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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 IIIA-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 Mt*Zpt2-1* 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 mM 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) genes (see Experimental procedures). Six thousand 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 identifaction 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 $1.0E^{-30}$ 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 XIIB (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 of rapidly recoverable and slowly recoverable conditions (4n and 7n, 4 and 7 days of 150 mM NaCl treatment) from *M. truncatula* roots and aerial parts (Figure 1b), and (ii) RNAs from roots treated with 100 mM of 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.

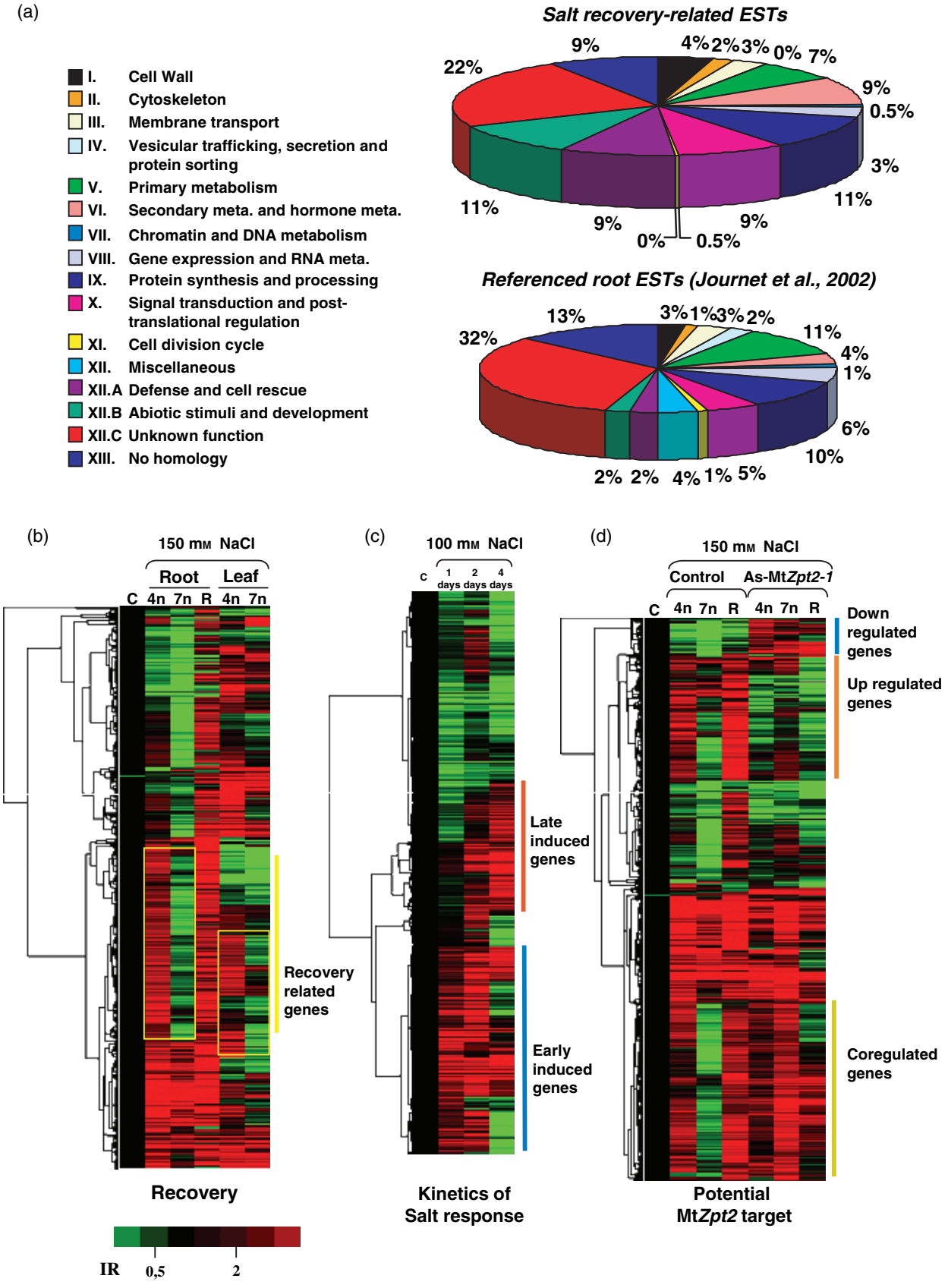
To monitor gene expression based on this macroarray dedicated to salt stress, only replicates (belonging to one or two independent biological experiments) with a high determination coefficient ($r^2 > 0.8$) were conserved for further analysis (Beissbarth *et al.*, 2000; Ramirez *et al.*, 2005). Moreover, 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 7n-treated 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).



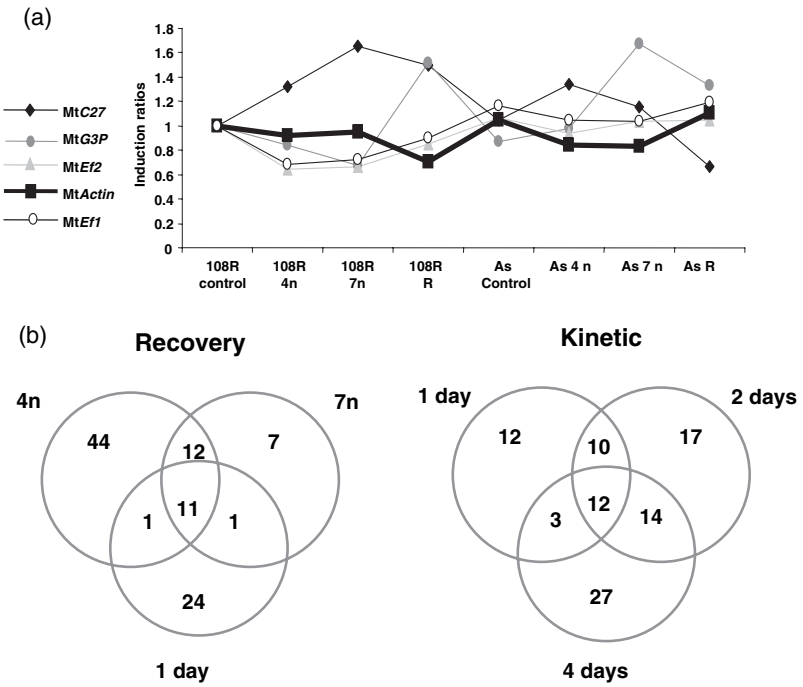


Figure 2. Expression analysis revealed sets of genes induced by the different salt treatments.
(a) Expression summary of constitutive genes present in the dedicated macroarray. The bold line highlights the *MtActin* gene expression showing the lowest variations under all tested conditions among all other 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 FPF1 (*MtFpf1*, Flowering Promoting Factor 1; Kania *et al.*, 1997). The new RNA-binding protein *MtRbp2* is homologous to the *At5 g04600* gene (E-value $2E^{-60}$) 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

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 Mt*Zpt2-1* roots; C, control condition used for normalization (roots of non-treated plants); 4n and 7n, treatment for 4 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).
Intensity ratios (IR) were calculated between the salt-treated and untreated samples (control condition). Six representative examples of expression pattern clusters 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- and upregulated genes showing different responses in antisense plants are indicated. Co-regulated genes indicate genes showing a 'recovery' expression pattern in wild-type plants not found in the antisense plants.

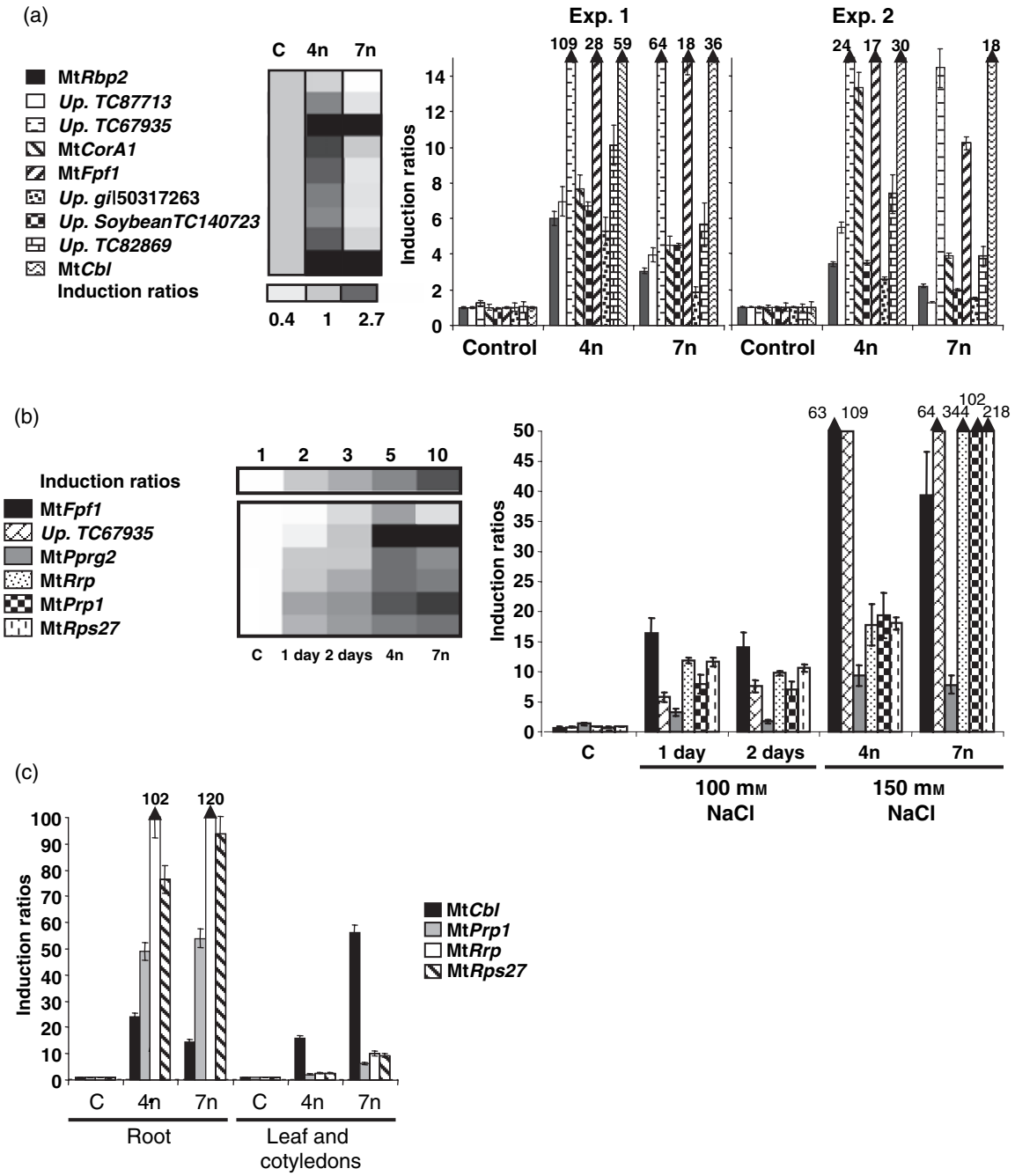


Figure 3. Real-time RT-PCR analysis of gene expression during salt-stress responses and recovery.
(a) Comparison of the expression patterns of selected genes associated with the recovery process using a macroarray (left panel) or qRT-PCR (right graph) in two new biological repetitions (Exp. 1 and Exp. 2) C, roots of untreated plants; 4n or 7n, roots of plants stressed for 4 or 7 days with 150 mM NaCl.
(b) Analysis of gene expression of selected early salt-induced genes using macroarray (left panel) or real-time RT-PCR (right panel). C, roots of untreated plants; 1d, 2d, roots of plants treated with 100 mM NaCl for 1 or 2 days, respectively.
(c) Expression pattern during the recovery process of four genes induced by salt both in roots and aerial parts. (4n, 7n: see above). Numbers above the expression bars indicate the fold induction of gene expression (higher than the *Y*-axis scale). TIGR TC accession numbers are shown only for genes encoding for proteins of unknown function (Up.). For genes names, see Table S4. Error bars indicate standard deviation.

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 $3.4E^{-51}$) 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 *M. truncatula* in comparison with cold stress (Figure 4b,c). Another potential

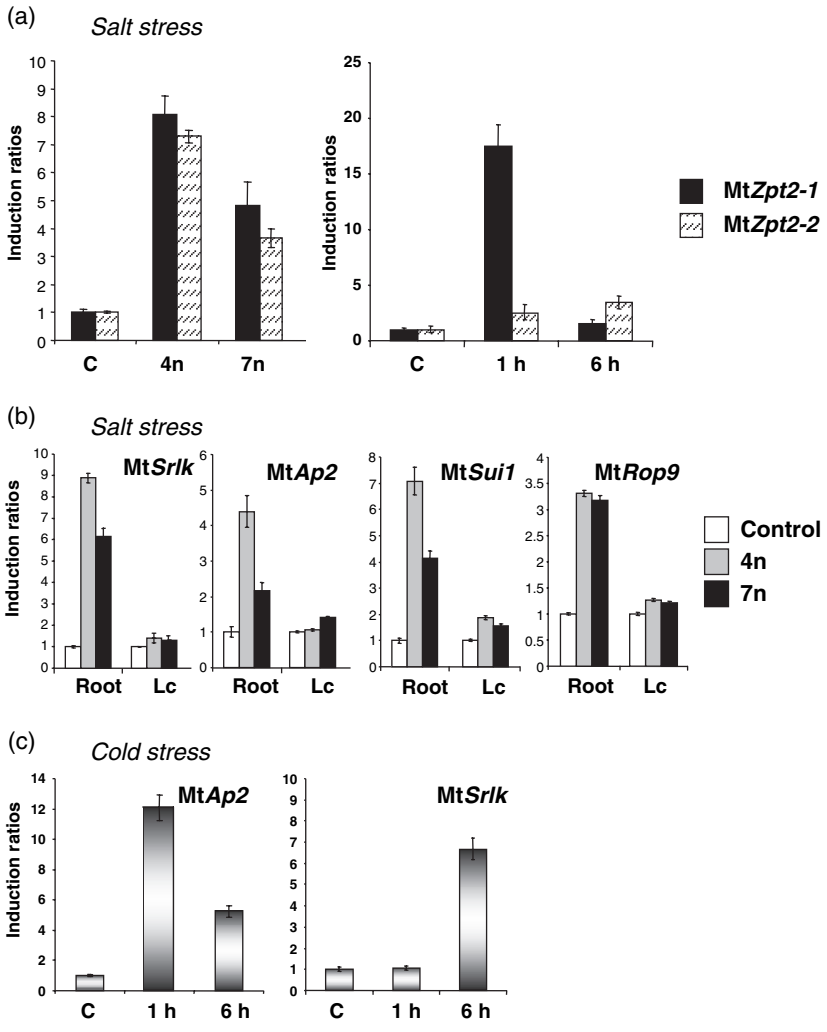


Figure 4. Expression of potential regulatory genes in response to salt or cold stress in *M. truncatula*.
(a) Induction of *MtZpt2-1* and *MtZpt2-2* genes that encode putative TFs in roots of *M. truncatula* 108R plants treated with 150 mM NaCl (C, control plants; 4n, 7n, plants treated for 4 and 7 days 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 *RR*s and cytokinin receptors by salt stress also suggests a possible cross-talk between environmental and cytokinin signaling.

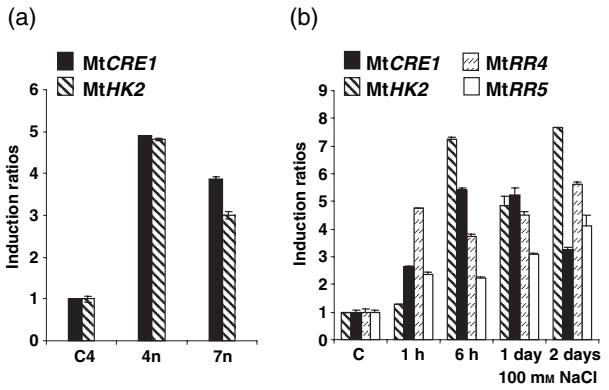


Figure 5. Regulation of cytokinin-signaling genes by salt stress.
Expression of two histidine kinases (*MtCRE1* and *MtHK2*) and two A-type response regulators (*MtRR4* and *MtRR5*) using qRT-PCR during salt-recovery processes (a: 4n and 7n, roots of plants treated for 4 and 7 days respectively with 150 mM NaCl) or in a salt-stress kinetic (b: roots treated with 100 mM NaCl for 1 or 6 h and 1 or 4 days). C, control. Error bars indicate standard deviation.

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. Macroarray 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.

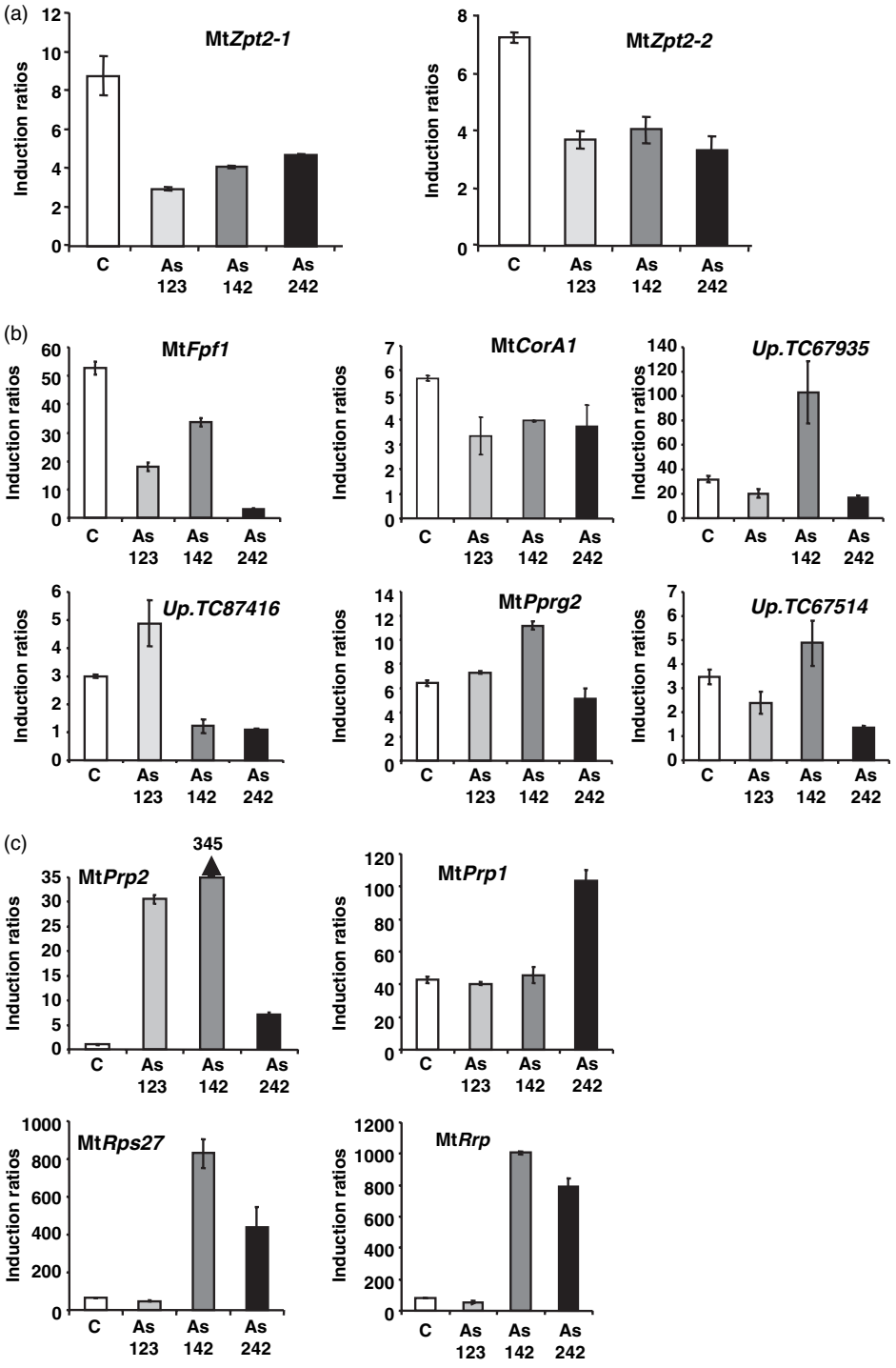


Figure 6. Identification of potential targets of *MtZpt2* transcription factors by comparing control and antisense plants.
(a) Expression analysis by qRT-PCR of *MtZpt2* genes in roots of control and three independent antisense lines (lines 1.2.3, 1.4.2, 2.4.2).
(b) Expression of putative positively 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

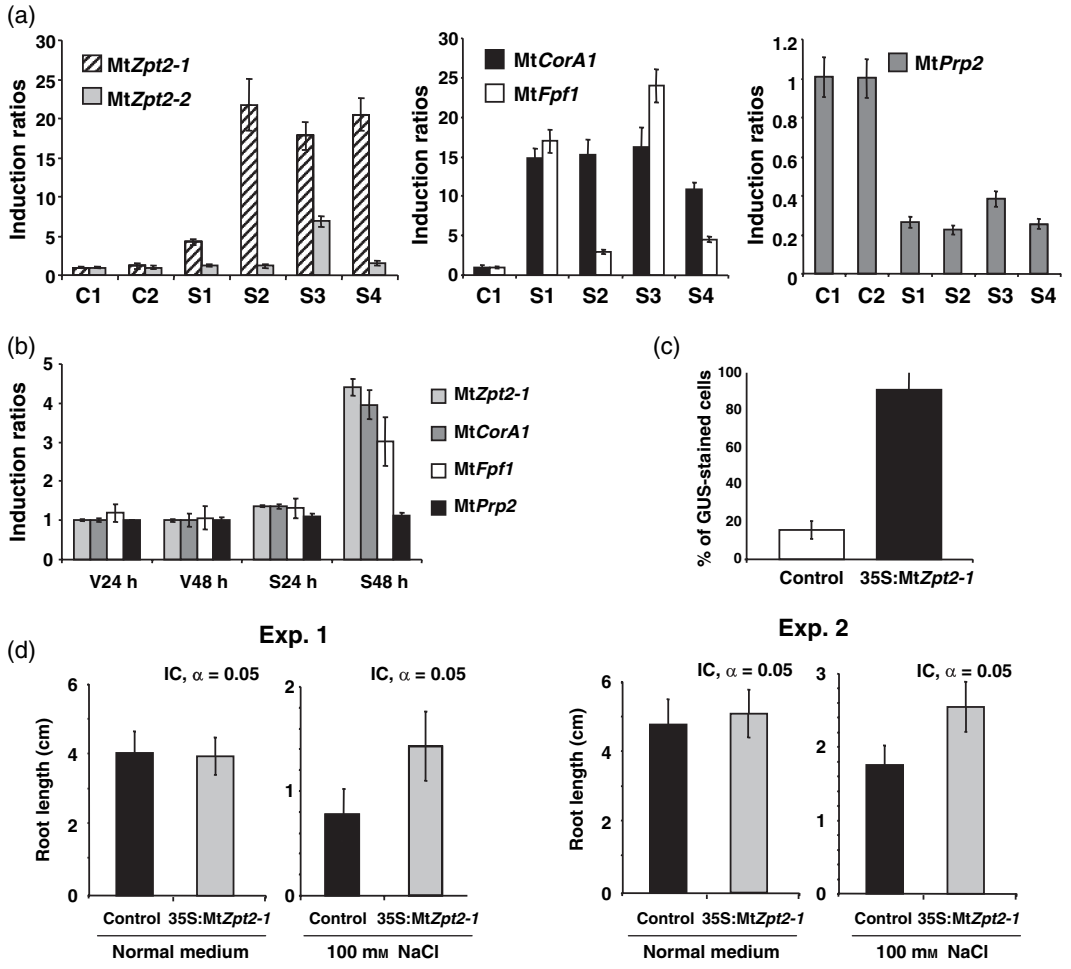


Figure 7. Overexpression of *MtZpt2-1* affects expression of at least three putative target genes and salt tolerance of roots.
Composite plants were prepared as described in Boisson-Dernier *et al.* (2001) using a control empty vector and a 35S CaMV:Mt*Zpt2-1* construct.
(a) Left panel: roots from two 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:Mt*Zpt2-1* (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:Mt*Zpt2-1* 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 *trans*-activation 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 35SCaMV: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 (Burkle *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/FDLNL/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 *CORA* 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 reverse the effects of salt stress on roots. 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 PCR-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 ('tester' 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 XL10-Gold® Ultracompetent 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 ^{33}P-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 (*MtEf1*, *MtEf2* and *MtEf3*), *Mtc27*, *Mtactin*, *Mttubulin*, *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 PCRs 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.genomicsolutions.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 ^{33}P-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 ^{33}P-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 SuperScriptII buffer (Gibco BRL, http://www.gibcobrl.com) in the presence of 2 μl of 0.1 M DTT and 8 μl of [α-^{33}P]-dCTP (code no. AH9905, Amersham Pharmacia Biotech, http://www5.amershambiosciences.com/) at 42°C for 2 min. Then, 1 μl of SUPERSCRIPT® II first-strand synthesis system (Invitrogen, http://www.invitrogen.com/) was added and incubated at 42°C for 1 h to synthesize ^{33}P-labeled single-stranded cDNA. The reaction was terminated by heating at 70°C for 15 min and gel filtration through a ProbeQuantTM G-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 replicas. Only those replicas for which the linear model could explain at least 80% of the variation (determination coefficient $r^2 > 0.8$) were considered further. This process yielded a total of seven well-correlated replicas for 4n and 7n root hybridization experiments; and three replicas, 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 replicas. 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 mg 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 Mt*Zpt2-1* 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 pMF2 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 *MtFpf1*, *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-100/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 pre-fixation step was omitted). One day after bombardment, onion epidermal cells were analyzed in light and fluorescent microscopy (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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