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A Krüppel-like transcription factor gene is involved in salt stress responses in *Medicago* spp.

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Abstract

Legume plants are able to fix nitrogen in symbiotic association with rhizobia and, like many crops, are sensitive to high salt conditions. However, very few molecular markers can be associated to stress tolerance in legume crops. A Krüppel-like transcription factor, *Mtzpt2-1*, required for the formation of the nitrogen-fixing region, confers salt tolerance to yeast cells. Here, legume responses to salt stresses were studied using alfalfa and its close relative *Medicago truncatula*, a model legume species. Salt stress induces the *Mszpt2-1* gene both in roots and root harbouring nodules. In addition, *Sinorhizobium meliloti* strains tolerating up to 700 mM NaCl, were used in nodulation assays to assess salt tolerance of the symbiotic response of *M. truncatula*. Few nodules, mainly in the upper part of the root, could be detected in plants treated with 200 mM NaCl, suggesting that nodule initiation was particularly sensitive to salt stress. We have also defined for *M. truncatula* the threshold of NaCl tolerance after which recovery of stressed plants is irreversible under laboratory conditions. After analysing several times of salt treatment (150 mM NaCl), *M. truncatula* 108R plants stressed for 7 days could not recover (less than 5%), whereas a 4-day treatment allowed at least 75% recovery. Transgenic *M. truncatula* plants expressing *Mtzpt2-1* in antisense configuration are more sensitive to 'recover' from salt stress than the wild type. These results identify *Mtzpt2-1* as a molecular marker potentially linked to stress tolerance in *M. truncatula* and suggest its participation in a transcriptional program induced in these plants to cope with salt stress.

Introduction

Salinity problems in agriculture represent a major constraint in the productivity of crops and pastures such as alfalfa. It constitutes an important threat in several areas of the planet, notably the tropics and Mediterranean regions (Frommer et al., 1999; Hasegawa et al., 2000). Increasing salt concentrations in soils lead to marked changes in the growth pattern of plants and in legumes, additionally, affect the symbiotic nitrogen fixation process due to the interaction with the soil bacterium rhizobia. Legumes, like most plants, are very sensitive to salt levels in soils and experience water deficit due to osmotic stress, coupled to biochemical perturbations induced by the influx of sodium ions. There are only very few molecular markers identified that can be associated to salt tolerance in legumes (Arrese-Igor et al., 1999; Zahran, 1999).

The formation of nitrogen-fixing nodules results from an interactive process between the legume and the rhizobia, in which signal molecules discovered in the last decade, play a decisive role. Flavonoids present in the root exudate induce the expression of rhizobial nodulation genes (*nod*) that produce nodulation (Nod) factors, which in turn induce nodule organogenesis in the legume root (Crespi and Galvez,

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2001). The bacteria of the genera *Rhizobium* and *Bradyrhizobium*, are generally more salt tolerant than their legume hosts. Growth of *Sinorhizobium meliloti* strains were found to tolerate up to 700 mM NaCl, concentrations strongly affecting plant growth (Del Papa et al., 1999). On the plant side, varieties of single plant species exhibit a high degree of variation in salt tolerance suggesting that only few key genes might confer salt tolerance to plants. The physiological consequences of environmental stresses in legumes had been investigated (Arrese-Igor et al., 1999). During salt stress, water scarcity leads to stomatal closure preventing water loss by transpiration. However, increased stomatal resistance to gas diffusion also diminishes O_2 and CO_2 uptake and consequently, respiration and photosynthesis. This results in reduced photosyntate production influencing the carbon metabolism of the root nodule. Furthermore, nodules have an O_2 diffusion barrier located in the cortical cell layers that is also affected by salt stress, an effect that also might be due to the diminished carbon metabolism in the nodule (Hunt and Layzell, 1993). On the other hand, very little is known about the molecular mechanisms involved in these physiological responses. Salt-stressed plants induce specific gene responses in order to adapt cells and tissues for this restrictive condition. Genes induced during salt stress are good candidates to be involved in osmotolerant responses in plants, particularly those putative regulatory genes such as transcription factors or protein kinases as has been shown in other organisms (Hasegawa et al., 2000). Putative zinc-finger transcription factors were found among the salt-inducible transcripts in several plants suggesting that specific osmotolerance programs are induced in order to cope with salt stress (Shinozaki et al., 1997; Winicov et al., 1999).

We have identified a Krüppel-like transcription factor in alfalfa and *M. truncatula* that is induced in vascular tissues of roots and nodules. Expression of this gene confers salt tolerance to yeast cells. *M. truncatula* transgenic lines expressing this gene in an antisense configuration are unable to develop nitrogen-fixing nodules (Frugier et al., 2000). Here, we show that this gene is induced by salt stress in ground tissues and that the antisense *Mtzpt2-1* lines are less able to trigger a recovery process after salt stress. These results suggest that *Mtzpt2-1* is involved in a salt-stress transcriptional program induced in these plants.

Materials and methods

Plant material and treatment

M. sativa cv. Sitel, *M. truncatula* cv. 108-R seeds were sterilised as described and germinated in low nitrogen i medium (Charon et al., 1999). Seedlings were grown vertically in growth chamber at 24 °C under a 16-h light period. Treatments of 3-day-old plantlets (roots measured between 2.5 and 3.5 cm) were done in fresh medium supplemented with different concentrations of NaCl (50 to 150 mM) or mannitol (150 mM). Material was collected at the indicated time points and immediately frozen in liquid nitrogen for further analysis.

For recovery experiments, *M. truncatula* seedlings were grown for 3 days on porous paper on agar i medium to allow plants to be transferred easily (without breaking root hairs) to a new medium containing 100 or 150 mM NaCl at variable times. After different days of osmotic stress, plants were transferred to fresh medium. To establish the point of recovery, root length was measured all along these treatments. Three independent transgenic antisense *Mtzpt2-1* lines (Frugier et al., 2000) were used to compare recovery experiments.

For measuring the effect of salt concentration on nodulation of *M. truncatula* plants by the salt-tolerant *S. meliloti* strain LP63 (Del Papa et al., 1999), eight 2-day-old seedlings were transferred to gamma-irradiated sterilized plastic pots containing vermiculite. This support was previously washed and embedded in low nitrogen medium two times in the absence or presence of different NaCl concentrations. Five days later, primary roots were inoculated by dipping 0.2 mL of bacterial suspension (DO 0.6). After 4 weeks plants were unearthed and the number and distribution of nodules was scored on all plants.

For salt effects on nodule expression, plants containing 3-week nodules were unearthed and planted in hydroponic conditions. After two washings and 1 day of recovery, salt treatment by adding medium with NaCl 150 mM for different times. Root nodules were collected and frozen for Northern analysis.

RNA extraction and analysis

Total RNAs were extracted from frozen organs of treated *M. sativa* roots as described (Charon et al., 1999). RNA from roots from *M. sativa* and *M. truncatula* were prepared as described (Jimenez-Zurdo et al., 2000) and used to synthesize cDNA or for North-

ern analysis. Semiquantitative RT-PCR has been done as described (Charon et al., 1999). Briefly, total RNA (2 μg) pre-treated with Dnase (RQ1 Rnase-free Dnase, Promega, France) was reverse transcribed with Superscript reverse transcriptase (Superscript II Rnase H-Reverse Transcriptase, Gibco-BRL, France) using an oligo-dT primer. cDNAs were amplified with specific primers for different PCR cycles depending on the gene being analysed (17–20 for *Msc27*, 25 for *Mszpt2-1*). Primers for *Mszpt2-1* were:

ZN5: 5'-CTT GCG TAA CGC TAA CTA ACT CT-3'

ZN3: 5'-AAG TCC GGA AAA GCC GGG AG-3'

Msc27 was used as a constitutive control of gene expression in *Medicago* ssp. roots. Amplified cDNA were subjected to electrophoresis, blotted to nylon membrane (Hybond-N, Amersham, France) and hybridised to specific radio-labelled DNA probes. Probes were generated by random priming using the Megaprime labelling system kit (Amersham Pharmacia Biotech, Uppsala, Sweden) from PCR products amplified from *Mszpt2-1* clone (Frugier et al., 2000). All RT-PCR experiments concerning osmotic stress treatments were done at least in duplicate.

Results

A Krüppel-like transcription factor is induced by salt stress in roots

Previous results suggested that the *Mszpt2-1* gene may be involved in osmotolerance in yeast (Frugier et al., 2000). We have studied the expression pattern of this gene in germinating alfalfa seedlings using RT-PCR. Strong expression was detected early after germination (Figure 1) during root development, whereas a significant reduction in transcript levels could be detected 6 h after plating onto agar plates, an expression level that remained low in roots. Then, 3-day-old seedlings (T0) were submitted to various environmental stresses and examined in a time course sampling. Both salt and osmotic stresses induced accumulation of *Mszpt2-1* transcripts in roots. Induction by salt stress was detected 6 h after the treatment (Figure 1) with 150 mM NaCl, being maximal at 24 h. About 10-fold increase in transcript levels was detected in comparison to the *Msc27* control. Under these conditions, root elongation and growth were completely arrested (Figure 1A). In all cases, the level of gene induction was normalised using the *Msc27* gene as a control of constitutive expression. This response of *Mszpt2-1* to salt stress was found to be detectable in a range of NaCl concentration between 50 and 150 mM (Figure 1B). In addition, induction was also detected in response to hyperosmotic stress provoked by treatment of the seedlings with 150 mM mannitol, which did not affect root growth. This induction was evident from 3 h of treatment and was maximal at 24 h (Figure 1C). Thus, the *Mszpt2-1* gene codes for a transcription factor involved in osmotic stress responses and early steps of germination of alfalfa.

Genes are rapidly induced by salt stress in nodules

Since salt stress also significantly affects the function of the nodule, we explored the expression of *Mtzpt2-1* in nodulated roots from the model legume *M. truncatula* treated with salt for different times (Figure 2). We also studied the expression of the genes sucrose synthase *Mtsusy* and carbonic anhydrase *Mtca1*. These are two nodule molecular markers which are associated with different functional processes in nodules; carbon metabolism and the function of the inner cortex cell layer, respectively (Coba de la Pena et al., 1997; Hohnjec et al., 1999). Induction levels were normalised against *Mtc27*. Roots harbouring 3-week-old nodules (from plants grown under normal conditions) were transferred to a liquid medium containing 150 mM NaCl. Strong induction of *Mtzpt2-1* was rapidly induced 30 min after treatment. The induction was so high that expression could be directly monitored by Northern analysis, whereas transcript levels were undetectable in these root and nodule samples at the initial time point. In addition, *Mtsusy* was also induced by salt treatment in these samples. Induction of its expression could be detected after 30 min (Figure 2). In contrast, a slight induction of *Mtca1* was found, notably at earlier time points. This induction was much reduced in comparison with the *Mtzpt2-1* and *Mtsusy* genes.

These results indicated that *Mtzpt2-1* is rapidly induced by high levels of salt also in *M. truncatula* nodulated roots.

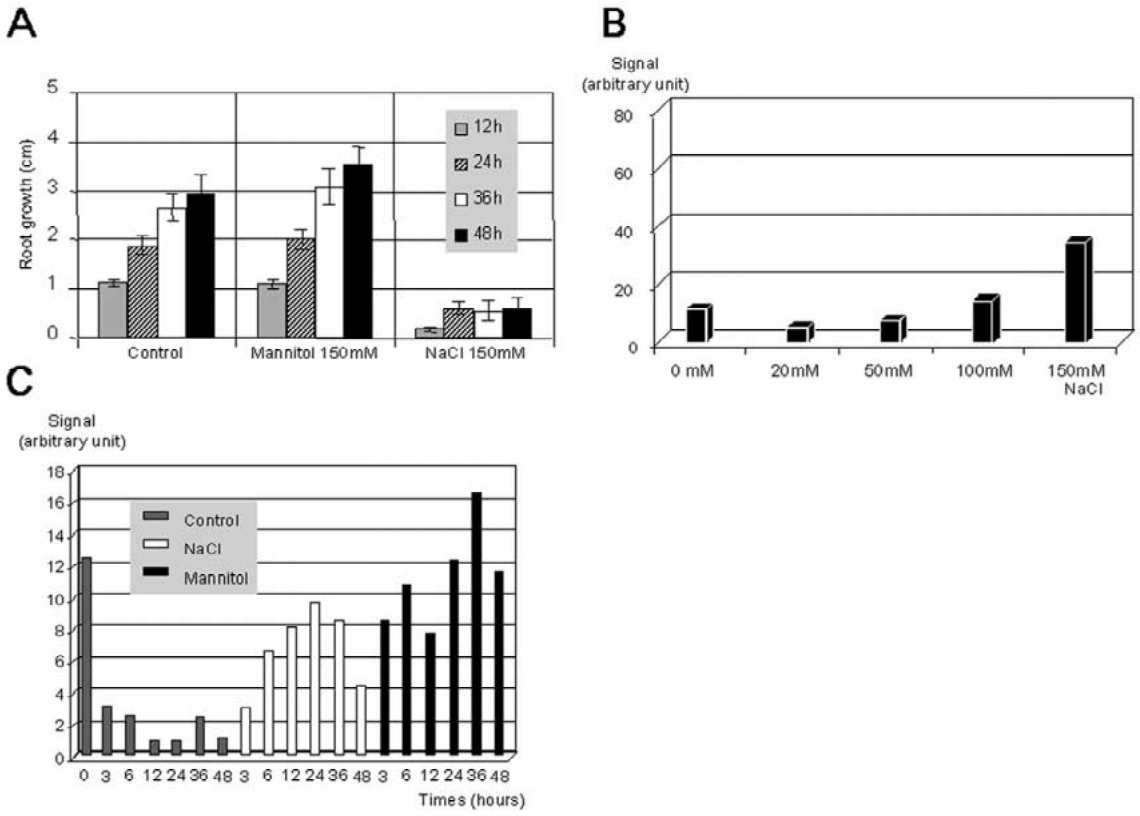

Figure 1. Induction of *Mszpt2-1* expression during osmotic stress in alfalfa. (A) Root elongation of plantlets submitted to stresses on agar plates. Roots were measured 12, 24, 36 and 48 h after plating on medium supplemented with NaCl (150 mM) and mannitol (150 mM). (B) *Mszpt2-1* expression after treatment of *M. sativa* seedlings with different stresses. *Mszpt2-1* expression was analysed by RT-PCR of root RNA samples collected 48 h after treatment with the indicated NaCl concentrations. *Msc27* expression was used as constitutive control. Histograms show relative quantification of *Mszpt2-1* transcript levels in arbitrary units normalised through the *Msc27* signal. (C) RT-PCR analysis of *Mszpt2-1* expression of root RNA samples collected 3, 6, 12, 24, 36 and 48 h after a 150 mM NaCl or 150 mM mannitol treatment. Histograms show relative quantification of *Mszpt2-1* in same conditions as (B).

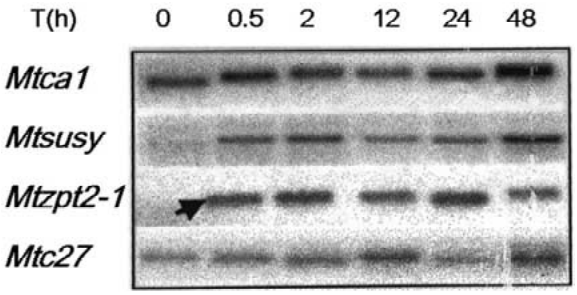

Figure 2. Effects of salt stress on root nodule gene expression. A mixture of roots and nodules from plants 3 weeks after inoculation was treated with 150 mM NaCl for different times. Northern analysis against the indicated probes using 10 μg of total RNA is depicted. Arrows indicate maximal induction levels for *Mtzpt2-1*.

Salt stress affects root growth and the symbiotic interaction in M. truncatula

To better understand the negative effect of salt stress on nodulation of the model plant *M. truncatula*, we have examined root growth and nodulation capacity in response to a salt stress treatment.

M. truncatula plants cv. 108R were highly sensitive to the presence of salt in agar plates using a paper-based growth system. Root growth was completely inhibited by treatment with 150 mM (Figure 3A). However, after removing these plants from this medium and planting them on normal medium agar plates, growth could be fully restored.

In order to study the nodulation capacity of these plants under salt stress, *M. truncatula* plants were grown in the presence of different NaCl concentrations and inoculated with a salt-tolerant *S. meliloti* strain,

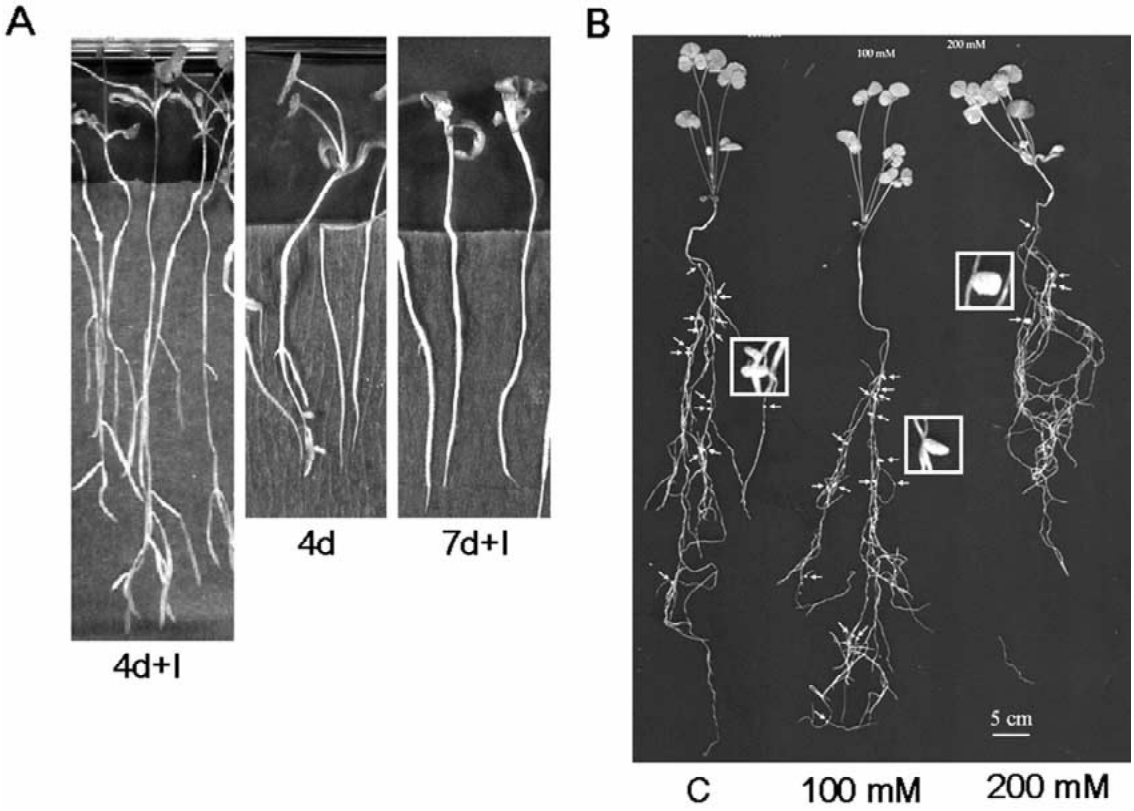

Figure 3. Salt stress affects root growth and the symbiotic interaction in the model legume *M. truncatula.* (A) *M. truncatula* 108R plants were grown on porous paper deposited onto agar plates for 3 days (time zero). Plantlets were divided in two groups: (1) plants treated for 4 days with salt medium (4d) and subsequently transferred for 3 days to normal low nitrogen medium (4d + I, meaning 7 days after time zero); (2) plants treated for 7 days in salt medium and then transferred for 3 days to growing medium without salt (7d + I, meaning 11 days after time zero). Plants 7 days on salt (not shown) have a similar size and morphology as the plants 4d. After 4 days of salt root growth could be recovered, whereas 7 days of salt treatment prevented further root growth on normal medium. (B) Differences in number, form and distribution of nodules produced in *M. truncatula* under salt stress. Representative plants grown under 0, 100 and 200 mM NaCl are shown. Nodules are indicated by arrows. Amplification of the biggest nodule in each plant is shown closed to the corresponding root. Note that roots in 200 mM treated-plants are shorter than control ones.

LP63 (Del Papa et al., 1999). Three weeks after inoculation, the plants were assessed for root nodulation. Under the plant growth conditions applied (vermiculite), roots could develop even with 200 mM NaCl; however, roots were shorter than control non-treated plants (Figure 3B). As shown in Figure 4, a negative effect on nodule formation was clearly observed when increasing salt concentration in the medium. Particularly, a drastic reduction in the total number of nodules was observed in the roots maintained at 200 mM of salt. Roots submitted to high concentrations of salt (100 and 200 mM of NaCl) presented less nodule clusters than control plants. Moreover, the few nodules produced in the presence of 200 mM NaCl had a large size and were localised mainly in the upper part of the root (Figure 3B, arrows). This suggests that nodule initiation is affected by salt stress in roots. In addition, at high salt levels (200 mM NaCl), plants displayed typical symptoms of salt stress such as shorter roots, smaller size and yellow leaves, symptoms likely associated to the poor nitrogen fixation of these plants.

The arrest of root growth looked to us as a convenient way of monitoring salt responses and plant recovery from stress in *M. truncatula*. We have then defined for *M. truncatula* 108R, the point marking the level of general damage beyond which the plant tissue can not longer be recovered from the salt stress. This experiment was performed by treating plants for several days with salt (150 mM NaCl), and subsequently transferred them to normal growth medium to test their ability to recover from this stress. In three independent experiments, plants stressed for 4 days showed around

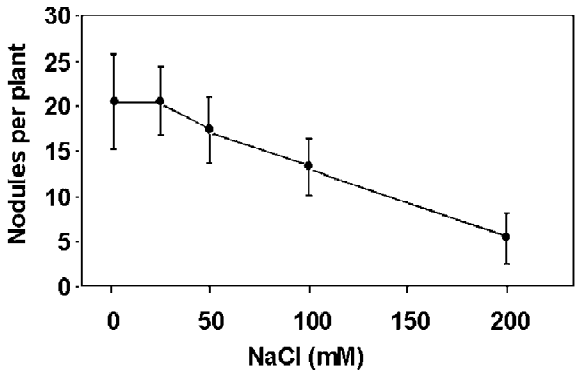

Figure 4. Salt stress affects nodulation on *M. truncatula.* The effect of salt concentration on nodulation of *M. truncatula* by the salt-tolerant *S. meliloti* strain LP63 is given as mean of the total nodule number observed 21 days post inoculation. *M. truncatula* plants were grown under different salt concentration (0, 25, 50, 100 and 200 mM NaCl). Standard deviations of the values are indicated.

a 95% recovery when transferred to normal medium (Figure 5), whereas a 7-day treatment yield a majority of plants that could not recover (more than 75%). A representative pattern of root growth is illustrated in Figure 3A. Intermediate time points yield more variable percentages of recovery (Figure 5 and data not shown). Hence, this treatment was used to compare the response of *M. truncatula* cv. Jemalong, which is known to be salt-tolerant. After 7 days of treatment, Jemalong plants resumed normal root growth in contrast to 108R plants (Figure 5).

Thus, we have determined the effects of salt stress on root growth and nodulation capacity of the model legume *M. truncatula*. Using these conditions we monitor the recovery capacity of different cultivars and confirm that cv. Jemalong is more tolerant than 108R.

Antisense Mtzpt2-1 *transgenic plants recover more slowly than control plants*

Earlier we have generated antisense *Mtzpt2-1* transgenic *M. truncatula* plants and showed that they were unable to form nitrogen-fixing nodules (Frugier et al., 2000). Hence, nodulation capacity could not be used to monitor their differential response to salt stress. We then monitored root growth of these plants in comparison with control plants (transformed with an empty vector) under normal conditions and during salt stress treatments and recovery processes. Even though the antisense plants had slightly better growth under normal conditions, no significant differences could be detected between control and *Mtzpt2-1* antisense plants after 5 days of salt treatment (Figure 6). After treatment with salt, both types of plants were transferred to normal medium. Using three independent transgenic lines, we observed that root growth was significantly reduced starting from 2-day of recovery in the antisense plants. This effect lasted for at least 4 days (Figure 6B and data not shown). These results strongly suggest that *Mtzpt2-1* is required for the recovery process from salt stress in *M. truncatula*.

Discussion

Possible approaches to improve crop productivity in saline soils require a better understanding of the mechanisms involved in the response to salt stress. Several genes that respond to high salt levels and other environmental stresses have been recently identified, and their encoded proteins were thought to play a role in protecting cells from these stresses (Hasegawa et al., 2000). The plant responses and adaptation involve a range of biochemical and gene expression changes, including the synthesis of stress hormones like abscisic acid. A particular set of genes, encoding salt-inducible transcription factors regulates expression of many other salt-inducible genes (Espartero et al., 1994; Shinozaki et al., 1997; Winicov et al., 1999). In this work we were able to demonstrate that the *M. truncatula* gene *Mtzpt2-1*, which codes for a Krüppel-like transcriptional factor was activated under conditions of high salt concentrations and osmotic stress both in roots containing nodules and young seedling roots. Even though we used a semiquantitative RT-PCR approach, the strong observed differences at several time points were very significant. As it was demonstrated for the case of other plant genes, cell viability assays in yeast cells allowed to demonstrate *Mszpt2-1* to be functional in conferring osmotolerance to yeast (Frugier et al., 2000; Matsumoto et al., 2001). These results and those previously reported on numerous signal and signal-like molecules identified as putative mediators of osmotic adaptation, provide a view on the complexity of plant signalling factors involved in the salt response of the plant (Hasegawa et al., 2000). Recently, the *alf1* transcription factor was shown to be able to induce osmotolerance programs by overexpression in alfalfa cells and transgenic plants (Winicov et al., 1999). Nevertheless, the use of constitutive promoters for expression of a transcription factor has been shown to affect plant development in non-specific ways by cross-interaction on the regulat-

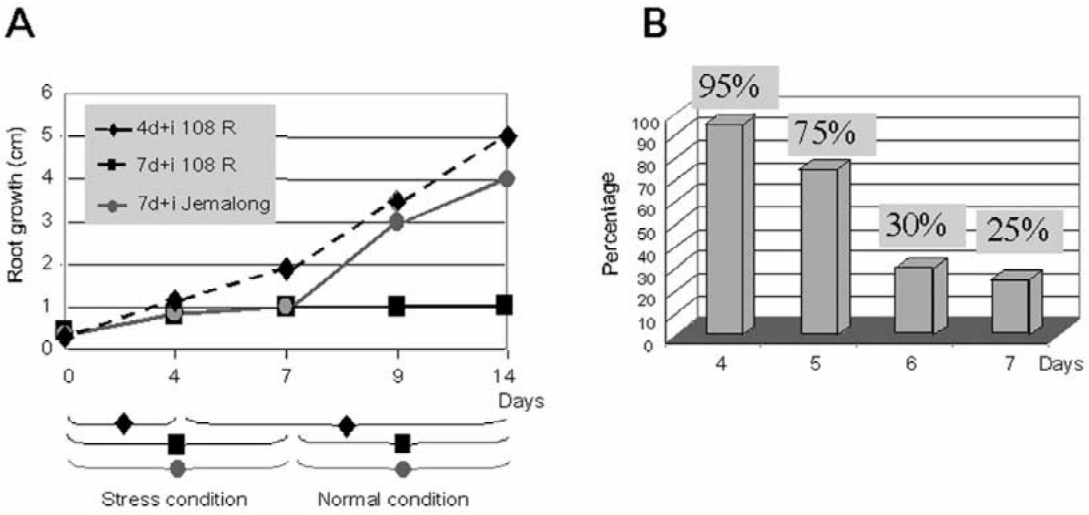

Figure 5. Recovery curves for 108R and Jemalong. (A) *Medicago* plants cv. 108R grown in salt medium for 4 or 7 days, were subsequently transferred to i medium (black discontinuous and black lines, respectively). Plants from cv. Jemalong were grown during 7 days in salt medium and then transferred to medium i. These plants resumed root growth after 7 days of salt stress, in contrast to 108R plants. (B) Percentage of plants showing the recovery response shown in (A) after several days of salt treatment. Histograms show percentage of *M. truncatula* plants that recovered after treatment with NaCl 150 mM for different days.

ory networks of the cell (Liu et al., 1998). This has been overcome by using strong stress-inducible promoters (Kasuga et al., 1999). Indeed, overexpressing *Mszpt2-1* plants are sterile and yield non-viable reproductive organs (Frugier et al., 2000). Nevertheless, by examining the level of transcripts during salt stress and using antisense transgenic plants, we demonstrate that *Mszpt2-1* is part of a signal transduction pathway required, at least, for plant recovery after salt stress. It has also been shown that antioxidant compounds also help to improve recovery of stressed plants, undermining the role of oxidative stress in cell damage due to salt stress (Shalata and Neumann, 2001).

The pattern of activation and suppression of gene expression during salt stress and plant recovery will lead to a better understanding of the interrelationships of the multiple signalling systems that control stress-adaptive responses in plants. Significant progresses have been or are being accomplished at the genetic and genomic levels of the model legume *M. truncatula*, such as macro- and microarray analysis allowing the screening of large number of transcripts and detailed genetic mapping of crosses between salt-tolerant and sensitive varieties (Oldroyd and Geurts, 2001). Different *M. truncatula* cultivars show variable levels of recovery for salt stress, which may serve to characterise gene responses in a comparative way. These approaches coupled to transgenic strategies on selected genes may serve to define useful markers for studying salt stress in this legume, that could be later exploited in agriculturally important legumes.

Legumes are interesting candidates for improving saline soil fertility due to their capacity to grow on nitrogen-poor soils through the symbiotic interaction with rhizobia (Crespi and Galvez, 2001). However, this interaction can be specifically affected in saline soils, as shown for several legumes (Arrese-Igor et al., 1999; Zahran, 1999). Nodulation capacity and nodule function are adversely affected even by mild stress conditions which have no adverse effect on plant growth depending on combined nitrogen. The identification of markers to assist breeders in the selection of varieties more tolerant to salinity, and able to perform efficient symbiosis, may represent ways to overcome these limitations. Genes induced in nodules during salt stress are good candidates to be involved in osmotolerant responses in this organ, particularly those putative regulatory genes such as transcription factors or protein kinases as has been shown in other organisms (Hasegawa et al., 2000). We think that *Mszpt2-1*, a gene involved in the salt-induced response of roots and nodules, could be used to screen alfalfa cultivars for salt tolerance and potentially offer new perspectives for plant breeding. Since about 40% of the worlds land surface can be categorized as having potential salinity problems, any improvement in salt tolerance by plants will have a signficant impact worlwide.

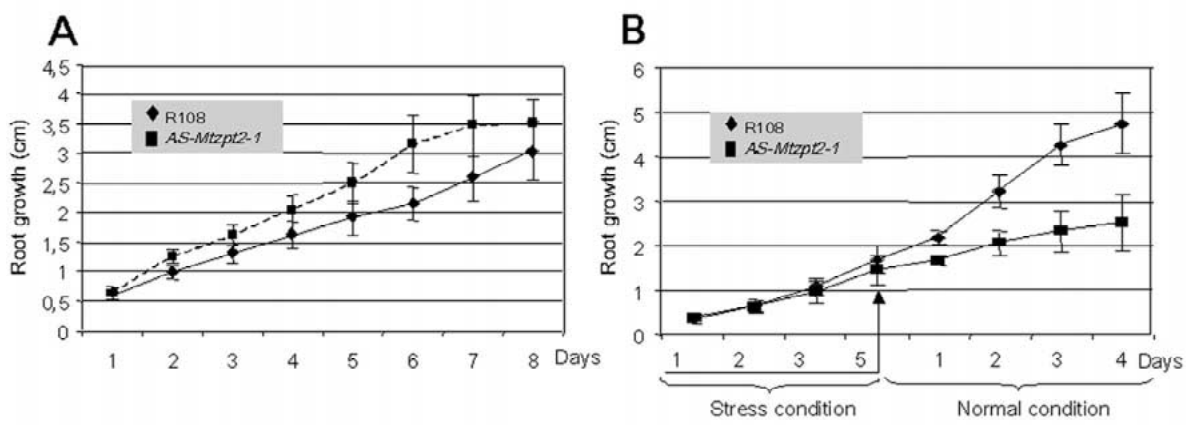

Figure 6. Mtzpt2-1 antisense plants recover more slowly than control plants. (A) *M. truncatula* cv. 108R and *AS-Mtzpt2-1* plants were grown in i medium (under normal conditions) for 8 days. Root growth of antisense plants is slightly better than that of control plants. (B) *M. truncatula* cv 108R (as control) and *AS-Mtzpt2-1* plants were grown in medium supplied with 100 mM NaCl for 5 days and then transferred to i medium for 4 days. Root growth was diminished in antisense plants. These experiments gave very similar results using three independent transgenic antisense lines. We show a representative curve for one series of transgenics.

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