Cloning and Expression of Pyrroline-5-carboxylate synthetase from Eucalyptus camaldulensis (Dehnh.) under Salt Stress

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ABSTRACT

Salt stress is one of the most significant factors that negatively affect plant growth and development. Proline has been reported to be an osmoprotectant that confers tolerance to salinity in various plant species. A cDNA for Δ¹-pyrroline-5-carboxylate synthetase (P5CS), a key enzyme involved in proline biosynthesis was isolated and characterized from E. camaldulensis (Dehnh.) (clone T5), now designated EuP5CS. The full-length EuP5CS gene has 2,944 bp containing an open reading frame of 2,142-bp that encodes for 713 amino acids. The deduced EuP5CS protein structure exhibited a high homology to the P5CS of other plant species, and was predicted to possess a glutamate 5-kinase domain at its N-terminal and a gamma-glutamyl phosphate reductase domain at its C-terminal. Semi-quantitative reverse transcription polymerase chain reaction analysis revealed that the transcriptional expression level of the EuP5CS gene was considerably up-regulated by up to about 50% in response to NaCl treatments. This result indicated that EuP5CS is a salt-inducible gene and plays an important role in proline biosynthesis in E. camaldulensis clones subjected to salt stress.

Keywords: salt stress, Δ¹-pyrroline-5-carboxylate synthetase, eucalypt, gene expression

INTRODUCTION

Eucalyptus camaldulensis (Dehnh.) is recognized as an economic tree species that can acclimatize to a wide range of unfavorable environments due to its tolerance of salinity, waterlogging and drought (Marcar and Termaat, 1990; Gibson et al., 1991). It suitably adapts to surrounding soil salinity during reclamation of salt-affected soils, has a high survival percentage and grows successfully in northeast Thailand (Terra et al., 2004).

Salt stress is one of the most important abiotic stresses that are having a serious effect on global plant growth and crop productivity in the agricultural sector. To maintain normal cellular metabolism, plants have to develop various protective mechanisms to overcome the deleterious effects of salt stress. One of these defense mechanisms is the synthesis and accumulation of compatible solutes that are thought to play an important role in the process of adjusting to the higher osmotic potential. These organic solutes are low molecular weight, electrically neutral molecules and usually nontoxic to cells at high concentrations (Rontein et al., 2002).
The amino acid proline is an important osmolyte accumulated in high levels in response to osmotic stress that results from salt and drought stress (Hsu et al., 2003; Taji et al., 2004; Kishor et al., 2005). Many functions have been assigned to proline such as being an osmoprotectant that plays a role in counteracting the osmotic stress effects, a redox potential buffer, a subcellular structural stabilizer and also as a free radical scavenger (Schobert and Tschesche, 1978; Handa et al., 1986; Hong et al., 2000).

In higher plants, proline is synthesized by two possible pathways using either glutamate or ornithine as a precursor. The glutamate pathway is considered to be dominant under conditions of stress. In the glutamate pathway, the $\gamma$-glutamyl kinase activity of $\Delta^1$-pyrroline-5-carboxylate synthetase (P5CS; EC 2.7.2.11) represents the rate-limiting step in proline biosynthesis and also can feature feedback inhibition by proline. Since P5CS is a bifunctional enzyme, glutamate is first phosphorylated by the kinase activity to produce $\gamma$-glutamyl-phosphate which is then reduced by the reductase activity to glutamate-$\gamma$-semialdehyde (GSA). This intermediate spontaneously cyclizes to pyrroline-5-carboxylate (P5C) which is then reduced by the $\Delta^1$-pyrroline-5-carboxylate reductase (P5CR; EC 1.5.1.2) to proline (Delauney and Verma, 1993). Proline catabolism on the other hand involves its oxidization to glutamate by a two-step reaction including two mitochondrial enzymes, proline dehydrogenase (PDH; EC 1.5.99.8) and $\Delta^1$-pyrroline-5-carboxylate dehydrogenase (P5CDH; EC 1.5.1.12) (Kiyosue et al., 1996; Verbruggen et al., 1996; Deuschle et al., 2001).

Proline accumulation in response to osmotic stress is a result of a reciprocal regulation, the activation of proline biosynthesis and repression of its catabolism (Verbruggen et al., 1996). Accumulation of proline in many plant species under salt stress has been correlated with stress tolerance, and its concentration has been shown to be generally higher in stress-tolerant than in stress-sensitive plants (Petrusa et al., 1997).

In a previous study, Kulsongkunakorn, (2007) reported that the accumulation of proline in *E. camaldulensis* (clone T5) under salt stress was increased dramatically as a result of an increase in the activity at the chloroplastic P5CS and a decrease in the activity of PDH. In order to better understand the molecular regulation of proline biosynthesis, the objective of the current study was to isolate a full length *EuP5CS* gene from *E. camaldulensis* by reverse transcription polymerase chain reaction (RT-PCR) and walking to get the full length of the gene by RACE, and to examine the gene expression of *EuP5CS* by semi-quantitative RT-PCR (semi-qRT-PCR) in response to salt stress.

**MATERIALS AND METHODS**

**Plant materials and salinity treatment**

*In vitro* culture of *E. camaldulensis* (clone T5) was propagated on the modified Murashige and Skoog agar medium, MS6 (Murashige and Skoog, 1962) supplemented with 20 mg.L$^{-1}$ kinetin, 50 mg.L$^{-1}$ 6-benzylaminopurine, 10 mg.L$^{-1}$ biotin and 10 mg.L$^{-1}$ panthothenic acid in a culture room (25 °C under 16 h.d$^{-1}$ photoperiod with 335 mmol.m$^{-2}$.s$^{-1}$ photosynthetic photon flux density). For salinization treatments, 45-day-old plantlets were transferred to MS6 media supplemented with 0, 50, 100, 200, 300, 400 and 500 mM NaCl. The whole multiple shoots of plantlets were harvested for *EuP5CS* expression measurements after 0, 1, 3, 5 and 7 d of treatment.

**RNA isolation and first strand cDNA synthesis**

*E. camaldulensis* shoots were ground in liquid nitrogen. The total RNA of ground shoots was extracted with a Plant Total RNA Mini Kit (Real Biotech Corporation; Banqiao, Taiwan) and kept at -80 °C until analysis. The quality and concentration of the extracted RNA was analyzed by electrophoresis on agarose gel and
spectrophotometry, respectively. First strand cDNA was synthesized using the ImProm-IITM Reverse transcription System Kit (Promega; Madison, WI, USA) according to the manufacturer’s instructions.

**Isolation of EuP5CS by reverse transcription polymerase chain reaction and rapid amplification of cDNA ends**

To obtain the partial DNA sequence of EuP5CS, the degenerated P5CS forward primer 5′TGGGATAAYGAYGYTTRGC3′ and the reverse primer 5′AGYTTRTTRCTNCCTTGG3′, were designed based on multiple amino acid sequence alignments using the ClustalW program (Thompson et al., 1997) from eleven different plant species, *Arabidopsis* (GenBank accession no. CAB81586.1), *Brassica* (GenBank accession no. AAK01360.1), *Chorispora* (GenBank accession no. AAV67896.1), *Mesembryanthemum* (GenBank accession no. AAC18862.1), *Glycine* (GenBank accession no. AAR86688.1), *Lycopersicon* (GenBank accession no. AAB67875.1), *Actinidia* (GenBank accession no. AAC14481.1), *Vitis* (GenBank accession no. CAB40834.1), *Triticum* (GenBank accession no. AAX35536.1), *Oryza* (GenBank accession no. AAS9034.1), *Saccharum* (GenBank accession no. ABM30223.1), all data being obtained from the National Center for Biotechnology Information (NCBI) database (Pruitt et al., 2007).

For RT-PCR, the reaction mixture of 10 μL containing 1X Taq DNA polymerase reaction buffer, 1.25 mM MgCl2, 0.25 mM dNTP mix, 1 μM each of the degenerated P5CS primers, 0.5 U Taq DNA polymerase and about 100 ng cDNA template was placed in a thermal cycler followed by preheating at 94 °C for 5 min, 35 cycles of denaturing at 94 °C for 1 min, annealing at 52.7 °C for 1 min, extension at 72 °C for 1.30 min, and a final extension step at 72 °C for 10 min.

To obtain the full-length cDNA fragment, rapid amplification of cDNA ends (RACE) was introduced to isolate the 5′ and 3′ ends of the gene using the SMART™ RACE cDNA amplification kit (Clontech; Mountain View, CA, USA). For the 5′-RACE, an aliquot of 1 μg total RNA was reverse-transcribed to the 5′-ready cDNA using Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) following the manufacturer’s protocol with a modified lock-docking oligo(dT) primer and the SMART II™ A oligonucleotide. The resulting 5′-ready cDNA was used for 5′ RACE PCR amplification with a specific P5CS reverse primer (5′GGATAAGCCC ATCAGCCAGCTCCGTTCTC3′), designed based on the sequence of previous RT-PCR products (Figure 1B), and a UPM primer (provided in the kit). For the 3′ RACE, the 3′-ready cDNA was synthesized with 1 μg total RNA using MMLV-RT and a modified lock-docking oligo (dT) primer following the manufacturer’s protocol. The 3′-ready cDNA was used for 3′ RACE PCR amplification with a specific P5CS forward primer (5′CTCGTGTAGGGAGAGGTGGTAT GACTGCG3′) designed based on the sequence of previous RT-PCR products (Figure 1B) and a UPM primer. The PCR program was set up as 5 cycles of 94 °C for 30 sec and 72 °C for 3 min, followed by 5 cycles of 94 °C for 30 sec, 70 °C for 30 sec, 72 °C for 3 min, followed finally by 25 cycles of 94 °C for 30 sec, 68 °C for 30 sec, 72 °C for 3 min according to the Marathon® RACE instruction (Clontech; Mountain View, CA, USA). The complete full-length EuP5CS cDNA sequence was obtained by linking the above three sequences using Sequencher software version 4.0.5 (Gene Codes; Ann Arbor, MI, USA).

**Cloning and sequencing of polymerase chain reaction products**

All RT-PCR and RACE products were analyzed by electrophoresis on 1.5% (w/v) agarose gel and visualized by staining with EtBr. The expected DNA fragments were cut and purified from the agarose gel using the Nucleospin Extraction Kit (MACHEREY-NAGEL GmbH & Co.; Düren, Germany), then cloned into the
pGEM-T vector (Promega; Madison, WI, USA) and transformed into *Escherichia coli* DH5α cells (Invitrogen; Carlsbad, CA, USA). Plasmids were extracted using the AccuPrep® plasmid DNA extraction kit (Bioneer; Alameda, CA, USA) and sequenced by the Macrogen DNA sequencing service (Seoul, South Korea).

**Multiple alignments and bioinformatics analyses**

Sequence comparison with other databases was performed through the NCBI (http://www.ncbi.nlm.nih.gov) via the basic alignment search tool (BLAST; Altschul *et al.*, 1997). Multiple sequence alignments and analysis were conducted by ClustalW (Thompson *et al.*, 1997). The phylogenetic relationship from the deduced amino acid sequences was constructed by the neighbor-joining method (Saitou and Nei, 1987) with 1,000 bootstrap replicates using the MEGA version 5.1 software program (Tamura *et al.*, 2011). Comparisons of the deduced amino acid sequences were performed using the GeneDoc program in the MEGA software.

**Semi-quantitative reverse transcription polymerase chain reaction**

Total RNA was extracted from 100 mg of multiple shoots of *E. camaldulensis* after they had been treated with various NaCl concentrations for 0, 1, 3, 5, and 7 d. First strand cDNA of each time course was synthesized from 1 μg of total RNA. The specific P5CS forward and reverse primers were used to detect EuP5CS transcripts by RT-PCR. The expression of EuP5CS was controlled with a constitutively expressed *E. camaldulensis*’s 26S rRNA gene (GenBank accession no. AF190363). The PCR reaction of 10 μL containing 1× Taq DNA polymerase reaction buffer, 1.25 mM MgCl2, 0.25 mM dNTP mix, 0.2 μM each primers, 0.5 U Taq DNA polymerase and 100 ng cDNA template was placed in a thermal cycler that preheated the samples to 94 °C for 5 min, then 30 cycles (except for the 26S rRNA gene that used 20 cycles) of denaturing at 94 °C for 1 min, annealing at 70 °C (P5CS) or 50 °C (26S rRNA gene) for 30 sec, extension at 72 °C for 1.30 min, and a final extension step at 72 °C for 10 min. The RT-PCR products of EuP5CS and the 26S rRNA gene were analyzed by electrophoresis on a 1.5% agarose gel and stained with EtBr and photographed by a Gel Document (ImageMaster® VDS; Amersham Biosciences, Piscataway, NJ, USA). Densitometry values of the DNA bands were analyzed with the ImageMaster® 1D Elite computer software (Amersham Biosciences, Piscataway, NJ, USA). The data were statistically analyzed using the one-way analysis of variance method according to Duncan’s multiple range test (Sokal and Rohlf, 1981).

**RESULTS**

**Cloning and characterization of EuP5CS**

A 970 bp fragment was amplified by RT-PCR with the degenerated P5CS primers. After sequencing, it had 948 nucleotides (Figure 1). This nucleotide sequence showed similarities to other P5CS plant genes as revealed by a BLAST tool. Based on this partial EuP5CS sequence, the specific P5CS primers were designed and used to amplify a 5′ RACE and 3′ RACE PCR product generating 1.3 kb and 2 kb fragments, respectively (Figure 2). By overlapping, these three sequences were assembled into a 2,944 bp full-length cDNA of EuP5CS, that has been registered at the NCBI (GenBank accession no. EU254744).

The full-length cDNA sequence contained a 337-bp 5′-untranslated region, a 465-bp 3′-untranslated region and a 1,142-bp open reading frame (ORF) beginning with a methionine codon (ATG) at nucleotide position 338-340 and ending with a termination codon (TAA) at nucleotide position 2,477-2,479 (Figure 3). The deduced EuP5CS protein consisted of 713 amino acid residues with a calculated theoretical molecular weight (MW) of 77.2 kDa and predicted isoelectric point (pI) of 5.79 using the Compute
**Figure 1** Polymerase chain reaction (PCR) product and partial sequence of EuP5CS: (A) Electrophoresis of PCR product amplified by degenerated P5CS primers. Lane M: 100 bp ladder and lane 1: reverse transcriptase-PCR product; (B) Partial EuP5CS nucleotide sequences. The underlined nucleotides indicate the specific primer sequences used for rapid amplification of cDNA ends (RACE)-PCR.
PI/MW tool (ExPASy is the SIB Bioinformatics Resource Portal which provides access to scientific databases and software tools at http://expasy.org/). Sequence analysis with the BLAST algorithm of this deduced amino acid sequence showed a high homology with *Gossypium arboreum* P5CS at 84 % (GenBank accession no. ACI62865.1), *Boehmeria nivea* P5CS at 83 % (GenBank accession no. AEV46825.1), and *Jatropha curcas* P5CS at 83 % (GenBank accession no. ABX54880), respectively.

To identify conserved domains within the EuP5CS using PROSITE (ExPASy), EuP5CS was shown to be a bifunctional enzyme with two catalytic domains—an N-terminal glutamate 5-kinase domain (at amino acid position 228-245, consensus pattern; \[\text{[GSTNAD-} \text{X}_{\text{i}}]}\text{-[LIVAMTCK]-X-}[\text{HWFY}]-[\text{IM}]-\text{X}_{\text{j}}] -[\text{HYWNRFT}] -[\text{GSNT}]-[\text{STAG}]-\text{X}_{(0,1)}] -\text{H}-[\text{ST}]-[\text{DE}]-\text{X}_{(1,2)}] -1\)—as shown in Figure 3. In addition, other conserved regions found in the sequence of EuP5CS included a putative ATP-binding site (at amino acid position 57-66), an NAD(P)H-binding site (at amino acid position 426-463), Leu-rich regions (at amino acid positions 173-201 and 554-581).

To investigate the evolutionary relationship of the EuP5CS protein and the P5CS from other species, a phylogenetic tree was constructed based on the deduced amino acid sequences of P5CSs retrieved from GenBank (Figure 4). The phylogenetic tree clearly classified groups of P5CS into monocotyledonous and dicotyledonous clades. The results indicated that EuP5CS was similar to other dicotyledon P5CS clusters and was closely related to the P5CS of *Euonymus japonicus*, *Populus euphratica*, and *P5CS* of *Gossypium arboreum*. In addition, a phylogenetic analysis was used to determine the evolutionary relationship of the EuP5CS protein and the P5CS from other species, a phylogenetic tree was constructed based on the deduced amino acid sequences of P5CSs retrieved from GenBank (Figure 4). The phylogenetic tree clearly classified groups of P5CS into monocotyledonous and dicotyledonous clades. The results indicated that EuP5CS was similar to other dicotyledon P5CS clusters and was closely related to the P5CS of *Euonymus japonicus*, *Populus euphratica*, and *Gossypium arboreum*. In addition, a phylogenetic analysis was used to determine the evolutionary relationship of the EuP5CS protein and the P5CS from other species, a phylogenetic tree was constructed based on the deduced amino acid sequences of P5CSs retrieved from GenBank (Figure 4). The phylogenetic tree clearly classified groups of P5CS into monocotyledonous and dicotyledonous clades. The results indicated that EuP5CS was similar to other dicotyledon P5CS clusters and was closely related to the P5CS of *Euonymus japonicus*, *Populus euphratica*, and *Gossypium arboreum*.
Expression of EuP5CS under salt stress

Total RNAs were isolated from multiple shoots of E. camaldulensis, which had been grown in vitro for 7 d in MS6 agar medium supplemented with various NaCl concentrations of 0, 50, 100, 200, 300, 400 and 500 mM for the salt stress experiment. The isolated total RNAs had an A_260/A_280 ratio (the optical density at 260 nm—for detecting nucleic acid contents and 280 nm—for detecting protein) of 1.8–2.0 suggesting that the RNAs had high quality without any contamination (Sambrook and Russell, 2001). Yields of the RNA were approximately 0.5 to 1 μg total RNA per milligram of tissue. First strand cDNAs were synthesized from 1 μg total RNAs. The EuP5CS transcript level in whole multiple shoots under salt stress was investigated using semi-qRT-PCR analysis (Figure 5). The EuP5CS expression levels on the first day were all significantly increased compared to the control with all NaCl concentrations used but the increases were larger and similar with all NaCl concentrations above 50mM. These levels except for the 50 mM samples decreased slightly at the third and fifth day of growth but were still significantly higher than the controls, On the seventh day, the EuP5CS...
Figure 4  Phylogenetic analyses of aligned amino acid sequences deduced from \textit{EuP5CS} conserved cDNA with other plant species \textit{P5CS}, \textit{Gossypium arboreum} (GenBank accession no. ACI62865.1), \\textit{Boehmeria nivea} (GenBank accession no. AEV46825.1), \textit{Jatropha curcas} (GenBank accession no. ADK37758.1), \textit{Euonymus japonicus} (GenBank accession no. ACF19677.1), \textit{Cucumis melo} (GenBank accession no. AEO27874.1), \textit{Malus hupehensis} (GenBank accession no. AEO51062.1), \textit{Populus euphratica} (GenBank accession no. ABP63534.1), \textit{Solanum torvum} (GenBank accession no. AEN04068.1), \textit{Phaseolus vulgaris} (GenBank accession no. ABY61079.1), \textit{Apocynum venetum} (GenBank accession no. ABO70348.1), \textit{Brassica napus} (GenBank accession no. AAK01361.1), \textit{Nicotiana tabacum} (GenBank accession no. ADL61840.1), \textit{Medicago truncatula} (GenBank accession no. AET35478.1), \textit{Vigna unguiculata} (GenBank accession no. BAI22477.1), \textit{Aegiceras corniculatum} (GenBank accession no. ABG74923.1), \textit{Oryza sativa} (japonica cultivar-group) (GenBank accession no. BAA19916.1), \textit{Arabidopsis thaliana} (GenBank accession no. CAB81586.1), \textit{Saccharum arundinaceum} (GenBank accession no. ABV03819.1), \textit{Triticum aestivum} (GenBank accession no. AAX35536.1), \textit{Chorispora bungeana} (GenBank accession no. AAV67896.1), \textit{Puccinellia chinampoensis} (GenBank accession no. ADU02859.1), \textit{Sorghum bicolor} (GenBank accession no. ACU65227.1). Construction of the phylogenetic tree and bootstrap analysis (1,000 replicates) were performed with the neighbor-joining method of the MEGA 5.1 version. The phylogenetic tree was divided into two major groups, (I) for the dicotyledonous group, and (II) for the monocotyledonous group.
expression levels from the samples treated with 400 mM and 500 mM NaCl were lower than the controls because the salt-stressed plantlets at this time showed wilting and death, but for all others grown with below 400 mM, the NaCl levels were still higher than for the control but reduced from their levels at days 3 and 5.

**DISCUSSION**

Proline is known to be one of the compatible solutes that plays an important role in the process of adjusting the osmotic pressure when plants experience severe environmental conditions such as drought and salinity. It has been suggested that the control of proline levels involves both the synthetic and catabolic pathways, mediated primarily by an up-regulation of P5CS and P5CR gene expression, and a concomitantly down-regulation of the expression of the PDH gene (Verbruggen and Hermans, 2008).

In a previous study, Kulsongkunakorn, (2007) reported the accumulation of proline in an *E. camaldulensis* (clone T5) that increased after *E. camaldulensis* was treated for 5 d with NaCl at a concentration over 200 mM and reached a maximum after treatment with 400 mM NaCl. This directly resulted from an increase of the chloroplastic P5CS enzyme activity as has been likewise reported in other plant species such as leguminous plants (alfalfa, soybean and pea; Tramontano and Jouve, 1997) and sugar beet (Ghoulam *et al*., 2002), where the level of proline was dramatically enhanced in response to salt stress.

In order to clarify the proline biosynthesis regulation under salt stress in the higher plant model of *E. camaldulensis* (clone T5), a complete EuP5CS cDNA was isolated encoding a putative 713 amino acid protein having an overall similarity to other plant P5CSs. Analysis for specific domains indicated that EuP5CS has two domains—an N-terminal glutamate 5-kinase domain at amino acid position 228-245 and a C-terminal gamma-glutamyl phosphate reductase domain at amino acid position 603-625. These two domains are highly conserved in other reported plant P5CSs. Sequence analyses showed a high protein identity

![Figure 5](image-url)  
**Figure 5** Expression patterns of the EuP5CS gene under salt stress over a 7-day period. Relative expression profiles of the EuP5CS gene from a densitometric analysis of the gels are expressed as a percentage of pixel density. The data represent a mean ± SE of three replications.
with the P5CS from *Gossypium arboreum* (84%), *Boehmeria nivea* (83%) and *Vitis vinifera* (82%), respectively.

Analysis of gene expression showed that *EuP5CS* is one of the salt-inducible genes. The *EuP5CS* transcript level was significantly increased in response to salt stress and this was positively correlated with an increasing chloroplastic *EuP5CS* enzyme activity, and resulted in the extensive accumulation of proline as previously reported (Kulsongkunakorn, 2007). From the current study, it can be concluded that the *EuP5CS* gene plays an essential role in regulating proline synthesis under salt stress. The rapid activation of the *EuP5CS* transcript level within 1 d, in response to high salinity may result from the *EuP5CS* promoters that respond rapidly to an elevated salt concentration. However, the remarkable increase in their proline content was exhibited over much longer time periods of salt exposure (Kulsongkunakorn, 2007).

The accumulation of proline was preceded by an increase of the *P5CS* transcript level and the increase was related to the degree of salt tolerance in *A. thaliana*, *M. sativa*, *O. sativa* and *O. streptacantha* (Yoshida et al., 1995; Peng et al., 1996; Igarashi et al., 1997; Yoshida et al., 1997; Claudia et al., 2008). Furthermore, over-expression of the *P5CS* gene in transgenic tobacco (Kishor et al., 1995), rice (Zhu, 2001; Anoop and Gupta, 2003; Su and Wu, 2004), potato (Hmida-Sayari et al., 2005) and wheat (Vendruscolo et al., 2007) resulted in the accumulation of proline and conferred stress tolerance on the plant. In addition, an Arabidopsis plant treated with antisense transgenic *AtP5CS1* cDNA showed hypersensitivity to osmotic stress (Nanjo et al., 1999).

Cloning of the *P5CS* cDNA of *E. camaldulensis* will provide a novel opportunity to study the structure and function of this gene in further studies. Moreover, the *EuP5CS* gene expression profile provides a good starting point for understanding the stress-induced changes in the molecular mechanism of *EuP5CS* and may reveal how *Eucalyptus* species adapt to salt stress.

**CONCLUSIONS**

A complete full-length cDNA of *EuP5CS* gene was isolated. The full-length *EuP5CS* gene is 2,944 bp with an open reading frame of 2,142-bp encoding 713 amino acids. The expression of the *EuP5CS* gene was measured in whole multiple shoots subjected to various NaCl concentrations. According to this semi-qRT-PCR analysis, an increasing gene expression was positively correlated with increasing NaCl concentrations, especially at low NaCl concentrations (50, 100 and 200 mM) on the first, third and fifth day of growth. However, on the seventh day, the expression levels at high NaCl conditions (400 and 500 mM) were decreased to lower than the level of the control because of the death of the cultures. This experiment further confirmed that *EuP5CS* plays a critical role in proline biosynthesis and the extensive accumulation of proline was correlated with increased *EuP5CS* expression, and is one of the important tolerance mechanisms of *E. camaldulensis* in response to salt stress. Moreover, *EuP5CS* could be used as a good candidate gene for genetic engineering of other plants to improve their salt stress tolerance.

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