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Evaluation of the role of genes encoding for Δ^1-pyrroline-5-carboxylate synthetase (P5CS) during drought stress in arbuscular mycorrhizal *Glycine max* and *Lactuca sativa* plants

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Abstract

In this study, we have determined whether the arbuscular mycorrhizal (AM) symbiosis is able to alter the pattern of Δ^1-pyrroline-5-carboxylate synthetase (*p5cs*) gene expression under drought stress and whether such possible alteration functions in the protection of the host plants against drought. To achieve this, we cloned a P5CS-encoding gene from *Glycine max* (*gmp5cs*) and another from *Lactuca sativa* (*lsp5cs*) and analyzed their contribution to the response against drought in control and AM soybean and lettuce plants. The analysis of *gmp5cs* and *lsp5cs* gene expression showed that these genes were up-regulated by drought stress. The highest gene expression was found in non-inoculated plants subjected to drought. A contrasting result was obtained in soybean plants singly inoculated with *Bradyrhizobium japonicum*, where the *gmp5cs* gene showed little up-regulation in roots under drought stressed conditions. Moreover, both soybean and lettuce AM plants showed lower *p5cs* transcript accumulation under drought stress than non-inoculated plants. The present results indicate that the induction of *p5cs* gene is not a mechanism by which the AM symbiosis protects their host plant against drought.

Keywords: Arbuscular mycorrhizal symbiosis; Drought stress; Proline; Δ^1-Pyrroline-5-carboxylate synthetase (P5CS)

1. Introduction

Plants are, in nature, frequently exposed to adverse environmental conditions that have a negative effect on plants physiology. Water deficit is one of the most common environmental stress factors experienced by soil plants. It interferes with both normal development and growth and has a major adverse effect on plant survival and productivity [13,36].

The arbuscular mycorrhizal (AM) fungi are widespread microorganisms able to establish a symbiotic association with the roots of most terrestrial plants. AM plants have improved ability for nutrient uptake and tolerance to biotic and abiotic stresses while the fungus acquires a protected ecological niche and plant photosynthates [64]. The AM symbiosis is present in all natural ecosystems, even in those affected by adverse environmental conditions [7], and it can be defined as a specialized system for nutrient uptake and transfer, more efficient than roots alone [66]. Nevertheless, the physiological role of the AM symbiosis is not limited to uptake and transfer of nutrients to the host plant. Many other beneficial effects for the host plant and for ecosystems have been described [64], including enhancement of tolerance to drought stress [3,5,49,51,53,56]. It is currently accepted that the contribution of the AM symbiosis to plant drought tolerance results from a combination of physical, nutritional and cellular effects [51].

Plants, can also respond to drought stress at morphological, anatomical and cellular levels with modifications that allow the plant to avoid the stress or to increase its tolerance [11]. The typical first response of all living organisms to water deficit is osmotic adjustment. To counter with drought stress, many plants increase the osmotic potential of their cells by synthesizing and accumulating compatible osmolytes such as proline that participates in the osmotic adjustment [32,35,42].

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However, proline performs also an important function as a protective compatible osmolyte in scavenging of free radicals and facilitating a correction of altered redox potential by replenishment of the NADP+ supply [23,26]. Accumulation of proline is due primarily to de novo synthesis, although a reduced rate of catabolism has also been observed [35]. The first two steps of proline biosynthesis are catalized by Δ^1-pyrroline-5-carboxylate synthetase (P5CS) by means of its γ-glutamil kinase and glutamic-γ-semialdehyde dehydrogenase activities. Subsequently, the Δ^1-pyrroline-5-carboxylate (P5C) formed is reduced by P5C reductase (P5CR) to proline [32]. The rate-limiting step in this pathway is represented by the γ-glutamil kinase activity of P5CS, which is sensitive to feedback inhibition by relatively low levels of proline [63]. In addition, in *Arabidopsis*, the P5CS-encoding gene is induced by drought stress, salinity and ABA, but P5CR is not [68]. The overexpression of the P5CS-encoding gene in transgenic tobacco plants has been shown to increase proline production and to confer tolerance of such plants to osmotic stress [35]. Hence, the P5CS-encoding gene is of key importance for the biosynthesis of proline in plants [1].

Investigations carried out so far on osmorregulation in the AM symbiosis are scarce and somewhat contradictory. While some studies have shown an increase in proline accumulation in mycorrhizal plants subjected to drought [6,22,53], the same studies also demonstrated that the increase in proline accumulation was quite variable depending on the AM fungus involved. For instance, while plants colonized by *G. deserticola* accumulated 120 nmol of proline per g fresh weight, plants colonized by *G. intraradices* only accumulated 41 nmol proline per g fresh weight [53]. It has also been shown that under low Ca in the medium AM plants accumulated more proline than non-AM plants when subjected to PEG-induced drought stress, while under high Ca in the medium this was not so [52]. On the contrary, other studies regarding drought [50] or salt stress [54] have shown a lower proline accumulation in AM plants than in non-AM ones.

Although in recent years there has been an increase in the understanding of the water relations of AM plants and the physiological processes involved in the enhanced tolerance of mycorrhizal plants to water limitation, there are still many unknown aspects which must be elucidated [51]. Moreover, a molecular basis for the tolerance to water stress in AM plants remains far from being understood. The establishment of the expression pattern of genes such as *p5cs* in AM plants under osmotic stress conditions, should provide an insight into the role of the AM symbiosis in the process of osmotic adjustment during drought stress. As a first approach, in the present study we have cloned a P5CS-encoding gene from *Glycine max* (*gmp5cs*) and another from *Lactuca sativa* (*lsp5cs*) and analyzed their contribution to the response against drought in mycorrhizal soybean and lettuce plants.

2. Materials and methods

2.1. Experimental designs

2.1.1. First experiment (with Glycine max)

When considering experiments with legume plants the control plants are not only the non-inoculated plants, but also the plant singly inoculated with the corresponding *Rhizobium*. For that reason, this experiment was designed to cover the different inoculation combinations of *Bradyrhizobium japonicum* and the two AM fungi used. The experiment consisted of six inoculation treatments: (1) plants inoculated with the nitrogen-fixing bacteria *Bradyrhizobium japonicum*, strain USDA 110 (Br), (2) plants inoculated with the arbuscular mycorrhizal (AM) fungus *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe and *B. japonicum* (Gm+Br), (3) plants inoculated with the AM fungus *Glomus intraradices* (Schenck and Smith) and *B. japonicum* (Gi+Br), (4) plants inoculated with *G. mosseae* (Gm), (5) plants inoculated with *G. intraradices* (Gi), and (6) non-inoculated control plants (NI). Twelve replicates of each treatment were done totalling 72 pots (one plant per pot) so that half of them were cultivated under well-watered conditions throughout the entire experiment, while the other half were drought-stressed for 10 days before harvest.

2.1.2. Second experiment (with Glycine max)

The experiment consisted of a randomized complete block design with three inoculation treatments: (1) plants inoculated with the nitrogen-fixing bacteria *Bradyrhizobium japonicum*, strain USDA 110 (Br), (2) plants inoculated with the AM fungus *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe and *B. japonicum* (Gm+Br) and (3) non-inoculated control plants (NI). For each treatment plants were cultivated at four time intervals: 5, 12, 20 or 35 days after inoculation (dai). A variable number of replicates of each treatment was done, ranging from 12 for plants to be harvested after only 5 dai to 6 for plant to be harvested after 35 dai, totalling 108 pots (one plant per pot) so that half of them were cultivated under well-watered conditions throughout the entire experiment, while the other half were drought-stressed for 5 days (for plants harvested 5 dai) or for 10 days (for the rest of treatments) before harvest.

2.1.3. Third experiment (with Lactuca sativa)

The experiment consisted of a randomized complete block design with three inoculation treatments: (1) plants inoculated with the AM fungus *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe (Gm), (2) plants inoculated with the AM fungus *Glomus intraradices* (Schenck and Smith) (Gi), and (3) non-inoculated control plants (NI). Ten replicates of each treatment were done totalling 30 pots (one plant per pot) so that half of them were cultivated under well-watered conditions throughout the entire experiment, while the other half were drought-stressed for 10 days before harvest.

2.1.4. Fourth experiment (with Lactuca sativa)

The experiment consisted of a randomized complete block design with two inoculation treatments: (1) plants inoculated with the AM fungus *Glomus intraradices* (Schenck and Smith) (Gi) and (2) non-inoculated control plants (NI) and two rates of proline added to the medium: (1) 10 mM proline (+Pro) or no proline added (−Pro). Ten replicates of each treatment were done totalling 80 pots (one plant per pot) so that half of them were cultivated under well-watered conditions throughout the entire experiment, while the other half were drought-stressed for 10 days before harvest.

2.2. Soil and biological materials

Loamy soil was collected from the Zaidin Experimental Station (Granada, Spain), sieved (2 mm), diluted with quartz-sand (<1 mm) (1:1, soil:sand, v/v) and sterilized by steaming (100 °C for 1 h for 3 days). The soil had a pH of 8.1 (water); 1.81% organic matter, nutrient concentrations (mg kg^{-1}): N, 2.5; P, 6.2 ($NaHCO_3$-extractable P); K, 132.0. The soil texture was made up of 35.8% sand, 43.6% silt and 20.5% clay.

Soybean (*Glycine max* L. cv Williams) seeds were sterilized in a 15% H_2O_2 solution for 8 min, then washed several times with sterile water to remove any trace of chemical that could interfere in seed germination, and placed on sterile vermiculite at 25 °C to germinate. Three-day-old seedlings were transferred to plastic pots containing 600 g of sterilized soil/sand mixture (1:1, v/v). When appropriate, a suspension (2 ml seed) of the diazotrophic bacterium *Bradyrhizobium japonicum*, strain USDA 110 (10^8 cell ml^{-1}) was sprinkled over the seedling at the time of planting.

Three seeds of lettuce (*Lactuca sativa* L. cv. Romana) were sown in pots containing 500 g of the same soil/sand mixture as described above and thinned to one seedling per pot after emergence.

Mycorrhizal inoculum for each endophyte was bulked in an open-pot culture of *Allium cepa* L. and consisted of soil, spores, mycelia and infected root fragments. The AM species were *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe, isolate BEG 122 and *Glomus intraradices* (Schenck and Smith) isolate BEG 121. Ten grams of inoculum of the two *Glomus* isolates, possessing similar infective characteristics (about 115 infective propagules per gram, according to the MPN test), were added to appropriate pots at sowing time just below soybean seedlings or lettuce seeds.

Uninoculated control plants for each microbial treatment received the same amount of autoclaved rhizobial and/or mycorrhizal inoculum.

2.3. Growth conditions

Plants were grown in a controlled environmental chamber with 70–80% RH, day/night temperatures of 25/15 °C, and a photoperiod of 16 h at a Photosynthetic photon flux density (PPFD) of 460 μmol m^{-2} s^{-1} (Licor, Lincoln, NE, USA, model LI-188B).

Soil moisture was measured with a ML2 ThetaProbe (AT Delta-T Devices Ltd, Cambridge, UK), which measures volumetric soil moisture content by responding to changes in the apparent dielectric constant of moist soil. Water was supplied daily to maintain constant soil water content close to field capacity (17% volumetric soil moisture, as determined experimentally using a pressure plate apparatus and applying a pressure of one third atmosphere for 48 h, and then determining the volumetric soil moisture) during the first 4 weeks of plant growth (first experiment). At this time half of the plants were allowed to dry until soil water content reached 70% field capacity (two days needed), which corresponded to 10% volumetric soil moisture (also determined experimentally in a previous assay), while the other half was maintained at field capacity. Plants were maintained under such conditions for additional 10 days. In order to control the level of drought stress, the soil water content was daily measured with the ThetaProbe ML2 (at the end of the afternoon) and the amount of water lost was added to each pot in order to return soil water content at the desired 10% of volumetric soil moisture (70% of field capacity). However, during the 24h-period comprised between each rewatering the soil water content was progressively decreasing until a minimum value of 60% of field capacity. For the second experiment, half of the plants was maintained at field capacity during the entire experiment, while the other half was drought stressed as indicated above for 5 days (plants harvested 5 dai) or for 10 days for the rest of harvests. In the third and fourth experiments plants were maintained at field capacity during 5 weeks and then half of the plants was subjected to drought stress (70% of field capacity) for two weeks, while the other half remained under well-watered conditions.

Each week throughout the experiment, soybean plants received 10 ml of Hewitt's nutrient solution lacking N and P [29]. Three weeks after planting, plants received nutrient solution amended with N and/or P as follows [21]: 0.18 mM K_2HPO_4 and 2 mM NH_4NO_3 (NI plants), 0.35 mM K_2HPO_4 (Br plants), 3 mM NH_4NO_3 (Gm and Gi plants). Nutrient concentrations were chosen in an attempt to obtain well-watered plants of similar size and nutrient contents in all the microbial treatments.

Each week throughout the experiment, uninoculated control lettuce plants received 10 ml of Hewitt's nutrient solution [29], modified to contain 4 mM N + 1 mM P. Mycorrhizal plants did not receive nutrient solution. The use of such fertilization level for non-mycorrhizal plants was meant to obtain well-watered control plants of similar size and nutrient contents to the AM plants tested in this assay.

In the second experiment with lettuce, proline was added as an aqueous solution at a rate of 30 ml per week, so that at

the end of the experiment the total amount of proline added was 10 mM per pot.

2.4. Symbiotic development

The percentage of mycorrhizal root infection in soybean and lettuce plants was estimated by visual observation of fungal colonization after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactic acid (v/v), according to Phillips & Hayman [47]. The extent of mycorrhizal colonization was calculated according to the gridline intersect method [20]. Nodule number in soybean roots was determined using a binocular microscope.

2.5. Proline content

Free proline was extracted from 0.3 g of fresh tissue and determined by spectrophotometric analysis at 515 nm of the ninhydrin reaction, according to Bates et al. [8].

2.6. RNA isolation and synthesis of first strand cDNA

Total RNA was isolated from soybean or lettuce roots by phenol/chloroform extraction [34]. DNase treatment of total RNA was performed according to Promega's recommendations. Total RNAs (2.5 μg) from soybean and from lettuce roots subjected to drought stress were reverse transcribed to first strand cDNA using AMV-RT enzyme (Finnzymes, Espoo, Finland) and oligo$(dT)_{15}$ primer (Promega, Madison, WI), in a final volume of 25 μl with the buffer and temperature recommended by the enzyme supplier.

Total RNA was also isolated from soybean nodules (0.3 g fwt) that had been previously separated from the roots at the harvest time and kept at −80 °C in order to be used for other biochemical determinations. The RNA from nodules was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany).

2.7. Cloning the gmp5cs and lsp5cs genes

Several stretches of conserved amino acids were apparent from the compilations of sequences for the P5CS protein in plants. Two stretches were used to design degenerate oligonucleotide primers as described by Numberg et al. [43]: primer forward 5′-GGT GT(TA) CTC CTG AT(TG) GT(TC) TTT GA-3′ and primer reverse 5′-CC (TC)TC AAC (TC)CC (TG)AC (TA)GG (AT)CC-3′. Using cDNA from either soybean or from lettuce roots subjected to drought stress as template, a cDNA fragment of about 840 bp was amplified with these primers and the polymerase chain reaction (PCR). PCR was carried out in a final volume of 50 μl containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.25 mM dNTPs, 100 pmoles of each primer and 2 units of *Taq* DNA polymerase (Sigma, St Louis, MO, USA). A Perkin/Elmer thermocycler (model 2400) was employed with the following values: initial denaturation at 95 °C for 5 min, followed by 35 denaturation cycles at 95 °C for 45 s, annealing at 52 °C for 45 s, elongation at 72 °C for 75 s, and a final elongation at 72 °C for 5 min. The amplified cDNA was purified following electrophoresis through a 1.2% agarose gel with the QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into pGEM plasmid (Promega). Recombinant plasmids were used to transform competent *E. coli* DH-5α cells. Positive clones screened by PCR were subcultured and plasmid DNA isolated using QIAprep^R Spin Miniprep kit (Qiagen).

2.8. Sequencing the cloned cDNA and analyses

Sequencing was performed by the dideoxy-sequencing method [57] using fluorescent dye-linked universal M13 primers and a Perkin-Elmer ABI Prism model 373 DNA sequencer. Similarity searches were carried out in the EMBL databank, using the BLAST software or the FASTA program available on-line from the National Centre for Biotechnology Information (NCBI).

2.9. Northern blot analysis

Total RNA (15 μg) was separated by electrophoresis on 1.2% agarose gel containing 2.2 M formaldehyde and blotted onto Hybond-N^+ nylon membranes (Amersham, Little Chalfont, UK) by capillarity [55]. Equal RNA loading and transfer were verified by methylene blue staining of nylon membranes before hydridization [28]. Blots were prehybridized 2–3 h at 42 °C in 5X Denhardt's solution, 5X SSC, 0.5% SDS and hybridized with *gmp5cs* or *lsp5cs* specific probes obtained by radioactive PCR labelling of plasmid inserts. Unincorporated ^{32}P was removed using Mini Quick Spin^{TM} columns (Boehringer Manheim, Indianapolis, IN). A total of 10^7 cpm probe was heat-denatured and used to hybridize the blots overnight at 65 °C under standard conditions [55]. After washing twice for 5 min at room temperature in 2X SSC and 0.1% SDS, and twice for 15 min at 65 °C with 0.5X SSC and 0.1% SDS, membranes were exposed overnight to Kodak X-RAY-OMAT at −70 °C. Signals on autoradiograms were analyzed and quantified using Quantity One software (BioRad, Hemel Hempstead, UK). Transcript accumulation levels for each gene probe were normalized according to the amount of rRNA in the corresponding membrane, which had been also quantified with Quantity One software. Each quantification of signals on autoradiograms and of rRNA was repeated three times and the average value was used for normalization. Northern blot analyses were repeated twice with different set of plants.

2.10. Nucleotide sequence accession number

The nucleotide sequences corresponding to *gmp5cs* and *lsp5cs* cDNAs have been deposited in the EMBL database

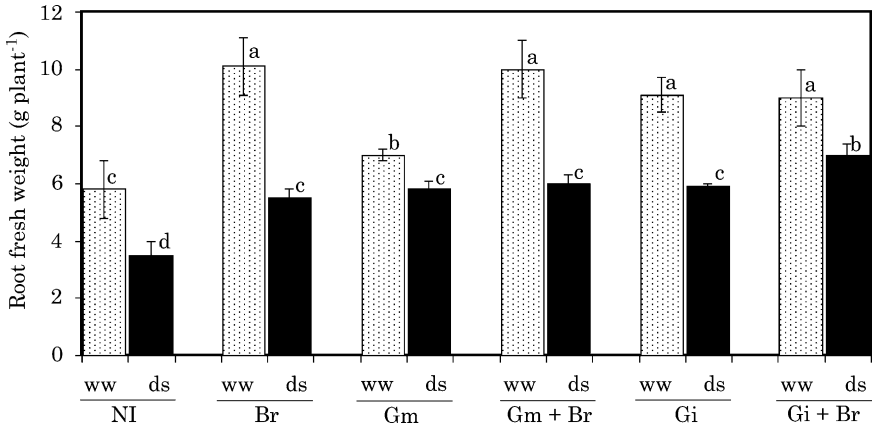

Fig. 1. Root fresh weight of soybean plants. Treatments are designed as NI, non-inoculated controls; Br, *Bradyrhizobium japonicum*; Gm, *Glomus mosseae*; Gm + Br, *G. mosseae* plus *B. japonicum*; Gi, *Glomus intraradices*; Gi + Br, *G. intraradices* plus *B. japonicum*. Plants were either well-watered (ww) or drought stressed (ds) for 10 days.

under accession numbers AJ715851, and AJ715852, respectively.

2.11. Statistical analysis

Data on proline content and root fresh weight were subjected to analysis of variance (ANOVA) with microbial treatment, water regime and microbial treatment–water regime interaction as sources of variation, and followed by Duncan's multiple range test [18].

3. Results

3.1. Symbiotic development in soybean and lettuce plants

No AM colonization or nodules were observed in non-inoculated soybean plants. The percentage of AM infection in plants from the first experiment ranged from 86 to 92% and the number of nodules in *B. japonicum*-inoculated plants from 30 to 50 (data not shown). In the time-course experiment, mycorrhizal infection inside roots and nodule formation were visible 12 dai and both symbioses were progressing until the last harvest (35 dai). The AM colonization at 12 dai was 17% (ww) and 15% (ds) of mycorrhizal root length, at 20 dai it reached 30% (ww) and 25% (ds) and at 35 dai it was 55% (ww) and 47(ds), while the number of nodules ranged from 20 to 30 in *B. japonicum*-inoculated plants (data not shown).

The percentage of AM colonization was about 80% in both lettuce experiments and resulted unaffected by drought stress and by proline addition (data not shown).

3.2. Root development

The root fresh weight of soybean plants (experiment 1) is shown in Fig. 1. Drought stress reduced root fresh weight in all treatments. Plants dually inoculated with *G. intraradices* plus *B. japonicum* showed the highest root development under drought stress conditions, while non-inoculated plants showed the lowest root development.

The root fresh weight of lettuce plants (experiment 1) also was reduced by drought stress in all treatments (Fig. 2). However, the reduction in root development was lower in both AM treatments than in the non-AM plants.

3.3. Cloning gmp5cs and lsp5cs genes

The use of the degenerate primers designed on conserved segments of P5CS allowed to obtain several clones containing inserts of the expected size using cDNA from soybean and from lettuce roots subjected to drought stress. The sequencing of several of the clones obtained from soybean cDNA showed that one of them contained a cDNA insert putatively encoding for a P5CS protein. The clone was named *gmp5cs*. Such clone contained a cDNA fragment of 831 bp and the putative protein encoded gave 76% identity with a P5CS protein from *Vigna unguiculata* (accession Q9AYM4, $e = 1 \times 10^{-114}$).

In the case of lettuce, we also sequenced several clones and only one of them contained a cDNA insert putatively encoding for a P5CS protein. Such clone was named *lsp5cs*.

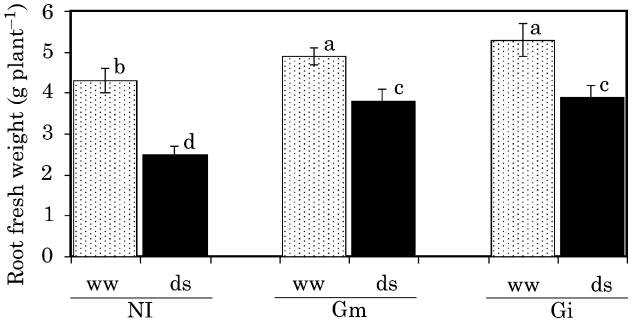

Fig. 2. Root fresh weight of lettuce plants. Treatments are designed as NI, non-inoculated controls; Gm, *Glomus mosseae*; Gi, *Glomus intraradices*. Plants were either well-watered (ww) or drought stressed (ds) for 10 days.

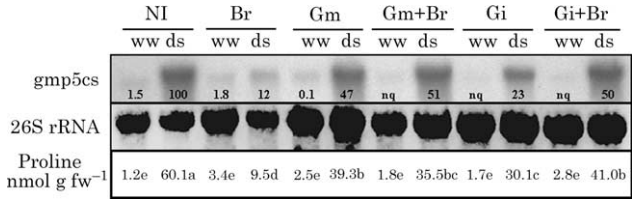

Fig. 3. Northern blot of total RNA (15 μg) from soybean roots. Treatments are designed as NI, non-inoculated controls; Br, *Bradyrhizobium japonicum*; Gm, *Glomus mosseae*; Gm + Br, *G. mosseae* plus *B. japonicum*; Gi, *Glomus intraradices*; Gi + Br, *G. intraradices* plus *B. japonicum*. Plants were either well-watered (ww) or drought stressed (ds) for 10 days. The percentage of gene expression is indicated by numbers close to each northern (nq means 'not quantifiable'). The second panel shows the amount of 26S rRNA loaded for each treatment (methylene blue staining). The third panel shows the proline content in these roots.

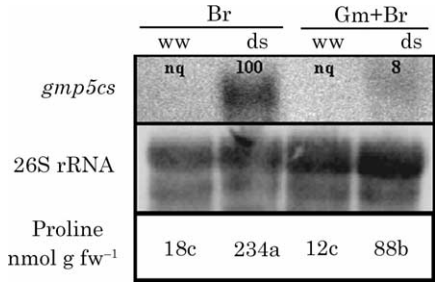

Fig. 4. Northern blot of total RNA (10 μg) from soybean nodules. Treatments are designed as Br, *Bradyrhizobium japonicum* and Gm + Br, *G. mosseae* plus *B. japonicum*. Plants were either well-watered (ww) or drought stressed (ds) for 10 days. The percentage of gene expression is indicated by numbers close to each northern (nq means 'not quantifiable'). The second panel shows the amount of 26S rRNA loaded for each treatment (methylene blue staining). The third panel shows the proline content in these nodules.

It contained a cDNA fragment of 839 bp. The putative protein encoded by that cDNA gave 72% identity with the P5CS from *Mesembryanthemum crystallinum* (accession O65361, $e = 1 \times 10^{-113}$).

3.4. Northern blot analysis with soybean RNAs and proline content

The cDNA insert from soybean (*gmp5cs*) was used as probe in northern blot analyses with RNA from soybean roots from a variety of microbial treatments (see Section 2.1). The results obtained are shown in Fig. 3. The higher gene expression was found in non-inoculated plants subjected to drought that was set as 100% in arbitrary units after normalization of northern according to the amount of ribosomal RNA loaded in the blots. Under well-watered conditions, all the treatments showed slight but constitutive gene expression, which ranged from 1.8% for plants singly inoculated with *B. japonicum* to 0.1% for plants singly inoculated with *G. mosseae* or with *G. intraradices*. Drought stress up-regulated the level of *gmp5cs* transcript accumulation in all treatments. Only plants singly inoculated with *B. japonicum* showed a reduced level of up-regulation in *gmp5cs* gene expression under drought stress conditions (only 12% of non-inoculated plants). Plants singly inoculated with *G. mosseae* showed up-regulation of *gmp5cs* gene expression under drought stress (47% of the level found in non-inoculated plants), and a similar level of gene expression was found in those dually inoculated with *B. japonicum* plus *G. mosseae* under drought stress conditions (51% of non-inoculated plants). Plants singly inoculated with *G. intraradices* only showed detectable *gmp5cs* gene expression under drought stress conditions, but this expression was lower than in the other fungal treatment (23% of non-inoculated plants). Finally, plants dually inoculated with *B. japonicum* plus *G. intraradices* also exhibited *gmp5cs* gene expression under drought stress conditions (50% of non-inoculated plants).

The proline content was also maximum in non-inoculated plants subjected to drought stress (Fig. 3), followed by all the AM plants subjected to drought. However, plans singly inoculated with *G. intraradices* or with *B. japonicum* accumulated less proline under drought stress than the rest of treatments (as also happened with the *gmp5cs* transcripts). The level of proline accumulated in roots from well-watered plants was low in all treatments.

RNA from soybean root nodules was also extracted and used for northern blot analysis of *p5cs* transcript accumulation. Fig. 4 shows results obtained with *gmp5cs* probe in nodules from plants inoculated only with *B. japonicum* or dually inoculated with *B. japonicum* plus *G. mosseae* (the fungus that had showed a more normal behaviour in relation to *gmp5cs* gene expression as compared to *G. intraradices*, see Fig. 3). It is clearly visible that in nodules of *B. japonicum*-inoculated plants there was no expression of *gmp5cs* gene in well-watered plants, while the expression increased notably in nodules of these plants when subjected to drought stress (which was set as 100%). In nodules from plants dually inoculated with both symbiotic microorganisms the level of *gmp5cs* gene expression under drought stress conditions was lower than in the non-AM plants (8% of Br plants).

Proline also accumulated considerably more in nodules of non-AM plants subjected to drought than in the corresponding AM plants.

Following the low up-regulation of *gmp5cs* gene expression and low proline accumulation in roots from plants singly inoculated with *B. japonicum* and subjected to drought, we planed a time-course experiment in order to study the expression level of *gmp5cs* gene at different time intervals in non-inoculated soybean plants or after inoculation with either *B. japonicum* or with *B. japonicum* plus *G. mosseae* (the fungus that had showed a more normal behaviour in relation to *gmp5cs* gene expression as compared to *G. intraradices*, see Fig. 3). All plants harvested at 5 dai showed a low but constitutive level of

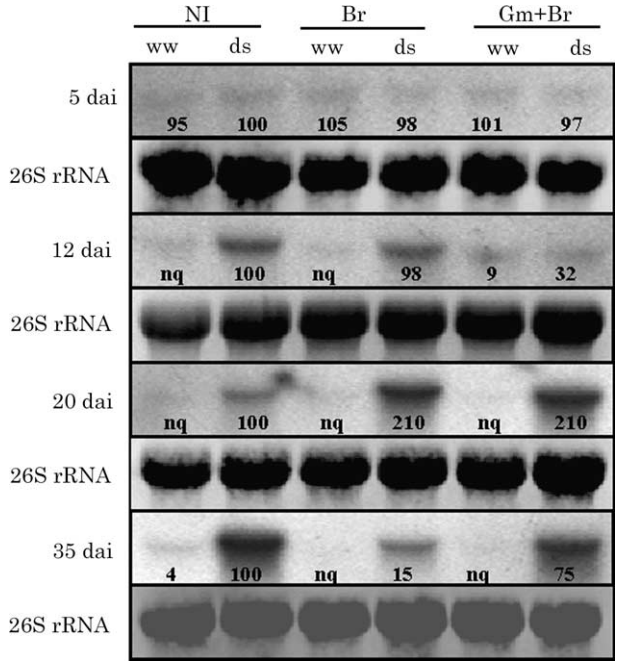

Fig. 5. Northern blot of total RNA (15 μg) from soybean roots with *gmp5cs*. Plants were harvested 5, 12, 20 or 35 days after inoculation (dai). Treatments are designed as NI, non-inoculated controls; Br, *Bradyrhizobium japonicum*; Gm+Br, *G. mosseae* plus *B. japonicum*. Plants were either well-watered (ww) or drought stressed (ds) for 10 days. The percentage of gene expression is indicated by numbers close to each northern (nq means 'not quantifiable'). The panel under each northern shows the amount of 26S rRNA loaded for each treatment (methylene blue staining).

gmp5cs transcript accumulation under whatever conditions (Fig. 5). At 12 dai, *gmp5cs* transcript accumulation was up-regulated in the three treatments when subjected to drought. However, plants dually inoculated with *B. japonicum* plus *G. mosseae*, showed a considerably lower up-regulation of gene expression. After normalization, the level of gene expression in arbitrary units was 100% for non-inoculated plants, 98% for *B. japonicum*-inoculated plants and 32% for dually inoculated plants. At 20 dai, again *gmp5cs* transcript accumulated in the tree treatments subjected to drought, but, surprisingly, the level of expression was considerably lower in the non-inoculated plants (set as 100%) than in the other two treatments (about 210% of non-inoculated plants). Finally at 35 dai, the pattern of *gmp5cs* transcript accumulation returned similar to that observed in plants from the first experiment (Fig. 3) since non-inoculated plants exhibited the highest levels of transcript accumulation under drought stress (set as 100%), while plants inoculated only with *B. japonicum* showed little *gmp5cs* gene expression under drought conditions (15% of non-inoculated plants). Plants dually inoculated with *B. japonicum* plus *G. mosseae* also showed enhanced gene expression under drought stress, but lower than in non-inoculated plants (75% of non-inoculated plants).

3.5. Northern blot analysis with lettuce RNA and proline content

In order to test the behaviour of the P5CS gene in a non legume plant and avoid the interference of AM symbiosis with that of *Bradyrhizobium* symbiosis, the cDNA clone from lettuce (*lsp5cs*) was also used for northern blot analysis with RNA from non-inoculated of AM lettuce roots cultivated under well-watered or drought stressed conditions. Results (Fig. 6) showed that under well-watered conditions there was a low but constitutive *lsp5cs* gene expression the three treatments assayed (more visible in non-inoculated plants). Drought stress induced *lsp5cs* transcript accumulation in the three treatments. However, the level of induction was higher in roots from non-inoculated plants than in roots from both AM treatments. After normalization to the corresponding RNA loaded in the blots, the level of *lsp5cs* gene expression in non-inoculated plants was set as 100%, the level of *lsp5cs* gene expression in plants colonized by *G. mosseae* was 70% as compared to non-inoculated plants, and the level of expression in plants colonized by *G. intraradices* was 72% as compared to the non-inoculated plants.

The highest accumulation of proline was observed in non-inoculated plants subjected to drought stress, followed by both AM stressed treatments (Fig. 6). Non-inoculated plants accumulated slightly higher levels of proline under well-watered conditions than well-watered AM plants.

We planed a second experiment with lettuce plants added or not of proline (the osmolyte in whose biosynthesis participates the P5CS). In that case, since both AM fungi tested affected in a similar way the expression of *lsp5cs* gene, we used *G. intraradices* as the colonizing AM fungus because in previous experiments with lettuce plants it has shown to be very effective protecting the host plants against drought [41]. The expression of *lsp5cs* genes was analyzed in roots of AM or control lettuce plants added or not of 10 mM proline during the growth period. Results obtained (Fig. 7) showed that in root tissues of non-inoculated plants *lsp5cs* transcripts accumulated both under well-watered and under drought stressed conditions (regardless of proline

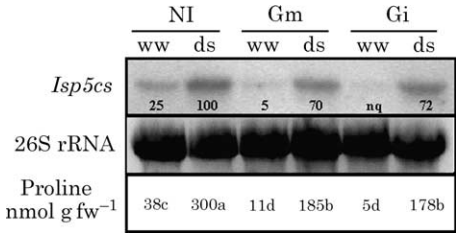

Fig. 6. Northern blot of total RNA (15 μg) from lettuce roots. Treatments are designed as NI, non-inoculated controls; Gm, *Glomus mosseae*; Gi, *Glomus intraradices*; plants were either well-watered (ww) or drought stressed (ds) for 10 days. The percentage of gene expression is indicated by numbers close to each northern (nq means 'not quantifiable'). The second panel shows the amount of 26S rRNA loaded for each treatment (methylene blue staining). The third panel shows the proline content in these roots.

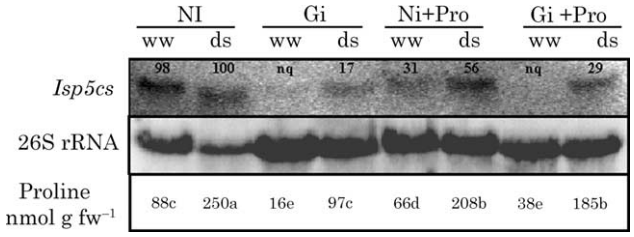

Fig. 7. Northern blot of total RNA (15 μg) from lettuce roots added or not of 10 mM proline (+Pro). Treatments are designed as NI, non-inoculated controls; Gi, *Glomus intraradices*; plants were either well-watered (ww) or drought stressed (ds) for 10 days. The percentage of gene expression is indicated by numbers close to each northern (nq means 'not quantifiable'). The second panel shows the amount of 26S rRNA loaded for each treatment (methylene blue staining). The third panel shows the proline content in these roots.

addition), while in *G. intraradices*-colonized plants, the expression was only detected under drought stressed conditions (regardless of proline addition). The highest expression of *lsp5cs* gene was found in roots from non-inoculated plants without proline addition, both under well-watered and under drought stress conditions. The two values of non-inoculated plants, after normalization according to the RNA loaded in the membrane, were set up as 98 and 100%, respectively. Plants colonized with *G. intraradices* and not supplied with proline only showed 17% of *lsp5cs* transcript accumulation under drought stress conditions. The addition of proline to non-inoculated plants decreased the level of *lsp5cs* transcript accumulation to 31% under well-watered conditions and to 56% under drought stress conditions. The addition of proline to plants colonized by *G. intraradices* slightly enhanced the accumulation of *lsp5cs* transcript until 29% as compared to the same plants not supplied with proline.

Non-inoculated plants subjected to drought and not supplied with proline accumulated the highest amount of proline (Fig. 7). The two plant treatments added of proline and subjected to drought also accumulated important amounts of proline. Curiously, the non-inoculated plants cultivated under well-watered conditions also showed significant levels of proline and accumulated a similar amount of proline as the stressed AM plants not supplied with proline.

4. Discussion

As a soil dries out and its water potential becomes more negative, plants must decrease their water potential to maintain a favourable water flow gradient from soil into roots. The most important mechanism to achieve such an effect, known as osmotic adjustment or osmoregulation, is to decrease the plant osmotic potential by active accumulation of organic ions or solutes [30,42]. Of these metabolites, proline is probably the most widespread in plants [2,44,65,69]. It has been shown that proline accumulates under conditions of water shortage, high salinity, chilling, heat and heavy metal exposure. It plays a major role in osmorregulation and osmotolerance [15]. Moreover, it has been shown to protect enzymes from inactivation by salinity, heat, chilling and dilution in vitro [16,59].

In previous studies, we have shown several physiological and biochemical mechanisms by which the AM symbiosis protected the host plants against the detrimental effects of drought [51]. In contrast, molecular investigations in AM symbiosis have been far less common to date than physiological studies. In this study, we have extended previous physiological observations by studying at the molecular level the possible participation of genes encoding for P5CS, the enzyme catalyzing the rate-limiting step in proline biosynthesis [2,35,71], in the enhanced tolerance to drought stress in mycorrhizal soybean and lettuce plants. In fact, several investigations on the relationship between the expression of the key gene involved in the synthesis of proline and the accumulation of proline under water stress indicate that the level of proline in plants is mainly regulated at the transcriptional level during water stress [1,2,14,32,68,69,70].

The analysis of *gmp5cs* and *lsp5cs* gene expression showed that, in general, these genes responded to drought and were up-regulated in drought stressed treatments, suggesting that they are important for the plant response against stresses involving water deficit [24,35,46,69]. A contrasting result was obtained, however, in soybean plants singly inoculated with *B. japonicum*, where the *gmp5cs* gene showed little up-regulation in roots under drought stressed conditions. This result was latter confirmed in the time-course experiment at 35 dai. Hence, a question raises; why the level of *gmp5cs* gene expression did not increase at these plant stages in nodulated non-AM plants after drought stress? To answer this question it must be considered that drought stress also induces synthesis and accumulation of abscisic acid (ABA), which mediates the plant responses to water deficiency, mostly by promoting stomatal closure [25], but also by inducing the expression of water deficit-responsive genes such as *p5cs* [1,10,11,12,62]. In addition, it has been demonstrated that the expression of *p5cs* genes has two regulatory pathways, an ABA-dependent and an ABA-independent pathway, and that both can act simultaneously [1,19,58,61,70]. Hence, a possible answer to the above question is that nodulation itself can be affecting one of these regulatory pathways for P5CS accumulation (i.e. the ABA-independent pathway), avoiding the accumulation of *p5cs* transcripts. In contrast, the mycorrhization of nodulated plants restore, at least in part, the normal *p5cs* transcripts accumulation pattern by compensating in some way such ABA-independent pathway.

In nodules of plants singly inoculated with *B. japonicum*, the pattern of *gmp5cs* gene expression was the expected one, namely up-regulation under drought stress conditions. An elevated rate of proline biosynthesis in nodules has been suggested to stimulate ureide synthesis in legumes and to help transfer redox potential from the nodule cytoplasm to the bacteroids [37]. Our results also showed that the amount

of proline accumulated in nodules was higher than in soybean roots. Proline may also act as a carbon and nitrogen source for the bacteroids. An additional role of proline in nodules may be its involvement in osmoregulation [14]. In fact, the osmoticum in infected nodule cells is known to be 4- to 5-fold higher than in root cells [67]. Hence, the up-regulation of *gmp5cs* in nodules of droughted plants may represent an osmoregulatory adaptation to increased concentration of solutes. What is not explained by that hypothesis is why the expression of the *gmp5cs* gene in nodules from soybean plants dually inoculated with the *G. mosseae* and with *B. japonicum* was considerably lower than in the corresponding non-mycorrhizal plants. However, it has been proposed that mycorrhization can alter the levels of ABA in the host plant and that under drought stress the levels of ABA are lower in AM than in non-AM plants [17,21,39]. Hence, an ABA-dependent regulation pathway could explain the decrease in *gmp5cs* gene expression in nodules of these double inoculated plants as compared to those of single nodulated soybean plants.

The last mechanism may explain, in the same way, why the levels of *gmp5cs* and *lsp5cs* gene expression are lower in roots from droughted soybean and lettuce AM plants than in roots from droughted soybean and lettuce non-inoculated plants.

The regulation of gene expression by ABA or water stress varies depending on the tissue or on the developmental stage of plant [33,60]. Furthermore, *p5cs* genes can be also under developmental regulation [2,70]. These findings can explain the little *gmp5cs* gene expression during the first stages of the time-course experiment and why the gene expression increased in subsequent stages of the time-course.

Proline has been demonstrated to ameliorate dehydration-induced perturbation in proteins [45], and exogenously supplied proline confers osmotic tolerance to the plants and cultured cells [44]. In this study, the addition of proline to lettuce plants reduced the expression of *lsp5cs* gene in all cases, except in *G. intraradices*-inoculated plants subjected to drought. It is known that P5CS protein (and, probably, *p5cs* gene expression) is feedback inhibited by proline [63]. Such feedback inhibition of P5CS by proline is lost under drought stress due to conformational changes in the P5CS protein [9,27,31,40,71]. However, it has been proposed that in some circumstances the feedback inhibition of P5CS protein (and we assume that also of *p5cs* gene, as mentioned above) is not completely eliminated under drought stress conditions [31], which could explain that *lsp5cs* gene did not increase significantly the transcript accumulation levels after addition of proline in non-inoculated or in *G. intraradices*-inoculated plants.

In any case, a consistent effect observed both in soybean and in lettuce plants is that, contrarily to our initial hypothesis expecting a possible increase of *p5cs* gene expression in AM plants and contributing to the alleviation of drought stress by the AM symbiosis, the expression of *gmp5cs* and *lslp5cs* genes decreased in drought stressed AM plants as compared to non-inoculated plants. As mentioned above, this effect can be related to an hormonal or other developmental change induced in AM plants, but it is also possible that AM plants were simply less strained by drought stress and by that reason the level of *p5cs* genes expression was lower in AM than in non-AM plants. In previous studies in which we and other authors have found physiological or biochemical mechanisms involved in the enhanced tolerance to drought stress in AM plants it has also been proposed that primary drought-avoidance mechanisms (i.e. direct water uptake by hyphae) or increased water uptake related to mycorrhizal changes in root morphology [38] or soil structure [4,5] might have contributed to the AM protection of host plants against drought [48]. Also, a recent study has revealed that, apart from direct hyphal water uptake, it seems that first the AM symbiosis enhances osmotic adjustment in roots, that can contribute to maintain a water potential gradient favourable to the water entrance from soil into the roots. This enables higher leaf water potential in AM plants during drought and keeps the plants protected against oxidative stress, and all these cumulative effects increases the plant tolerance to drought [49].

In conclusion, our results demonstrate that the cloned *p5cs* genes responded clearly to drought stress and were up-regulated under drought conditions in soybean and lettuce plants, contributing to their protection against drought. Mycorrhization of these plants with either *G. mosseae* or *G. intraradices* did not induce the expression of the *p5cs* genes analyzed. Moreover, the levels of *p5cs* transcript accumulation in mycorrhizal treatments subjected to drought were considerably lower than in the corresponding non-mycorrhizal plants, indicating that the induction of *p5cs* gene is not a mechanism by which the AM symbiosis protects their host plant, and suggesting that AM plants were less strained by drought than non-AM plants due to primary drought-avoidance mechanisms.

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