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Heterologous expression of *P5CS* gene in chickpea enhances salt tolerance without affecting yield

S. KIRAN KUMAR GHANTI[1]*, K.G. SUJATA[2], B.M. VIJAY KUMAR[3], N. NATARAJA KARBA[2], K. JANARDHAN REDDY[4], M. SRINATH RAO[1] and P.B. KAVI KISHOR[5]

Department of Botany, Gulbarga University, Gulbarga, Karnataka-585106, India[1]
Department of Crop Physiology, University of Agricultural Science, Bengaluru, Karnataka-560065, India[2]
Indian Institute of Science, Bengaluru, Karnataka-560012, India[3]
Department of Botany[4] *and Department of Genetics*[5], *Osmania University, Hyderabad-500007, India*

Abstract

Vigna Δ^1-pyrroline-5-carboxylate synthetase (*P5CS*) cDNA was transferred to chickpea (*Cicer arietinum* L.) cultivar Annigeri *via Agrobacterium tumefaciens* mediated transformation. Following selection on hygromycin and regeneration, 60 hygromycin-resistant plants were recovered. Southern blot analysis of five fertile independent lines of T0 and T1 generation revealed single and multiple insertions of the transgene. RT-PCR and Western blot analysis of T0 and T1 progeny demonstrated that the *P5CS* gene is expressed and produced functional protein in chickpea. T1 transgenic lines accumulated higher amount of proline under 250 mM NaCl compared to untransformed controls. Higher accumulation of Na^+ was noticed in the older leaves but negligible accumulation in seeds of T1 transgenic lines as compared to the controls. Chlorophyll stability and electrolyte leakage indicated that proline overproduction helps in alleviating salt stress in transgenic chickpea plants. The T1 transgenics lines were grown to maturity and set normal viable seeds under continuous salinity stress (250 mM) without any reduction in plant yield in terms of seed mass.

Additional key words: *Agrobacterium tumefaciens*, *Cicer arietinum*, Δ^1-pyrroline-5-carboxylate synthetase, NaCl, proline accumulation, RT-PCR, Southern blot, Western blot.

Introduction

Adaptation or tolerance of plants to osmotic stress involves the accumulation of compatible, low molecular mass osmolytes, such as proline (Pro) and glycine betaine (Greenway and Munns 1980). High accumulation of Pro (up to 80 % of the amino acid pool) under a variety of stress conditions is due to increased synthesis and decreased degradation (Kavi Kishor *et al*. 2005). In addition to acting as an osmoprotectant, Pro also serves as redox potential regulator (Saradhi and Saradhi 1991) or a hydroxyl radical scavenger (Smirnoff and Cumbes 1989), and protects macromolecules against denaturation (Schobert and Tschesche 1978) due to cell acidity reduction (Venekamp *et al*. 1989). The Pro biosynthesis pathway was first elucidated in *Escherichia coli* (Leisinger *et al*.1987). Starting from an ATP-dependent phosphorylation of L-glutamic acid by the γ-glutamyl kinase (γ-GK), the γ-GK product is then reduced to glutamic-γ-semialdehyde (GSA) by GSA dehydrogenase that cyclizes spontaneously to Δ^1-pyrroline-5-carboxylate (P5C), and finally is reduced to Pro by P5C reductase (P5CR). In plants, proline is synthesized from either glutamate or ornithine. However, the glutamate pathway is the primary route used under osmotic stress or nitrogen limitation whereas the ornithine pathway is prominent under high nitrogen input (Delauney and Verma 1993). Mothbean (*Vigna aconitifolia*) cDNA clone encoding P5CS was first isolated by a functional complementation technique using *E. coli* mutants (Hu *et al*. 1992). P5CS is a bifunctional enzyme (EC 2.7.2.11/1.2.1.41) that catalyses the first two steps of proline biosynthesis

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Abbreviations: Chl - chlorophyll; EC - electrical conductivity; PCR - polymerase chain reaction; PMSF - phenylmethylsulfonyl fluoride; Pro - proline; RT-PCR - reverse transcription PCR; SDS - sodium dodecylsulphate.
* Author for correspondence present address: Department of Plant Physiology, University of Agricultural Science, Bengaluru, Karnataka-560065, India; fax: (+91) 80 23636713, e-mail: ghantikiran@rediffmail.com

in plants. Several studies demonstrated increased tolerance to abiotic stress in transgenic plants engineered for overproduction of proline (Kavi Kishor *et al*. 1995, Zhu *et al* 1998, Sawahel and Hassan 2002, Han and Hwang 2003, Sayari *et al*. 2005). Chickpea (*Cicer arietinum* L.) is very sensitive to salinity (Lauter and Munns 1987). Previous results showed that no chickpea cultivar could grow at electrical conductivity (EC) higher than 6 dS m^{-1} (Dua 1992, Dua and Sharma 1995). With the aim to improve the adaptation of chickpea to saline soils, *Vigna P5CS* cDNA was transferred into chickpea cultivar Annigeri under the control of CaMV 35S promotor.

Materials and methods

Plants, transformation and selection of transgenic shoots: Seeds of chickpea (*Cicer arietinum* L.) cv. Annigeri were obtained from the Agricultural Research Station, Gulbarga, Karnataka, India. Seeds were surface sterilized according to Kiran Ghanti *et al*. (2009). Cotyledonary nodes were excised aseptically from 5 to 6-d-old seedlings and inoculated on to Murashige and Skoog's (1962; MS) medium. The plasmid pCAMBIA 1301 (Fig. 1*A*) was used for transformation *via Agrobacterium tumefaciens* strain LBA4404. This construct contains *hptII* gene for resistance to hygromycin sulphate, *GUS* reporter gene, encoding β-glucuronidase, and the *Vigna aconitifolia P5CS* cDNA. *Vigna P5CS* cDNA was cloned into a site of *Hind III* of PRT 100 vector and subsequently into pCAMBIA 1301, a binary vector under the control of CaMV 35S promoter. It is terminated by the nopaline synthase (*nos*) terminator. To prepare the inoculum, a single colony of *Agrobacterium* was grown in 2 cm^3 of liquid LB medium with antibiotics overnight and then suspended in 50 cm^3 of AB minimal medium supplemented with kanamycin (50 mg dm^{-3}) for 48 h at 28 °C (Sanchayita *et al.* 1996). Bacteria were then collected by centrifugation at 1845 *g* for 5 min. Pellet was resuspended in 20 cm^3 of ½ MS medium and cotyledonary node explants were incubated at room temperature for 4 - 5 min. A total of 680 explants were used for transformation. Putative transgenic plants were successfully transferred to poly-cups and later to the pots. Out of 60 independent primary transgenic chickpea, five transgenic lines were selected in T0 and T1 progeny and were analyzed for *GUS* expression (Jefferson 1987).

PCR, Southern blot, RT-PCR and Western blot: Genomic DNA from leaves of T0 and T1 progeny of five chickpea lines was extracted according to Dolly and Dolly (1989). PCR reaction was performed with purified genomic DNA (30 ng) using two primers that are complementary to the *P5CS* cDNA (5'-CAAGGC TTGCTTCACAAACC-3' and 5'-GACGGGGCCA GAGGAGACGAGTA-3'). Amplification reactions consisted of one cycle of 4 min at 94 °C, followed by 25 cycles (60 s, 94 °C, 45 s 58 °C, 2 min 72 °C) and finally an extension cycle of 10 min at 72 °C. PCR products were separated on 1 % agarose gel and stained with ethidium bromide.

A 30 µg DNA sample from transgenic lines and untransformed controls were digested with *Hind III* and probed with 887 bp DNA, resolved on 1 % agarose gel and blotted on a *Hybond* N^+ (*Amersham Intermational*, Little Chalfont, Bucks, UK) membrane. The blot was probed with the ^{32}P-radio labeled *P5CS* cDNA (Sambrook *et al*. 1989).

Total RNA from leaf tissue was isolated from both transgenic and untransformed control plants according to Verwoerd *et al*. (1989). Reverse transcription was performed with *Superscript III* first strand synthesis system (*Invitrogen*, Carlsbad, CA). cDNA products were further amplified with *P5CS* sense primer 5'-GGTGGATCCTTCTCGGGGGTTCA-3' and anti-sense primer 5'-GACGGGGCCAGAGGAGACGAGT-3' to detect transcription of the *P5CS* gene. An actin depolymerizing factor (sense primer: 5'-GCAGCATCA GGTATGGCAGT-3' and antisense primer: 5'-TTCCAT CAAGTTCCCCTCTT-3'), was amplified with all cDNA products to check their integrity. Negative control reactions without reverse transcriptase were performed for each sample to test DNA contamination (Sambrook *et al*. 1989).

For Western blot analysis, 1 g fresh leaves from transgenic and untransformed chickpea plants were homogenized in 5 cm^3 of extraction buffer (10 mM β-mercaptoethanol and 1 mM PMSF in 50 mM Tris/HCl, pH 7.8). Protein (10 µg), was separated by 10 % SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and immunoblotted using antibodies raised against purified *Vigna P5CS* protein.

Contents of proline, chlorophyll, sodium and relative electrical conductivity measurements: Two-week-old transgenic and wild-type (WT) chickpea seedlings were grown in hydroponic tanks in the greenhouse. A diluted solution of MS salts (1/8) supplemented with different concentrations of NaCl was applied on alternate days. The concentrations of NaCl were increased stepwise by 50 mM every 4 d. The effect of salt-stress on plant growth was monitored 15 d after reaching the final concentration. Young, old leaves and seeds were collected from each line and thoroughly rinsed in deionized water. The fresh mass of each sample was determined. Samples were dried at 65 °C for 24 h, and

the dry mass of each sample was measured. The dry material was digested in concentrated HNO_3 overnight. Samples then were picked in 2 M HCl and analyzed for Na^+ content using simultaneous inductively coupled argon-plasma emission spectrometry (ICP trace analyzer, *Labtam*, Braeside, Australia).

Leaf tissue (1 g) was quickly frozen and ground in liquid nitrogen. Then the tissue was extracted with 10 % (m/v) sulfosalicylic acid and after centrifugation (10 000 *g*), the supernatant was used for determining proline content according to Bates *et al*. (1973).

Leaf total chlorophyll content was monitored in acetone extract by spectrophotometer (*Shimadzu UV-160*, Tokyo, Japan) according to Arnon *et al*. (1974).

Membrane permeability was determined as relative electrical conductivity (REC) measured by conductivity bridge (*Elico*, Hyderabad, India) according to Leopold and Willing (1984).

Data were subjected to analysis of variance (*ANOVA*). Experiments were repeated twice, each time with 3 replicates.

Results and discussion

A total of 680 explants were used for transformation. Explants co-cultivated with *Agrobacterium* were grown on regeneration medium containing MS salts with B5 vitamin supplemented with 2.0 mg dm^{-3} N^6-benzyl-aminopurine + 0.2 mg dm^{-3} indole-3-butyric acid without any antibiotics for three days. They were then transferred into the same regeneration medium containing 250 mg dm^{-3} cefotaxim and 60 mg dm^{-3} hygromycin for shoot initiation (Fig. 1*B*) and proliferation (Fig. 1*C*). Untransformed shoots in the selection medium turned necrotic and died subsequently. Dwarf shoots were subcultured onto MS medium fortified with 1.0 mg dm^{-3} gibberellic acid plus 60 mg dm^{-3} hygromycin (Fig. 1*D*). Elongated shoots were transferred into 1/2 MS medium containing 0.5 mg dm^{-3} phenyl acetic acid along with 40 mg dm^{-3} hygromycin for rooting (Fig. 1*E*). Independent putative transgenic plants were transferred to pots (Fig. 1*F*).

GUS analysis was carried out with different parts of putative transgenic plants. The tissue was examined under stereomicroscope for blue colour in shoot (Fig. 1*G*), cotyledon, mid rib and vein regions of the leaf (not shown). PCR was performed for the presence of *P5CS* gene in the five selected T0 lines (1, 8, 16, 24, 35) and T1 progeny of five lines (1- 6, 8-14, 16-4, 24-20, 35-28) using purified genomic DNA as template. PCR analysis of chickpea plants using primers specific to *P5CS* gene showed the presence of 887 bp amplicon thus proving the transformed nature of the plants. Southern blot analysis was carried out in all PCR positive lines (Fig. 1*H*) using genomic DNA isolated from T0 and T1 plants. DNA was digested using Hind III and a hybridization signal of expected size of about 0.8 kb was detected. One to two copies of the transgene was observed in the selected five transgenic lines. RT-PCR analysis was carried out for all Southern positive lines of chickpea using specific internal primers. The cDNA obtained from reverse transcription of RNA extracted from Southern blot positive plants was used as a template. Transgenics showed high *P5CS* expression when compared to wild type (Fig. 1*I*). Plants expressing high levels of *P5CS* transcript also accumulated high contents of P5CS protein as detected by Western blotting using *P5CS* polyclonal antibodies. A protein band of 72 kDa was detected in transgenic lines while no signal was noticed in control plants (Fig. 1*J*). These results confirmed that *Vigna P5CS* is expressed in transgenic chickpea lines.

To examine the basic mechanism resulting in salt-tolerant phenotypes of *Vigna P5CS* overexpressing lines, Na^+ contents in leaves and seeds of T1 plants growing in the presence and absence of NaCl were measured. Approximately, five times more Na^+ was accumulated in old leaves of transgenic plants growing in the presence of 250 mM NaCl as compared to plants grown without NaCl, a significant difference was observed also in the young leaves and T1 seeds (Table 1). This finding suggests that older leaves seem to function as ion sinks, thus keeping the young leaves and seeds essentially free from the additional Na^+. It was reported that Na^+ is always higher in plant leaves (xylem fed) than in their fruits or seeds (phloem fed), thereby indicating that plants maintain lower Na^+ concentrations in the seeds by controlling the loading of Na^+ (Garg *et al*. 2002, Singla-Pareek *et al*. 2003).

Before NaCl treatment, contents of proline in roots and leaves of transgenic plants were 10-folds higher than in the wild type (WT). After NaCl treatment, proline contents increased in both controls and transgenics (Table 2). A comparison of proline content between transgenic and WT plants at 150 mM NaCl was not possible because no WT plant survived. The response of plants to salt stress consists of a coordinated function of several biochemical pathways. Therefore salt tolerance is based on complex multigenic traits that are difficult to establish in crops. Several genes such as barley *HVA1* (Xu *et al*. 1996), *Brassica GlyI* (Veena Reddy *et al.* 1999), rice *CDPK* (Saijo *et al.* 2000), alfalfa *Alfin1* (Winicov 2000) and tobacco *NPK1* (Kovtun *et al.* 2000) have been ectopically expressed in transgenic plants to enhance their stress tolerance. Several reports suggest that transfer of a single gene can also improve stress tolerance to some extent in plants (Kasuga *et al.* 1999, Saijo *et al.* 2000, Zhang *et al.* 2001, Ermawati *et al.* 2009).

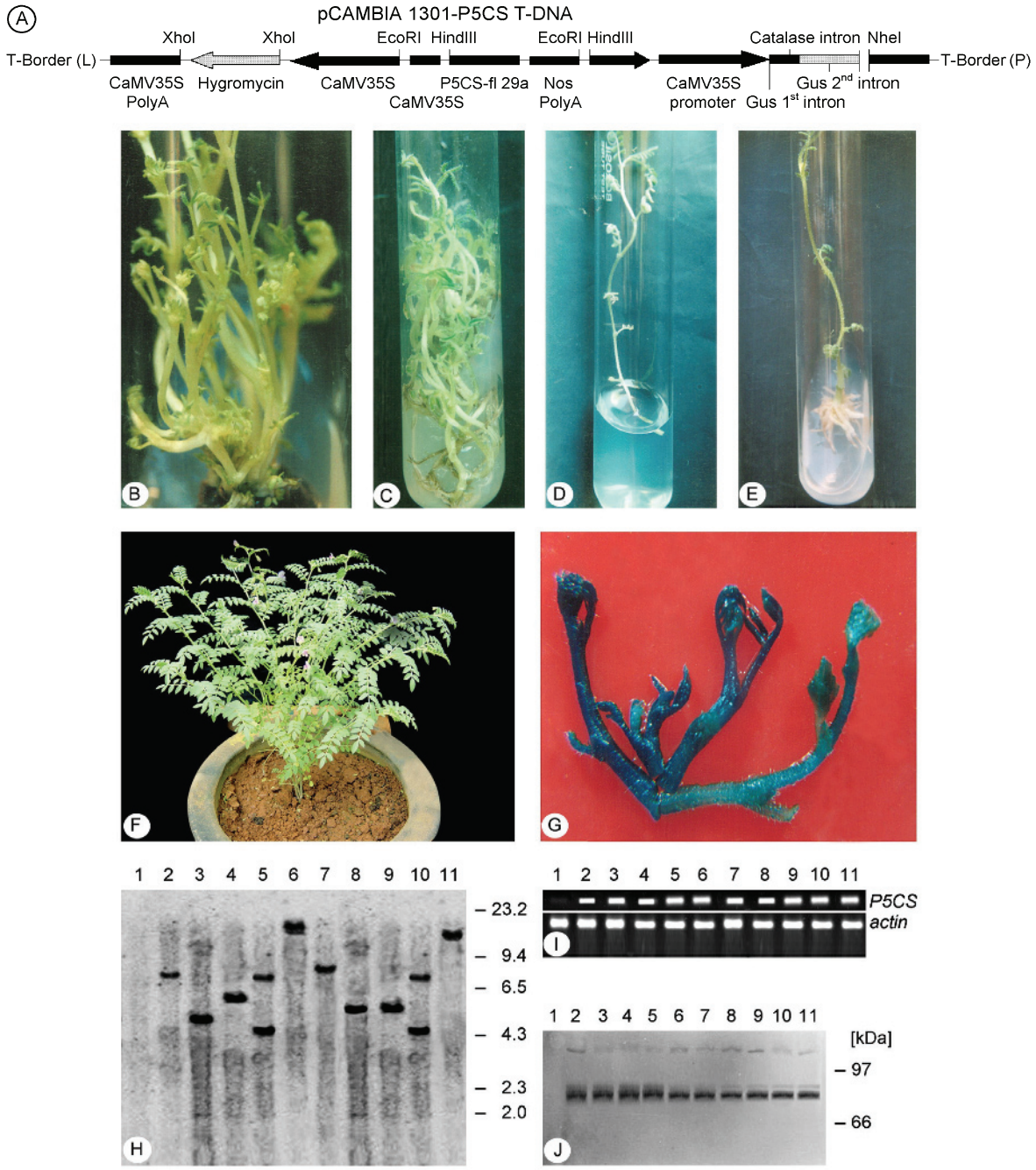

Fig. 1. Transgenic plants obtained from *Agrobactrium* mediated transformation with *Vigna P5CS*. *A* - Construction of the T-DNA region of the binary plasmid pCAMBIA 1301 that contains *Vigna aconitifolia P5CS* cDNA, *hptII* for resistance to hygromycin sulphate and *GUS* reporter gene, encoding β-glucuronidase. *B* - Initiation of putative transgenic chickpea plantlets cultured on selection medium containing 2.0 mg dm^{-3} BAP + 0.2 mg dm^{-3} IBA + 60 mg dm^{-3} hygromycin + 250 mg dm^{-3} cefotaxime, after 9 d of inoculation. *C* - Shoot proliferation of putative transgenic chickpea plantlets on selection medium containing 2.0 mg dm^{-3} BAP + 0.2 mg dm^{-3} IBA + 60 mg^{-1} hygromycin + 250 mg dm^{-3} cefotaxime after 17 d of inoculation. *D* - Shoot elongation of putative transgenic chickpea plantlets on selection medium containing 1.0 mg dm^{-3} GA_3 + 60 mg dm^{-3} hygromycin+ 250 mg dm^{-3} cefotaxime after 30 d of inoculation. *E* - Rooting of putative transgenic chickpea plantlets on selection medium containing ½ MS medium, 0.5 mg dm^{-3} phenylacetic acid + 40 mg dm^{-3} hygromycin after 45 d of inoculation. *F* - Putative transgenic chickpea plant in pot. *G* - GUS assay in shoot. *H* - Southern blot analysis of genomic DNA from transgenic plants of T0 and T1 generation after digestion by Hind III. The blot was hybridized with ^{32}P-labeled 0.8 kb *Vigna P5CS* cDNA. *I* - RT-PCR screening for *Vigna P5CS* transcription in T0 and T1 progenies. *J* - Western blot analysis of total soluble proteins from chickpea plants. *Line* 1 - non-transgenic plant, *lines* 2 to 6 - transgenic T0 plants; *lines* 7 to 11 - transgenic T1 plants.

Table 1. Total Na^+ content [μg g^{-1}(d.m.)] of young, mature leaves and seeds of wild type (WT) and T1 transgenic lines growing in water, 100, and 250 mM NaCl (means ± SE, n = 3).

T1 lines	Young leaf				Mature leaf				Seed			
	WT		T1		WT		T1		WT		T1	
	water	100 mM	100 mM	250 mM	water	100 mM	100 mM	250 mM	water	100 mM	100 mM	250 mM
1-6	1180±16	1190±15	1230±12	1200±19	1190±18	1900±16	3125±14	5521±35	615±12	620±11	630±14	654±13
8-14	1225±14	1245±14	1256±10	1290±22	1245±19	1950±19	2950±17	5612±38	610±16	615±13	665±12	635±18
16-4	1245±13	1310±10	1289±14	1345±23	1352±16	2089±14	3210±16	5635±32	612±12	628±15	651±15	652±10
24-20	1254±19	1260±12	1324±15	1294±24	1349±17	2045±13	3100±20	5596±30	620±11	622±12	630±12	621±12
35-28	1275±14	1310±11	1378±13	1350±21	1395±16	1930±10	3280±19	5624±28	635±14	642±18	645±14	686±15

Table 2. Proline content [pmol g^{-1}(d.m.)] in roots and shoots of WT and five T1 transgenic chickpea plants at various NaCl concentrations (means ± SE, n = 3, ns - not survived).

NaCl [mM]	WT		Transgenic plants									
			1-6		8-14		16-4		24-20		35-28	
	roots	leaves	roots	leaves	roots	leaves	roots	leaves	roots	leaves	roots	leaves
0	165±2	150±5	1689±5	1402±35	1741±2	1410±5	1785±6	1521±5	1658±6	1451±6	1796±7	1451±3
10	1352±12	1100±6	2945±12	2751±11	3014±4	2841±8	3114±9	2910±11	2912±13	2886±12	3132±14	2886±12
50	1645±13	1452±13	3958±14	3685±14	3895±11	3710±12	4012±11	3790±13	4042±16	3825±14	4189±17	3825±15
100	2256±21	1952±18	7125±19	6985±19	7190±14	6862±14	7215±18	6713±19	7250±19	6868±18	7289±15	6778±15
150	ns	ns	8521±21	7856±24	8589±15	7985±16	8624±21	7915±18	8654±21	8145±16	8695±19	8145±19
200	ns	ns	11145±25	9562±22	11195±22	9645±18	11252±19	9445±21	11296±22	9698±20	11385±23	9578±23
250	ns	ns	13945±28	11326±23	14019±24	12445±21	13098±23	12395±23	14185±20	12454±22	13245±24	11354±21

Table 3. Electrolyte leakage (EL) [%] and total chlorophyll (Chl) content [mg g^{-1}(f.m.)] in roots and shoots, respectively, of WT and five T1 transgenic chickpea plants at various NaCl concentrations (means ± SE, n = 3, ns - not survived).

NaCl [mM]	WT		Transgenic plants									
			1-6		8-14		16-4		24-20		35-28	
	Chl	EL	Chl	EL	Chl	EL	Chl	EL	Chl	EL	Chl	EL
0	1.86±0.10	0	2.10±0.11	0	2.40±0.15	0	2.20±0.18	0	2.35±0.16	0	2.30±0.09	0
10	1.64±0.10	20	1.99±0.13	4	2.31±0.13	3	2.10±0.16	6	2.08±0.14	5	2.10±0.12	4
50	1.35±0.15	40	1.88±0.14	15	2.25±0-16	14	1.90±0.14	11	1.80±0.19	13	2.00±0.14	9
100	1.11±0.12	90	1.65±0.1	18	1.95±0.14	20	1.80±0.13	16	1.65±0.15	16	1.75±0.13	16
150	ns	ns	1.32±0.13	25	1.61±0.12	26	1.35±0.12	23	1.45±0.14	21	1.52±0.12	19
200	ns	ns	1.12±0.12	30	1.45±0.15	32	1.32±0.13	29	1.31±0.12	26	1.25±0.13	23
250	ns	ns	0.89±0.10	38	1.10±0.19	36	1.05±0.14	34	1.20±0.13	32	0.95±0.16	34

Table 4. Comparison of various growth parameters of WT and T1 transgenic chickpea plants grown at various NaCl concentrations (means ± SE, n = 3, ns - not survived).

NaCl	Shoot dry mass [g]						Root dry mass [g]						Number of seeds [plant^{-1}]						Seed mass [g plant^{-1}]					
[mM]	WT	1-6	8-14	16-4	24-20	35-28	WT	1-6	8-14	16-4	24-20	35-28	WT	1-6	8-14	16-4	24-20	35-28	WT	1-6	8-14	16-4	24-20	35-28
0	10	13	12	13	13	14	2.1	3.0	3.2	3.0	3.5	2.6	40	46	46	43	43	46	7	8.4	7.8	7.3	7.9	7.9
10	34	14	12	12	13	15	2.2	3.2	3.1	3.2	3.2	2.8	42	45	43	44	42	47	5	7.2	7.9	7.4	7.6	7.7
50	24	12	13	12	14	15	1.6	2.8	3.3	3.4	2.9	2.6	30	44	45	45	44	48	4	6.5	6.8	7.5	7.1	7.4
100	7	12	12	12	12	14	1.0	2.5	2.8	2.9	2.6	2.5	10	45	44	42	41	46	2	7.5	6.6	6.9	6.9	7.1
150	ns	11	11	12	12	14	ns	2.4	2.6	2.8	2.8	2.4	ns	42	43	39	42	45	ns	7.2	6.7	6.6	6.7	7.3
200	ns	11	11	11	12	13	ns	2.2	2.4	2.3	2.5	2.3	ns	44	42	41	40	43	ns	6.9	6.8	6.7	6.5	6.4
250	ns	11	10.5	11	11	12	ns	2.1	2.2	2.0	2.4	2.2	ns	43	42	40	39	45	ns	6.8	6.7	6.5	6.4	6.8

Fifteen days after subjecting the plants to different NaCl concentrations, leaf total chlorophyll content was monitored. The data showed significant differences between T1 transgenic lines and untransformed controls. Compared to untransformed plants, transgenics showed an increase in chlorophyll content revealing protection of chlorophyll in transgenics under NaCl stress (Table 3). However, there existed variation in chlorophyll content among independent transgenic lines. The electrical conductivity of the cell leachate indicates the extent of damage caused to the cell membrane during salt stress. With increasing NaCl concentration from 150 to 250 mM, transgenic lines showed 16 to 38 % damage to cell membrane, while untransformed controls showed 90 % damage at 100 mM (Table 3).

Under salt stress, WT plants displayed progressive chlorosis, reduced leaf size and a reduction in growth and died at 150 mM NaCl. In transgenics, inhibition of growth increased progressively with increasing NaCl concentrations (Table 4) but they survived even at 250 mM NaCl. Although transgenic seedlings that were exposed to 250 mM NaCl stress showed slower growth rate, the plants reached maturity, flowered and set seeds. However, flowering time in NaCl-stressed plants was delayed by a week when compared to plants grown without stress, but there was no change in the flower morphology and seed yield (Table 4).

Chickpea plants over expressing *Vigna P5CS* cDNA showed increased tolerance to salt stress as indicated by the presence of higher chlorophyll content and accumulation of proline in the leaf and root of salt-stressed plants. These data suggest that tolerance in transgenics is conferred not only by over-expression of *P5CS* under CaMV35S promoter but also by regulating the protein turnover under salt stress. It was observed that a single copy of *P5CS* in transgenic lines (16-4, 35-28) also exhibited salt tolerance, clearly indicating that position of integration or copy number may not influence the expression pattern under stress. This observation also indicates that there must be a minimum threshold of overexpressed protein above the untransformed levels that is sufficient to confer tolerance.

In order to investigate the inheritance of salt tolerance, primary transgenic (T0) chickpea plants were self-pollinated and the T1 seeds obtained were screened for hygromycin resistance, examined for the presence of *Vigna P5CS* cDNA by PCR and tested for salt tolerance. The progenies of all the 5 independent lines exhibited salt tolerance at 250 mM NaCl. The segregation of salt tolerance among progenies was in the Mendelian ratio of 3:1 (tolerant : sensitive), as tested by χ^2 analysis ($\chi^2 = 0.078$, $P < 1$ %). These results demonstrate a marked, heritable enhancement of salt tolerance of chickpea expressing *Vigna P5CS*. Thus, it might be possible to increase salt tolerance by genetic manipulation of proline biosynthetic pathway genes that allow plants to accumulate more proline under stress.

In conclusion, expression of *P5CS* transgene resulted in the overproduction of *P5CS* enzyme in transgenic chickpea plants as well as an increase in proline accumulation. This is consistent with recent reports indicating that the accumulation of osmoprotectants may increase tolerance to osmotic stress and sodium toxicity. Thus, overproduction of proline makes the transgenic plants suitable for agricultural use in saline soils.

References

Arnon, D.I., McSwain, B.D, Tsujimoto, H.Y., Wada, K.: Photochemical activity and components of membrane preparation from blue-green algae. I. Coexistence of two photosystems in relation to chlorophyll a and removal of phycocyanin. - Biochem. Biophys. Acta. **357**: 231-245, 1974.

Bates, L.S., Waldren, R.P., Teeare, I.D.: Rapid determination of free Pro for water-stress studies. - Plant Soil **39**: 205-207, 1973.

Delauney, A.J.,Verma, D.P.S.: Proline biosynthesis and osmoregulation in plants.- Plant J. **4**: 215-223, 1993.

Doyle, J.J., Doyle, J.L.: Isolation of plant DNA from plant fresh tissue. - BRL Focus. **12**: 13-14, 1989.

Dua, R.P.: Differential response of chickpea (*Cicer arietinum*) genotypes to salinity. - J. agr. Sci. **119**: 367-371, 1992.

Dua, R.P., Sharma, P.C.: Salinity tolerance of Kabuli and Desi chickpea genotypes. - Int. Chickpea/Pigeonpea News Lett. **2**: 19-22, 1995.

Ermawati, N., Liang, Y.S., Cha, J.Y., Shin, D., Jung, M.H., Lee, J.J., Lee, B.H., Han, C.D., Lee, K.H., Son, D: A new TIP homolog, *ShTIP*, from *Salicornia* shows a different involvement in salt stress compared to that of TIP from *Arabidopsis*. - Biol. Plant. **53**: 271-277, 2009.

Garg, A.K., Kim, J.K., Owens, T.G., Ranwala, A.P., Choi, Y.D., Kochian, L.V., Wu, R.J.: Trehalose accumulation in rice plants confers high tolerance levels to different abitoic stresses. - Proc. nat. Acad. Sci. USA **99**: 15898-15903, 2002.

Greenway, H., Munns, R.: Mechanisms of salt tolerance in non halophytes. - Annu. Rev. Plant Physiol. **31**: 149-190, 1980.

Han, K.H., Hwang, C.H.: Salt tolerance enhanced by transformation of a *P5CS* gene in carrot. - J. Plant Biotechnol. **l5**: 149-153, 2003.

Hu, C., Delauney, A., Verma, D.: A bifunctional enzyme (Δ^1- pyrroline-5-carboxylate synthetase) catalyzes the first two steps in proline biosynthesis in plants. - Proc. nat. Acad. Sci. USA **89**: 9354-9358, 1992.

Jefferson, R.A.: Assaying chimeric genes in plants the GUS gene fusion system. - Plant mol. Biol. Rep. **5**: 387-405, 1987.

Kasuga, M., Liu, Q., Miura, S.,Yamaguchi-Shinozaki, K., Shinozaki, K.: Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. - Nat. Biotechnol. **17**: 287-291, 1999.

Kavi Kishor, P.B., Hong, Z., Miao, G.H, Hu, C.A.A., Verma, D.P.S.: Over expression of Δ^1-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. - Plant Physiol. **108**: 1387-1394, 1995.

Kavi Kishor, P.B., Sangam, S., Amrutha, R.N, Sri Laxmi, P., Naidu Rao, K.R, Sreenath Rao, Reddy, K.J., Theriappan, P., Sreenivasulu, N.: Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: its implications in plant growth and abiotic stress tolerance. - Curr. Sci. **88**: 422-443, 2005.

Kiran Ghanti, S., Sujata, K.G., Srinath Rao, M.: The effect of phenyl acetic acid on shoot bud induction, elongation and rooting of chickpea. - Biol. Plant. **53**: 779-783, 2009.

Kovtun, Y., Chiu, W.L., Tena, G., Sheen, J.: Functional analysis of oxidative stress-activated MAPK cascade in plants. - Proc. nat. Acad. Sci. USA **97**: 2940-2945, 2000.

Lauter, D.J., Munns, D.N.: Salt sensitivity of chickpea during vegetative growth and at different humidities. - Aust. J. Plant Physiol. **14**: 171-180, 1987.

Leisinger, T.: Biosynthesis of proline. - In: Neidhart, F.C., Ingraham, J.L., Low, K.B., Magasanik, B., Schaechter, M., Umbarger, H.E. (ed.): *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology. Pp. 346-351. American Society for Microbiology, Washington 1987.

Leopold, A.C., Willing, R.P.: Evidence for toxicity effects of salt on membrane. - In: Staples, R.C., Tonnessen, G.H. (ed.): Salinity Tolerance in Plants. Strategies for Crop Improvement. Pp. 67-76. Wiley and Sons, New York 1984.

Murashige, T, Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Saijo, Y., Hata, S., Kyozuka, J., Shimamoto, K., Izui, K.: Over-expression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. - Plant J. **23**: 319-327, 2000.

Sambrook, J., Fritish, E.F, Maniatis, T.: Molecular Cloning: a Molecular Manual. 2nd Ed. - Cold Spring Harbor Laboratory Press. Cold Spring Harbor - New York 1989.

Sanchayita, K., Tony, M.J., Pritilata, N., Sen, S.K.: Efficient transgenic plant regeneration through *Agrobacterium-mediated* transformation of chickpea (*Cicer arietinum* L.). - Plant Cell Rep. **16**: 32-37, 1996.

Saradhi, A., Saradhi, P.P.: Proline accumulation under heavy metal stress. - J. Plant Physiol. **138**: 554-558, 1991.

Sawahel, W.A., Hassan, A.H.: Generation of transgenic wheat plants producing high levels of the osmoprotectant proline. - Biotechnol. Lett. **24**: 721-725, 2002.

Sayari, A.H., Bouzid, R.G., Bidani, A., Jaoua, L., Savoure, A., Jaoua, S.: Over expression of Δ^1-pyrroline-5-carboxylate synthetase increases proline production and confers salt tolerance in transgenic potato plants. - Plant Sci. **169**: 746-752, 2005.

Schobert, B., Tschesche, H.: Unusual solution properties of proline and its interaction with proteins. - Biochim. Biophys. Acta **541**: 270-277, 1978.

Singla-Pareek, S.L., Reddy, M.K., Sopory, S.K.: Genetic engineering of the glyoxalase pathway in tobacco leads to enhanced salinity tolerance. - Proc. nat. Acad. Sci. USA. **100**: 14672-14677, 2003.

Smirnoff, N., Cumbes, Q.J.: Hydroxyl radical scavenging activity of compatible solutes. - Phytochemistry **28**: 1057-1060, 1989.

Veena Reddy, V.S., Sopory, S.K.: Glyoxalase I from (*Brassica juncea)* molecular cloning, regulation, and its over expression confer tolerance in transgenic tobacco under stress. - Plant J. **17**: 385-395, 1999.

Venekamp, J.H., Lampe, J.E.M., Koot, J.T.M.: Organic acids as sources of drought induced proline synthesis in field bean plants (*Vicia faba* L.*)* - J. Plant Physiol. **133**: 654-659, 1989.

Verwoerd, T.H.C., Dekker, B.M.M., Hoekema, A.: A small-scale procedure for the rapid isolation of plant RNAs. - Nucl. Acids Res. **6**: 23-62, 1989.

Winicov, I.: Alfin1 transcription factor over expression enhances plant root growth under normal and saline conditions and improves salt tolerance in alfalfa. - Planta **210**: 416-422, 2000.

Xu, D., Duan, X., Wang, B., Hong, B., Ho, T.H.D., Wu, R.: Expression of a late embryogenesis abundant protein gene, *HVA1*, from barley confers tolerance to water deficit and salt stress in transgenic rice.- Plant Physiol. **110**: 249-257,1996.

Zhang, H.K., Hodson, J.N., Williams, J.P., Blumwald, E.: Engineering salt-tolerant (*Brassica*) plants: characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium accumulation. - Proc. nat. Acad. Sci. USA **98**: 12832-12836, 2001.

Zhu, B., Su, J., Chang, M., Verma, D.P.S., Fan, Y.L., Wu, R.: Overexpression of a Δ^1-pyrroline-5-carboxylate synthetase gene and analysis of tolerance to water and salt-stress in transgenic rice. - Plant Sci. **139**: 41-48, 1998.