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Isolation and characterization of two different cDNAs of Δ^1-pyrroline-5-carboxylate synthase in alfalfa, transcriptionally induced upon salt stress

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Abstract

Two different cDNA clones, *MsP5CS-1* and *MsP5CS-2*, encoding Δ^1-pyrroline-5-carboxylate synthase (P5CS), the first enzyme of the proline biosynthetic pathway, were isolated from a λZap-cDNA library constructed from salt stressed *Medicago sativa* roots. *MsP5CS-1* (2.6 kb) has an open reading frame of 717 amino acids, as well as a non-spliced intron at a position corresponding to the evolutionary fusion point of the bacterial *proA* and *proB* genes. *MsP5CS-2* (1.25 kb) is a partial clone. The clones share 65% identity in nucleotide sequences, 74% homology in deduced amino acid sequences, and both show a high similarity to *Vigna aconitifolia* and *Arabidopsis thaliana P5CS* cDNA clones. Southern blot analysis confirmed the presence of two different *P5CS* genes. The effect of salinity on the transcription of *MsP5CS-1* and *MsP5CS-2* in roots was studied, using northern blot analysis and a RT-PCR approach. A rapid increase in the steady-state transcript level of both genes in roots was observed by RT-PCR upon exposure of hydroponically grown 6-day old seedlings to 90 mM NaCl, suggesting that both are salt-inducible genes, yet a higher response was observed for *MsP5CS-2*.

Introduction

Alfalfa (*Medicago sativa*) is an important leguminous forage crop worldwide. Salt stress imposed by 50–200 mM NaCl significantly limits productivity of alfalfa, via its adverse effects on growth, interaction with *Rhizobium meliloti*, formation of nodules and symbiotic nitrogen-fixation capacity [2]. Salt stress increases the amino acid concentration in alfalfa nodules and roots, with proline being the most highly accumulated; its endogenous concentration increases from less than 3% under normal conditions to at least 12% of the total amino acid content during two weeks of 150 mM NaCl stress [7]. This dramatic increase in intracellular proline has been observed in a wide variety of osmotically stressed plants [28], suggesting that this amino acid is a compatible osmolyte that plays a role in counteracting the stress effects [5, 9]. In higher plants, proline is synthesized from glutamate as well as from ornithine [5]. Under conditions of osmotic stress, the glutamate pathway predominates over the ornithine pathway, and plants convert more glutamate to proline than transaminate ornithine to Δ^1-pyrroline-5-carboxylate (P5C) [5, 15].

Proline is synthesized from glutamate via two successive reductions. In *Escherichia coli*, γ-glutamyl kinase (γ-GK; encoded by the *proB* gene) phosphorylates glutamate to γ-glutamyl phosphate, which is then

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X98421 (*MsP5CS-1*) and X98422 (*MsP5CS-2*).

reduced to glutamic-γ-semialdehyde (GSA) by GSA dehydrogenase (encoded by the *proA* gene). GSA cyclizes spontaneously into P5C, which is then reduced to proline by pyrroline-5-carboxylate reductase (P5CR; encoded by the *proC* gene) [17]. In plants such as *Vigna aconitifolia* [10] and *Arabidopsis thaliana* [23, 30], reduction of glutamate to its semialdehyde intermediate is catalyzed by a single bifunctional enzyme, Δ^1-pyrroline-5-carboxylate synthase (P5CS), whose transcription is induced in roots subjected to salt stress. Furthermore, overexpression of *V. aconitifolia* P5CS (VaP5CS) in transgenic tobacco plants increased the proline level and rendered plants less sensitive to osmotic stress [15]. In contrast, the expression of soybean *P5CR* cDNA in transgenic tobacco, although resulting in increased P5CR activity, did not affect the level of proline [27], indicating that P5CR is not the rate-limiting enzyme in proline biosynthesis in plant cells.

In the present work, cDNAs representing two different *P5CS* genes were identified. Structural analysis suggested that one of them still contained a unique unspliced intron localized at the site corresponding to intron 7 of *A. thaliana P5CS* genes and to the evolutionary fusion point of *Escherichia coli proB* and *proA* genes. While both of the alfalfa *P5CS* genes were found to be activated by salt in roots, *MsP5CS-1* was more responsive.

Materials and methods

Plant growth, stress treatment and proline evaluation

Alfalfa (*Medicago sativa* L. cv. Gilboa) seedlings were grown hydroponically under axenic conditions, using a sterile medium [12] under a 12 h photoperiod (white light) at 27 °C with aeration. Six-day-old seedlings were subjected to salt stress by adding NaCl to a final concentration of 90 mM or maintained on NaCl-free medium for 72 h. Roots of 100 treated or control seedlings were collected 6, 24, 48 and 72 h after salt application, in the middle of the light period to eliminate photoperiodic effects, frozen in liquid nitrogen and stored at −80 °C.

Root proline content was evaluated as described by Kapulnik and Heuer [13].

Cloning of M. sativa-P5CS *cDNAs*

Total RNA was isolated from 6 h NaCl-treated roots [14]. The poly(A)$^+$ fraction was purified by chromatography on oligo(dT) cellulose as described by Sambrook *et al.* [22] except for the omission of SDS from the solutions. A λZap-cDNA library was constructed (Stratagene, USA) and screened by plaque hybridization using *Arabidopsis P5CS* cDNA [26] as a probe. Five positive clones were isolated from 50 000 plaques and converted to pBluescript SK(−) phagemids by *in vivo* excision, according to the Stratagene manual.

PCR amplification of specific MsP5CS *sequences from the cDNA library*

PCR amplification was performed with 100 ng DNA of the λZap-cDNA library as a template, and 40 pmol of direct and reverse primers corresponding to nucleotides 727–748 and 1506–1487 of the *MsP5CS*-1 cDNA sequence, respectively. After denaturation for 3 min at 95 °C, 30 amplification cycles of 45 s at 94 °C, 45 s at 50 °C and 45 s at 72 °C with an 5 min extended elongation step were applied in a PTC-100 Programmable Thermal Controller (MJ Research, USA). PCR product was gel-purified, cloned in pUC57 (Fermentas) and sequenced.

DNA sequence analysis

The two strands of each cDNA clone in pBluescript SK(−) and *MsP5CS*-1 PCR clone in pUC57, were sequenced by the dideoxy chain termination method with an automated DNA sequencer, dye terminators, and the standard T3 and T7 primers or clone-specific primers based on already sequenced regions. Computer analyses of nucleotide and amino acid sequences were carried out using software from the GCG/EGCG package of the University of Wisconsin running under a UNIX system.

Southern blot analysis

Genomic DNA was isolated from leaves of 3-week old *M. sativa* plants grown in soil [4]. For Southern blot analysis, 20 μg DNA was digested with *Eco*RI or *Hin*dIII, separated on a 0.8% agarose gel in Tris-borate (TBE) buffer and blotted onto a Hybond-N$^+$ membrane (Amersham, UK). The membrane was hybridized with the ^{32}P-1.02 kb *Eco*RI-*Bam*HI 5′ fragment of *MsP5CS-1* cDNA labelled by random priming. Hybridization was carried out at 65 °C in 0.263 M Na_2HPO_4, 1% (w/v) BSA, 7% (w/v) SDS and 1 mM EDTA. Washes were performed twice for 15 min at 65 °C with the following solutions: (1) 0.263 M

Na_2HPO_4, 1% SDS (2) 2× SSC, 1% SDS (3) 1× SSC, 0.1% SDS.

Evaluation of P5CS *transcript steady-state levels by northern blot analysis and RT-PCR*

Total RNA was isolated from 6, 24, 48 and 72 h salinized and control *M. sativa* roots using the Tri-Reagent kit (Molecular Research Center, USA). For evaluation of the overall level of *MsP5CS* transcripts by northern analysis, samples of 10 μg total RNA were separated on a 1.1% formamide-agarose gel [22], blotted onto a nylon membrane (Hybond N, Amersham, UK), and hybridized either with ^{32}P-*MsP5CS-1* cDNA or with 1 kb tomato ^{32}P-rDNA fragment. The blots were washed twice with 2× SSC, 0.1% (w/v) SDS at room temperature for 10 min, and twice with 0.2× SSC, 0.1% (w/v) SDS at 60 °C for 10 min and exposed to either X-ray film (Fujifilm, Fuji, Japan) with an intensifying screen at −70 °C or a Phosphor-Imager screen. The Phosphor-Imager Program (Fujix BAS 1500, Fuji, Japan) was used for radioactivity quantification.

Evaluation of the steady-state levels of *MsP5CS*-1 or *MsP5CS*-2 transcripts were performed by reverse-transcription-PCR-coupled reactions (RT-PCR) using specific primers for each gene. Roots total RNA (1 μg) samples from salt and control treatments were subjected to a reverse transcription reaction performed with 200 units of M-MLV RT (RNase H-Minus, Promega, USA), 0.5 unit RNasin (Promega), 200 μm dNTPs and 40 pmol specific primers, in a total volume of 20 μl. The reverse primer 3, 5′-TTCATGATCTTCTGTG, was specifically used for *MsP5CS-1* (nucleotides 2068–2052) and reverse primer 4, 5′-CTCTGATTCAACTGATTGC, was specific for *MsP5CS-2* (nucleotides 872–854). Primers were annealed to mRNA templates at 50 °C for 10 min prior to the addition of the enzyme. The RT reactions were performed at 42 °C for 30 min and stopped by heat inactivation of the enzyme at 94 °C for 5 min. The reactions were then diluted 10-fold and samples corresponding to 5, 25 and 50 ng RNA were PCR-amplified several times at different number of cycles to calibrate template dependent linearity. According to this calibration, samples corresponding to 15 ng total RNA initially taken for the RT reaction, and shown to be within the linearity range, were PCR-amplified in 50 μl of a reaction mix consisting of 10 mM Tris-HCl pH 8.8, 50 mM KCl, 0.08% (v/v) NP-40, 1.5 mM $MgCl_2$, 100 μM dNTPs, 25 pmol of each direct and reverse primer and 1 unit *Taq* DNA polymerase (Promega). The PCR reaction specific for *MsP5CS-1* contained reverse primer 3 and the direct primer 1, 5′-AACGAACTGAGCTTGCAGAG (nucleotides 1328–1347), whereas in the reaction specific for *MsP5CS-2*, primer 4 served as the reverse primer and primer 2, 5′-GCAGCCTGCAATGCAATGG (nucleotides 310–328), as the direct primer. Twenty amplification cycles of 45 s at 94 °C, 45 s at 50 °C and 45 s at 72 °C were applied in a PTC-100 Programmable Thermal Controller (MJ Research, USA). PCRs were repeated twice with replicates. RT-PCR products were separated on a 1.5% agarose gel in TBE buffer, transferred onto a Hybond-N$^+$ membrane and hybridized with ^{32}P-random-primer-labelled *MsP5CS-1* or *MsP5CS-2* probes to detect the specific products. Hybridization conditions were as described for Southern analysis. ^{32}P-labelled bands were quantified by the Phosphor-Imager program. To normalize the RT-PCR results per equal amount of rRNA, a slot blot

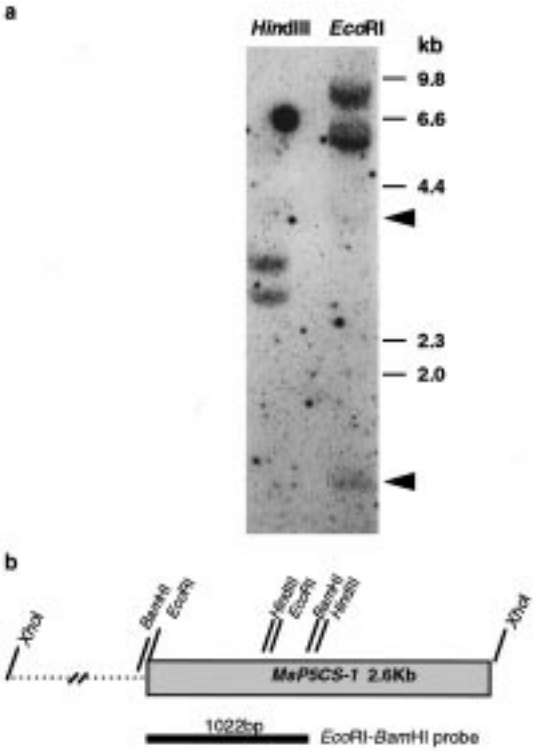

Figure 1. Estimation of the number of *P5CS* genes in the alfalfa genome by Southern blot analysis. a. DNA (20 μg) was digested with *Hin*dIII or *Eco*RI. Hybridization was carried out with a 1.02 kb *Eco*RI-*Bam*HI 5′ fragment of the *MsP5CS-1* clone. Size markers are indicated on the right-hand side. Arrows denote the weakly labelled *Eco*RI bands. b. Physical map of the *MsP5CS-1* cDNA clone indicating the restriction sites and the probe (below) used for hybridization. Dotted line represents pBluescript SK(−) vector.

was prepared using similar RNA samples and probed with a ^{32}P-labelled 1 kb tomato rDNA (28S) fragment under identical conditions. The radioactivity of the hybridization signals was similarly evaluated. The Phosphor-Imager values of the RT-PCR blot were normalized to equal amounts of rRNA and presented in Phosphor-Imager arbitrary units.

Results

Isolation and characterization of M. sativa-P5CS *cDNAs*

A cDNA library in λZap was prepared from root RNA of hydroponically grown 6-day-old seedlings which had been exposed to 90 mM NaCl for 6 h. The library was screened using the 1.6 kb *Bgl*II fragment of *A. thaliana P5CS* (*AtP5CS-2)* cDNA as a probe [26]. Five clones out of 50 000 plaques were identified. The largest cDNA clone was sequenced and designated *MsP5CS-1*. The 2603 bp sequence had a single putative open reading frame.

To estimate the number of *P5CS* genes present in the alfalfa genome, a Southern blot analysis of *M. sativa* DNA was performed using the 1.0 kb *Eco*RI-*Bam*HI fragment, corresponding to the 5′ half of the *MsP5CS-1* cDNA, as a probe (Figure 1b). Four bands were observed in DNA digested with *Eco*RI and only two bands following *Hin*dIII digestion (Figure 1). Two of the *Eco*RI bands (7 kb, 5 kb) and both *Hin*dIII bands (2.8 kb, 3.5 kb) were evenly and strongly labelled and corresponded to two 5′ fragments of two different genes. Two other, weakly labelled *Eco*RI bands (4 kb, 1.1 kb, indicated by arrows) probably represented two 3′ fragments of two different genes. By screening *Eco*RI-digested DNA patterns of the initially identified λZap clones, an additional cDNA clone was isolated. The second clone was designated *MsP5CS-2* and was fully sequenced. Sequence comparison of the two isolated clones showed that *MsP5CS-2* represents only the 3′ part of a second *P5CS* gene, comprising the last 281 codons and a 336 bp 3′-untranslated region.

Complementation experiments with the *MsP5CS-1* clone were performed in *E. coli* JM83 (Δ*proAB*) harboring pACYC184-*proA* (H. Stein, unpublished). The isolated *MsP5CS-1* clone and a modified *MsP5CS-1* clone starting from its translation start site, both driven by the *lacZ* promoter in pBluescript, were examined for γ-GK complementation ability. Both clones failed to complement native *proB* deficiency in the presence of IPTG (isopropylthio-β-D-galactoside).

An overall 65% identity in nucleotide sequences and 74% identity in deduced amino acid sequences within the last 281 amino acid residues, were found between the two alfalfa-*P5CS* cDNA clones. Comparison of *MsP5CS-1* and *MsP5CS-2* to nucleotide and amino acid sequences of two *Arabidopsis* cDNA clones (accession numbers X87330, X86777, Y09355) [23, 26], and the *V. aconitifolia* cDNA [10] is summarized in Figure 2 and Table 1. Unexpectedly, the alfalfa *MsP5CS-1* cDNA was more homologous to the *Vigna* cDNA than to the second alfalfa cDNA (Table 1), while 82% identity is found between the two *A. thaliana* cDNAs. Comparison of the entire deduced amino acid sequences of MsP5CS-1 and MsP5CS-2 with those of *E. coli* γ-GK and GSA dehydrogenase showed low identity (Table 1), as had previously been shown for VaP5CS [10] and AtP5CS [23].

A seemingly extraordinary coding sequence of 36 amino acid residues was present only in alfalfa *MsP5CS-1* (Figure 2). The sequence was located where the *E. coli* γ-GK-like region is fused to the GSA dehydrogenase-like region [26]. Comparison of that sequence with the *A. thaliana* genomic (X86778, X89414) and corresponding cDNA sequences (Y09355, X87330) showed that *MsP5CS-1* cDNA still contains a single unprocessed intron at a position corresponding to intron 7 of *AtP5CS-1,2* [23, 26]. To verify the presence of this intron, PCR specific amplification of the *MsP5CS-1* region, corresponding to *A. thaliana* sequence from exon 5 through exon 10 was carried out, using the whole λZap-cDNA library as the DNA template (Figure 3a). In parallel, similar reaction was performed with the cloned *MsP5CS-1* as the template. A major amplification product of 675 bp and a very low amount of 780 bp product (a faint band not visualized in Figure 3a) were obtained when the λZap-cDNA library served as the template, while a single 780 bp product resulted from the amplification of *MsP5CS-1* clone. The 675 bp fragment was sequenced and found to be identical to the *MsP5CS-1* sequence devoid of intron 7. Thus, a small number of *MsP5CS-1* mRNA molecules appear to have retained an intron, while all the other introns (19 in the case of the *Arabidopsis* genes) were correctly spliced.

Transcription of M. sativa P5CS *in roots during salt stress*

M. sativa plants exposed to salt stress accumulate proline in their shoots and roots [7, 13]. To verify whether salinity induces transcription of *P5CS* genes in the

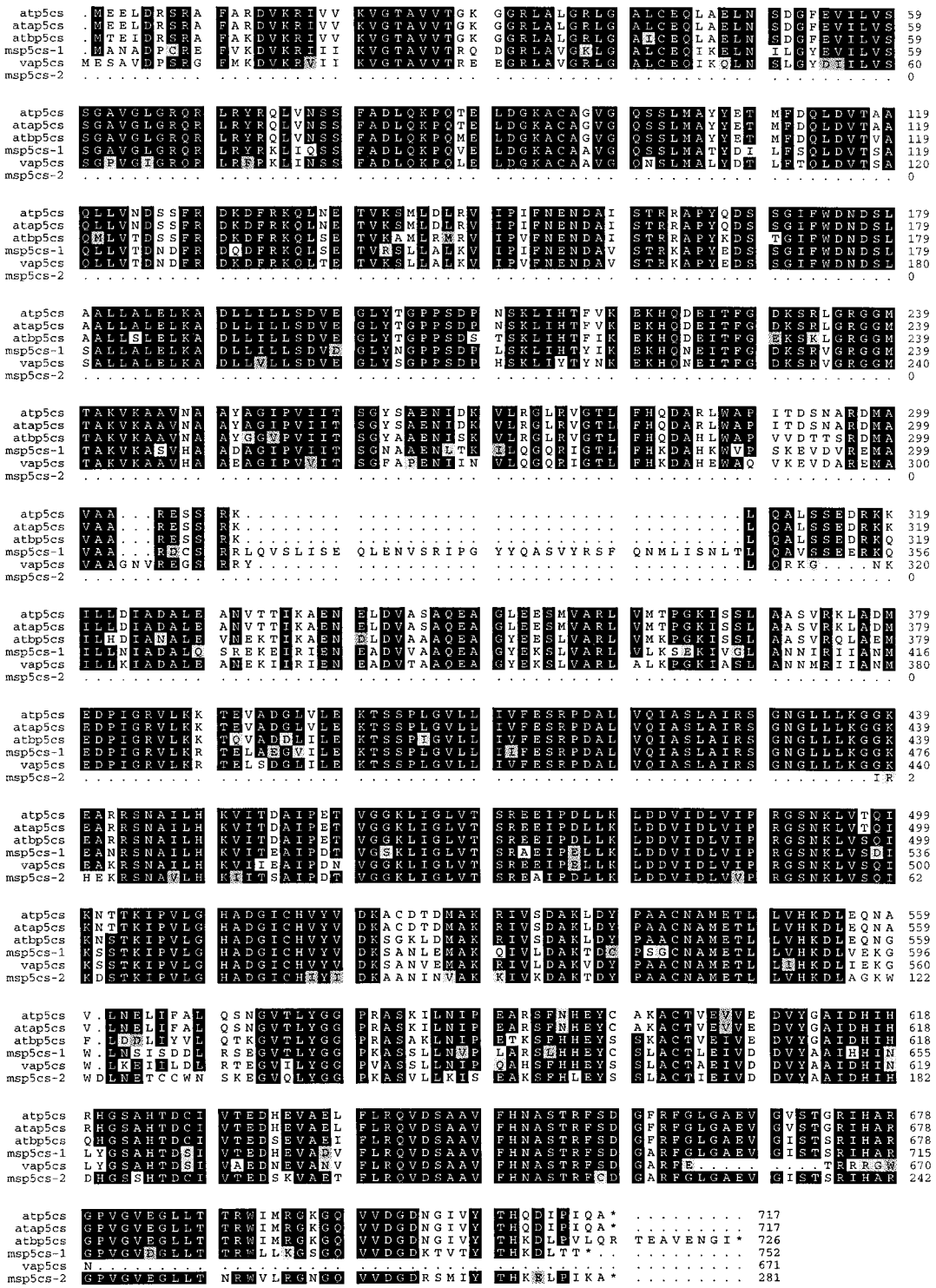

Figure 2. Alignment of the predicted amino acid sequences of the P5CS polypeptides from the following plants: *A. thaliana* (EMBL accession numbers: atp5cs, X87330; atap5cs, X86777; atbp5cs, Y09355), *M. sativa* (msp5cs-1 and msp5cs-2), and *V. aconitifolia* (vap5cs). A putative ATP-binding site and NAD(P)H-binding domain are located at amino acids 62–69 and 469–504 of msp5cs-1, respectively. Amino acid sequence from position 310 to 345 in *MsP5CS-1* display an open reading but, belong to an intron homologous to intron 7 of the *Arabidopsis* genes.

roots, six-day-old alfalfa seedlings were grown hydroponically in a medium containing 90 mM NaCl. Control plants were grown in a similar medium without salt. Root samples were taken periodically (6, 24, 48 and 72 h) and total RNA was extracted. Northern blot analysis showed an increase in the *MsP5CS* transcripts level after 48 h in the salt treatment and a decrease in the control (Figure 4). To specifically characterize each *MsP5CS* gene response to salt stress, a RT-PCR strategy was used. Specific reverse transcription of *MsP5CS-1* and *MsP5CS-2* was performed by using the gene-specific reverse primers 3 and 4, respectively, and equal amounts of total RNA as templates. *MsP5CS-1* cDNA was then PCR-amplified using the direct primer 1 and primer 3 corresponding to nucleotides 1328–1347 and 2052–2068 of the *MsP5CS-1* sequence, respectively. *MsP5CS-2* cDNA was PCR-amplified using primers 2 (direct) and 4 (reverse) corresponding to nucleotides 310–328 and 854–872 of the *MsP5CS-2* sequence, respectively. Southern blots of the amplified products, probed with the specific cDNAs, were quantified by the PhosphorImager program. Values obtained from control and salted roots after normalization per equal amount of rRNA were compared, enabling an evaluation of the effect of salt stress on steady-state *MsP5CS* transcript levels. Figures 5a and b compare the steady-state levels of *MsP5CS-1* (5a) and *MsP5CS-2* (5b) transcripts in normally grown roots vs. those exposed to 90 mM NaCl. Gene induction level was determined by calculating the ratio between values of the salt treatments and their corresponding controls (Figure 5c). The results show an immediate and consistent increase in steady-state mRNA level of the two genes following salinization (Figure 5a, b), with a higher induction of *MsP5CS-2* transcription (Figure 5c).

Proline content in salinized roots

Proline levels were compared in NaCl-treated and normally grown roots. During 72 h of growth with 90 mM NaCl, no significant change in proline content was observed (Figure 6).

Figure 3. Identification of the unprocessed intron in *MsP5CS-1* cDNA clone. a. Gel separation of PCR products resulted from amplification of the *MsP5CS-1* region corresponding to exon 5 through exon 10 of the *A. thaliana P5CS* genes [26], using λZap-cDNA library (lane 1) and the *MsP5CS-1* cDNA clone (lane 2) as templates. ϕX174 digested with *Hae*III is separated on the s.m. lane. b. Sequence comparison of the PCR fragment shown in lane 1 (denoted as PCR product) and lane 2 (denoted as P5CS-1) of panel a. The intron position correlates with intron 7 site of *AtP5CS* genes.

Discussion

In this paper we report the isolation and characterization of two different cDNA clones encoding P5CS from *M. sativa*. The clones *MsP5CS-1* and *MsP5CS-2* show 65% identity in their nucleotide sequences

Table 1. Comparison of the two alfalfa *P5CS* cDNAs and deduced proteins with their counterparts from *Vigna*, *Arabidopsis* and *E. coli*.

	MsP5CS-1 (X98421)[ab]		*MsP5CS-2* (X98422)[a]	
	cDNA[c]	protein[d]	cDNA[c]	protein[d]
MsP5CS-1	100	100	65	74
V. aconitifolia (M92276)[a]	81	83	66	74
A. thaliana (X86777)[e]	72	75	63	74
A. thaliana (Y09355)	70	75	65	75
E. coli γ-GK (P07005)	–	33	–	21
E. coli GSA- dehydrogenase (P07004)	–	38	–	37

Comparison was performed by the GAP program of the GCG. [a]EMBL/GenBank accession number. [b]*MsP5CS-1* sequence without the intron segment. [c]% nucleotide sequence identity. [d]% amino acid sequence identity. [e]Identical to X87330.

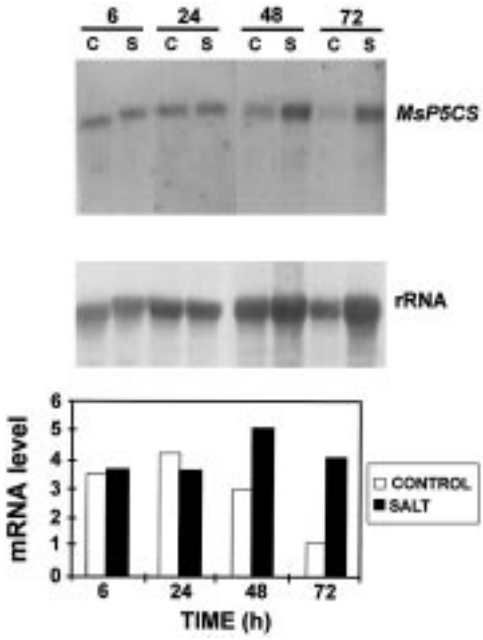

Figure 4. Evaluation of *MsP5CS* mRNA steady-state level. Total RNA samples (10 μg) extracted from seedling roots exposed to 90 mM NaCl for 6, 24, 48, and 72 h (S), and from control plants (C), were subjected to northern analysis using the full-length *MsP5CS-1* cDNA and tomato rDNA fragment as probes. The *P5CS* mRNA value in each treatment was normalized per equal rRNA content, using the Phosphor-Imager analytical program, and expressed in arbitrary units (lower panel).

and 74% identity in their deduced amino acid sequences. *MsP5CS-1* cDNA has an open reading frame of 717 amino acids showing identity of 83% with VaP5CS [10] and 75% to AtP5CS [23, 26]. Because the *MsP5CS-2* clone represents only the 3′-coding region of the corresponding gene, homology comparisons with known P5CS sequences were limited to the 3′ region, and showed 74–75% identity to all compared sequences. Furthermore, sequence analysis of *MsP5CS-1* cDNA revealed that the isolated clone, uniquely, contains an unprocessed intron. This intron is localized similarly to intron 7 of *Arabidopsis AtP5CS 1* and *2* genes and corresponds to the putative evolutionary fusion point of *E. coli proB* and *proA* homologues [10]. PCR amplification showed that most of the *MsP5CS-1* transcripts present in the cDNA library do not contain this intron and thus leading to the assumption that this intron is the last to be spliced. Comparison of *MsP5CS-1* intron 7-like sequence features with those of *AtP5CS-1* and *AtP5CS-2* [23, 26] showed that like all the 19 *Arabidopsis* introns, the alfalfa intron is devoid of 5′ and 3′ splicing consensus sequences [24], but maintains the URA branch point consensus (Figure 3). Similarly to the *Arabidopsis* introns, the alfalfa intron sequence is AT-rich and is therefore expected to be efficiently processed [24]. Thus, further evolutionary study is required to understand how the fused gene encoding the bifunctional P5CS enzyme has evolved in respect to its high intron frequency [26] and splicing pattern. The two cDNA clones described in this work indicate that two different *P5CS* genes are present in the alfalfa genome, as has been shown for *Arabidopsis* [26]. Whether this gene duplication leads to a generally higher level of transcription and/or each of the genes is separately controlled by different physiological conditions, as reported for *Arabidopsis* [26], is a question that awaits further elucidation.

In *Vigna*, *VaP5CS* transcription has been found to be more prominent in leaves than in roots [10]. However, increased transcription was observed in roots in response to salt stress, as has also been reported for *Arabidopsis AtP5CS* [23, 26, 30]. Similarly, our study using RT-PCR analysis, showed that the transcription

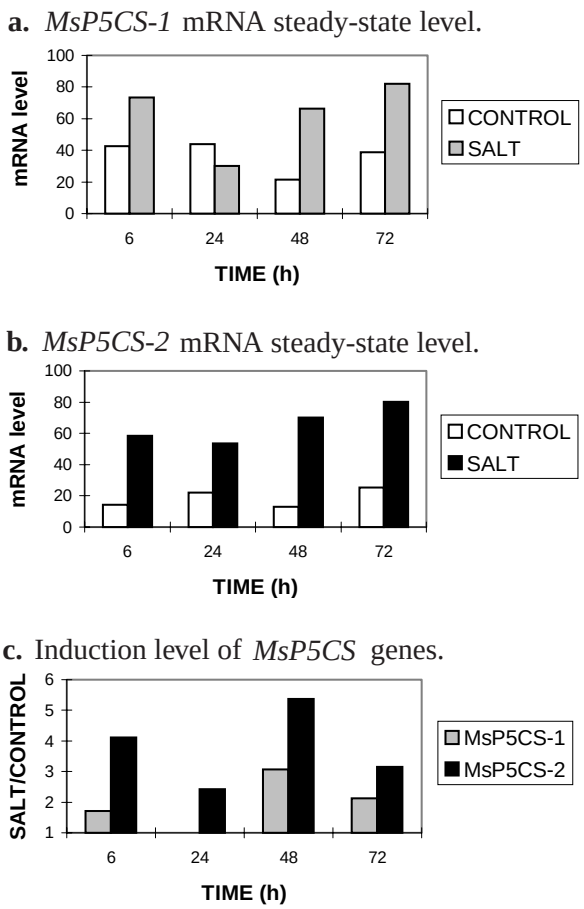

Figure 5. Induction of alfalfa *P5CS-1* and *P5CS-2* transcription by salt stress. Total RNA extracted from seedling roots exposed to 90 mM NaCl (gray and black boxes) and from control plants (white boxes) was subjected to RT-PCR analysis using *MsP5CS-1* or *MsP5CS-2* gene-specific primers. Southern blots of the PCR products were hybridized with their corresponding ^{32}P-cDNA clones and the radioactivity values were analyzed by Phosphor-Imager program. Values obtained were normalized per equal rRNA content in each sample. Panels a and b display mRNA steady-state levels of *MsP5CS-1* and *MsP5CS-2*, respectively. The relative induction of each gene is shown in c. The RNA samples were within the range of template dependent linearity.

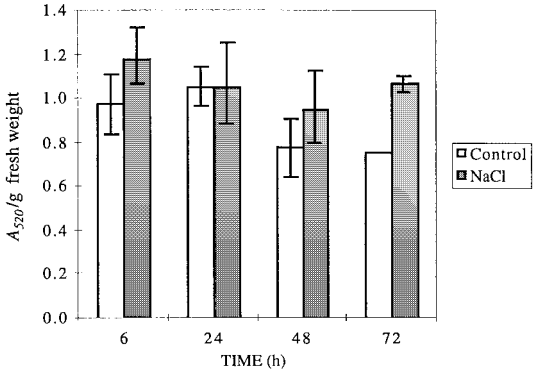

Figure 6. Proline content in alfalfa roots. Evaluation of proline levels in *M. sativa* roots exposed to 90 mM NaCl compared to control, unsalinized roots (four replicates in each treatment, two in the 72 h control).

of the two alfalfa *MsP5CS* genes is enhanced in roots exposed to NaCl. Salt induction of *MsP5CS-2* transcription was always higher than that of *MsP5CS-1*. Northern blot analysis does not show a significant increase in the level of the total *P5CS* transcripts during the first 48 h of salinity. This indicates that the salt induced increase during that period, observed by the sensitive RT-PCR approach, does not contribute much to the total *P5CS* transcript levels in the young roots. Also, it not linked to a significant change in proline content. Later on, when a slight reduction in *P5CS* transcript level is observed in the control, probably due to seedlings development, the salt induced increase in *P5CS* transcript level is more prominent.

The accumulation of proline in response to decreasing osmotic potential or water deficit has been observed in bacteria, algae and plants [1]. This accumulation is due primarily to *de novo* synthesis [21, 29] and is correlated with increased *P5CS* gene transcription in plants [30]. Irigoyen *et al.* [11] showed that the accumulation of organic acids and sugars precedes the increase in proline content and suggested that these organic acids provide the carbon skeleton for proline synthesis.

The elevation of the proline level in plant tissues involves a complex series of processes in addition to increased transcription of the *P5CS* genes. Under osmotic stress, proline degradation is inhibited as a result of the reduced transcription and activity of proline dehydrogenase (oxidase) [16, 19, 25]. Furthermore, whereas under normal conditions proline is also produced from ornithine, during osmotic stress the glutamate pathway predominates [5, 6]. The proline biosynthesis pathway via glutamate is not only transcriptionally controlled but is also feedback regulated; proline inhibits the γ-GK activity of P5CS [10], presumably by an allosteric effect [31]. P5CS activity has also been suggested to be regulated by an as yet unidentified protein inhibitor [31]. In addition, a salt-stress induced expression of proline membrane transporters, especially ProT2, has been observed in yeast [20]. This increase is coupled to a repression of the expression of a broad-specificity amino acid permease. Together, such changes may increase proline

content, as has been observed in the phloem sap of alfalfa shoots [8], as well as in all plant organs [13].

The delay in proline accumulation despite the rapid activation of *P5CS* has led to two engineering strategies designed to improve salt- and drought-stress tolerance by increasing the content of free proline in plant cells. The first is based on the removal of the proline feedback inhibition site of the *Vigna* VaP5CS by a single substitution of an alanine for a phenylalanine at position 129, in order to use it as a potent transgene [31]. The second intended to over-express the native *VaP5CS* cDNA in tobacco [15]. The resulting transgenic tobacco plants that contained more than one copy of *VaP5CS* accumulated proline to up to 14 times the control level under normal growth conditions. However, drought conditions induced proline accumulation in non-transgenic tobacco plants and consequently, the difference in proline content between the non-transgenic and transgenic plants decreased (73.06% and 78.95% of the total amino acid content, respectively). Nevertheless, some increased tolerance to osmotic stress was still observed in the transgenic plants [15].

The rapid change in *P5CS* transcript level in response to a high concentration of NaCl shows that the *P5CS* promoters respond rapidly to an elevated salt concentration. Strizhov *et al.* [26] found that such induction of *AtP5CS-1* transcription in *Arabidopsis* was strictly reduced in the *abi1-1* and *aba1-1* mutants. These mutants are involved in ABA signal transduction or biosynthesis, respectively [18, 26]. *AtP5CS-2* was found to be subjected to a different type of regulation and is more active in dividing cells. The differential induction of alfalfa *MsP5CS-1* and *MsP5CS-2* in the same organ, may indicate two additive regulatory mechanisms, which are involved in the induced synthesis of P5CS.

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