arabidopsis, Oono et al., 2003) but the present work examines this process using a large-scale SSH analysis in legumes. Interestingly, several of the regulatory genes identified, such as a salt-induced receptor-like kinase, a new RNA-binding protein and an AP2-transcription factor, display a downregulation when plants cannot be recovered. This suggests that their expression may be involved in a process allowing roots to restart growth. Certain of these genes could be linked to abiotic stress responses in Arabidopsis (Genevestigator data) but most were not previously associated with these responses. Moreover, these novel regulatory genes showing a ‘recovery’ pattern were not always associated with early salt-stress responses, suggesting that specific pathways may be activated in this process.
Our approach is therefore a useful alternative for identifying candidates that have a role in improving root growth under restrictive conditions. Nonetheless, we do not rule out the possibility that genes not showing this expression pattern could contribute to the reacquisition of root growth. Plants affected in the expression of the MtZPT2-1 transcription factor, which show a ‘recovery’ expression pattern, however, indicate that such genes can be crucial for root capacity to recover from salt stress.

Phytohormones have been linked to recovery responses in various plant tissues. Notably, in *Vigna radiata*, kinetin was able to overcome (to a certain extent) the adverse effects caused by NaCl stress (Chakrabarti and Mukherji, 2003). Furthermore, plants expressing ipt, a cytokinin biosynthetic gene, recovered more rapidly from flooding than did wild-type plants (Huynh et al., 2005), an effect that has been linked with the capacity of this hormone to retard senescence. Several hormones regulate root growth at different levels such as ethylene and ABA and interact with the auxin/cytokinin balance in response to abiotic stresses (Casimiro et al., 2003; Malamy, 2005). We recently characterized the *M. truncatula* cytokinin signaling genes MtCRE1, MtRR4 and MtRR5 that are upregulated a few hours after treatment with the cytokinin 6-benzyl-amino-purine (BAP). Moreover, MtCRE1 RNAi roots are cytokinin insensitive and affected in their architecture (Gonzalez-Rizzo et al., 2006). Now we show that MtCRE1 and MtHK2, two genes homologous to cytokinin receptors, are induced in roots during the salt recovery process. Furthermore, these MtHK as well as MtRR genes, homologous to downstream cytokinin signaling pathway genes, are rapidly induced by salt stress, suggesting a linkage between this phytohormone and stress responses in roots. In agreement, Urao et al. (1998) have shown that several ARR genes are induced by abiotic stresses in Arabidopsis. Cytokinin concentrations are very variable in different plant systems and there are at least 20 naturally occurring cytokinins in plants (Hare et al., 1997). In pea, the presence of salt induces a significant increase in cytokinin concentration in roots (Atanassova et al., 1999), which may explain the negative effect of salt treatment on primary root growth (Werner et al., 2003). Changes in the sensitivity to cytokinins during stress may also affect root growth, either through signaling to the aerial parts (Burke et al., 2003; Haberer and Kieber, 2002) or by affecting cell size and/or root differentiation. Endogenous phytohormones and regulatory genes sensing the soil environment interact to adapt and optimize the root architecture (number of lateral roots, distribution of root hairs, primary and lateral root length; Lopez-Bucio et al., 2003). Alteration of cytokinin perception by salt stress and recovery, may lead to a reorganization of root architecture.

Transcription factors are crucial elements for the regulation of development and adaptation to abiotic stresses in plants, and overexpression of specific TFs leads to increased tolerance to cold or salt stresses (Davletova et al., 2005; Guo et al., 2004; Kim et al., 2001, 2004; Mukhopadhyay et al., 2004; Vogel et al., 2005). The MtZPT2-1 TF is induced by osmotic and salt stress (Merchan et al., 2003) and, as shown here, is also induced by cold stress. To understand the regulatory pathway involving this TF, we performed a comparative analysis of gene expression patterns in antisense plants and overexpressing *A. rhizogenes*-transformed roots, and correlated the changes in TF expression levels with the expression of three co-regulated genes. *Medicago truncatula* ZPT2-1 may be an activator of the MtFpf1 (Kania et al., 1997) and MtCorA1 (Laberge et al., 1993) genes, and a repressor of MtPrp2 gene. Interestingly, overexpression of FPF1 affected flower formation in Arabidopsis and root development in rice (Xu et al., 2005). The SCOF1 TF containing the L/DFNLN/FxP sequence (similar to MtZPT2-1) also induces the transcription of *COR* genes through interaction with the SGBF-1 TF and ABA-responsive element (ABRE) promoter sequences (Kim et al., 2001). In addition, analysis of a mutant affected in the sos response (sos3; Gong et al., 2001) has shown that STZ (a closely related Arabidopsis homolog of MtZpt2-1) is negatively regulated by this signaling pathway and acts as a transcriptional repressor (Sakamoto et al., 2004). Indeed, this gene family is differentially induced both by diverse abiotic stresses (drought, cold and salt stress; Maruyama et al., 2004; Sugano et al., 2003) and during flower development (Takatsuji, 1998). Depending on the signaling pathway involved, these genes may act as activators or repressors of transcription. Similarly to AtAZF2 and AtSTZ in *A. thaliana* (Sakamoto et al., 2004), MtZPT2-1 may also act as a transcriptional repressor, at least for the MtPrp2 gene. Recently, it has been shown that AtZAT12, another Arabidopsis homolog of MtZPT2, plays a central role in oxidative and abiotic stress signaling pathways, and putative target genes both activated and repressed in plants overexpressing this TF have been identified (Davletova et al., 2005). Interestingly, as shown for the latter Arabidopsis homolog, overexpressing MtZpt2-1 in *Medicago* roots enhanced their growth under salt-restrictive conditions.

In the *M. truncatula* genomic sequences available, we only found promoter regions of three *COR* genes containing several copies of the AGT core sequences (separated by 10–12 bp) which are bound by ZPT2-type TFs in vitro (Takatsuji and Matsumoto, 1996; Takatsuji et al., 1994). In two of them, we also found the A(G/C)T-X3-4-A(G/C)T cores described by Sakamoto et al. (2004). Both sets of data support our in planta expression analysis and the results obtained in the transient assays, strongly suggesting that the MtCorA1 gene may be a direct target of the MtZPT2-1 TF.

Our results allow speculations about the regulatory mechanisms involved in the process of the recovery of roots from a salt stress. Repressing auxin-induced responses through MtZPT2 TFs (as suggested by MtPrp2
expression analysis), together with enhancement of cytokinin sensitivity (mediated by MtCRE1), may be involved in affecting root growth and architecture during recovery responses after salt stress (e.g. by limiting primary root growth, controlling the emergence of lateral roots or the root apical dominance in response to environmental conditions; Aloni et al., 2006). Moreover, other pathways involving several levels of gene regulation at transcriptional (MtAp2) and post-transcriptional (MtRbp2 and MtSui1) levels may contribute to the restoration of root salt stress. Finally, several developmentally related genes have also been linked to these responses, such as MtFpf1 and MtSrlk, which may contribute to the reacquisition of root growth, notably through the emergence of lateral roots. We think that this work offers interesting perspectives for the analysis of the signaling pathways involved in the recovery of root growth in response to abiotic stresses in legumes, plants which have major ecological and agronomic impacts worldwide.

Experimental procedures

Plant growth conditions and RNA extraction

Medicago truncatula 108-R seeds were sterilized and germinated as described in Charon et al. (1999). Seedlings were grown vertically in a growth chamber at 24°C under 6-h light for 4 days, and treated for salt stress and recovery experiments as described previously in Merchan et al. (2003). Roots were collected at the indicated time points, and immediately frozen in liquid nitrogen for RNA extraction. For the different experiments (except macroarray hybridizations), a Total RNA Isolation Kit (Macherey-Nagel, http://www.macherey-nagel.com/) was used. Macroarray hybridizations were done as described in the 'EMBO Medicago truncatula Practical Course' manual (http://www.isv.cnrs-gif.fr/EMBO01/manuels/pdf/module5.pdf)

Suppression subtractive hybridization (SSH)

Suppression subtractive hybridization was carried out using a SMART PCR cDNA Synthesis Kit and a SMART-Select Subtractive Hybridization Kit (Clontech, http://www.clontech.com/). Experimental and control samples for each treatment were processed simultaneously. All procedures were performed according to the manufacturers' recommendations. For construction of the salt-induced library, tester and driver cDNAs were synthesized using RNA extracted from roots of plants grown in salt (150 mM NaCl) medium for 4 days (4n) and control plants (grown for 4 days without salt, 4i), respectively. For construction of the recovery library, cDNAs were synthesized from RNA extracted from roots of 4n plants ('driver' cDNA), and from roots of plants grown in salt (150 mM NaCl) medium for 7 days (7n, 'driver' cDNA). As indicated by the manufacturer, we estimated the efficiency of subtraction by comparing the abundance of a known stress-induced and recovery gene, MtZpt2-1 (Merchan et al., 2003), and a non-differentially expressed gene, MtActin, before and after subtraction (using qRT-PCR, Light Cycler, Roche Diagnostics, http://www.roche.com/). Medicago truncatula Zpt2-1 was enriched twofold and eightfold, respectively, in the recovery and salt-induced SSH libraries whereas MtActin was reduced 6.5 times in both subtracted cDNAs.

Molecular cloning techniques

The PCR products derived from subtracted cDNAs were cloned using the pGEM-T Easy T/A Cloning Vector System (Promega, http://www.promega.com/) according to the manufacturer's instructions. Ligation products were transformed into XL1-Blue® Competent Cells (Stratagene, http://www.stratagene.com/). About 3000 white colonies per library were picked using a QPix II robot (Genetix; http://www.genetix.com) grown in 384-well plates in Luria–Bertani (LB) medium with 100 mg l⁻¹ ampicillin and subsequently spotted on Immobilon-N membranes (Millipore, http://www.millipore.com/) using a Biogrid robot (BioRobotics Ltd) placed on 22 cm × 22 cm Petri dishes (Nalge Nunc International, http://www.nalgenunc.com/) again containing LB medium with 100 mg l⁻¹ ampicillin. The presence and size of inserts was checked by amplification of a hundred random clones. Deoxyribonucleic acid from grown bacteria was released by alkaline lysis onto a nitrocellulose filter (Sambrook and Russell, 2001). Membranes were hybridized in Church Buffer (Church and Gilbert, 1984) containing 0.1 mg ml⁻¹ denatured salmon sperm DNA (overnight at 65°C) with 33P-labeled probes generated from the driver and tester SMART cDNAs. Membranes were finally washed with 0.1 SSC/0.1% SDS for 15 min at 65°C. Plasmids from 418 clones selected by their differential expression were sequenced.

Sequence analysis

Cycle-sequencing reactions were prepared using BigDye Terminator (GenPak, http://www.genpakdna.com), and nucleotide sequences were determined in an automatic laser sequencer 373A (Applied Biosystems, http://www.appliedbiosystems.com/). Sequences were used to query the TIGR Medicago Gene Index database (http://www.tigr.org/) using the BLASTN sequence-comparison algorithm. Sequences that failed to show significant homologies in databases were used to query BLASTN and BLASTX on the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/).

Polymerase chain reaction amplification and DNA array construction

The T7, T3, SP6 and specific 20-mer primers for all genes (indicated in Table S4) were used to amplify DNA from bacterial clones. Polymerase chain reaction products corresponding to M. truncatula elongation factors (MtEF1α, MtEF2 and MtEF3), MtC27, Mtactin, Mtubulin, Mtg3p (Table S1) were used as internal 'constitutive' controls. Inserts of bacterial clones (ranging in size between 100 and 500 bp) were amplified by PCR. Cycling conditions (30 cycles) were as follows: 30 sec of denaturing at 95°C, 30 sec of annealing at 55°C, and 50 min of elongation at 72°C. The quality of the PCR products was checked by electrophoresis on agarose gels. Sequencing of 20 PCR fragments provided a second quality control to check that all generated sequences corresponded to the expected gene. The PCR products (approximately 10 μg) were denatured with DMSO and spotted on Immobilon-N membranes using the GeneTAC G3 robot (Genomic Solutions, http://www.genetix.com). Each clone was printed in quadruplicate with spots of 0.6 mm in diameter, together with distilled water and vector DNA as negative controls. After air drying, membranes were denatured in 0.6 M NaOH for 3 min, neutralized
in 0.5 M 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS)-HCl (pH 7.5) for 3 min, and rinsed in distilled water for 30 sec. The DNA was cross-linked to membranes using UV radiation (125 mJ) in a standard UV chamber (Stratagene), and membranes were stored at 4°C.

Ribonucleic acid extraction and 32P-labeled target preparation

Total RNA was extracted from roots and leaves using a RNeasy Plant Mini Kit (Qiagen, http://www1.qiagen.com/). For preparation of 32P-labeled target cDNA, 20 µg of total RNA was heat-denatured at 95°C for 5 min and 1.4 µg of oligo(dT)12–18, 4 mM dATP, 4 mM dGTP and 4 mM dTTP in 5 µl SuperScript II buffer (Gibco BRL, http://www.gibcobrl.com) in the presence of 2 µl of 0.1 M NaOH and 0.1 SSC/0.5% SDS for 30 min. The reaction was terminated by heating at 70°C for 15 min and gel filtration through a ProbeQuantG 50 Micro Column (Amersham Pharmacia Biotech) was performed according to the manufacturer’s instructions.

Hybridization procedure

Macroarrays in nylon filters were pre-hybridized in Church buffer (Church and Gilbert, 1984) for 2 h at 65°C. The cDNA probe was heated for 5 min at 95°C, rapidly cooled on ice, and added to 5 ml of Church buffer. Hybridization was carried out for 17 h at 65°C and each filter was washed in 40 mM sodium phosphate, 0.1% SDS, pH 7.2 for 20 min at 65°C. Filters were exposed to a phosphorimager-screen for 16–48 h to allow near-saturation of the most intense spots. For rehybridization, membranes were washed at 42°C successively with 0.1 M NaOH for 30 min and 0.1 SSC/0.5% SDS for 30 min.

Data acquisition and analyses

Hybridization signals on the phosphorimager-screen imaging plates (Molecular Dynamics, http://www.gelifesciences.com) were detected using Storm 860 (Amersham Pharmacia Biotech) with 50-mm resolution. The signal intensity of each spot was determined using ‘Array Vision’ (Media Cybernetics, http://www.mediacy.com/). Local backgrounds calculated in the corners between individual spots were subtracted to obtain raw signal intensities.

In order to work with highly reproducible experiments, linear regression analysis was performed for each pair of membrane replicates. Only those replicates for which the linear model could explain at least 80% of the variation (determination coefficient r2 > 0.8) were considered further. This process yielded a total of seven well-correlated replicates for 4n and 7n root hybridization experiments; and three replicates, for salt kinetics experiments as well as experiments with leaves. False positives showing high ratios but very low absolute intensity signals were discarded by selecting intensity/background ratios greater than 1.5.

Values were then normalized relative to expression levels of at least six ‘house-keeping’ genes present in the macroarray. Single expression values per treatment were then calculated as the gene’s mean expression in sets of correlated replicates. Many useful normalization strategies have been developed to correct for the systematic biases in macroarray data analysis (e.g. Student’s t-test; El Yahyaoui et al., 2004; Hirai et al., 2003; Ramirez et al., 2005), even though no single normalization method has become a standard (Finkelstein et al., 2002). We checked the significance of the differential expression levels by two statistical tests: (i) a moderated Student’s t-test using raw intensities to identify transcripts significantly induced or repressed (P < 0.05) and (ii) conformity with the standard deviation separation criteria (Bey et al., 2004). Lists of regulated genes according to these two statistical criteria, together with their signal intensity ratios in selected conditions, are available in Table S1. Hierarchical cluster analyses were performed using CLUSTER and TreeView software (http://rana.lbl.gov/EisenSoftware.htm; Eisen et al., 1998).

Quantitative RT-PCR studies

For qRT-PCR, first-strand cDNA was synthesized from 1.5 µg of total RNA using the SUPERSCRIPT® II first-strand synthesis system (Invitrogen). One-tenth of the cDNAs was used as a template in 10 µl PCR reactions. The PCR was performed with a Light Cycler apparatus and the ‘LC FastStart DNA Master SYBR Green IR (Roche Diagnostics) in a standard PCR reaction according to the manufacturer’s instructions. Gene-specific PCR primers were designed according to the cDNA sequences using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), and are indicated in Table S4. Parallel reactions to amplify Mtactin were used to normalize the amount of template cDNA. The reproducibility of the assay was monitored by running technical duplicates, and standard deviations for two independent cDNA preparations (from the same RNA sample) are shown as errors bars in all figures. Two biological replicates were performed per condition, and primer combinations had at least 90% efficiency.

Transient expression assays and preparation of A. rhizogenes-transformed roots

Agrobacterium rhizogenes-transformed M. truncatula roots of ‘composite’ plants were prepared as described in Boisson-Dernier et al. (2001). Briefly, 2 weeks after inoculation of seedling roots without apices with A. rhizogenes (Arqua1 strain), plants developed transgenic roots under kanamycin selection (25 µg l−1). The resulting composite plants were transferred to Fahreus medium containing a brown filter paper (from a growth pouch, Mega International, http://www.mega-international.com/) to allow easy transfer to a new plate with salt-containing medium (100 mM NaCl in Fahreus). The position of the root apex at the moment of transfer was labeled and root length measured 1 week after transfer. For overexpression of MtZpt2-1 we used a pG3.3-derived vector (referred as pMF2 vector) containing a 35S CaMV promoter-uidA (with an intron) cassette as well as a 35S CaMV promoter after which the MtZpt2-1 full-length cDNA was introduced. A pM2F empty vector was used as a control.

For transient expression assays in M. truncatula, Agrobacterium tumefaciens strains carrying the same constructs were used for vacuum infiltration of M. truncatula 108R 14-day-old plants as described in Kapila et al. (1997). Plants were then grown in a growth chamber at 24°C under 16-h light for 2 days. Leaves were collected 24 and 48 h after infiltration for total RNA extraction to perform qRT-PCR studies. The expression of MtPfp1, MtCorA1, MtPrp2 and MtZpt2-1 was normalized using the MtActin (as a control of constitutive expression). To control transformation efficiency, the uidA expression level was shown to be comparable between samples.
A second transient assay in heterologous epidermal onion cells was used to study directly the trans-activation of the MtCorA1 promoter by MtZPT2-1. Constructs containing a 35S-MtZpt2-1 fusion or an empty vector were co-bombarded with a fusion of the MtCorA1 promoter pMtCorA1 (a 2 kb 5’ upstream fragment from the MtCorA1 ORF) to uidA reporter gene, and a 35S:GFP construct (to evaluate transformation efficiency). All these constructs were cloned into the pBluescript SK+ vector (Stratagene). Particle bombardment using a Biolistic PDS-1000/He particle gun (Bio-Rad, http://www.bio-rad.com/) was performed and the number of GFP fluorescent cells and β-glucuronidase (GUS) stained cells determined as described before (Campalans et al., 2004; the fixation step was omitted). One day after bombardment, onion epidermal cells were analyzed in light and fluorescent microscope (Leica, Rueil-Malmaison, France) to determine: (i) the total number of transformed cells (GFP fluorescence), (ii) the number of GUS-stained cells from this total (percentage of GUS-stained cells relative to GFP-stained cells). Three independent experiments were performed with a minimum of 400 GFP-transformed cells per experiment.

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Root development of M. truncatula 108-R seedlings during salt stress and salt recovery.

Table S1 List of all macroarray genes
Table S2 Legume-specific ESTs identified in SSH libraries
Table S3 List of 156 recovery-related genes classified according to their increasing P values
Table S4 Early-induced genes also showing salt recovery-related expression
Table S5 Genes selected for qPCR studies and their corresponding optimized oligonucleotides, product size and figures showing data
Table S6 List of genes having high expression in 7n compared to 4n plants
Table S7 List of putative regulated target genes of MtZPT2 transcription factors

This material is available as part of the online article from http://www.blackwell-synergy.com.

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