RESEARCH ARTICLE

Two *P5CS* genes from common bean exhibiting different tolerance to salt stress in transgenic *Arabidopsis*

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Abstract

Many plants accumulate proline in response to salt stress. Δ-pyrroline-5-carboxylate synthetase (P5CS) is the rate-limiting enzyme in proline biosynthesis in plants. Plasmid DNA (pCHF3-PvP5CS1 and pCHF3-PvP5CS2) containing the selectable neomycin phosphotransferase gene for kanamycin resistance and *Phaseolus vulgaris P5CS* (*PvP5CS1* and *PvP5CS2*) cDNA was introduced into *Arabidopsis* plants using *Agrobacterium*-mediated gene transfer. Southern blot, northern blot and RT-PCR analyses demonstrated that the foreign genes were integrated into *Arabidopsis* chromosomal DNA and expressed. Single-gene transformants were analysed in this study. Transgenic plants expressed higher levels of *PvP5CS1* and *PvP5CS2* transcripts under salt stress conditions than under normal conditions. When treated with 0, 100 and 200 mM NaCl, the average proline content in leaves of transgenic plants was significantly higher ($P < 0.01$) than control plants. The average relative electrical conductivity (REC) of transgenic lines was significantly lower ($P < 0.01$) than control plants under salt stress condition. Biomass production of transgenic lines was significantly higher ($P < 0.05$) than control plants under 200 mM NaCl stress treatment. These results indicated that introducing *PvP5CS1* and *PvP5CS2* cDNA into transgenic *Arabidopsis* caused proline overproduction, increasing salt tolerance. Although the expression of *PvP5CS1* in L4 lines and *PvP5CS2* in S4 lines was the same under salt stress condition, the S4 lines accumulated 1.6 and 1.9 times more proline than the L4 lines under 100 and 200 mM NaCl treatments, respectively. The REC of S4 plants was 0.5 (100 mM NaCl) and 0.6 times (200 mM NaCl) that of L4 plants. The biomass production of S4 plants was 1.6 times (200 mM NaCl) more than in L4 plants. Total P5CS enzyme activity of S4 was significantly higher than that of L4. These results implied that the PvP5CS2 protein had stronger capacity to catalyze proline synthesis than PvP5CS1 under salt stress condition.

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Introduction

Salt is an important environmental challenge that causes osmotic stress, negatively impacting plant growth and crop productivity (Qin *et al.* 2011). Plant adaptation to salt stress involves the accumulation of low molecular mass osmolytes, such as proline and glycine betaine (Djilianov *et al.* 2005; Hossain and Fujita 2010). Proline accumulation has been reported in response to drought, high salinity, high light, heavy metals, oxidative stress and biotic stresses (Kavi Kishor *et al.* 2005; Wang *et al.* 2007). In many species, this accumulation of proline has been correlated with stress tolerance, and many researchers have reported higher levels of proline accumulation in salt-tolerant genotypes than in their salt-sensitive counterparts. For example, Igarashi *et al.* (1997) found that the salt-tolerant rice cultivar DGWG steadily increased its proline level under salt-stressed conditions, whereas level in the salt-sensitive cultivar IR 28 increased only slightly. Compared with a control, the salt-tolerant wheat cultivar KRL-19 exhibited 93% and 148% more proline, three and six days after salt treatment (DAT) with 100 mM NaCl, respectively, whereas a salt-sensitive cultivar, WH-542, had only 86.3% and 114% more, respectively (Mandhania *et al.* 2010). An increase in proline concentration under salt stress has been observed in crops as diverse as mulberry (Kumar *et al.* 2003), green gram (Misra and Gupta 2005) and sorghum (Jogeswar *et al.* 2006). Proline accumulation in plant cytosol not only increases cell osmotic potential, stabilizes membrane proteins, protects cells against oxidative damage and maintains appropriate NAD/NADH ratios (Szabados and Savouré 2009), but can also induce

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expression of salt-stress responsive genes (Chinnusamy *et al.* 2005).

In higher plants, the main proline synthesis is glutamate pathway, which has two reaction steps. The first step is catalyzed by a bifunctional enzyme, pyrroline-5-carboxylate synthetase (P5CS), which is a rate-limiting enzyme in the pathway. Recently, the *P5CS* gene was isolated from *Vigna aconitifolia* (Hu *et al.* 1992), *Arabidopsis thaliana* (Strizhov *et al.* 1997), *Lycopersicon esculentum* (Fujita *et al.* 1998), *Oryza sativa* (Igarashi *et al.* 1997), *Medicago truncatula* (Armengaud *et al.* 2004), and other species. Expression assays indicated that *P5CS* from plants could be induced by various abiotic stresses. For example, the *AtP5CS1* gene was upregulated by dehydration, high salinity and abscisic acid (ABA) treatments in most plant organs but silent in dividing cells (Strizhov *et al.* 1997). Rice *P5CS1* was induced by salt, drought, ABA and cold treatment but not by heat treatment (Igarashi *et al.* 1997). In response to NaCl stress, mRNA of *tomPRO2* increased more than three-fold, whereas transcripts of *tomPRO1* were undetectable (Fujita *et al.* 1998). Manipulation of these *P5CS* genes has demonstrated that their overexpression increases proline production and confers salt tolerance in transgenic plants, including rice (Kumar *et al.* 2010), wheat (Vendruscolo *et al.* 2007), potato (Hmida-Sayari *et al.* 2005), tobacco (Yamchi *et al.* 2007) and chickpea (Ghanti *et al.* 2011).

We previously cloned two full-length cDNAs, *PvP5CS1* (GenBank: EU340347) and *PvP5CS2* (GenBank: EU407263), for Δ^1-pyrroline-5-carboxylatesynthetase, an enzyme involved in the biosynthesis of proline, from common bean, *Phaseolus vulgaris* (Chen *et al.* 2009). Nucleotide sequence analysis showed that *PvP5CS1* and *PvP5CS2* shared 73.1% and 73.2% homology with that of *AtP5CS1*, respectively, and shared 74.5% and 74.5% homology with that of *AtP5CS2*, respectively. The present study had two aims: (i) to introduce *PvP5CS1* and *PvP5CS2* cDNAs into *Arabidopsis* via *Agrobacterium tumefaciens* and (ii) to evaluate differences in expression of the transgenes in *Arabidopsis* and the tolerance of transgenic plants under salt stress.

Materials and methods

Plasmid construction

To transfer *PvP5CS1* and *PvP5CS2* into *Arabidopsis*, we first PCR-amplified the open reading frames of the two genes from common bean using specific primers. The primer pairs P1 (5′-CCC GGG ATG GAG AAC ACA GAT CCT TGTA GAC-3′ and 5′-GGA TCC TAA ACA TCT CTC CCC TCT ATT G-3′) and P2 (5′-GGT ACC GCT ATT GCT CGT ATC AGT GCT CAG-3′ and 5′-GTC GAC CAA AAG GAA TCA AAG CAC C-3′) were designed for *PvP5CS1* and *PvP5CS2*, respectively. PCR was performed for *P5CS* gene in a 20 μL reaction volume containing 2 μL common bean leaf cDNA template, 1× PCR buffer, 2.0 mM of $MgCl_2$, 0.25 μmol of each primers, 1.2 mM dNTPs and 1.6 U of *Taq* polymerase (Promega, Shanghai, China), respectively. The PCR was carried out using PTC-100^{TM} Programmable Thermal Controller (MJ Research, Watertown, USA) as follows: initial denaturation at 94°C for 3 min; 30 cycles of 94°C for 0.5 min, 55°C for 50 s, 72°C for 1.5 min; and a final extension at 72°C for 10 min. The PCR products were digested with *Sma*I/*Bam*HI and *Kpn*I/*Sal*I, and subcloned into the expression vector pCHF3, which contains a CaMV 35S promoter, the nopaline synthase terminator and the selectable neomycin phosphotransferase gene for kanamycin resistance, to create recombinant plasmids pCHF3-PvP5CS1 and pCHF3-PvP5CS2.

Generation of transgenic Arabidopsis plants

To transform *A. thaliana* ecotype Landsberg, the recombinant plasmids (pCHF3-PvP5CS1 and pCHF3-PvP5CS2) was transferred into *Agrobacterium tumefaciens* (GV3101) by the flower-dip electroporation transformation method (Clough and Bent 1998). The pCHF3 vector was transformed using the same procedure. Seeds from transformed *Arabidopsis* plants were harvested and termed T1 generation transformants.

Southern blot analysis

Genomic DNA was isolated from leaves of transgenic *Arabidopsis* plants by the hexadecyltrimethylammonium bromide (CTAB) method (Murray and Thompson 1980). Approximately 10 μg of DNA was digested with *Kpn*I and subjected to electrophoresis on a 0.8% agarose gel. The DNA was then blotted onto a nylon membrane (DuPont/NEN, Wilmington, USA). The membrane was probed with [^{32}P]dATP-labelled *PvP5CS1* and *PvP5CS2* coding regions at 55°C. All the other procedures were carried out according to the manufacturer's instructions.

Salt stress treatment of transgenic T3 generation plants

Wild type, pCHF3 and T3 transgenic *Arabidopsis* seeds were germinated in MS medium. After seven days, the young plants were transferred to four new MS medium plates containing 0, 100, 200 and 300 mM NaCl and grown in the greenhouse under a 16/8 light/dark cycle at 25°C for 5, 10 and 15 days. In salt stress assay, four *Arabidopsis plants* of each *Arabidopsis* type: wild type, pCHF3, L4 (transgenic with PvP5CS1) and S4 (transgenic with PvP5CS2) were transplanted in a plate. A total of 60 plates were treated in each salt stress condition.

RT-PCR

To determine the expression patterns of *PvP5CS1* and *PvP5CS2* in *Arabidopsis*, semiquantitative RT-PCR was carried out. Total RNA was extracted using Trizol reagent

(Invitrogen, Carlsbad, USA) from leaves of *Arabidopsis* plants treated with salt for 10 days, and cDNAs were synthesized using ImPromII^{TM} Reverse Transcriptase (Promega, Fitchburg, USA). The primers were P1 for *PvP5CS1* and P2 for *PvP5CS2*.

Northern RNA hybridizations

Total RNA (15 μg) of transformed and wild-type *Arabidopsis* plants treated with salt for 10 days were subjected to electrophoresis in 1.2% agarose gel, transferred to nylon filter (DuPont/NEN), and hybridized to *PvP5CS1* and *PvP5CS2* cDNA labelled with [^{32}P]dATP. Hybridization was carried out at 60°C and filters were exposed to X-ray film.

Determination of physiological parameters

Free proline content in fresh leaves of single transformed and wild-type *Arabidopsis* plants treated with salt for 10 days was determined according to Bates *et al.* (1973) and expressed as μg proline per gram fresh weight (μg/g, fresh weight).

Leaves from transformed and wild-type *Arabidopsis* plants treated with salt for 10 days were used for an electrolyte leakage assay as follows. First, leaves from plants were placed in glass tubes containing 10 mL of distilled water and the excess of air was removed from the tubes by vacuum pressure. Closed tubes were incubated in a shaker for 1 h to ensure the leakage of ions from the cells into water phase. The electrical conductivity of water extracts was measured with a conductivity meter (DDS-11A, Shanghai Precision and Scientific Instruments, Shanghai, China). Then, the tubes were incubated in a boiling water bath for 5 min, shaken for additional 20 min and the electric conductivity was measured again. The relative electrical conductivity (REC) was calculated according to the equation $I = (L_1/L_2) \times 100\%$, where L_1 is the conductivity of a sample after salt treatment and L_2 is the conductivity of a sample after boiling.

All leaves of a plant were used in the measurement of free proline content and REC. The whole plant was used in the measurement of biomass production of seedlings. For each physiological parameter, a total of 60 plants, which come from each stress-treated plate, were used.

P5CS activity assay

The activity of P5CS was assayed following the method described by Špoljarević *et al.* (2011). Frozen leaf tissue samples (0.5 g) were ground to a fine powder with liquid nitrogen and homogenized in the appropriate extraction buffer. The ratio of buffer volume:g tissue was 2:1. The extraction buffer used for P5CS assays was 50 mM Tris-HCl (pH 7.5) containing 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 4 mM DTT, 2 mM PMSF, 1 mM EDTA and 2% PVPP. The extracts were centrifuged at 4°C for 15 min at 20,000 g and the resulting supernatant was used as the enzyme source. The assay mixture contained 50 mM Tris-HCl buffer (pH 7.0), 20 mM $MgCl_2$, 50 mM L-glutamate, 100 mM hydroxylamine-HCl and 10 mM ATP. The reaction was initiated by the addition of enzyme extract. After incubation at 15 min for 37°C, the reaction was stopped by adding 1 mL of stop buffer (2.5 g $FeCl_3$ and 6 g trichloroacetic acid in a final volume of 100 mL of 2.5 M HCl). The precipitated proteins were removed by centrifugation (4°C for 15 min at 10,000 g) and the absorbance recorded (UV-VIS spectrophotometer Carry 50, Varian Medical Systems Inc., Palo Alto, USA) 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 of 250 M^{-1} cm^{-1} reported for the Fe^{3+} hydroxamate complex of the compound (Hayzer and Leisinger 1980). One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of γ-glutamyl hydroxamate per minute. Total enzyme activity was expressed per g of frozen tissue (fresh weight).

Statistical analysis

Statistical analyses were performed using SAS statistical software (North Carolina State University, USA). The Fisher's protected LSD test was used to determine the differences among the treatments. Significance was declared at $P < 0.05$.

Results

Production of transgenic Arabidopsis plants

The full-length cDNA sequences of *PvP5CS1* and *PvP5CS2* were separately subcloned into pCHF3 vector and transformed into wild-type *Arabidopsis* via *A. tumefaciens*. *Arabidopsis* seeds (T1) produced by the transformed plants were screened on plates (MS medium containing 50 μg/mL kanamycin). Transformed seeds could germinate and grow. Five T1 positive transgenic individuals transformed with *PvP5CS1* (denominated L1 to L5) and with *PvP5CS2* (denominated S1 to S5) were transplanted from screening plates to pots filled with soil and cultured in the greenhouse. RT-PCR using leaf cDNA from each positive T1 seedling as template showed that *PvP5CS1* and *PvP5CS2* had been inserted into the *Arabidopsis* genomes and were expressed normally in the transgenic plants (figure 1). The T2 seeds obtained from selfpollinated T1 seedlings were again germinated on screening plates, transplanted to pots in the greenhouse and used to generate seeds (T3). Positive transformants with the pCHF3 vector were screened on plates and used as control plants in this study.

Southern blot analysis of transgenic plants

Genomic Southern blot analysis of the 10 T3-generation transgenic plants showed that one to four copies of the

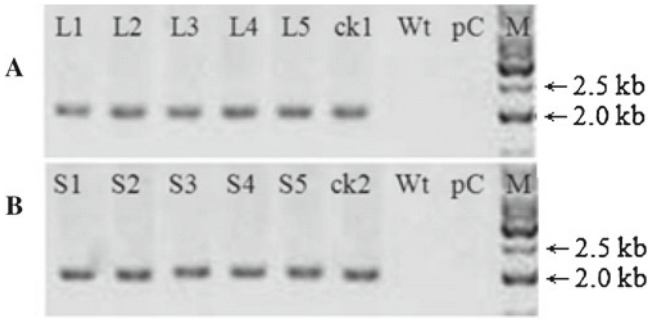

Figure 1. Expression of two *PvP5CS* genes in transgenic T1 plants. (A) RT-PCR analysis *PvP5CS1* gene using primer pair P1. (B) RT-PCR analysis *PvP5CS2* gene using primer pair P2. L1, L2, L3, L4 and L5 represent the individual of PvP5CS1-transgenic T1 plant. S1, S2, S3, S4 and S5 represent the individual of PvP5CS2-transgenic T1 plant. ck1, ck2, Wt and pC represent pCHF3-PvP5CS1 vector, pCHF3-PvP5CS2 vector, nontransformed plant and plant transformed with vector pCHF3. 500 bp marker (M) was used in this assay.

PvP5CS1 and *PvP5CS2* cDNAs were inserted into their genomes. Each line had a specific hybridization pattern, with different higher molecular-weight bands, suggesting that these transgenic lines were derived from independent transformation events (figure 2). The single-gene transformant L4 and S4 lines were selected for further analysis.

Salt stress tolerance of PvP5CS1 and PvP5CS2 transgenic Arabidopsis plants

To investigate the functional roles of *PvP5CS1* and *PvP5CS2* in salt stress tolerance, the L4 and S4 lines were analysed. Wild type and pCHF3 plants were used as controls in the salt stress experiment. Result showed that there was no apparent phenotype difference among the transgenic *Arabidopsis* seedlings after five days salt treatment, and that all the transgenic *Arabidopsis* seedlings were dead after 15 days salt treatment. Seedlings after being treated for 10 days, exhibited obvious phenotype differences. After treatment for 10 d, 100 mM NaCl inhibited plant growth and 300 mM NaCl had a more severe inhibitory effect (figure 3). In the 100 mM NaCl treatment, growth of the control plants (Wt and pCHF3) was inhibited, but the transgenic plants (L4 and S4) grew normally; the control plant leaves were also more yellow than those of transgenic plants. Although 200 mM NaCl treatment inhibited the growth of both control and transgenic plants, the latter grew better. The S4 plants had stronger growth than L4 plants. In the 300 mM NaCl treatment, all of the control plants died. The transgenic plants survived, but their growth was severely inhibited. These results indicated that *PvP5CS1* and *PvP5CS2* overexpression could confer strong salt tolerance, and S4 plants exhibited stronger salt tolerance than L4 plants.

REC and biomass production of T3 transgenic plants under salt stress

The REC in transgenic lines was lower than that of the control plants (table 1). Under normal culture conditions (MS + 0 mM NaCl), the REC was not significantly different among wild type, pCHF3, transgenic L4 line and transgenic S4 line plants. The average REC values of S4 lines decreased to 77.8% (100 mM NaCl) and 58.6% (200 mM NaCl) of those of Wt plants. The average REC values of L4 lines decreased to 55.4% (100 mM NaCl) and 28.9% (200 mM NaCl) of those of Wt plants. Biomass production by T3 transgenic plants under salt stress in terms of seedling fresh weight was

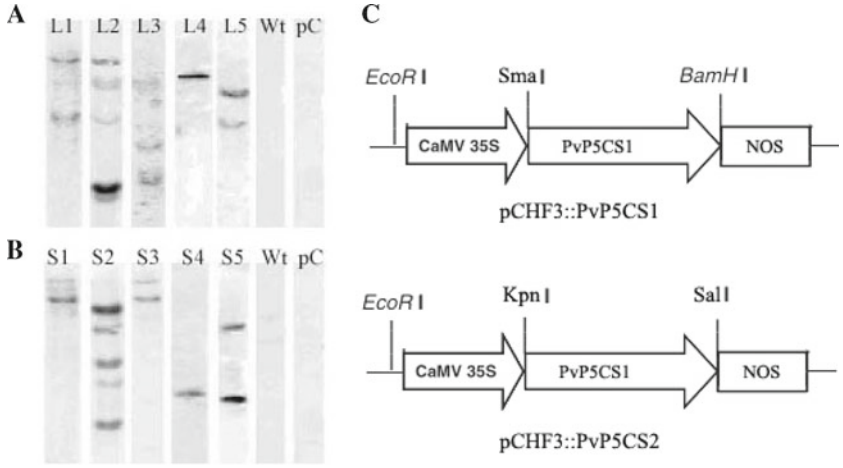

Figure 2. Southern blotting of transgenic T3 *Arabidopsis* lines. (A) The blot was probed by ^{32}P[dATP]-labelled PvP5CS1 cDNA. (B) The blot was probed by ^{32}P[dATP]-labelled PvP5CS2 cDNA. (C) Structure of the vector map of pCHF3::PvP5CS1/2. L1, L2, L3, L4 and L5 represent the individual of PvP5CS1-transgenic T1 plant. S1, S2, S3, S4 and S5 represent the individual of PvP5CS2-transgenic T1 plant. Wt represents nontransformed plant and pC represents plant transformed with vector pCHF3. Genomic DNA was digested by *kpn*I and resolved on 0.8% agarose gel.

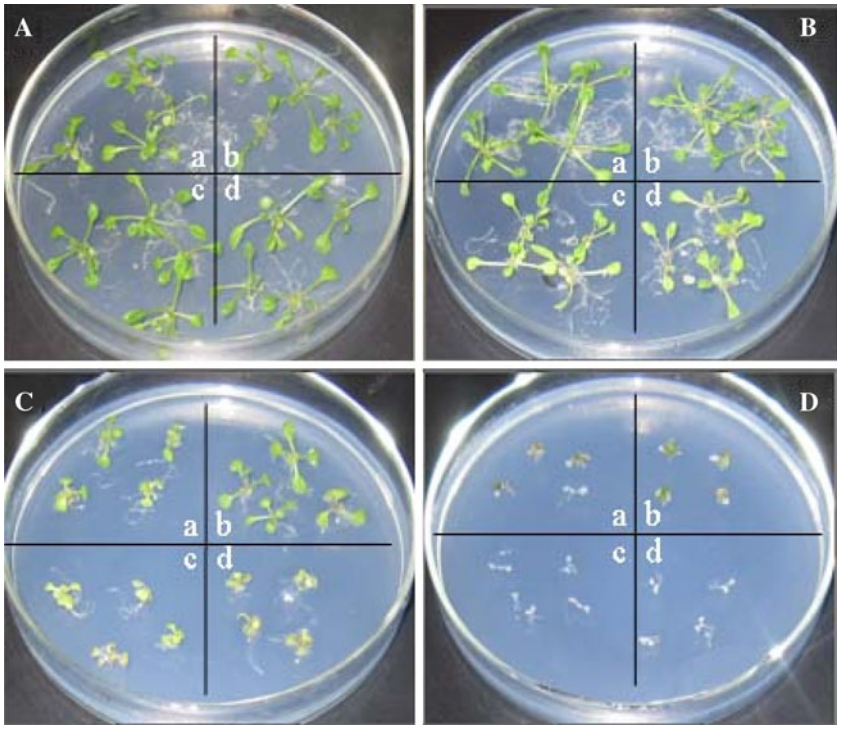

Figure 3. Salt tolerance of PvP5CS1 and PvP5CS2 overexpressed *Arabidopsis* plants under different concentration NaCl treatment. A, B, C and D represent *Arabidopsis* plants cultured on MS + 0 mM NaCl, MS + 100 mM NaCl, MS + 200 mM NaCl and MS + 300 mM NaCl plates for 10 days, respectively. a, b, c and d represent PvP5CS1-transgenic line (L4), PvP5CS2-transgenic line (S4), wild type and pCHF3 plant, respectively.

measured (table 2). Seedling growth did not differ between the untreated control and transgenic plants. However, growth of the transgenic lines was less inhibited than growth of the control plants under salt stress. Biomass production of S4 lines was 36.1% and 192.6% higher than that of Wt plants in the 100 and 200 mM NaCl treatments, respectively. Biomass production of L4 lines was 24.4% and 81.8% higher than that of Wt plants in the 100 and 200 mM NaCl treatments, respectively. These results indicate that *PvP5CS1* and *PvP5CS2* overexpression can confer strong salt tolerance.

The REC did not differ between L4 and S4 plants in the 0 mM NaCl treatments. However, a significant difference ($P < 0.01$) occurred under stress condition. The REC of S4 plants was 0.5 times (100 mM NaCl) and 0.6 times

Table 1. Relative electrical conductivity of transgenic and Wt *Arabidopsis* plants.

	0 mM	100 mM	200 mM
Wt	4.24 ± 0.89a*	28.04 ± 1.24a	61.79 ± 4.38a
pC	4.10 ± 0.70a	24.48 ± 1.67a	62.04 ± 4.80a
L4	3.96 ± 0.86a	12.51 ± 2.74b	43.90 ± 2.99b
S4	4.30 ± 1.00a	5.95 ± 0.69c	25.60 ± 3.0c
(Wt−S4)/Wt	0	0.788	0.586
(Wt−L4)/Wt	0	0.554	0.289
S4/L4	1.1	0.5	0.6

Wt, wild type; pC, pCHF3 plants; L4, PvP5CS1-transgenic line; S4, PvP5CS2-transgenic line.
*The values shown here present the mean of three measurements together with the standard deviation.
a, b and c represent difference significance ($P < 0.01$) statistical analysis using least significant difference methods. Means with the same letter are not significantly different.

Table 2. Biomass production of transgenic and Wt *Arabidopsis* plant seedlings.

	0 mM	100 mM	200 mM
Wt (g)	0.99 ± 0.07A	0.77 ± 0.06B	0.18 ± 0.03C
pC (g)	1.03 ± 0.06A	0.80 ± 0.05B	0.17 ± 0.01C
L4 (g)	1.00 ± 0.03A	0.96 ± 0.10A	0.33 ± 0.03B
S4 (g)	0.98 ± 0.02A	1.05 ± 0.03A	0.52 ± 0.09A
(S4−Wt)/ Wt	0	0.361	1.926
(L4−Wt)/ Wt	0	0.244	0.818
S4/L4	1.0	1.0	1.6

Wt, wild type; pC, pCHF3 plants; L4, PvP5CS1-transgenic line; S4, PvP5CS2-transgenic line.
*The values shown here present the mean of three measurements together with the standard deviation.
A, B and C represent difference significance ($P < 0.05$) statistical analysis using least significant difference methods. Means with the same letter are not significantly different.

(200 mM NaCl) that of L4 plants. The biomass production of seedlings did not differ between L4 and S4 plants in the 0 and 100 mM NaCl treatments. A significant difference ($P < 0.05$) occurred under 200 mM NaCl stress condition. The biomass production of S4 plants was 1.6 times that of L4 plants. The result implied that S4 plants exhibited stronger salt tolerance than L4 plants under stress conditions.

Proline accumulation in T3 transgenic plants

A significant difference ($P < 0.01$) in proline content was seen between the control and transformed plants under different culture conditions (table 3). Overall, in the 0, 100 and 200 mM NaCl stresses, the average proline content in leaves of S4 plants increased by 175.7%, 402.9% and 598.1%, respectively, compared with Wt plants. The average proline content in leaves of L4 plants increased by 180.5%, 223.1% and 268.9% compared with Wt plants under 0, 100 and 200 mM NaCl stresses, respectively. There was no obvious difference in proline content between the L4 and S4 lines under normal (0 mM NaCl) conditions. However, significant differences in proline content ($P < 0.01$) between L4 and S4 lines occurred under salt-stress conditions (table 3). In summary, overexpression of one copy of *PvP5CS2* resulted in 1.6 and 1.9 times more proline accumulating than did overexpression of one copy of *PvP5CS1* in transgenic *Arabidopsis* plants under 100 and 200 mM NaCl treatments, respectively.

Expression of the PvP5CS2 cDNA in transgenic Arabidopsis plants

RT-PCR analysis was carried out on L4 and S4 transgenic lines (figure 4A). Although transgenic plants expressed higher levels of *PvP5CS1* (and *PvP5CS2*) transcripts under salt stress conditions (lanes 2 and 3) than under normal culture condition (lane 1), no notable difference was seen between the 100 and 200 mM salt stress conditions. The expression of *PvP5CS1* in L4 lines was the same as the

Table 3. Proline content and total P5CS enzyme activity in leaves of transgenic and wild *Arabidopsis* plants.

		0 mM NaCl	100 mM NaCl	200 mM NaCl
Proline content	Wt (μg/g FW)	28.86 ± 3.40b*	69.60 ± 2.79c	40.23 ± 6.70c
	pC (μg/g FW)	29.05 ± 4.52b	70.63 ± 9.51c	39.37 ± 0.65c
	L4 (μg/g FW)	80.95 ± 2.04a	224.89 ± 2.90b	148.43 ± 6.38b
	S4 (μg/g FW)	79.57 ± 2.58a	350.10 ± 1.67a	280.85 ± 6.38a
	(S4−Wt)/ Wt	1.757	4.029	5.981
	(L4−Wt)/ Wt	1.805	2.231	2.689
	S4/L4	1.0	1.6	1.9
Total P5CS enzyme activity	Wt (μg/g FW)	0.164 ± 0.004B	0.328 ± 0.007C	0.236 ± 0.007C
	pC (μg/g FW)	0.158 ± 0.006B	0.331 ± 0.004C	0.241 ± 0.006C
	L4 (μg/g FW)	0.451 ± 0.004A	0.690 ± 0.009B	0.583 ± 0.008B
	S4 (μg/g FW)	0.462 ± 0.005A	0.898 ± 0.007A	0.751 ± 0.003A

Wt, wild type; pC, pCHF3 plants; L4, PvP5CS1-transgenic line; S4, PvP5CS2-transgenic line.
*The values shown here present the mean of three measurements together with the standard deviation.
a, b, c and A, B, C represent difference significance ($P < 0.01$) statistical analysis using least significant difference methods. Means with the same letter are not significantly different.

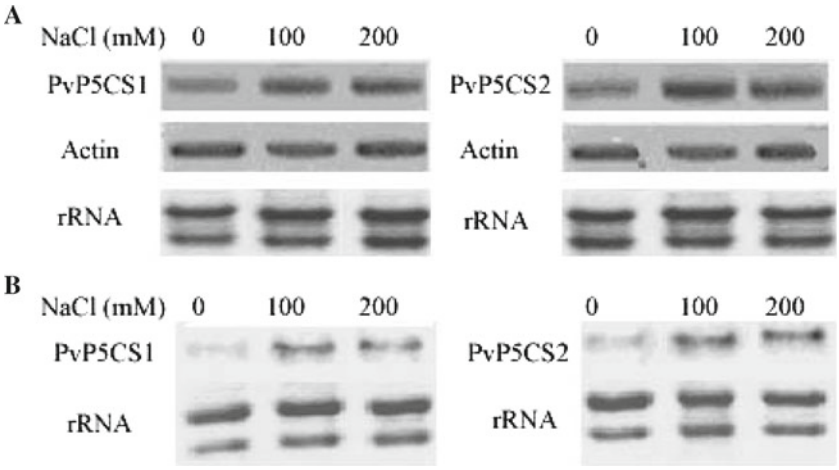

Figure 4. RT-PCR (A) and Northern blot (B) analysis of the expressions of PvP5CS1 and PvP5CS2 in transgenic *Arabidopsis* L4 and S4 plants, respectively. Total RNA used for RT-PCR and Northern blot analysis was extracted from transgenic lines (L4 and S4) cultured on MS + 0 mM NaCl, MS + 100 mM NaCl, MS + 200 mM NaCl plates for 10 days.

expression of *PvP5CS2* in S4 lines under normal (lane 1) and identical salt stress (lanes 2 and 3) conditions. The results were supported by the Northern blot assay (figure 4B).

P5CS enzyme activity in transgenic Arabidopsis plants

The result of enzyme activity assay showed that both under 100 mM NaCl salt stress condition and 200 mM NaCl salt stress conditions, total P5CS enzyme activity of S4 was significantly higher than that of L4, and there was no obvious difference between S4 an L4 under normal conditions (table 3).

Discussion

In plants, key enzymes of metabolic pathways are generally encoded by redundant genes, which may have been generated by historical gene duplication events. Afterwards, duplicated genes often incur sequence alterations, causing changes in transcriptional regulation (Turchetto-Zolet *et al.* 2009). Thus far, several duplicate *P5CS* genes have been found in *Arabidopsis* (Strizhov *et al.* 1997), rice (Igarashi *et al.* 1997), tomato (Fujita *et al.* 1998) and common bean (Chen *et al.* 2010). These duplicate *P5CS* genes originated from a single common ancestral gene (Turchetto-Zolet *et al.* 2009). Though many authors reported that transcriptional control of the duplicate *P5CS* genes in plants had been shown to be different in different organs and under different osmotic stresses, no previous report had compared the capacity of duplicate *P5CS* proteins in catalyze proline synthesis. We previously reported that the duplicate *PvP5CS* genes presented 15.2% divergence in amino acid sequences. Especially, there were three amino acids divergence in Glu-5-kinase catalytic domain and two amino acids divergence in GSA-DH catalytic domain between PvP5CS1 and PvP5CS2 (Chen *et al.* 2010). Amino acids divergence probably changes catalytic ability of PvP5CS1 and PvP5CS2 in proline synthesis. Here, we found that under same stress conditions, although no obvious differences existed between the expression of *PvP5CS1* and *PvP5CS2* in transgenic plants, the *PvP5CS2* transgenic plants accumulated more proline than the *PvP5CS1* transgenic plants. And total P5CS enzyme activity of S4 was significantly higher than that of L4. These results indicated that the PvP5CS2 protein had stronger capacity to catalyze proline synthesis than the PvP5CS1 protein under salt stress condition.

In this study, the induction of *PvP5CS1* and *PvP5CS2* transcripts also occurred in transgenic plants under salt stress. The mRNA levels of *PvP5CS1* and *PvP5CS2* in transgenic lines were higher when plants were salt stressed than when they were not. This phenomenon was also found in transgenic potato plants transformed with *A. thaliana P5CS* cDNA triggered by the cauliflower mosaic virus 35S RNA promoter (Hmida-Sayari *et al.* 2005). More *AtP5CS* transcripts accumulated in potato transgenic lines cultivated in the presence of 100 mM NaCl than in those cultivated without NaCl. Hong *et al.* (2000) comparison of P5CS protein levels between control and salt-treated plants indicated an increase of about 50% under salt stress conditions (200 mM NaCl). Recently, real-time quantitative PCR analysis indicated that *PvP5CS1* and *PvP5CS2* mRNA transcript levels in common bean seedling leaves steadily increased under cold, drought and salt stress (Chen *et al.* 2009). These results imply that some molecular mechanism in *Arabidopsis* upregulates *PvP5CS1* and *PvP5CS2*.

Genetic engineering strategies for abiotic stress tolerance in plants depend on the expression of genes involved in signalling and regulatory pathways, that encode proteins conferring stress tolerance, or that encode enzymes in pathways that synthesize structural metabolites and osmolytes (Goel *et al.* 2010). Various earlier researchers have reported higher

proline accumulation and subsequent abiotic stress tolerance of transgenic plants overexpressing *P5CS*; the genes transformed were mainly from *Vigna aconitifolia* P5CS (Su and Wu 2004; Kumar *et al.* 2010; Karthikeyan *et al.* 2011). This study verified the function of two *P5CS* genes from common bean to improve plant stress tolerance by genetic engineering. Our results demonstrated that the successful insertion and functional expression of *PvP5CS1* and *PvP5CS2* in the genome of *Arabidopsis*. That the average proline content in transgenic plants increased by 177.1% relative to control plants under normal culture conditions demonstrated that exogenous *PvP5CS1* and *PvP5CS2* functioned in *Arabidopsis*.

Under adversity, plants produce large amounts of oxygen free radicals that directly or indirectly peroxidize membrane lipids, damaging or destroying the membrane, reducing the cell membrane's selectivity, and increasing electrical conductivity. Electrical conductivity in transgenic plants was lower than in control plants, implying that transgenic plants suffered less membrane destruction than control plants under salt stress and that increased proline accumulation in the transgenic plants protected the cell membrane. The alleviation of salt stress in transgenic *Arabidopsis* plants was evidenced by phenotypic characters, relative conductivity rate and biomass production. The transgenic approach to improve plant salt tolerance via overproducing proline has shown promise. For example, Su and Wu (2004) observed significantly higher tolerance to NaCl or drought stress by *P5CS*-containing transgenic rice with higher proline accumulation. Karthikeyan *et al.* (2011) also reported that almost four times more proline content in *P5CS*-transgenic rice lines under salt stress; the transgenic plants grew well in the presence of up to 200 mM NaCl, while control plants died within 10 days under these conditions. All growth characters of canola plants decreased with increasing salinity stress, but applying proline (200 mg/L) and glycine betaine (400 mg/L) alleviated these negative effects (Sakr *et al.* 2012). Thus, the increase in proline content contributed to alleviating the adverse effects of salt stress on plants (Ashraf and Foolad 2007). Our results with free proline-containing transgenic lines under saline conditions also supported the hypothesis of a positive correlation between proline accumulation and salt stress tolerance of plants.

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