Transgenic Medicago truncatula plants that accumulate proline display nitrogen-fixing activity with enhanced tolerance to osmotic stress


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ABSTRACT

Legume root nodule nitrogen-fixing activity is severely affected by osmotic stress. Proline accumulation has been shown to induce tolerance to salt stress, and transgenic plants over-expressing Δ1-pyrroline-5-carboxylate synthetase (P5CS), which accumulates high levels of proline, display enhanced osmotolerance. Here, we transformed the model legume Medicago truncatula with the P5CS gene from Vigna aconitifolia, and nodule activity was evaluated under osmotic stress in transgenic plants that showed high proline accumulation levels. Nitrogen fixation was significantly less affected by salt treatment compared to wild-type (WT) plants. To our knowledge, this is the first time that transgenic legumes have been produced that display nitrogen-fixing activity with enhanced tolerance to osmotic stress. We studied the expression of M. truncatula proline-related endogenous genes M. truncatula Δ1-pyrroline-5-carboxylate synthetase 1 (MtP5CS1), M. truncatula Δ1-pyrroline-5-carboxylate synthetase 2 (MtP5CS2), M. truncatula ornithine δ-aminotransferase (MtOAT), M. truncatula proline dehydrogenase (MtProDH) and a proline transporter gene in both WT and transgenic plants. Our results indicate that proline metabolism is finely regulated in response to osmotic stress in an organ-specific manner. The transgenic model allowed us to analyse some of the biochemical and molecular mechanisms that are activated in the nodule in response to high salt conditions, and to ascertain the essential role of proline in the maintenance of nitrogen-fixing activity under osmotic stress.

Key-words: nodule; P5CS; salt stress.

INTRODUCTION

Arid and semi-arid regions offer optimal light and temperature conditions for most crops, but insufficient precipitation causes extensive reliance on irrigation. Irrigated lands are particularly prone to salinization (Szabolcs 1994), and salinity has profound effects on crop production. Reducing the spread of salinization and increasing the salt tolerance of high-yielding crops is becoming important global issues. Mineral nitrogen deficiency is another important limiting factor for plant growth in arid and semi-arid zones, and Rhizobium–legume symbioses are the primary sources of fixed nitrogen in these areas (Zahran 1999). Consequently, the development of legumes with improved osmotic tolerance in affected areas will lead to economic and environmental benefits, derived from increased yields and a reduction in the use of nitrogenous fertilizers.

The response of plants to osmotic stress is complex; several physiological and biochemical changes take place and an array of genes is induced. Several of these genes and their products have been identified; some are involved in the biosynthesis of osmolytes such as proline. Plants accumulate proline under osmotic stress (Singh, Aspinall & Paleg 1972; Rhodes 1987). The role of this amino acid in osmotolerance in plants has been widely discussed and remains controversial (Hare & Cress 1997; Nanjo et al. 1999b). Transgenic plants that accumulate high levels of proline are reported to display increased tolerance to salt and osmotic stress, as well as to cold and frost (Kishor et al. 1995, 2005; Zhu et al. 1998; Nanjo et al. 1999a; Hong et al. 2000; Han & Hwang 2003; and references therein). Various protection mechanisms have been proposed for proline. It is suggested to function as a mediator in osmotic adjustment (Handa et al. 1986), as a stabilizer of sub-cellular structures (Schobert & Tschesche 1978), as a free-radical scavenger (Smirnoff & Cumbes 1989; Saradhi et al. 1995; Hong et al. 2000), as a heavy metal detoxifier (Sharma, Schat & Vooijs 1998; Rai 2002), as a sink for energy (Alia & Saradhi 1991) and as a signalling/regulatory molecule able to activate multiple responses (Maggio et al. 2003).

In higher plants, proline is synthesized from both glutamic acid and ornithine. Under stress conditions, proline was proposed to be synthesized preferentially from glutamic acid (Delauney et al. 1993; Delauney & Verma 1995) via two intermediates, glutamic-γ-semialdehyde (GSA) and Δ1-pyrroline-5-carboxylate (P5C). Two enzymes, P5C synthetase (P5CS) and P5C reductase (P5CR) catalyse this pathway. P5CS is a rate-limiting enzyme in proline biosynthesis in higher plants (Delauney et al. 1993).
Transgenic plants over-expressing the P5CS gene from *Vigna aconitifolia* accumulate high proline levels and are more tolerant to osmotic stress (Kishor et al. 2005 and references therein). Genes encoding the P5CS enzyme have been identified in several plant species and are reported to be up-regulated in response to water deprivation and/or salinization (Hare & Cress 1997; Hare, Cress & Van Staden 1998). The legume *Medicago truncatula* accumulates proline in response to osmotic stress. Two P5CS genes have been isolated from *M. truncatula*: *M. truncatula* Δ^2^-pyrroline-5-carboxylate synthetase 1 (*MtP5CS1*) is reported to encode a developmental ‘housekeeping’ enzyme, while *M. truncatula* Δ^2^-pyrroline-5-carboxylate synthetase 2 (*MtP5CS2*) acts as a shoot-specific osmoregulated isoform (Armengaud et al. 2004).

Under osmotic stress conditions, efficiency in the establishment of the *Rhizobium*–legume symbiosis and nitrogen-fixing activity is dramatically affected (Sanchez-Diaz et al. 1982; Zahran & S Trent 1986; Durand, Sheehy & Minchin 1987; Delgado, Ligero & Lluch 1994; Serraj, Sinclair & Purcell 1999; Swaraj & Bishnoi 1999; Coba de la Peña et al. 2003; and references therein), reducing the legume’s productivity and soil-enriching capacity. Symbiotic nitrogen fixation markedly decreases upon exposure to mild saline conditions that have no adverse effect on soil nitrogen-dependent plant growth, and nodule function is affected by mild drought earlier and more severely than is photosynthesis (Djekoun & Planchon 1991). Salt stress involves a water deficit induced by the salt concentration in the rhizosphere, and an ionic toxicity due to excess ions (Zhu 2001). In short-term salinization experiments typically used in the study of the highly sensitive nitrogen-fixing activity, the major stress component is osmotic rather than ionic.

Legume nodules subjected to salt stress accumulate proline (Fernandez-Pascual et al. 1996; Swaraj & Bishnoi 1999). The nitrogen-fixing rhizobia, or bacteroids, display enhanced proline dehydrogenase (ProDH) activity, the main proline catabolic enzyme, when subjected to salt stress (Zhu, Shearer & Kohl 1992; Kohl, Straub & Shearer 1994) showed that proline supplied to soybean plants stimulates acetylene-reducing activity to a similar extent as do succinate or glutamate, and demonstrated that while the proline level in the host cell is high, it is low in the bacteroid. As proline oxidation renders high energy levels, it is suggested that proline could be used as an energy source for nitrogen fixation.

The relevant role of proline, both in legume nodule metabolism and in plant tolerance to osmotic stress, might well imply that legumes that accumulate high levels of proline could exhibit nitrogen-fixing activity with enhanced tolerance to osmotic stress. Here, we report the production of transgenic *M. truncatula* plants expressing the *V. aconitifolia* P5CS gene, which leads to proline accumulation, and we analyse the response of the transgenic plants to salt treatment. This is, to the best of our knowledge, the first description of transgenic legumes that display nitrogen-fixing activity with enhanced tolerance to osmotic stress.

The analysis of nitrogen fixation and nodule metabolism indicators, proline-related gene expression and nodule structure allows us to discuss the biochemical and molecular mechanisms that are activated in the nodule, as well as the role of proline in the maintenance of nitrogen-fixing activity under osmotic stress.

**MATERIAL AND METHODS**

**Plant material and growth conditions**

*Medicago truncatula* Gaertn. R-108-1 (c3) (Trinh et al. 1998) seeds were scarified with sandpaper, sterilized in 70% ethanol and placed on 0.9% agar in Petri dishes. The seeds were incubated for 5 d under growth chamber conditions (180 μmol photon m^-2^ s^-1^, 23 °C, 16 h photoperiod) to allow germination. Seedlings were transferred to growth pouches (CYG seed germination pouches; Mega International, Minneapolis, MN, USA) and were grown in the chamber. Bacterial inoculation was performed at sowing with *Sinorhizobium meliloti* strain 41 (Prakash et al. 1980) grown to exponential phase. After 28 d, plants were submitted to salt stress by adding 100 mM NaCl to the nutrient solution for 3 d. Control plants were maintained in a NaCl-free nutrient solution.

Ten plants per treatment were used to record morphometric parameters and to assess nitrogenase activity. Leaves, nodules and roots from 10 plants per treatment were frozen in liquid nitrogen immediately after harvesting and stored at −80 °C for RNA extraction, enzymatic assays and proline determination. Fresh nodules of 3 plants per treatment were used for microscopic analysis.

**Generation of transgenic plants**

An EcoRI fragment containing *V. aconitifolia* P5CS cDNA (Hu, Delyguy & Verma 1992) under the control of the cauliflower mosaic virus 35S promoter was excised from the binary vector pBI–P5CS (Kishor et al. 1995) and inserted in the EcoRI site of the binary vector pBI121-bar (Trieu et al. 2000). The resultant pBI121-bar–P5CS construct was introduced into *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993) by electroporation. *Medicago truncatula* leaf explants were transformed with *A. tumefaciens* containing pBI121-bar–P5CS by the *in vitro* transformation–regeneration method developed by Trinh et al. (1998). The *bar* gene, which confers phosphinotricin resistance, was used as the selective agent. T1 seeds were sown on vermiculite and analysed to confirm the presence of the transgene. Homozygous T2 seeds were harvested and used for subsequent experiments.

**Southern blot analysis**

Southern analysis was performed to confirm and select transformants and determine transgene copy number. Genomic DNA was isolated from leaf tissue by standard approaches (Dellaporta, Wood & Hicks 1983). Genomic
DNA samples (7 µg) were digested with EcoRV, separated by gel electrophoresis on a 0.7% agarose gel, denatured and blotted onto a nylon membrane (Zeta-Probe GT; Bio-Rad, Hercules, CA, USA) according to standard procedures (Sambrook, Fritsch & Maniatis 1989). The membrane was hybridized with an 892 bp digoxigenin-labelled probe containing an internal sequence of the V. aconitifolia P5CS cDNA. The probe was produced by PCR (PCR DIG Probe Synthesis Kit; Roche, Mannheim, Germany) using forward primer 5′-GCTCCCTATGAGGATTCTTC-3′ and reverse primer 5′-CAACATTATCTGGTATGG CCTC-3′. Hybridization and immunodetection were performed with the DIG High Prime Labeling and Detection Starter Kit I (Roche), as recommended by the manufacturer.

**Free proline content**

Leaves, roots and nodules were collected from the control and stressed plants. Samples (100 mg) pooled from at least 10 plants were ground and processed for free proline measurement as described by Bates, Waldren & Teare (1973).

**Nitrogenase activity**

The nitrogenase assay was determined by the acetylene reduction assay (ARA) (Hardy et al. 1968). Although the use of a ‘closed’ system for measuring acetylene reduction can lead to an acetylene-induced decline in nitrogenase activity (Minchin et al. 1983), it is appropriate for comparative measurements, especially when the time of assay is short.

**Low-temperature scanning electron microscopy (LTSEM)**

Nodule samples were examined under LTSEM. The samples were fixed onto the specimen holder of a cryotransfer system (Oxford CT1500; Oxford Instruments, Oxford, UK), plunged into sub-cooled liquid nitrogen, then transferred to a preparation unit via an airlock transfer device. Frozen samples were cryofractured and transferred directly via a second airlock to the microscope cold stage, where they were etched (2 min, −90 °C). After ice sublimation, etched surfaces were sputter coated with gold in the preparation unit. The samples were subsequently transferred to the cold stage of the scanning electron microscope chamber. Fractured surfaces were observed with a DSM 960 Zeiss scanning electron microscope (Zeiss, Jena, Germany) at −135 °C.

**Phosphoenolpyruvate carboxylase (PEPC) and sucrose synthase (SS) activity assays**

Sucrose synthase [enzyme class (EC) 2.4.1.13] activity was measured by monitoring NAD+ reduction (Morell & Copeland 1985). The reaction mixture included 100 mM sucrose, 2 mM uridine 5′-diphosphate (UDP), 1.5 mM NAD+ and 5 U of uridine 5′-diphosphate-glucose (UDPG) dehydrogenase in 100 mM bicine–KOH (pH 8.5). Nodule extracts (0.2–0.5 g mL−1) were prepared in 50 mM K-phosphate buffer (pH 8.0) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 20% ethylene glycol (Morell & Copeland 1985). The homogenate was centrifuged (10 000 g, 4 °C, 30 min) and the supernatant was used for the activity assay.

Phosphoenolpyruvate carboxylase (EC 4.1.1.31) activity was assayed spectrophotometrically by monitoring NADH oxidation (Lang, Martin & Golzano 1993). The reaction mixture included 1 mM NADH, 10 mM NaHCO3, 2 mM phosphoenolpyruvate (PEP) and 5 U of malate dehydrogenase (MDH) in 50 mM tris(hydroxymethyl)aminomethane (Tris)–HCl (pH 8.0) containing 10 mM MgCl2 and 5 mM dithiothreitol (DTT). Nodule extracts (0.2–0.5 g mL−1) were prepared in 50 mM Tris–HCl (pH 8.0) containing 10 mM MgCl2, 5 mM DTT and 25% ethylene glycol. The homogenate was centrifuged (10 000 g, 4 °C, 30 min) and the supernatant was used for the activity assay.

Protein content was estimated by the Bradford assay (Bio-Rad), using bovine serum albumin as standard (Bradford 1976).

**Quantitative real-time PCR analysis**

Total RNA was isolated from leaves, roots and nodules from the control and stressed plants using RNeasy plant mini kit (Qiagen, Valencia, CA, USA), to have melting temperatures of 57–62 °C and generate PCR products of approximately 100 bp Mtc27, a homolog of alfalfa constitutively expressed Msc27 (Pay, Heberle-Bors & Hirt 1992), was used as internal standard. Primer sequences were as follows: VaP5CS (forward, 5′-GCAAAAGTTGATTACCGGCAC-3′; reverse, 5′-TTGGAGTTAAGTAAGTAGATCC-3′); MtP5CS1 (forward, 5′-TCAATTGGAGAGGTAATTT GC-3′; reverse, 5′-CAATTTCCTAAGTGGAAGG AAC-3′); MtP5CS2 (forward, 5′-CAGTAGGAGTCGAGGTCTTTGTT-3′; reverse, 5′-CTTGTTGGATATTAACATCA TACTTCGATCG-3′); M. truncatula oryntine δ-amino transferase (MtOAT) (forward, 5′-GCAGTTGCAT TGCCCTCACC-3′; reverse, 5′-AGCAGACCAGGAAGCT CACC-3′); ProDH homolog (forward, 5′-AGGGTAGTGG AAGATGAAAC-3′; reverse, 5′-CCCTGCAATTTGC AAAAGCA-3′); proline transporter homolog (forward, 5′-ACAGAAATGGGCGGCTCTG-3′; reverse, 5′-TTGGAGT CACTGTCAATCTGCC-3′); and Mtc27 (forward, 5′-CACCCAAACTAGATGCAGAGAAACA-3′; reverse, 5′- CAAAGAATTTGAGTCCCTGAC-3′).

Gene sequences at the European Molecular Biology Laboratory (EMBL) nucleotide database have the
following accession numbers: M92276, AJ278818, AJ278820 and AJ278819 for VaP5CS, MtP5CS1, MtP5CS2 and MoOAT, respectively. Gene sequences at The Institute for Genomic Research (TIGR) database, *M. truncatula* Gene Index (MtGI) have the following tentative consensus (TC) report numbers: TC94264, TC107834 and TC85136 for ProDH, proline transporter and Mtc27, respectively.

Each PCR reaction contained 7.5 µL SYBR Green PCR master mix (PE Applied Biosystems), 5 µL cDNA and 0.4 µM (final concentration) of each primer, in a final volume of 15 µL. Real-time PCR reactions were run in an ABI Prism 7900HT Sequence Detection System (PE Applied Biosystems). The initial denaturing time was 10 min, followed by 40 PCR cycles of 95 °C, 15 s; 60 °C, 90 s; and 72 °C, 90 s. A melting curve was run after PCR cycles. Relative quantification was performed using the comparative Ct method with the ABI Prism 7900HT SDS v2.2 RQ software (PE Applied Biosystems). Experiments were performed six times in triplicate. Mean values ± SD error bars are represented.

**RESULTS**

Proline accumulation in transgenic *M. truncatula* plants expressing *V. aconitifolia* P5CS cDNA

To obtain transgenic *M. truncatula* plants overproducing proline and to evaluate their tolerance to osmotic stress, we transformed *M. truncatula* with the P5CS cDNA from *V. aconitifolia* (Hu et al. 1992). We confirmed integration and copy number in the plant genome using Southern blot. Two homozygous transgenic lines, arising from two different transformation events and containing one (p18) and two (p2) copies of the transgene, respectively, were selected for further experiments (Fig. 1a).

To verify transgene expression, VaP5CS transcript accumulation was determined in wild-type (WT) and transgenic plants by quantitative real-time RT–PCR analysis (Fig. 1b). Transgene expression was detected in leaves, roots and nodules of p2 and p18 plants. The highest expression levels were recorded in leaves, approximately 10-fold higher than those in roots and nodules. VaP5CS expression was stronger in line p2 than in line p18 in all organs tested. No expression was detected in WT plants, as specific primers were designed to selectively amplify VaP5CS and avoid cross amplification of endogenous MtP5CS1 or MtP5CS2 transcripts.

Free proline content in leaves, roots and nodules of WT and transgenic plants was analysed under control and salt stress conditions. Both transgenic lines expressing *V. aconitifolia* P5CS accumulated higher levels of proline in leaves, roots and nodules than WT plants (Fig. 2). Proline levels increased in WT and transgenic plants following salt treatment. The highest proline content was found in the roots of salt-stressed transgenic plants, with p2 roots containing up to 30 times more proline than WT salt-stressed roots.

**Effects of salt stress on nitrogen fixation and nodule ultrastructure in WT and transgenic *M. truncatula* plants**

Over-expression of P5CS in different plant species has been reported to provide increased tolerance to salt stress and water deficit (Kishor et al. 2005). In this study, our major interest was on nitrogen fixation, and in order to estimate the tolerance of nodule activity to salt stress in both transgenic lines, experimental stress conditions were designed in preliminary experiments such that the highly sensitive nitrogen-fixing activity would be affected to a significant, but not an extreme degree in salt-stressed plants.

To analyse the effect of salt stress on WT and transgenic plants, we measured several parameters related to plant growth, including height, fresh (FW) and dry weight (DW) of roots and aerial parts and number and FW of nodules (not shown). As expected, the stress conditions imposed were not severe enough to affect plant growth parameters substantially.

Conversely, under salt stress, the nitrogen-fixing activity measured by the ARA decreased considerably in WT
plants, compared to non-stressed plants (Fig. 3). Compared to WT plants, transgenic lines showed higher activity values when subjected to salt stress. The p2 plants were not affected by stress treatment, as the slight decrease observed was not statistically significant. The p18 plants showed a more marked decrease in nitrogen-fixing activity than the p2 plants under salt stress, but still retained a high activity.

To study control and salt-stressed nodules at the ultrastructural level, LTSEM was performed on WT and transgenic p2 nodules. No remarkable differences were observed in nodule structure between WT (Fig. 4a) and p2 (Fig. 4c) non-stressed plants. Nodules from salt-stressed WT plants showed structural alterations in most infected cells. Stress induced severe modifications in bacteroids, which appeared to be collapsed and devoid of internal matrix (Fig. 4b). On the contrary, salt-stressed nodules of p2 plants (Fig. 4d) showed no significant ultrastructural differences in comparison to untreated plants.
Carbon metabolism in nodules of WT and transgenic plants

The SS enzymatic activity, responsible for sucrose breakdown in the nodule, showed higher values (approximately twofold) in WT than in transgenic plants (Fig. 5a). Salt stress significantly inhibited the SS activity in both WT and transgenic plants to a similar extent, with values always lower in the transgenic lines. The PEPC activity increased significantly in nodules of WT and transgenic plants following salt treatment (Fig. 5b). Nodules of salt-stressed WT plants displayed a PEPC activity that was up to fivefold higher than that observed under control conditions. In both transgenic lines, values increased approximately 16-fold when subjected to salt stress. Transgenic p18 plants showed the highest PEPC activity following stress and control treatments.

Expression of proline metabolism-related genes in WT and transgenic M. truncatula plants under salt stress

To determine organ specificity and elucidate the contribution of each gene in proline accumulation patterns under control and salt stress conditions, and to ascertain whether VaP5CS expression induced changes in the expression pattern of proline biosynthesis-related genes, we used quantitative real-time PCR to determine transcript accumulation levels of the endogenous M. truncatula genes MtP5CS1, MtP5CS2, MtOAT, ProDH and a proline transporter gene in the leaves, roots and nodules of WT and transgenic plants.

MtP5CS1 expression was higher in non-stressed roots and nodules than in non-stressed leaves (Fig. 6a). Expression levels were significantly diminished by salt stress in the non-aerial organs, however, while they remained unaffected in leaves. No major differences were detected in transcript accumulation between WT and transgenic lines. In non-stressed plants, the highest MtP5CS2 transcript accumulation levels were observed in roots and nodules (Fig. 6b). Salt stress induced a strong induction of expression in leaves, whereas we found repression of gene expression in roots and nodules. No major differences were detected between WT and transgenic plants.

The enzyme ornithine δ-aminotransferase is involved in proline synthesis from ornithine, and catalyses the loss of the ornithine Δ-amino group to give P5C, which is further reduced to proline by P5CR. In WT non-stressed plants, MtOAT expression levels were similar in leaves, roots and nodules (Fig. 6c). Transcript accumulation increased approximately fourfold in leaves and roots and up to six- to sevenfold in nodules following salt stress. MtOAT expression levels in roots and nodules of transgenic plants were similar to those in WT plants, and increased similarly after salt stress. In leaves, MtOAT transcripts accumulated at higher levels in transgenic compared to WT plants. Significant repression in gene expression was observed in both transgenic lines after salt stress, in contrast to the induction observed in WT leaves.

We also quantified the expression levels of a ProDH homolog. ProDH is the first enzyme in the proline degradation pathway; it is bound to the inner membrane of the mitochondria, and catalyses the oxidation of proline to P5C. In non-stressed plants, higher ProDH transcript levels were found in leaves of both WT and transgenic plants than in roots or nodules (Fig. 6d). Salt stress induced a certain degree of repression in leaves, and a slight induction was detected in roots. A strong up-regulation was observed in nodules of both WT and transgenic salt-stressed plants.

In the absence of salt stress, a proline transporter homolog was expressed at higher levels in leaves than in roots and nodules (Fig. 6e). Salt stress induced some repression in all organs. No major differences were detected in transcript accumulation between WT and transgenic lines.

DISCUSSION

P5CS catalyses the first two steps of proline biosynthesis from glutamic acid. Over-expression of P5CS is reported to increase proline levels in osmotically stressed plants (Kishor et al. 1995), and several studies have demonstrated that proline overproduction in transgenic plants results in
Proline preserves legume nodule activity under osmotic stress

Expression of *V. aconitifolia* P5CS cDNA in *M. truncatula* led to proline accumulation in leaves, roots and nodules of the transgenic plants. Whereas the highest transcript accumulation levels were detected in the leaves, proline accumulated preferentially in roots and, to a lesser extent, in nodules of the transgenic plants under osmotic stress. Proline transport from the leaves, as well as organ-dependent transcript stability and enzyme activity, could account for this organ-specific accumulation pattern. Under control conditions, no significant differences were detected in nitrogen fixation between WT and transgenic plants. In salt-stressed WT plants, nitrogen-fixing activity decreased significantly, whereas transgenic lines retained high activity values.

*VaP5CS* over-expression increased proline production as well as the salt stress tolerance of the nitrogen-fixing activity in transgenic *M. truncatula* plants. Various roles described for proline can explain its beneficial effects under stress conditions. Oxidative pentose phosphate pathway (OPPP), one of the antioxidant defence mechanisms, is dependent on NAD(P)⁺ availability and is inhibited by NADPH. Proline biosynthesis generates NADP⁺, and is proposed to enhance OPPP activity, which would provide precursors to support the demand for increased secondary metabolite production during stress (Khedr et al. 2003). Hong et al. (2000) demonstrated that proline has a role in scavenging free radicals in transgenic tobacco plants overproducing proline. It has been established that a molecular signalling system able to sense proline levels acts in plant cells to control gene expression (Shinozaki & Yamaguchi-Shinozaki 1996; Garcia et al. 1997; Hellmann et al. 2000). As a part of an adaptation process, proline may act as a signalling/regulatory molecule able to activate multiple responses (Iyer & Caplan 1998; Khedr et al. 2003; Maggio et al. 2003).

In nodules, proline is imported to the bacteroid where it is metabolized (Zhu et al. 1992). Although proline normally crosses the peribacteroid membrane more slowly than succinate or malate (Udvardi & Day 1997), under osmotic stress there is an increase in the rate of proline uptake into symbiosomes (Pedersen, Feldner & Rosendahl 1996). High proline concentration was suggested to protect nodule metabolism by avoiding protein denaturalization and maintaining cell pH levels (Irigoyen, Emerich & Sanchez-Diaz 1992). Straub et al. (1997) suggested that proline catabolism in bacteroids might provide an agronomically significant yield advantage under mild stress conditions. Kohl et al. (1991) described that drought-stressed nodules show a severe reduction in nitrogenase activity, high proline levels and enhanced ProDH activity in bacteroids, which remains elevated during recovery from stress. It was suggested that rapid recuperation of nitrogen-fixing activity of nodules from re-watered soybean plants can be explained as the ability of bacteroids to use proline to maintain nitrogen fixation until normal nodule metabolism is attained (Kohl et al. 1991). Salt stress induces increased proline and proline

In legumes, proline is involved in nodule metabolism under osmotic stress (Kohl et al. 1991), and its accumulation could exert a protective effect on the nodule’s nitrogen-fixing activity.

**Figure 6.** Transcript accumulation of *Medicago truncatula* endogenous genes in leaves, roots and nodules of wild-type (WT) and transgenic p2 and p18 plants under control (white bars) and salt stress (100 mM NaCl, 3 d) (grey bars) conditions, as determined by quantitative real-time PCR. (a) Δ²-pyrroline-5-carboxylate synthetase 1 (*MtP5CS1*). (b) Δ¹-pyrroline-5-carboxylate synthetase 2 (*MtP5CS2*). (c) Ornithine Δ-amino transferase (*MtOAT*). (d) *M. truncatula* proline dehydrogenase (*MtProDH*). (e) *Proline transporter* homolog. Gene expression is given relative to *Mic27* mRNA levels. Data are the means of six replicates ± SD.

In legumes, the reaction system of proline synthesis and catabolism is a defensive mechanism that protects the plant against stress, as shown by its increased tolerance to osmotic stress (Kishor et al. 2005). In legumes, proline is involved in nodule metabolism under osmotic stress (Kohl et al. 1991), and its accumulation could exert a protective effect on the nodule’s nitrogen-fixing activity.

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betaine (PB) concentrations in the bacteroids and the cytosol of alfalfa nodule host cells (Trinchant et al. 2004).

In the absence of stress, the structure of bacteroids from WT and transgenic p2 plants appeared similar in LTSEM. Salt treatment caused major alterations in the bacteroids of many infected cells in WT plants, whereas p2 plant bacteroids showed hardly any changes in comparison to untreated plants. Elevated proline concentration in transgenic plants apparently supported bacteroid structure under salt stress. It was reported that proline-rich proteins (PRPs) provide mechanical support for cells under stress conditions (Showalter 1993; Muñoz, Dopico & Labrador 1998). Transcripts encoding PRPs accumulate in bean nodules as a response to salt and drought stress (Verdoy et al. 2004), and increased proline levels could lead to enhanced PRP synthesis.

Proline accumulation led to some alterations in nodule carbon metabolism. Both transgenic lines had lower SS activity than WT plants. Proline accumulation in nodules of transgenic plants could favour a lower demand for substrates, such as sucrose, for bacteroid metabolism and thus lead to diminished SS activity. The SS activity in nodules of both WT and transgenic plants subjected to salt stress was lower than the SS activity in nodules under control conditions. Under stress, the impairment of sucrose metabolism in the nodule has been reported to be responsible for the decline in nitrogen fixation (Gonzalez et al. 1995; Gordon et al. 1997, 1999). This could explain the severe reduction in nitrogenase activity in WT plants submitted to salt stress. The maintenance of nodule function under stress in the transgenic lines could be a consequence of proline availability as a metabolic substrate that might be used as an alternative to sucrose.

Phosphoenolpyruvate carboxylase activity was stimulated in nodules from WT and transgenic plants by salt stress. Induction of PEPC activity in salt-stressed root nodules was reported previously (Irigoyen et al. 1992; Delgado et al. 1993; Soussi, Ocaña & Lluch 1998). The p2 and p18 nodules displayed increased PEPC activity compared to WT nodules. It was suggested that proline biosynthesis stimulates OPPP (Hare & Cress 1997), and that the end products of this pathway can be metabolized through glycolysis to produce phosphoenolpyruvate. Transgenic plants that accumulate proline might display intense activity of the OPPP, which could lead to a higher PEP concentration in the cell. High substrate levels might induce enhanced PEPC activity.

P5CS is subject to feedback inhibition by proline, and feedback regulation of P5CS plays a role in controlling the level of proline in plants under both normal and stress conditions (Hong et al. 2000). Despite this regulation, plants over-expressing P5CS accumulate high proline levels (Hong et al. 2000). The possibility exists that P5CS over-expression might induce changes in endogenous gene expression that lead to the reported proline accumulation patterns.

Medicago truncatula endogenous P5CS genes, MtP5CSI and MtP5CS2, were cloned and characterized by Armengaud et al. (2004) in several organs of non-nodulated M. truncatula plants. Our results on expression levels of both genes in leaves concur with those of these authors. Following salt stress treatment, MtP5CSI transcript levels did not change in leaves, while considerable repression was observed in roots and nodules. Whereas Armengaud et al. (2004) described MtP5CSI as a housekeeping gene, MtP5CSI appears to be osmotically down-regulated in roots and nodules under our experimental conditions.

MtOAT transcript accumulation patterns suggest that the ornithine pathway plays a very significant role in proline synthesis in M. truncatula leaves, roots and nodules under salt stress. In leaves, elevated proline synthesis following stress appears to be due to the additive effects of enhanced MtP5CS2 and MtOAT expression, while salt stress-induced proline biosynthesis in roots and nodules appears to be a consequence of ornithine pathway activation. Proline synthesis seems to be sufficient to counteract the increased proline catabolism derived from stress-induced ProDH up-regulation in the nodule. Our results concur with those of Armengaud et al. (2004), who reported stress-induced enhanced MtOAT expression in leaves and roots, and are contrary to the idea that proline is synthesized mainly from glutamate in plants under stress conditions (Delauney et al. 1993; Delauney & Verma 1993). Our data also reinforce the suggestion that the ornithine pathway for proline biosynthesis is linked to nitrogen metabolism in nodules (Kohl et al. 1988).

In general, proline catabolism is reported to be repressed during osmotic stress (Kishor et al. 2005). ProDH is down-regulated during water stress in Arabidopsis, and is induced during rehydration or by proline accumulation (Kiyosue et al. 1996; Peng, Lu & Verma 1996). Transgenic tobacco and Arabidopsis plants bearing antisense ProDH show higher proline content and increased tolerance to salt stress and freezing (Nanjo et al. 1999a; Mani et al. 2002; Kochetov et al. 2004). In this study, we analyse the expression of a plant ProDH homolog in legume nodules. Specific primers were used to discard amplification of bacterial ProDH. ProDH transcript levels have been reported to decrease in a fast, salt concentration-dependent manner in alfalfa roots (Miller et al. 2005). However, we observed up-regulation in roots and nodules of salt-stressed plants. It is possible that our stress conditions were too mild to repress ProDH gene expression and that induction by proline was still predominant. ProDH activity has been reported to increase in bacteroids from drought-stressed nodules, probably supplying reducing equivalents through proline oxidation and sustaining nitrogen fixation (Kohl et al. 1988, 1991). The remarkable increase in gene expression observed in salt-stressed nodules suggests active proline catabolism and consequent generation of energy and reducing equivalents through OPPP and glycolysis.

Proline transport appears to be very essential under stress conditions. In Arabidopsis, proline is synthesized mainly in roots, although it does not accumulate in them in large amounts (Hua et al. 1997). It was reported that several proline-specific transporters are osmotically stress induced in different plant species, implying a crucial role for proline
distribution under such conditions (Rentsch et al. 1996; Ueda et al. 2001; Waditee et al. 2002). Girousse, Bour- 
coville & Bonnemain (1996) described a high proline con-
centration in the phloem sap of alfalfa plants subjected to 
water deficit. We studied the expression pattern of an M. 
*truncatula* proline transporter homolog to elucidate its role 
in proline transport in leaves, roots and nodules during salt 
stress. This gene sequence presents high similarity with 
proline/glycine betaine transporters from several plants. 
According to our results, this gene does not appear to be 
involved in proline transport during salt stress in M. *trun-
catula* under the conditions applied. Proline transporters 
constitute a multigene family, however, and it is likely that 
still-unidentified putative homologs may be involved in 
specific proline transport in *M. truncatula* during salt stress. 
Stress-induced post-transcriptional or post-translational 
modifications cannot be discarded for this or any of the 
other genes studied. Such modifications, that remain to be 
investigated, might modulate enzymatic activities and con-
tribute to the proline accumulation patterns that we have 
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