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Research article

Salt stress increases the expression of *p5cs* gene and induces proline accumulation in cactus pear

Claudia O. Silva-Ortega [a], Ana E. Ochoa-Alfaro [a], Juan A. Reyes-Agüero [b], Gerardo A. Aguado-Santacruz [c], Juan F. Jiménez-Bremont [a,*]

[a] *División de Biología Molecular, Instituto Potosino de Investigación Científica y Tecnológica, Camino a la Presa de San José 2055, Apartado Postal 3-74 Tangamanga, 78210 San Luis Potosí, San Luis Potosí, Mexico*
[b] *Instituto de Investigación en Zonas Desérticas, Universidad Autónoma de San Luis Potosí, Altair 200, Fracc. del Llano, 78377 San Luis Potosí, San Luis Potosí, Mexico*
[c] *Campo Experimental Bajío, Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias, Km. 6.5 Carretera Celaya-San Miguel de Allende, 38110 Celaya, Guanajuato, Mexico*

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Abstract

Proline (Pro) is one of the most accumulated osmolytes in salinity and water deficit conditions in plants. In the present study, we measured the Pro content, the activity and the expression level of delta 1-pyrroline-5-carboxylate synthetase (P5CS: γ-glutamyl kinase, EC 2.7.2.11 and glutamate-5-semialdehyde dehydrogenase, EC 1.2.1.41), a key regulatory enzyme involved in the biosynthesis of Pro, in cactus pear (*Opuntia streptacantha*) subjected to 6, 9 and 11 days of salt stress. Treatment with NaCl of *O. streptacantha* young plants resulted in a decrease in the cladode thickness and root length, and in a significant and gradual accumulation of Pro in young cladodes, in a time- and concentration-dependent manner. P5CS activity, studied as γ-glutamyl kinase, was reduced at all times as a consequence of salt treatment, except at the sixth day at 75 and 150 mM of NaCl, where a slight increase was observed. We isolated an open reading frame (ORF) fragment of *p5cs* gene. The deduced amino acid sequence of the P5CS protein exhibited 90.4% of identity with the P5CS protein from *Mesembryanthemum crystallinum*. RT-PCR analysis revealed that the *Osp5cs* gene of *O. streptacantha* was induced by salt stress at 9 and 11 days of treatment. Furthermore, ABA-induced *Osp5cs* gene expression was observed in cladodes of cactus pear young plants. We observed an evident correlation between the transcript up-regulation and the Pro accumulation under salt stress; however, these results do not parallel with the changes in P5CS enzymatic activity. This Pro accumulation might function as an osmolyte for the intracellular osmotic adjustment and might be playing a critical role in protecting photosynthetic activity in *O. streptacantha* plants under salt stress.

Keywords: Abscisic acid; Delta 1-pyrroline-5-carboxylate synthetase; *Opuntia streptacantha*; Proline; Salt stress

1. Introduction

Salinity is considered a major factor in limiting plant development and productivity, mainly of important crops. Today, there is increasing use of saline water in agriculture in arid and semiarid regions where the availability of fresh water is scarce. Saline stress causes inhibition of plant growth due to a reduction in water availability, sodium ion accumulation, and mineral imbalances, leading to cellular and molecular damage. Proline (Pro) is one of the most accumulated osmolytes found in plants with salinity and water deficit conditions [8,21,44]. Pro acts as osmoprotectant, it can also function as a protein stabilizer, it functions as a hydroxyl radical scavenger, stabilizes cell membranes by interacting with phospholipids,

Abbreviations: ABA, abscisic acid; ORF, open reading frame; *Osp5cs*, *Opuntia streptacantha* delta 1-pyrroline-5-carboxylate synthetase; PCR, polymerase chain reaction; Pro, proline; P5CS, delta 1-pyrroline-5-carboxylate synthetase; RT-PCR, reverse transcriptase-polymerase chain reaction.

* Corresponding author. Tel.: +52 444 8 342000; fax: +52 444 8 342010.
E-mail address: jbremont@ipicyt.edu.mx (J.F. Jiménez-Bremont).

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and serves as a source of carbon and nitrogen [22]. In plants, Pro biosynthesis occurs via two pathways from either glutamate or ornithine; however, Pro biosynthesis from glutamate appears to be the predominant pathway, especially under stress conditions [8]. The key enzyme delta 1-pyrroline-5-carboxylate synthetase (P5CS: γ-glutamyl kinase, EC 2.7.2.11; glutamate-5-semialdehyde dehydrogenase, EC 1.2.1.41) catalyses the first two steps of Pro biosynthesis from glutamate in plants [19]. *p5cs* gene has been isolated from several plants [11,14,19,20,38] and the correlation between the induction of *p5cs* gene and the accumulation of Pro has been shown in *Arabidopsis thaliana* and rice [20,46].

The finding that abiotic stress increases the accumulation of Pro in numerous plant species, together with the demonstration that it is possible to enhance osmotic stress tolerance in plants by Pro overproduction [39], gave us the motivation for the isolation and analysis of *Opuntia streptacantha* delta 1-pyrroline-5-carboxylate synthetase (*Osp5cs*) gene in Cactaceae, like *Opuntia*.

Opuntia spp. has become important in the semiarid areas of Mexico, where it plays a strategic role in subsistence agriculture [32]. Cactus pear is edible as a fruit and vegetable, and also is planted as an ornamental and a forage crop. As a crassulacean acid metabolism plant, *Opuntia* spp. is characterized by high water use efficiency. Because of this and its tolerance to drought, this plant can adapt to a variety of climatic conditions; therefore it has been cultivated in the arid and semiarid regions of many countries [24]. On the other hand, *Opuntia* species are sensitive to salinity [2,13,28,29,42]. The accumulation of osmolytes in *Opuntia* under abiotic stress is little known; only a few reports have been documented [9,31,10]. In *Opuntia megacantha* submitted to heat stress (37 and 47 °C) the Pro content increased as a response to this stress condition [9]. In *Opuntia ficus-indica* and *Opuntia humifusa* submitted to low temperatures a general increase in cladode concentrations of putative cryoprotectants such as fructose, glucose, sucrose, mannitol, sorbitol, and total amino acids including Pro was observed [31]. In cladodes and roots of *O. ficus-indica*, a slight increase in Pro content at 150 mM NaCl was recently reported [10].

Nevertheless, it is not known if Pro accumulation under abiotic stress in cactus pear correlates with the *p5cs* transcript induction. For the above reasons, the objective of the present investigation was to determine the content of Pro under salt stress in *O. streptacantha*, and to establish the relationship between the Pro accumulation, the P5CS activity and the expression of *Osp5cs* gene. Based on previous reports, where the abscisic acid (ABA) treatment increased *p5cs* transcript in *A. thaliana* and rice [20,41,46], we considered the possibility that the *Osp5cs* might be regulated by ABA, and so an additional aim was to determine if this gene was regulated.

2. Results and discussion

To determine whether the *p5cs* gene encoding the P5CS enzyme and the Pro content are affected in the salt stress response in *O. streptacantha*, cactus pear young plants of 9 weeks old were treated with 0, 75, 150, 250 and 350 mM NaCl for a period of 11 days. In addition, some physiological parameters were evaluated: cladode length, cladode thickness and root length at 0, 6, 9 and 11 days of treatment with NaCl.

2.1. Salt stress effect on cladode thickness and root length

No visible damage was observed in the stressed young plants except for a pronounced decrease in succulence, which resulted in flaccid cladodes at 9 days of stress at 350 mM NaCl, and at 250 and 350 mM NaCl at 11 days of stress (Fig. 1C, 1–4). Cladode growth, measured as total length, did not show significant changes (data not shown) due to the short period of salt stress. The growth reduction is a characteristic of salt sensitive plants and has been shown in *Opuntia* submitted to months of salt stress [10,28,29]. We observed a decrease of about 25% in cladode thickness of the young plants that were treated with the higher concentrations (250 and 350 mM) of NaCl during the complete period in comparison to the control cladodes (Fig. 1A). In addition, the root length showed a considerable decrease under salt stress. At the ninth day of stress at 75 and 150 mM NaCl, a 45% decrease in root length was observed, while at 250 and 350 mM NaCl, a decrease of 30% was observed from the beginning of the sixth day of stress (Fig. 1B). Silverman et al. [42] showed that the application of NaCl in two populations of *O. humifusa* (with 50 mM increments up to 400 mM) every 3 days for 6 weeks caused the cladodes to decrease up to 30% in thickness. The same effect was observed in *O. ficus-indica* where cladode thickness decreased with increasing salinity [28]. With regard to the root physiology, a rapid and transient reduction in growth rates has been documented after sudden increase in NaCl [34]. Root growth of agaves and cacti is drastically inhibited by saline stress [30], specifically in the *Opuntia* genus the reduction in the root length has already been reported, for example in *O. humifusa* and *O. ficus-indica* the root length was significantly affected by salinity [28,42]. Gersani et al. [13] reported that in *O. ficus-indica* roots exposed to 30 and 100 mM NaCl for 28 days, cortical cells became shorter and lateral root development was substantially reduced as salinity was increased. The diminution in cladode thickness and root length showed in this work suggests that the *O. streptacantha* plants are affected by saline stress; moreover the marked decrease in succulence, as a consequence of water reduction in response to salinity, possibly indicates that the *O. streptacantha* young plants are under osmotic stress, suggesting the possibility of the accumulation of compatible osmolytes such as Pro, to act as osmoprotectant and take part in the osmotic adjustment.

2.2. Effect of salt stress on the proline content

Pro content in young cladodes of *O. streptacantha* plants treated with 0, 75, 150, 250 and 350 mM NaCl during 11 days is shown in Fig. 2. In general Pro accumulation increased gradually with rising concentration of NaCl and days of

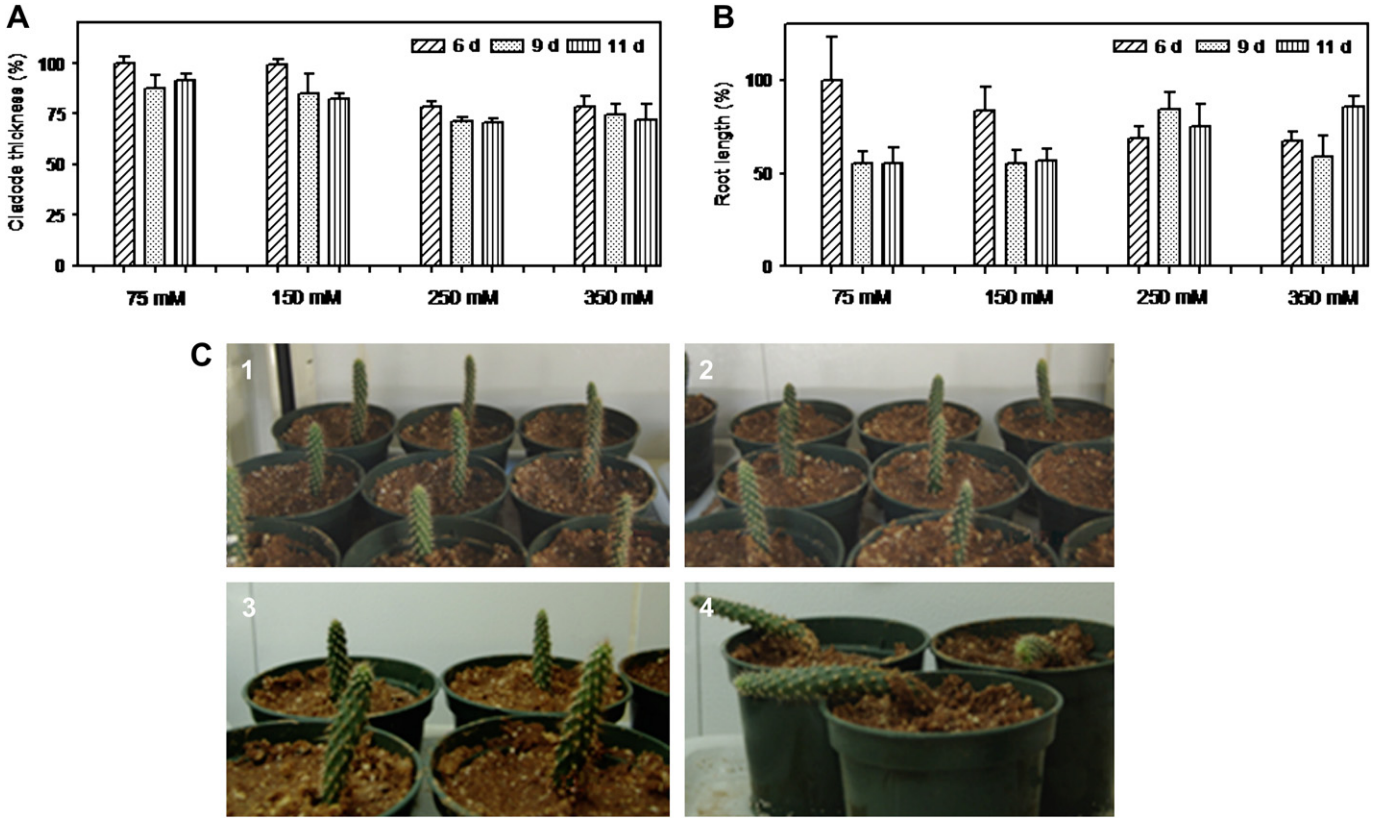

Fig. 1. Effect of salt stress in 9-week-old *Opuntia streptacantha* young plants. Percentage of (A) cladode thickness and (B) root length of plants subject to 75, 150, 250 and 350 mM NaCl during 6, 9 and 11 days. The reduction in the percentage of cladode thickness and root length of salt stressed plants was calculated according to 100% of the control plants. Data are mean ± SD, $n = 3$. (C) Photographs of *Opuntia streptacantha* plants at 0 (1), 6 (2), 9 (3) and 11 (4) days of treatment with 350 mM NaCl.

treatment. At 6 days of stress the major increase (fivefold) in the Pro content in young cladodes was observed at 350 mM NaCl, in comparison to the non-treated cladodes. At 9 days of treatment the Pro accumulation was significantly stimulated at the high concentrations (250 and 350 mM NaCl); being approximately 15- and 20-fold to the control. At 11 days of stress the Pro accumulation in control cladodes was almost undetectable, and the most significant accumulation (20- and 30-fold) was found at 250 and 350 mM NaCl compared to the cladodes treated with 150 mM NaCl (Fig. 2). Environmental stresses such as high salt, low water availability, and low temperature can induce the accumulation of one or several compatible solutes such as Pro, glycinebetaine, polyols or sugars in plants [8,33]. Cacti family includes many species that occupy extreme habitats and produce many compounds that probably can confer tolerance to those environments; for example, anthocyanins are replaced for betalains [4]. Pro accumulation in drought and salinity stress in *Opuntia* species is a not very explored field. In *O. megacantha* cultivars exposed to heat stress, an increase in the Pro concentration in one of the cultivars at 47 °C, approximately twofold with regard to the control, was reported [9]. In *O. ficus-indica* Pro content was measured in basal and apical cladodes and roots. A slight increase in the amount of Pro at the highest concentration of NaCl (150 mM) was observed in the cladode as well as in the roots [10]. Pro interacts with enzymes to preserve protein structure and activities due to its characteristic of forming hydrophilic colloids in aqueous media with a hydrophobic backbone interacting with the protein [5]. For example, it has been

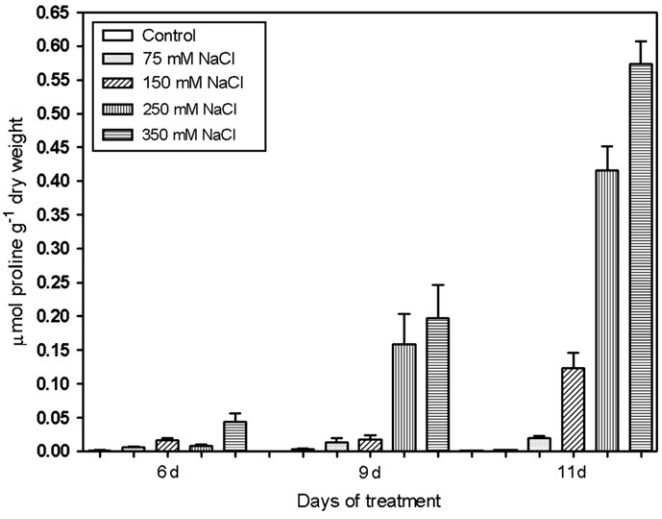

Fig. 2. Proline content in cladodes of *Opuntia streptacantha* young plants subjected to 0 (control), 75, 150, 250 and 350 mM NaCl during 6, 9 and 11 days. Data are mean ± SD, $n = 3$.

shown that Pro reduces in vitro enzyme denaturalization caused by heat and NaCl stress [16]. Our results, together with other reports, suggest that the Pro increase observed under salt stress conditions in *Opuntia* might function as a source of solute for intracellular osmotic adjustments in salt stress response.

2.3. Isolation and sequence analysis of an open reading frame (ORF) fragment corresponding to O. streptacantha delta 1-pyrroline-5-carboxylate synthetase gene

Plant P5CS sequences have highly conserved regions and thus provide several candidate sites for designing PCR primers of consensus sequence in order to clone related genes in a plant of our interest. Nested amplifications by PCR were carried out using three primers to obtain a partial cDNA sequence encoding P5CS from *O. streptacantha*. First amplification with primers 71 and 72 gave rise to a smear. A second nested amplification with primer 73, instead of 72, produced a single band of 1401 bp corresponding to an ORF fragment of *Osp5cs* from *Opuntia*. The sequence obtained (Fig. 3) was registered at the GenBank (GenBank accession no. EF527256) and it corresponds to all of the amino acids from 85 to 551 of the *Mesembryanthemum crystallinum* protein, which has 719 amino acids. We compared the partial cDNA sequence of the *O. streptacantha* P5CS reported in this work using *Clustal* analysis with the corresponding sequences reported from other plant P5CSs as observed in Fig. 3. The highest identity was to *M. crystallinum* with 90.4% (GenBank accession no. O65361), *Vitis vinifera* with 81.4% (GenBank accession no. CAB40834), *A. thaliana* with 78.8% (GenBank accession no. NP_181510) and *Oryza sativa* with 78.4% (GenBank accession no. BAA19916). A phylogenetic tree of P5CS from different plant species was constructed using MEGA 3.1 version [23] (Fig. 4). As expected, the highest homology was found with the *M. crystallinum* facultative halophyte, because this plant as well as the cactus pear belongs to the Caryophyllales order.

2.4. Differential expression of O. streptacantha delta 1-pyrroline-5-carboxylate synthetase gene under salt stress conditions

In order to determine whether induction of Pro accumulation by salt stress in cactus pear cladodes correlates with transcriptional regulation, *Osp5cs* transcript level was assessed by reverse transcriptase-polymerase chain reaction (RT-PCR), using the same samples as those described above for Pro analysis. In order to carry out a RT-PCR analysis, we amplified an actin cDNA fragment (see Section 4) of 630 bp corresponding to an ORF fragment of *actin* from *Opuntia*, to use it as a loading control. The sequence obtained was registered at the GenBank (GenBank accession no. EF527257). As shown in Fig. 5, at 6 days of stress the *Osp5cs* gene did not show changes in its expression. However, at 9 days of treatment, we observed that the *Osp5cs* gene was gradually up-regulated with increase in concentration of NaCl. In addition, at 11 days of stress, the major transcript accumulation was observed at 250 mM NaCl (Fig. 5). At 9 days of stress there was evident correlation between the transcript up-regulation and the Pro accumulation with increase in NaCl concentration. The same effect was observed at 11 days of treatment, up to 250 mM NaCl. In *A. thaliana* and rice the gene encoding for P5CS was induced by high salt and dehydration, and the simultaneous accumulation of Pro was observed as a result of both of the previous treatments in *A. thaliana* [46]. However, unlike in *A. thaliana* the accumulation of Pro in rice was observed only as a result of high salt treatment [20].

The increase in the Pro content can be due to de novo synthesis, reduction in the rate of proteins' synthesis and/or proteins' degradation. The finding that the salt stress induces the *Osp5cs* gene, that encodes for the key enzyme P5CS, which catalyses the first two steps of Pro biosynthesis from glutamate in plants, suggests that the Pro accumulation is due to the de novo synthesis without discarding other possibilities like diminution in the rate of proteins' synthesis and/or proteins' degradation.

2.5. Effect of abscisic acid on expression of O. streptacantha delta 1-pyrroline-5-carboxylate synthetase gene

In order to characterize the response of *Osp5cs* gene to ABA, we examined the effect of this plant growth regulator in 9-week-old *O. streptacantha* young plants by applying 0, 0.1 and 100 μM ABA in Hoagland's nutrient solution under light condition during 1.5 h by RT-PCR analysis (Fig. 6). Interestingly, the *Osp5cs* gene was regulated at the transcriptional level by 0.1 and 100 μM ABA treatments. Lower concentration of ABA (0.1 μM) induced the highest accumulation of the *Osp5cs* transcript in comparison to the non-treated control (Fig. 6). ABA has long been known to be a mediator in triggering plant responses to various environmental stresses, particularly drought and salinity. It also affects the patterns of expression of more than 1000 genes in a full-length cDNA microarray of around 7000 genes in *A. thaliana* [41]. In *A. thaliana* and rice it was found that the expression of *p5cs* is up-regulated by ABA [20,46]. With respect to cactus pear plants, it was demonstrated that the exogenous ABA application in *O. ficus-indica* (cold sensitive plant) and *Opuntia fragilis* (cold tolerant plant) increased the cold stress tolerance and that this effect accentuates in *O. fragilis* [26]. However, there are no studies on the effect of ABA in the transcriptional regulation of those genes involved in the osmolite biosynthesis in cactus pear. Our data reveal that in *O. streptacantha*, ABA is an *Osp5cs* gene inductor. It is important, since ABA is one of the main components of the signaling pathway during abiotic stress.

2.6. Effect of salt stress on the delta 1-pyrroline-5-carboxylate synthetase (P5CS) activity

P5CS activity, studied as γ-glutamyl kinase, in the same stressed cladodes of *O. streptacantha* plants as those described

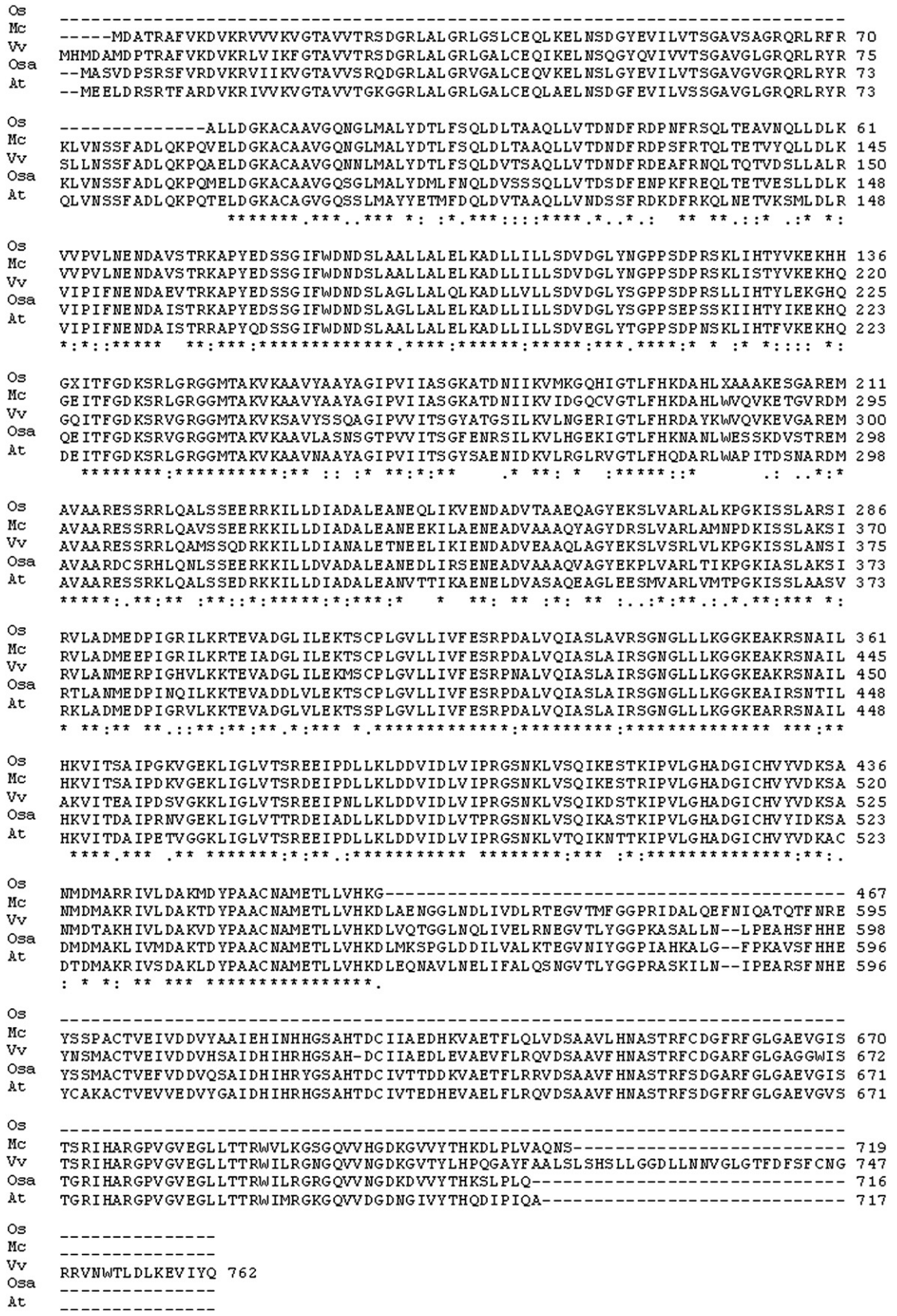

Fig. 3. Multiple amino acid alignment as derived by maximal identity of P5CS fragment from the *Opuntia streptacantha* (Os) with the corresponding protein sequences of other plant P5CSs: Mc, *Mesembryanthemum crystallinum* (GenBank accession no. O65361); Vv, *Vitis vinifera* (GenBank accession no. CAB40834); Osa, *Oryza sativa* (GenBank accession no. BAA19916) and At, *Arabidopsis thaliana* (GenBank accession no. NP_181510). Identical residues (asterisk) in the five proteins and conserved amino acid substitutions (dots) are indicated. Dashes show gaps in the amino acid sequences introduced to optimize alignment.

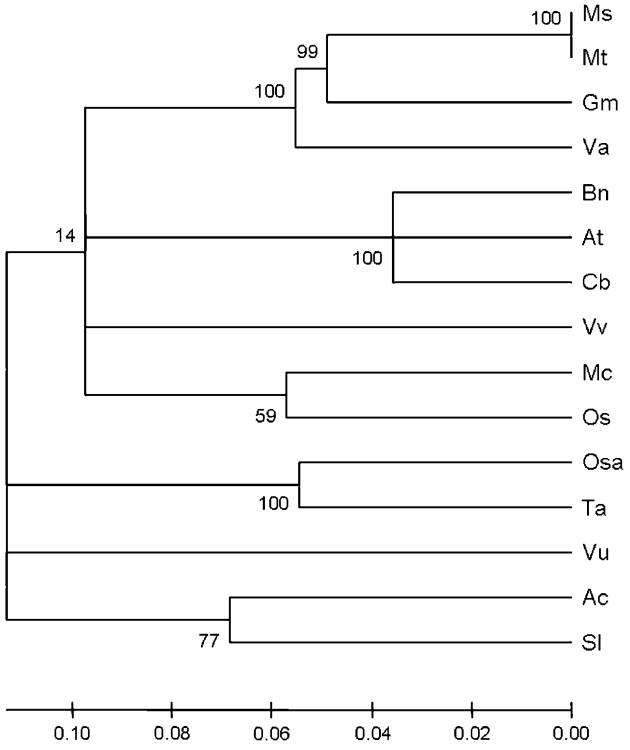

Fig. 4. Phylogenetic dendrograms of delta 1-pyrroline-5-carboxylate synthetase of Ms, *Medicago sativa* (GenBank accession no. CAA67069); Mt, *Medicago truncatula* (GenBank accession no. CAC82184); Gm, *Glycine max* (GenBank accession no. AAR86688); Va, *Vigna aconitifolia* (GenBank accession no. P32296); Bn, *Brassica napus* (GenBank accession no. AAK01361); At, *Arabidopsis thaliana* (GenBank accession no. NP_181510); Cb, *Chorispora bungeana* (GenBank accession no. AAV67896); Vv, *Vitis vinifera* (GenBank accession no. CAB40834); Mc, *Mesembryanthemum crystallinum* (GenBank accession no. O65361); Os, *Opuntia streptacantha* (GenBank accession no. EF527256); Osa, *Oryza sativa* (GenBank accession no. BAA19916); Ta, *Triticum aestivum* (GenBank accession no. BAD97364); Vu, *Vigna unguiculata* (GenBank accession no. BAB33037); Ac, *Actinidia chinensis* (GenBank accession no. O04015) and Sl, *Solanum lycopersicum* (GenBank accession no. Q96480). Construction of the phylogenetic tree and bootstrap analysis (1000 replicates) were performed with MEGA 3.1 version.

above for Pro analysis is shown in Fig. 7. We observed that the cactus pear plants have a tendency to maintain or mainly to reduce the P5CS activity as a consequence of salt treatment, except for the sixth day at the lower NaCl concentrations (75 and 150 mM), where a slight increase was observed. The reduction in the P5CS activity was more notable at 9 days of stress with regard to the control. In spite of the fact that at 9 and 11 days of salt stress *O. streptacantha* young plants treated with NaCl showed minor P5CS activity, they accumulated the major amount of Pro. Our result is in agreement with previous studies on aquatic macrophytes and wheat. Rout and Shaw [37] reported that in aquatic macrophyte *Hydrilla verticillata* the Pro levels in response to salt stress were increased nearly threefold, while the activity of the P5CS enzyme remained nearly unchanged. In leaves of wheat exposed to salt stress at 150 and 300 mM NaCl a significant increase in Pro content was found, however, the P5CS activity remained unchanged [48]. Nevertheless, other authors have reported that the P5CS activity increases in response to salinity [19,47]. Our results suggest that it is possible that salt stress only induces the synthesis and/or stabilization of *Osp5cs* transcript without leading to an increase in the activity of P5CS enzyme. Pro is synthesized from glutamate by two successive reductions, which are

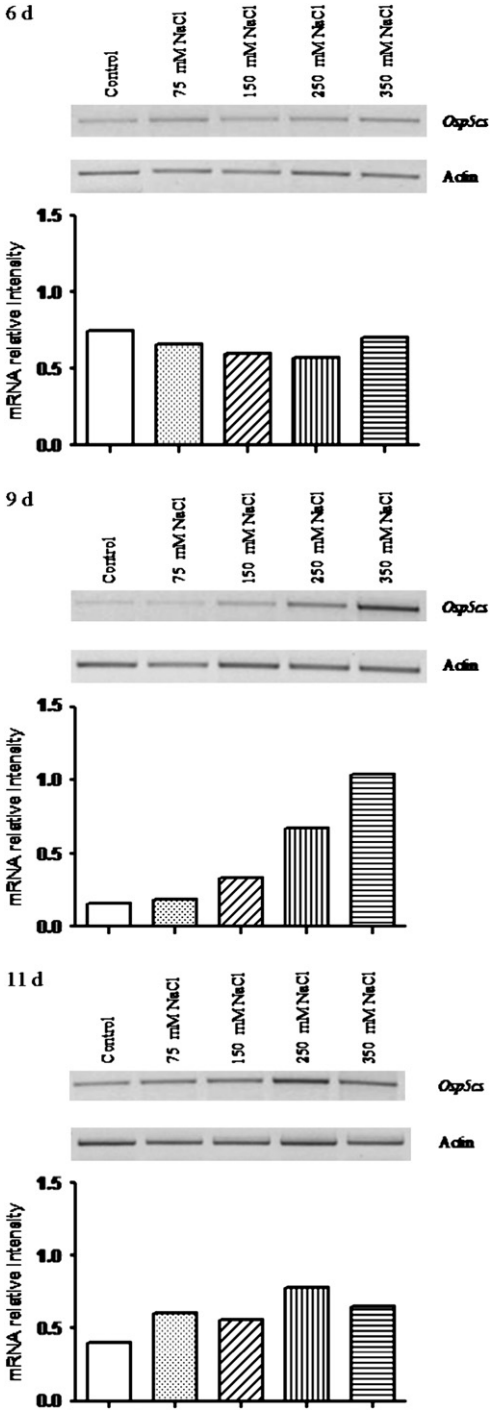

Fig. 5. Effect of salt stress on *Osp5cs* expression in cactus pear. Total RNA was isolated from young cladodes of 9-week-old *Opuntia streptacantha* plants subjected to 6, 9 and 11 days to different concentrations of NaCl (0, 75, 150, 250 and 350 mM). Each RNA sample (1 μg) was used for RT-PCR analyses. RT-PCR product (15 μL) was loaded on each lane and separated by electrophoresis on 1% (w/v) agarose gel. As loading control the actin product was used. The histogram shows relative *Osp5cs* cDNA's abundance after normalization with the actin signal.

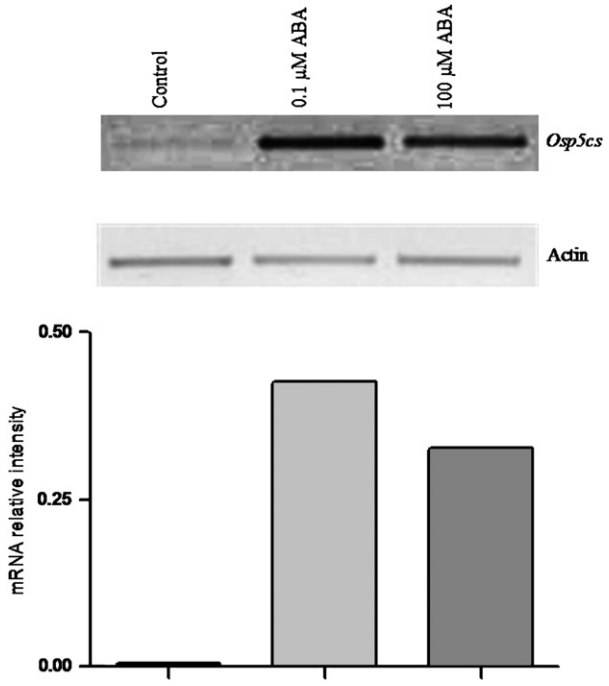

Fig. 6. Effect of ABA on the expression of the *Osp5cs* gene in cactus pear. Total RNA was isolated from young cladodes of 12-week-old *Opuntia streptacantha* plants subjected to 0 (control), 0.1 and 100 μM ABA during 1.5 h. Each RNA sample (1 μg) was used for RT-PCR analyses. RT-PCR product (25 μL) was loaded on each lane and separated by electrophoresis on 1% (w/v) agarose gel. As loading control the actin product was used. The histogram shows relative *Osp5cs* cDNA's abundance after normalization with the actin signal.

catalyzed by P5CS and pyrroline-5-carboxylate reductase (EC 1.5.1.2) [8]. In leaves of wheat an increase in the pyrroline-5-carboxylate reductase activity and Pro content by high salinity was observed, however, the P5CS activity did not show a specific response to salinity. The authors suggest that pyrroline-5-carboxylate reductase might be the rate-limiting factor in Pro synthesis from glutamate at high salinity [48]. It would be interesting to measure the pyrroline-5-carboxylate reductase activity to know the participation of this enzyme in Pro biosynthesis in *O. streptacantha* under salt stress.

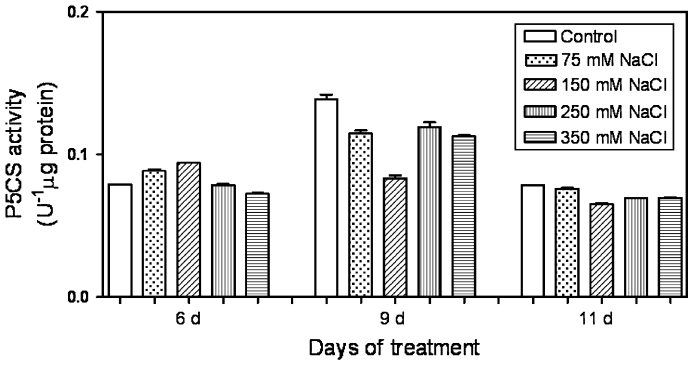

Fig. 7. Activity of P5CS (delta 1-pyrroline-5-carboxylate synthetase; U μg^{-1} protein), assayed as γ-glutamyl kinase, in cladodes of *Opuntia streptacantha* young plants after treatment with 0 (control), 75, 150, 250 and 350 mM NaCl during 6, 9 and 11 days. Data are mean ± SD, $n = 3$.

2.7. Salt stress effect on cladode chlorophyll content

Chlorophyll content is fundamental to understand the plant response to the environment in which it resides. To know if plants are damaged in the photosynthetic metabolism as a consequence of salt stress we decided to measure the cladode total chlorophyll content in the same stressed cactus pears as those described above for Pro analysis. Chlorophyll content measured in the young cladodes at 6 days of salt stress showed an increase of four and sevenfold approximately at 75 and 150 mM NaCl, respectively, compared to the control, while at 250 and 350 mM NaCl the increase was less being this of one and fourfold, respectively (Fig. 8). At 9 and 11 days of treatment the increase in chlorophyll content was increasing proportionally with the NaCl concentration. The most important augment in chlorophyll content was sixfold at 250 mM NaCl (9 days) and fivefold at 350 mM NaCl (11 days) (Fig. 8). Changes in leaf chlorophyll content can be used as an indicator of maximum photosynthetic capacity, leaf developmental stage, productivity and stress [7,15,25]. In salt susceptible plants such as tomato, potato, pea, and bean a decrease in the chlorophyll content has been shown [43]. On the other hand, the increase in the chlorophyll content during salt stress has previously been reported; for example, the chlorophyll content increased in *Amaranthus tricolor* treated with 300 mM NaCl for 7 days [45]. Our results show that in *O. streptacantha* the chlorophyll content increases rather than to reduce. Also graminaceous chlorophyllic cells subjected to osmotic stress developed substantially higher amounts of chlorophyll [12]. The increased chlorophyll accumulation in response to abiotic stress could be due to chloroplast development or to increased thylakoid number, as it has been reported [6,12]. On the other hand, compatible solutes play an important role in preventing thylakoid membrane damage during dehydration [40]. As Pro functions as a protein stabilizer, we suggest that the Pro might be protecting enzymes involved in the chlorophyll biosynthesis from stress

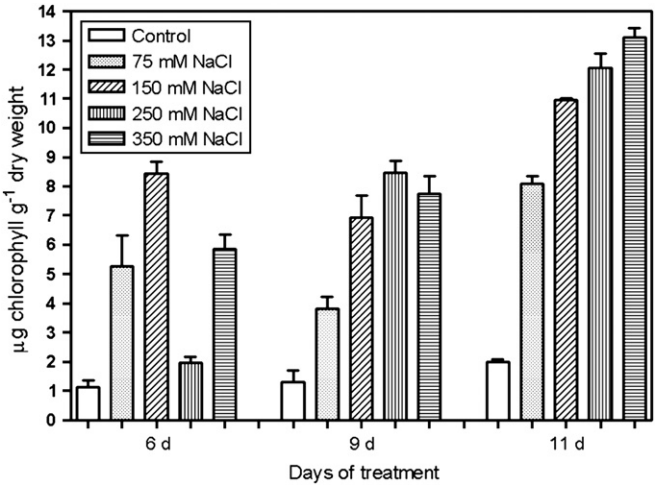

Fig. 8. Chlorophyll content in cladodes of *Opuntia streptacantha* young plants subject to 0 (control), 75, 150, 250 and 350 mM NaCl during 6, 9 and 11 days. Data are mean ± SD, $n = 3$.

conditions. We also suggest that the high Pro levels in *O. streptacantha* under salt stress could be involved in maintaining chlorophyll levels and the turgor.

3. Conclusion

In the present study, we have found Pro accumulation and transcriptional regulation of *Osp5cs* gene by salt stress. Due to the significant accumulation of Pro in *O. streptacantha* under salt stress, we suggest that the Pro might be a source of solute for intracellular osmotic adjustments. We observed an evident correlation between the transcript up-regulation and the Pro accumulation under salt stress; however, these results do not parallel with the changes in P5CS enzymatic activity. It is possible that salt stress only induces the synthesis and/or stabilization of *Osp5cs* transcript without leading to an increase in the activity of P5CS enzyme. In addition, ABA induces *Osp5cs* gene expression, and ABA signaling might mediate the regulation of this gene under stress. Finally, Pro might be playing a critical role in the turgor maintenance and protecting photosynthetic activity in *Opuntia* plants.

4. Methods

4.1. Plant materials and growth conditions

The *Opuntia* seeds gathered were taken from *O. streptacantha* cactus pears. The plant was growing in the semiarid region of Villa de Zaragoza in the state of San Luis Potosí, México. Mature cladodes of this plant were prepared as specimen and were deposited in the herbarium of the Autonomous University of San Luis Potosí. The seeds were washed and put to dry for 24 h. With the purpose to break the seeds latency, they were stored during 6 months at 25 °C [36]. Then they were placed in a seed germination bed in a growth chamber at 25 °C under 12 h (150 $\mu mol\, m^{-2}\, s^{-1}$) light and 12 h dark cycle. Emerging young plants were transferred to a Hoagland solution for 1 week to allow their adaptation before starting with the salt stress experiments.

4.2. Salt stress treatment

Nine-week-old *O. streptacantha* young plants with a cladode of an average length of 9.0 ± 0.5 cm were transferred to a perlite/sand support in semihydroponic conditions. Salt stress was imposed by application of the Hoagland nutrient solution [18] containing 0, 75, 150, 250 and 350 mM NaCl during 6, 9 and 11 days. After each salt treatment, young cladodes were transferred to liquid nitrogen for subsequent total RNA isolation, and estimation of Pro and total chlorophyll contents.

4.3. ABA treatment

Twelve-week-old *O. streptacantha* young plants with a cladode of an average length of 10.5 ± 0.5 cm were transferred to a perlite/sand support in semihydroponic conditions. ABA treatment was imposed by application of the Hoagland nutrient solution [18] containing 0, 0.1 and 100 μM ABA under light conditions during 1.5 h. After treatments, young cladodes were frozen in liquid nitrogen for subsequent total RNA isolation.

4.4. Cladode thickness and root length

Cladode length, cladode thickness and root length for each group of cactus pear young plants at 0, 6, 9 and 11 days of treatment in all the used NaCl concentrations were measured with a caliper to check for possible morphological differences. Measurements of control young plants in centimeter were considered to be 100%. To determine the percentage of the cladode length and thickness, and root length of the salt stress plants we used the following formula: % of cladode length or thickness or root length = (cm stress plant/cm control plant) × 100%.

4.5. Proline content

Free Pro was extracted by boiling 0.5 g of plant material in 2 mL of distilled water. Then 500 μL of 0.2 mM sodium citrate (pH 4.6) and 2 mL of 1% ninhydrin (acetic acid/water, 60/40, v/v) were added to 0.5 μL of the plant extract. Mixture was boiled 1 h, 2 mL of toluene was added for the extraction and then centrifuged. Pro content was measured spectrophotometrically (Beckman Du-530, Fullerton, USA) at 520 nm following the ninhydrin method described by Magne and Larher [27], using pure Pro (Sigma–Aldrich, Missouri, USA) as the standard. All of the experiments were carried out in triplicates using cladodes from three different plants and the data represent the mean ± SD.

4.6. RNA isolation and cDNA synthesis

Total RNA was extracted from *O. streptacantha* cladodes with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) as recommended by the manufacturer and treated with DNase I (Invitrogen, Carlsbad, USA). First-strand cDNA synthesis was performed in a total volume of 30 μL with SuperScript™ First-Strand Synthesis System for reverse transcriptase-polymerase chain reaction (RT-PCR; Invitrogen, Carlsbad, USA). Each reaction mixture contained 1 μg of total RNA, 0.3 mM dNTP mix, 0.5 μg $Oligo(dT)_{12-18}$, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 5 mM $MgCl_2$, 0.01 mM dithiothreitol (DTT), 40 U of Recombinant Ribonuclease Inhibitor (RNaseOUT™), and 50 U of SuperScript™ II RT. Reaction mixtures were incubated at 42 °C for 50 min, then at 70 °C for 15 min, and finally with 1 U of *Escherichia coli* RNase H at 37 °C for 20 min to remove RNA templates from the cDNA:RNA hybrid molecules. A control reaction for each sample was performed simultaneously without reverse transcriptase.

4.7. Osactin and Osp5cs ORF fragments' cloning and sequencing

Different sets of primers were designed to amplify each ORF fragment by RT-PCR: *actin* gene (GenBank accession

no. J01238) sense 5-Act 5′-AACTGGGATGACATGGAGAA-3′ and antisense 3-Act 5′-ATCACACTTCATGATGGAGTTG TA-3′ [35]. Primers (external or internal) of the *p5cs* gene were designed based on conserved regions of the corresponding enzymes from several plants. Nested PCR for the amplification of *Osp5cs* was started with the external primers 71 and 72 followed by further amplification using the external primer 71 and the internal primer 73. The primers synthesized to obtain the *Osp5cs* fragment were: 71 5′-CTTGATGGGAAAG CATGTGC-3′ (sense), 72 5′-CCTCGAGCATGAATCCTAC TT-3′ (antisense) and 73 5′-GTGTACAAGAAGTGTTTCCA TAGC-3′ (antisense) from conserved regions of cDNAs from: *M. crystallinum* (GenBank accession no. O65361), *Suaeda salsa* (GenBank accession no. AAM28630), *V. vinifera* (GenBank accession no. CAB40834), *Glycine max* (GenBank accession no. AAR86688) and *Medicago sativa* (GenBank accession no. CAA67069). PCR amplifications were performed in a 50 μL reaction mixture containing 1 μL of the cDNA as template obtained by reverse transcription using RNA isolated from cladode, 20 mM Tris–HCl pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 125 μM dNTPs, 0.2 μM each primer and 2.5 U Taq polymerase (Invitrogen, Carlsbad, USA). The PCR amplification conditions for *actin-1* gene were as follows: 5 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 57 °C and finally 60 s at 72 °C, followed by 72 °C for 7 min, and for *Osp5cs* gene were as follows: 5 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 60 s at 58 °C (nested PCR 1 *Osp5cs*) and 59 °C (nested PCR 2 *Osp5cs*) and finally 120 s at 72 °C, followed by 72 °C for 7 min. The samples were analyzed by electrophoresis on 0.8% agarose gels. Polymerase chain reaction (PCR) products of the expected size were cloned in the pCR4-TOPO vector (TOPO TA Cloning Kit, Invitrogen, Carlsbad, USA) and further sequenced with an ABI PRISM 377 DNA automated sequencer (Perkin–Elmer). Comparisons and protein sequence alignment were carried out using the ClustalW (http://www.ebi.ac.uk/clustalw/) program. For the construction of the dendrogram we used MEGA 3.1 version [23].

4.8. RT-PCR analyses of gene transcripts

Primers to amplify *Osp5cs* fragment by RT-PCR analyses were: 71 and 73. For synthesis of an actin fragment (loading control) the primers used were 5-Act and 3-Act. Equal RNA amounts were used for each RT-PCR of cladodes of *O. streptacantha* young plants treated with 0, 75, 150, 250 and 350 mM NaCl or treated with 0, 0.1 and 100 μM ABA. PCR amplifications were performed in a 50 μL reaction mixture containing 1 μL of the RT reaction product as template. The PCR amplification conditions for *Osp5cs* gene were as follows: 5 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 61 °C, 120 s at 72 °C and 7 min at 72 °C for the final extension. The PCR amplification conditions for *Osactin* gene were as follows: 5 min at 94 °C, 25 cycles of 30 s at 94 °C, 30 s at 57 °C, 60 s at 72 °C and 7 min at 72 °C for the final extension. Amplification was performed with different number of cycles to ensure a linear response in the PCR and was made in duplicates for each condition. PCR products were electrophoretically separated in 1% agarose gel. Quantification of signals was performed by Doc-It®LS Image Analysis Software. Each signal was normalized to the actin signal. Densitometry analyses were made with the Doc-It®LS Image Analysis Software.

4.9. P5CS activity assay

The activity of P5CS was assayed following the method described in Hayzer and Leisinger [17]. The extracts to measure the P5CS activity were obtained as follows: the cladodes were homogenized in an extraction buffer (pH 7.5, 100 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM EDTA, 10 mM β-mercaptoethanol, 4 mM DTT, 2 mM PMSF and 2% PVPP) in pre-chilled eppendorf tubes in a cold room. The extracts were centrifuged at 4 °C for 20 min at 10,000 × *g*. The supernatants were further clarified by centrifugation at 10,000 × *g* for 20 min at 4 °C. The activity of P5CS was determined as γ-glutamyl kinase in the enzyme extract by monitoring the formation of γ-glutamyl hydroxamate. The enzyme assay was carried out in a mixture contained the following in a final volume of 0.5 mL: Tris–HCl (50 mM, pH 7.0), 50 mM L-glutamate, 20 mM $MgCl_2$, 100 mM hydroxylamine·HCl, 10 mM ATP and the enzyme extract. The reaction mixture was incubated at 37 °C for 15 min and was stopped by adding 1 mL of the stop buffer (2.5 g of $FeCl_3$ and 6 g of trichloroacetic acid in a final volume of 100 mL of 2.5 N HCl). The precipitated proteins were removed by centrifugation and the absorbance was read at 535 nm against a blank identical to the above but lacking ATP. The amount of γ-glutamyl hydroxamate complex produced was estimated from the molar extinction coefficient 250 $mol^{-1}\,cm^{-1}$ reported for Fe^{3+} hydroxamate complex of the compound. The activity was expressed in U μg^{-1} protein which represents the amount of enzyme required to produce 1 μmol of γ-glutamyl hydroxamate min^{-1}. Total protein content was determined according to Bradford method [3].

4.10. Chlorophyll content

The photosynthetic pigment content of cladode was determined as described by Arnon [1]. Pigment was extracted in 80% cold acetone and the absorbance of the extract was measured spectrophotometrically (Beckman Du-530, Fullerton, USA) at 645 and 663 nm. All of the experiments were carried out in triplicates of cladode from three different plants and the data represent the mean ± SD.

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