A Krüppel-like zinc finger protein is involved in nitrogen-fixing root nodule organogenesis

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Mechanisms regulating plant host differentiation of the nitrogen-fixing root nodules remain mostly unknown. *Sinorhizobium meliloti* induces this process in *Medicago sativa* in which the *Mszpt2-1* gene is expressed in vascular bundles of roots and nodules. This gene codes for a Krüppel-like zinc finger protein, a class of transcription factors involved in many animal developmental processes. Expression of *Mszpt2-1* in yeast cells conferred osmotic tolerance. Antisense plants grew normally but developed nonfunctional nodules, in which differentiation of the nitrogen-fixing zone and bacterial invasion were arrested. Hence, a vascular bundle-associated Krüppel-like gene is required for the formation of the central nitrogen-fixing zone of the root nodule.

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Bacteria of the *Rhizobiaceae* family can induce the development of a symbiotic nodule on the roots of leguminous host plants. In this organ rhizobia are able to fix atmospheric nitrogen. Symbiotic nodule development is controlled by specific signal exchanges between the two partners (see Schultze and Kondorosi 1998, and references therein). This organogenesis starts with the entry of rhizobia into the root through infection threads and the division of inner cortical cells. Bacteria are then released from the infection threads into the cytoplasm of the primordium cells and differentiate into nitrogen-fixing bacteroids. In alfalfa, nodules of indeterminate type are formed where distinct zones can be observed, following a differentiation gradient: a persistent apical meristem (zone I), an invasion zone (II) where infection by *Sinorhizobium meliloti* occurs, an interzone II–III that accumulates amyloplasts, a central or symbiotic zone (III) where nitrogen fixation takes place in the bacteroids, and a senescent zone (IV; Vasse et al. 1990). These central tissues are surrounded by peripheral cell layers, containing vascular bundles connecting the nodule to the root.

Plant mutants affected in their symbiotic behavior were isolated in several leguminous species (Schultze and Kondorosi 1998), but the first regulatory gene has only been cloned very recently (Schauser et al. 1999). Numerous genes coding for transcripts induced at differ-

ent stages of nodule development, the so-called nodulin genes, have been characterized (Bladergroen and Spaink 1998). However, their role in nodulation has been investigated using functional approaches only in few cases (Cheon et al. 1993; Csanadi et al. 1996; Charon et al. 1999). Hence, little is known about regulatory genes controlling nodule organogenesis.

Krüppel-like (or TFIIIA) zinc finger proteins appear to be members of the largest family of eukaryotic transcription factors (300–500 are estimated to be encoded in the human genome; Bray et al. 1991), which play central roles in various tissue-specific differentiation as well as general housekeeping processes. In plants, several TFIIIA zinc finger proteins have been described and certain members of the petunia EPF-type family showed DNA-binding activity (Takatsuji 1998). Only two plant TFIIIA genes, *SUPERMAN* in *Arabidopsis* and *INDETERMINATE* in maize, have been functionally characterized. A mutation in *SUPERMAN* caused homeotic changes in floral organs (Sakai et al. 1995), whereas *INDETERMINATE* mutants showed delayed flowering, suggesting its involvement in the vegetative to floral transition of the meristem (Colasanti et al. 1998). Plant Krüppel-like genes are expressed predominantly in flowers, although transcripts of certain members were detected in vegetative tissues such as roots (Takatsuji 1998).

Here we demonstrate that a Krüppel-like zinc finger gene is strongly induced in the vascular tissues surrounding the symbiotic root nodule. Antisense *Mszpt2-1* plants were affected in the formation of the central ni-

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trogen-fixing region of this organ, suggesting that this gene is involved in a non-cell-autonomous mechanism controlling root nodule differentiation.

Results

Previously we have identified several transcripts induced during nodulation of alfalfa (*Medicago sativa*; Frugier et al. 1998). One of these, *Mscp17* (renamed *Mszpt2-1*), codes for a putative Krüppel-like Cys-2/His-2 zinc finger protein (Fig. 1A). Sequence analysis of the complete *Mszpt2-1* cDNA revealed best similarities to *Zpt2-3* from petunia (61% identity, 70% homology; Takatsui et al. 1994), to *Stz* and *Atzpt2-10* (renamed from *Zat10*) from *Arabidopsis* (53% identity, 65% homology; Lippuner et al. 1996; Meissner and Michael 1997), and to *Scof-1* from soybean (67% identity, 75% homology; Genbank accession no. U68763), indicating that it belongs to the EPF-type family. These genes code for Krüppel transcription factors divergent from *SUPERMAN* and *INDETERMINATE*, and contain two canonical Cys2/His2 zinc finger motifs (CX₂CX₃FX₅LX₂HX₃H consensus; Fig. 1A, residues 86–106 and 142–162), carrying the invariant QALGGH motif (residues 97–102 and 152–157) specific to plant EPF-type zinc finger proteins (Takatsui 1998). Mutation in this domain of the homologous EPF-

type gene *Zpt2-2* abolished the DNA-binding activity of the encoded protein (Kubo et al. 1998), whereas in *SUPERMAN*, it led to altered flower development (Sakai et al. 1995). Other features of the *Mszpt2-1* protein are (1) the presence of a hydrophobic region in the carboxy-terminal part (the DLNL box, residues 192–202) conserved in most TFIIIA plant zinc fingers and likely involved in protein–protein interactions, and (2) a putative nuclear localization signal in the amino-terminal part (NLS box, consensus: KR/KXR/K, Chelsky et al. 1989; residues 34–40). Southern blot analysis under stringent conditions with the *Mszpt2-1* probe revealed one strongly and another faintly hybridizing band in the diploid *Medicago truncatula*, whereas two main bands associated to two fainter ones were detected in the tetraploid *M. sativa* (data not shown). Thus, *Mszpt2-1* seems to be present as a single copy gene in the diploid species, even though a related gene might exist.

The closest *Arabidopsis* *Mszpt2-1* homolog, *Stz*, has been found to induce osmotic tolerance in yeast cells (Lippuner et al. 1996), a function that we tested by expressing *Mszpt2-1* (using the yeast-expressing vector pREP41; Maundrell et al. 1990) under the control of a promoter repressible by thiamine (data not shown). On selective minimum medium containing either 1.4 M NaCl (not shown) or 1 M LiCl (Fig. 1B), the strain expressing *Mszpt2-1* was able to grow efficiently, in contrast to the same strain grown under noninducing conditions, or the strain transformed with pREP41. This result indicates that ectopic expression of *Mszpt2-1* is able to increase osmotic stress tolerance in yeast. Moreover, transcriptional activation of the osmotic tolerant yeast gene *ena1*, coding for the major sodium/lithium efflux system (Rudolph et al. 1989), was detected in the *Mszpt2-1*-expressing strain (Fig. 1C).

Mszpt2-1 expression was analyzed in different alfalfa organs (Fig. 2A). Basal expression levels were detected in vegetative tissues, for example, roots, stems, and leaves. Higher levels of the *Mszpt2-1* transcript were present in flowers, as well as in spontaneous nodules and nodules induced by *S. meliloti* but not in roots infected with the nonnodulating strain ZB138 (Nod⁻). The expression pattern of *Mszpt2-1* in wild-type nodules, as well as in those arrested at different stages of development, was investigated by using the wild type (Sm41) and different mutants of *S. meliloti*. The surface polysaccharide mutant strain AK1492 (Eps⁻Kps⁻) is unable to invade root cortical cells and forms nodules devoid of bacteria, whereas the Sm8368 strain (Bac⁻) cannot differentiate into nitrogen-fixing bacteroids but is able to infect and invade plant cells (Kondorosi et al. 1984; Glazebrook et al. 1993). Upon infection with the invading strains Sm41 or Bac⁻, accumulation of the *Mszpt2-1* transcripts was strongly enhanced in the root region adjacent to the nodule (Fig. 2B) In contrast, the noninvading Eps⁻Kps⁻ strain showed similar *Mszpt2-1* transcript levels in both roots and nodule tissues. These results indicate that even though *Mszpt2-1* induction is associated with nodulation, the strong activation observed in the surrounding root tissues required bacterial invasion of the plant cells.

Figure 1. (A) Deduced amino acid sequence from the *Mszpt2-1* cDNA (EMBL accession no. Y18788). The NLS box corresponds to a putative nuclear localization signal (basic residues; consensus, KR/KXR/K). The two Krüppel-like or TFIIIA zinc finger motifs are shown (consensus, CX₂CX₃FX₅LX₂HX₃H); underlined amino acids indicate the highly conserved residues of EPF-type zinc fingers; asterisks show the plant-specific QALGGH motif. The DLNL box is a hydrophobic region corresponding to a putative protein–protein interaction motif. (B) Yeast cells carrying *Mszpt2-1* in pRep41 or only the empty vector (control) were grown in a medium with or without 1 M LiCl. (C) RT-PCR analysis of *ena1* expression in yeast cells corresponding to the strains shown in B grown in the absence of LiCl.

Figure 2. Northern blot analysis of *Mszpt2-1* expression in different organs of alfalfa plants. Total RNAs (10 μ g) were extracted from nodules and roots infected with a non-nodulating strain (Root Nod⁻) (A, left) and roots, mature *S. meliloti*-induced symbiotic nodules, spontaneous nodules (NAR phenotype: Nodulation in Absence of Rhizobium, Truchet et al. 1989), stems, and flowers (A, right), as well as roots grown on a nitrate-containing medium, and roots and nodules 3 weeks after infection with a wild-type Sm41, a Bac⁻ (Sm8368), or an Eps⁻Kps⁻ (AK1492) mutant strain (all infections were done in low nitrogen medium). (B) In all cases, *Msc27* was used as control of RNA loading (Pay et al. 1992), and histograms (below) show the relative quantification of *Mszpt2-1* transcript levels in arbitrary units (a.u.). The broken line indicates the transcript level corresponding to the uninoculated root RNA sample (1 a.u.).

The spatial expression pattern of *Mszpt2-1* was determined using in situ hybridization (Fig. 3). A signal associated with the vascular bundles from both roots and nodules was observed. Notably, the closest petunia homolog, *Zpt2-3*, is expressed in vascular bundles of floral tubes and sepals (Takatsui et al. 1994).

To analyze potential functions of this Krüppel-like gene in plant organogenesis, we constructed *M. truncatula* lines expressing *Mszpt2-1* in sense orientation or an antisense DNA fragment of the homologous *Mtzpt2-1* gene (for *M. truncatula*, see Materials and Methods), in both cases under the control of the strong constitutive 2x35S CaMV promoters. After in vitro regeneration, the primary transformants of either sense or antisense plants were not disturbed in their growth characteristics when compared with plants transformed with the control vector alone. However, 15 primary transformants (T_0) of 16 sense plants showed an abnormal flower phenotype. These flowers had an unusually long style compared to

the anther size that became dehydrated rapidly (data not shown), leading to inefficient pollinization. Only a few pods were produced (18 on average, vs. ~150 in the wild type) containing few seeds per pod (1 on average, vs. 4–7 in wild type). The antisense plants did not exhibit any abnormal flower morphology. These results suggest that the floral phenotype observed on the sense plants is likely due to the ectopic overexpression of *Mszpt2-1* in flowers.

Because *Mszpt2-1* expression was enhanced during nodule development and no alterations in growth or floral phenotypes were detected on the antisense plants, nodulation assays were performed on their progeny. Descendants (T_1) from eight independent T_0 lines (carrying one to three T-DNA insertion loci, as confirmed by Southern analysis; data not shown) were nodulated with Sm41. In three separate experiments, nodules from several T_1 plants (25%–29%) obtained from three independent T_0 lines were unable to fix nitrogen (Fig. 4A), in contrast to the control plants. Fifteen days after infection, both plant types had similar growth characteristics (Fig. 4A, left), and no significant differences either in leaf number or root length could be observed between antisense and control plants (data not shown). Nevertheless, only small white Fix⁻ nodules could be detected on roots of these antisense plants, whereas typical Fix⁺ nodules (that emerged about 12 d.p.i.) were seen on all plants carrying the control vector construct (Fig. 4B). The Fix⁻ phenotype was not due to any delay in nodulation, as even 1 month after infection, these plants showed severe starvation symptoms (Fig. 4A, right) and reduced weight of their aerial parts (0.18 ± 0.04 gram/plant in contrast to 1.1 ± 0.3 gram/plant for the controls), indicating strong limitations in their nitrogen nutrition. However, after transfer to a medium containing combined nitrogen, the plants were completely recovered and continued their normal development until flowering (data not shown). This indicated that the plant phenotype was due to nitrogen starvation induced by a defect in symbiotic nitrogen fixation.

The antisense fragment used spans the zinc finger regions of *Mtzpt2-1*. To analyze the specificity of our approach for *Mtzpt2-1*, (1) we searched for the expression of homologous gene(s) in nodules or root RNA samples by RT-PCR, Northern analysis, or library screening even under low stringency conditions (data not shown), and (2) Northern hybridizations with strand-specific probes were performed to measure the amounts of antisense and sense *Mtzpt2-1* transcripts in infected roots and nodules of several individuals originating from different plant lines. In these experiments, expression of homologous genes could not be detected in nodules. Moreover, a direct correlation was found between the Fix⁻ phenotype, the reduction of the sense transcript level, and the increase in antisense expression (Fig. 4C). A threshold level of *Mtzpt2-1* expression could be observed beyond which the plants were Fix⁻. The differences in the antisense and sense transcript levels between the Fix⁺ and Fix⁻ populations were shown to be statistically significant ($P < 0.01$, using Student's *t*-test). Furthermore, the coseg-

Figure 3. Localization of *Mszpt2-1* expression in alfalfa roots and nodules infected with Sm41 by in situ hybridization. Sections (8 μ m) were hybridized with digoxigenin-labeled *Mszpt2-1* riboprobe. (A–C) longitudinal sections of mature symbiotic nodules (20 d.p.i.), containing transversal sections of contiguous roots. (A) Epifluorescence illumination, showing the nitrogen-fixing zone (III, Vasse et al. 1990) and the root vascular bundles (VB); (B) Consecutive section of A hybridized with the *Mszpt2-1* antisense probe, viewed in bright-field illumination (arrow indicates signal localization); (C) the same section as A hybridized with the *Mszpt2-1* sense control probe, viewed in bright-field illumination. (D–F) Higher magnification of sections hybridized with the *Mszpt2-1* antisense probe, showing details of root and nodule vascular bundles. Under epifluorescence illumination (D,F), signals (arrows) appear as quenching of autofluorescence due to precipitation of the alkaline phosphatase product. (E) Bright-field illumination of section F. Bar, 50 μ m.

regation of the Fix^- phenotype with the T-DNA was studied. In the T_1 generation (54 plants from the 3 independent lines tested), all Fix^- plants (14) were also herbicide resistant. The phenotype was stable in the T_2 generation, and no intermediate phenotypes were detected in our experiments even 1 month after infection (in the same plant, only nitrogen-fixing or -nonfixing nodules were found). Overall, these results suggest strongly that inactivation of *Mtzpt2-1* was responsible for the arrest of root nodule development in these plants.

Further analysis of the nodulation phenotype was carried out using *S. meliloti* strains containing a constitutively expressed (δ -*ala*) or a bacteroid-specific (*nifH*) *lacZ* fusion (Leong et al. 1985; Szeto et al. 1987). Bacterial infection as well as the early stages of nodule development took place normally in these plants, whereas no *lacZ* expression was detected in the central zone of young and mature nodules for both strains (data not shown), suggesting that *S. meliloti* was not able to reach these regions or to express the nitrogenase gene *nifH*. This analysis confirmed the lack of nitrogen fixation at any nodule developmental stage. By histological analysis, no typical zone III containing strongly autofluorescent cells invaded by bacteroids was detected, even though only few infected cells were present in the apical region of these nodules (Fig. 5A,B). Using lugol staining, the central region of the Fix^- nodules was shown to contain a high number of amyloplasts, in contrast to Fix^+ nodules, where amyloplasts were observed only in the interzone II–III (Fig. 5C,D). Detailed analysis using optical (Fig. 5E,F) and electron (Fig. 5G,H) microscopy showed that in the Fix^- nodules, the bacteria present in

apical infected cells did not develop beyond the early stages of bacteroid differentiation and did not display the typical radial pattern organized around a central vacuole, observed for the nitrogen-fixing symbiosomes in the central region. In addition, no differences in the morphology of vascular bundles between Fix^+ and Fix^- nodules were detected. In both cases, xylem vessels, sieve elements, and transfer cells showing characteristic wall ingrowths (Joshi et al. 1993) were observed under electron microscopy (data not shown). These results indicate that expression of the *Mtzpt2-1* gene in antisense orientation affected nodule development by reducing bacterial invasion of the plant cells and preventing differentiation of the nitrogen-fixing zone III cells.

Discussion

The analysis of antisense plants indicates that *Mszpt2-1* is specifically involved in a vegetative organogenesis, the formation of the nitrogen-fixing nodule, and suggests that this transcription factor family may act in several plant-specific developmental programs (Takatsui 1998). Overexpression of *Mszpt2-1* likely affected, either by ectopic interaction or eventually by a cosuppression phenomenon, the action of homologous genes involved in flower differentiation. Most members of EPF-type TFIIIA zinc finger proteins are expressed predominantly in flower tissues and tightly regulated in specific floral organs (Takatsui et al. 1994). Our work also suggests that certain aspects of flower and nodule differentiation might share similar mechanisms. Nodulin genes might have derived from homologs expressed in other organs

Figure 4. Nodulation phenotype of *M. truncatula* expressing *Mszpt2-1* in antisense orientation. Plants were inoculated with the wild-type strain Sm41. (A) Whole view of a control vector-transformed plant (left) and a typical *Mszpt2-1* antisense plant (right), 15 d.p.i. (left). One month after infection, a Fix^- antisense plant displayed characteristic nitrogen-deprived appearance (right). Plants were grown in aeroponical conditions, optimal for root and nodule development. (B) Higher magnification of roots from plants 30 d.p.i. showing pink nitrogen-fixing nodules (left) on control plants, compared to white Fix^- nodules on a *Mszpt2-1* antisense plant (right). Bar, 0.5 cm. (C) Correlation of *Mszpt2-1* expression and Fix^+ or Fix^- nodule phenotype in individual plants from the progeny of several T_0 lines. Total RNAs (10 μg) from roots and nodules 30 d.p.i. were probed with an *Mszpt2-1* RNA probe, and *Msc27* was used as RNA-loading control. Points and inset histograms depict, respectively, individual values and averages of the relative quantification of *Mszpt2-1* antisense (left) or sense (right) transcript levels in the plants analyzed by Northern blot. Fix^+ (red) or Fix^- (blue) values are indicated. Progeny of the three different antisense Fix^- plant lines were tested. The broken line marks a threshold separating the two plant populations (Fix^- and Fix^+).

that were recruited to participate in nodule organogenesis (Bladergroen and Spaink 1998).

To maximize the specificity of our antisense approach we used a fragment of the *Mtzpt2-1* gene in *M. truncatula*, although we cannot completely exclude that inactivation of a related gene (undetectable by the different approaches undertaken; see Results) is responsible for the observed phenotype. Nevertheless, the fact that *Mtzpt2-1* transcript levels were specifically reduced in the antisense plants, together with the induction of this gene during this process, strongly suggests that *Mtzpt2-1* is the target of our approach. Moreover, the high frequency of detection of the Fix^- phenotype in independent antisense lines (three of eight) and the linkage of the phenotype with the transgene in the T_1 and T_2 genera-

tion confirmed that this antisense construction is responsible for the Fix^- phenotype. As a means of comparison, in a large scale transformation experiment carried out in our laboratory a maximum frequency of 1 Fix^- per 1000 plants was found. Numerous plant mutants affected in symbiotic nitrogen fixation have been isolated, indicating that different genes must be specifically involved in controlling this process. In *M. truncatula*, at least nine Nod^+Fix^- mutants have been isolated using γ -ray mutagenesis, but the reported phenotypes do not correspond to the defect induced by antisensing *Mtzpt2-1* (Sagan et al. 1995). The reduction of *Mtzpt2-1* expression might evoke the Fix^- phenotype also because of a slowly accumulating effect in these nodules, and a loss-of-function allele might produce a more severe phenotype. However, even in one plant showing very low *Mtzpt2-1* expression, we could not detect any alteration of the phenotype.

Histological analysis of Fix^- nodule development on the *Mszpt2-1* antisense plants suggests that initial root infection proceeds normally, whereas bacterial invasion of plant cells and nodule central zone differentiation are arrested. Accumulation of amyloplasts in the empty central region of these nodules indicates that the carbon source is not metabolized by the bacterial partner. These nodules resemble those obtained with surface polysaccharide mutant rhizobia where severe reduction of infection blocks nodule differentiation at early stages (Dickstein et al. 1988). Low levels of invasion occurring in the antisense plants were restricted to only a few cells in the nodule apex, further supporting that *Mtzpt2-1* is required for proper development of zone III. Because the expression of *nifH* (coding for the structural gene of the enzyme nitrogenase) was not detected at any developmental stage or in the mature nodules, we could discard any premature senescence process of the nodules formed on these plants.

The association of bacterial invasion events with the late induction of this gene in root and nodule vascular bundles, together with the phenotype of the antisense plants in the central region of the nodule, suggests a role of this transcription factor in a cell to cell communication process between these tissues required for the development of the nitrogen-fixing zone. This could be related to the translocation of specific metabolites in and out of the vascular phloem involved in sensing the carbon/nitrogen metabolic status of the plant (Streeter 1993). Phloem transfer cells are likely involved in the short distance transport of solutes (Joshi et al. 1993), and *Mszpt2-1* may be expressed in these cells. The exchange of metabolites through the vascular tissue may require osmotic adaptations of these cells. We showed that *Mszpt2-1* conferred salt tolerance in yeast, like its closest *Arabidopsis* homolog. Signaling mediated by carbon/nitrogen metabolites affecting osmotic conditions of the connecting vascular bundles might be crucial for the regulation of symbiotic nitrogen fixation (Streeter 1993). Interestingly, a function in a diffusible signaling process has been proposed for *INDETERMINATE*, another member of the plant Krüppel-like gene family, as it was ex-

Figure 5. Microscopy analysis of *Fix*⁻ nodules on *Mszpt2-1* antisense plants. (A–D) Longitudinal sections (8 μm) of *Fix*⁺ and *Fix*⁻ nodules observed by optical microscopy (paraffin embedding). (A) *Fix*⁺ nodule from a wild-type plant, showing typical zonation of nitrogen-fixing nodules (I, II, III; Vasse et al. 1990); (B) *Fix*⁻ nodule from an *Mtzpt2-1* antisense plant, showing abnormal central tissue differentiation and an apical infected zone (IZ), both viewed under epifluorescence illumination. (C,D) Higher magnifications of equivalent samples stained with lugol (amyloplast coloration), viewed in bright-field illumination: (C) A *Fix*⁺ nodule section, showing amyloplast accumulation (A) restricted to the interzone II–III; (D) a *Fix*⁻ nodule, showing amyloplast accumulation (A) in a large central region. (E,F) Semithin sections of nodules stained with toluidine blue showing cells from E, the central region (III) of a *Fix*⁺ nodule, and F, the apical region of a *Fix*⁻ nodule (IZ), indicated in A and B. Asterisks indicate one infected cell. Bar, 50 μm. (G,H) Ultrathin sections analyzed by electron microscopy showing details of one infected cell originating from the regions shown in E and F, respectively. (A) Amyloplast; (B) bacteroid. Bar, 1 μm.

pressed in leaves but influenced flower meristem formation (Colasanti et al. 1998). Although several explanations for the reported effects are possible, we propose that *Mtzpt2-1* may be also involved in a long-distance cell–cell communication process between root vascular bundles and the central region of the nodule.

Our results show that *Mtzpt2-1* is involved in symbiotic nodule organogenesis, permitting its invasion by rhizobia. Thus, its function in a vegetative developmental process indicates that Krüppel-like zinc finger proteins have various roles in plant organogenesis.

Materials and methods

Bacterial strains

S. meliloti mutant strains used were (1) the wild-type strain Sm41; (2) the AK1492 mutant strain (Eps⁻Kps⁻Fix⁻), a spontaneous Eps⁻ (*exoB*) derivative of Sm41, in which a Tn5 insertion in the *fix-23* region resulted in a Kps⁻ and Fix⁻ phenotype (Kondorosi et al. 1984); (3) the ZB138 Nod⁻ mutant strain, a derivative of Sm41 where all of the *nod* genes of the pSym megaplasmid are deleted (Kondorosi et al. 1984); and (4) the Sm8368 mutant strain (Bac⁻Fix⁻), a derivative of Sm1021 obtained by TnpH⁺ mutagenesis in the *bacA* gene (Glazebrook et al. 1993). A Sm41 strain carrying the pXLGD4 plasmid with a *δ-ala::lacZ* fusion (Leong et al. 1985) or an Sm1021 strain harboring a *nifH::lacZ* fusion (Szeto et al. 1987) have been used to follow bacterial infection and differentiation in transgenic plants. All strains were grown at 30°C on TA agar medium (Orosz et al. 1973).

Plants and nodulation assays

Alfalfa (*M. sativa* ssp. *sativa* cv. Sitel) or *M. truncatula* cv. 108-R4 seeds were sterilized, germinated, and grown for 1 week as described (Charon et al. 1999). Plants were then transferred to an aeroponic culture system (Coba de la Peña et al. 1997). After 1 additional week, plants were infected with a stationary-phase culture of a *S. meliloti* strain. Spontaneous nodules were obtained from *M. sativa* ssp. *sativa* genotype A2 lines as described (Coba de la Peña et al. 1997).

Nucleic acid techniques

Genomic DNA (15 μg), prepared from leaf material of *M. sativa* ssp. *varia* genotype A2 and *M. truncatula* 108-1, according to Sambrook et al. (1989), was used for Southern analysis. Total RNAs (10 μg) from different tissues, extracted as described in Sambrook et al. (1989), were used in Northern analysis. Hybridizations were carried in Church buffer (Church and Gilbert 1984) with a *Mszpt2-1* probe (the complete cDNA) overnight at 65°C, and washed under high stringent (65°C; 2× SSC; 0.1% SDS for 30 min; 1× SSC; 0.1% SDS for 30 min; 0.2× SSC; 0.1% SDS for 15 min) or low stringent (65°C; 2× SSC; 0.1% SDS for 30 min) conditions. Single-stranded RNA probes were synthesized with a DIG RNA labeling kit and revealed using chemiluminescence according to the manufacturer's instructions (Boehringer Mannheim Biochemica, Meylan, France). Sequencing was carried out with an automatic sequencer 373A (Applied Biosystems, Foster City, CA) using the dideoxy chain termination method (Pharmacia kit, Uppsala, Sweden). Sequence analysis was done using the GCG software package (Genetic Computer Group, University of Wisconsin, Madison, WI) and BLAST algorithm (Gish and States 1993).

Mszpt2-1 expression in yeast

Expression of *Mszpt2-1* in yeast was done by cloning the complete *Mszpt2-1* cDNA in the *NdeI-SalI* sites of the yeast expression vector pREP41 under the control of the thiamine-repressed promoter *nmt1* (Maundrell et al. 1990). Transformation of *Saccharomyces cerevisiae* strain YRG-2 (Stratagene) was done as described in Ausubel et al. (1989). Transformed yeast cells were selected for leucine auxotrophy (*leu2* marker) on agar plates and tested for osmotic tolerance by incubating for 6 days with or without 2 μ M thiamine in the presence or absence of 1.4 M NaCl or 1 M LiCl.

RT-PCR experiments (Charon et al. 1999) were done with total RNAs (extracted using RNeasy kit; Quiagen, Hilden, Germany) from yeast containing the control vector (pRep41) or expressing *Mszpt2-1*. cDNAs (6 ng) were PCR-amplified 30 cycles with specific oligonucleotides against the *ena1* gene (Rudolph et al. 1989). Membranes were hybridized to a *ena1* probe.

Sense and antisense expression in transgenic plants

For construction of transgenic plants a binary plasmid containing a strong constitutive chimeric promoter, comprising two 35S CaMV transcriptional enhancer elements and a translational enhancer from AMV, and the 3' nopaline synthetase region was used (Datla et al. 1991). Sense constructs were prepared using the complete *Mszpt2-1* cDNA (EMBL accession no. Y18788), whereas antisense constructs contained a 598-bp region (nucleotides 15–612) of the *M. truncatula Mtzpt2-1* gene cloned by RT-PCR. The sequence amplified was at least 88% identical to *Mszpt2-1* (data not shown), and no other *Mszpt2-1*-derived sequences could be detected in *M. truncatula* nodule RNA samples. *M. truncatula* 108-R4 plants were transformed with sense, antisense, or control vector according to Trinh et al. (1997).

In situ hybridization and microscopic analysis

In situ hybridization, starch staining, and histological techniques using paraffin-embedded material were performed as described in Coba de la Peña et al. (1997). In situ *lacZ* assays on nodule samples were done as described in Boivin et al. (1990). For electron microscopy, nodules were fixed for 1 hr under vacuum in 3% glutaraldehyde, 1% paraformaldehyde, 0.1 M Na cacodylate (pH 6.8), rinsed several times in cacodylate buffer, postfixed with 1% tannic acid for 1 hr, rinsed again and treated with 1% osmium for 1 hr. After washing, samples were incubated overnight at 4°C with 0.5% uranyl acetate, dehydrated using successive ethanol treatments (10%, 20%, 30%, 50%, 70%, 90%, each step 20 min), followed by 100% ethanol (30 min and 1 hr), and embedded in Spurr resin. After polymerization, either semithin (0.5 μ m; stained with toluidine blue) or ultrathin (80 nm) sections were cut using a Reichert Jung ultramicrotome UltracutE and analyzed with an electron microscope Philips CM10.

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