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ORIGINAL ARTICLE

Enhanced proline accumulation and salt stress tolerance of transgenic *indica* rice by over-expressing *P5CSF129A* gene

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Abstract Δ^1-pyrroline-5-carboxylate synthetase (P5CS) is a proline biosynthetic pathway enzyme and is known for conferring enhanced salt and drought stress in transgenics carrying this gene in a variety of plant species; however, the wild-type P5CS is subjected to feedback control. Therefore, in the present study, we used a mutagenized version of this osmoregulatory gene-*P5CSF129A*, which is not subjected to feedback control, for producing transgenic *indica* rice plants of cultivar Karjat-3 via *Agrobacterium tumefaciens*. We have used two types of explants for this purpose, namely mature embryo-derived callus and shoot apices. Various parameters for transformation were optimized including antibiotic concentration for selection, duration of cocultivation, addition of phenolic compound, and bacterial culture density. The resultant primary transgenic plants showed more enhanced proline accumulation than their non-transformed counterparts. This proline level was particularly enhanced in the transgenic plants of next generation (T_1) under 150 mM NaCl stress. The higher proline level shown by transgenic plants was associated with better biomass production and growth performance under salt stress and lower extent of lipid peroxidation, indicating that overproduction of proline may have a role in counteracting the negative effect of salt stress and higher maintenance of cellular integrity and basic physiological processes under stress.

Keywords *Indica* rice · Genetic transformation · Salt tolerance · *P5CSF129A* · Transgenic plants · Proline

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Introduction

Amongst various abiotic stresses, soil salinity is a major stress reducing the crop productivity globally to a great extent. Salinity is a serious problem in many coastal, arid and irrigated rice production systems and affects the crop production adversely (Kumar et al. 2009). However, despite the advances in the increase of plant productivity and resistance to a number of pests and diseases, improvement in salt tolerance of crop plants remains elusive, due to the fact that salinity affects almost every aspect of the physiology and biochemistry of plants. The yield of rice, especially Asian rice (*sativa*), is susceptible to salinity (Munns and Tester 2008). In India and especially in coastal rice fields of Maharashtra state, soil salinity is a major stress that reduces the rice productivity to a great extent.

Various mechanisms have been reported to be evolved by crop plants, particularly by rice in response to, and to

counteract, the consequences of salinity stress including accumulation of low molecular weight osmolytes such as proline and glycine betaine (Hmida-Sayari et al. 2005). Amongst these solutes, the accumulation, biosynthesis, transportation, and role of proline during salinity stress has been investigated thoroughly (reviewed by Kavi Kishor et al. 2005; Verbruggen and Hermans 2008). Rapid accumulation of free proline is a typical response to salt stress (Parida et al. 2008). When exposed to drought or a high salt content in soil, many plants have been observed to accumulate high amounts of proline, in some cases several times the sum of all other amino acids (Ali et al. 1999; Mansour 2000). Proline acts as an osmo-protectant, and plays an important role in osmotic balancing, protection of sub-cellular structures, enzymes and in increasing cellular osmolarity (turgor pressure) that provide the turgor necessary for cell expansion under stress conditions (Matysik et al. 2002; Sairam and Tyagi 2004). Proline is considered as the only osmolyte which has been shown to scavenge singlet oxygen, and free radicals including hydroxyl ions, and hence stabilize proteins, DNA, as well as membrane (Matysik et al. 2002). Proline is reported to reduce the enzyme denaturation caused due to heat, NaCl and other stresses. Proline also acts as a source of carbon, nitrogen and energy during, and recovery from, stresses (Kavi Kishor et al. 2005). Owing to these functions played by proline, higher proline accumulation is often related with the salt tolerance nature of plant species, and various researchers have reported higher proline accumulation in the salt-tolerant genotype than in their salt-sensitive counterparts including wheat (Sairam et al. 2005), mulberry (Kumar et al. 2003), green gram (Misra and Gupta 2005) and sorghum (Jogeswar et al. 2006).

In the recent past, manipulations of the genes involved in the biosynthesis of low molecular weight metabolites (osmolytes) including mannitol (Pujni et al. 2007), glycine betaine (Mohanty et al. 2002), and proline (Vendruscolo et al. 2007; Yamchi et al. 2007; Bhatnagar-Mathur et al. 2009) have resulted in enhanced tolerance to salt stress in transgenic plants. Amongst proline biosynthetic pathway genes, Δ^1-pyrroline-5-carboxylate synthetase (*P5CS*) seems to be heavily used and its over-expression in transgenics showed enhanced oxidative stress tolerance driven by drought and salt stresses. Several researchers have demonstrated that over-expression of *P5CS* genes increases proline production and confers salt tolerance in transgenics in a number of crop plants including rice (Anoop and Gupta 2003; Su and Wu 2004), wheat (Vendruscolo et al. 2007), potato (Hmida-Sayari et al. 2005) and tobacco (Yamchi et al. 2007). However, *P5CS*, being a rate-limiting enzyme in proline biosynthesis, is subjected to feedback inhibition by proline, and earlier reports suggested that proline accumulation in plants under stress might involve the loss of feedback regulation due to a conformational change in the *P5CS* protein (Hong et al. 2000). Therefore, Hong et al. (2000) removed the feedback inhibition by using site-directed mutagenesis to replace the *Phe* residue at position 129 in *P5CS* from *Vigna aconitifolia* with an *Ala* residue. The resulting mutated enzyme (*P5CSF129A*) was, therefore, no longer subject to feedback inhibition. Removal of this feedback inhibition resulted in two times more proline accumulation in *P5CSF129A* transgenics as compared to plants expressing wild-type *P5CS*, and this difference was further accentuated under 200 mM NaCl stress, and better protection of these plants from osmotic stress was observed (Hong et al. 2000). However, in spite of this fact, there are only a few reports where researchers have used this mutated version of *P5CS* gene (Hong et al. 2000; Molinari et al. 2004; Bhatnagar-Mathur et al. 2009).

The present study was undertaken with two aims, the first being to generate the transgenic *indica* rice plants over-expressing *P5CSF129A* gene and the second to evaluate their performance under NaCl stress. We are reporting herein an efficient and reproducible method for *Agrobacterium*-mediated transformation using callus as well as shoot apices as targeting materials for co-cultivation and recovery of transgenic rice cultivar (cv) Karjat-3 (KJT-3), a high yielding, early maturity cv. The resultant transgenics were evaluated for their growth performance, proline level and lipid peroxidation both with and without salt stress. This is the first report where transgenic *indica* rice plants have been produced using *P5CSF129A* gene.

Materials and methods

Plant transformation and selection of transformants

Agrobacterium tumefaciens strain LBA4404 harboring binary vector pCAMBIA 1301 carrying the mutagenized *V. aconotifolia P5CS cDNA* (*P5CSF129A*) under the control of *CaMV 35S* promoter was used for genetic transformation of *indica* rice cv KJT-3. In addition, the vector also contained the selectable marker (*hpt*II) and reporter (*uid*A) genes under the control of the same promoter. Bacteria were grown in 10 ml of liquid yeast extract mannitol (YEM) medium containing 50 mg l^{-1} kanamycin for 24 h (dark, 28°C) on a rotary shaker at 200*g*. *Agrobacteria* were then pelleted by centrifugation at 4,000*g* for 5 min followed by resuspension in MS liquid media (pH 5.8) supplemented with 100 µM acetosyringone (AS), and the cultures were allowed to attain an optical density (OD) of 0.6 at A_{600} nm.

Mature embryo-derived callus was produced as described earlier (Kumar et al. 2008). Briefly, the callus

induction medium (CIM) consisted of MS (Murashige and Skoog 1962) supplemented with 2 mg l^{-1} 2,4-D, 500 mg l^{-1} proline, 500 mg l^{-1} casein hydrolysate, 30 g l^{-1} sucrose, 7 g l^{-1} agar and pH 5.8. The cultures were kept in the dark for 4 weeks and then the obtained embryogenic-like compact hard callus was used for transformation. Callus pieces were immersed for 10–15 min in bacterial suspension with gentle shaking and were then dried with sterilized Whatman No. 1 filter papers followed by transfer onto cocultivation media consisting of CIM with 100 µM AS and incubated at 25°C in the dark for 3 days. After co-cultivation, calluses were rinsed thoroughly with sterile distilled water containing 250 mg l^{-1} cefotaxime, dried with sterile Whatman No. 1 filter paper and transferred onto CIM fortified with 250 mg l^{-1} cefotaxime and 20 mg l^{-1} hygromycin B for antibiotic selection and growth of hygromycin resistant calli. The cultures were maintained on the same media composition for 4 weeks and the calli were then transferred to shoot induction medium (SIM: MS plus 4 mg l^{-1} Kin plus 1.0 mg l^{-1} NAA) containing 20 g l^{-1} sorbitol, 250 mg l^{-1} cefotaxime and 20 mg l^{-1} hygromycin. The cultures were transferred to the same medium excluding sorbitol after 2 weeks. The microshoots obtained were transferred to ½ MS media supplemented with 250 mg l^{-1} cefotaxime and 20 mg l^{-1} hygromycin for rooting. Finally, the plantlets were transferred to plastic pots containing garden soil mixed with vermiculite and sand (1:1:1) for hardening, and subsequently transferred to green house conditions.

Three- or four-day-old shoot apices were isolated from in vitro raised plants. Roots were removed carefully and the shoot tip was completely cut out, leaving a shoot approximately 4–5 mm long with a thick basal portion. These shoot apexes were used as a targeting material for transformation and were immersed in the bacterial suspension for 5–10 min with gentle shaking. Inoculated tissues were then dried with sterilized Whatman No. 1 filter papers and transferred to co-cultivation media consisting of MS medium supplemented with 6 mg l^{-1} thidiazuron (TDZ) containing 100 µM AS and incubated for 3 days (24°C, dark) for co-cultivation. After co-cultivation, shoot apexes were rinsed thoroughly with 250 mg l^{-1} cefotaxime in sterile distilled water followed by drying with sterile Whatman No. 1 filter paper. The target tissues were then transferred onto MS medium fortified with 6 mg l^{-1} TDZ, 250 mg l^{-1} cefotaxime and 30 mg l^{-1} hygromycin. These cultures were maintained for 4 weeks and were then transferred to ½ MS media, containing 250 mg l^{-1} cefotaxime and 30 mg l^{-1} hygromycin, for rootingthe and were maintained for 2–3 weeks. Well-rooted plantlets were hardened off to green house conditions.

Histochemical GUS assay

Identification of transformed plant cells was done by detection of β-glucuronidase (GUS) activity with the substrate X-Gluc. The expression of GUS was performed by using a modified histochemical method of Jefferson et al. (1987). The callus tissues after two cycles of antibiotic selection were placed in GUS solution consisting of 1 mM X-Gluc (Sigma–Aldrich, USA), 100 mM Na-phosphate buffer solution (pH 7.0), 10 mM EDTA, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6 \cdot 3H_2O$ and 0.1% Triton X-100 and incubated in the dark overnight (12–18 h) at 37°C.

Molecular characterization of transformed plants

Genomic PCR was carried out using convergent primers complementary to *P5CSF129A* cDNA as follows: primers for the *P5CS* Gene (2.8 Kb): F: ACC ATA TGT GCT CTA AAG GCT ATT GC; R: GCG TCG ACG AAT TCC CGA TCT AGT AA. Total plant genomic DNA was isolated from young leaves of control and transgenic rice plants (T_0), using GeNei^{TM} Ultrapure Plant Genomic DNA Prep Kit (Bangalore Genei, India) by following the manufacturer's instructions and was used as template DNA for PCR analysis. The PCR reaction was performed using purified genomic DNA by following the standard method (Sambrook and Russell 2001) and the reaction was run in a thermocycler with the following conditions: initial denaturation at 94°C for 5 min, denaturation at 94°C for 1 min, annealing at 48°C for *P5CS* primers for 45 s and extension at 72°C for 2 min. These steps were repeated for 30 cycles followed by a final extension for 5 min at 72°C. Amplified DNA fragments (10 µl of post-amplification products) were loaded into 1.2% agarose gel, and gels were stored after staining with ethidium bromide. The image analysis of gels was done using the gel documentation system.

The integration of the *P5CSF129A* gene in transgenic plants (T_0) was confirmed by DNA gel blot hybridization analysis. For southern hybridization analysis, 15 µg of genomic DNA isolated from leaves of each PCR-positive primary transgenic plant along with non-transgenic plant was digested with *Eco*RI, and pCAMBIA-*P5CSF129A* plasmid digested with *Eco*RI restriction enzyme served as positive control. The samples were subjected to electrophoresis on 0.8% (w/v) agarose gel at 3 V cm^{-1} and transferred to nylon membrane (Hybond N^+; Amersham Biosciences) using standard protocols (Sambrook and Russell 2001). The membrane was UV cross-linked and probed with a 2.8-kb *P5CS* coding region labeled with [α-^{32}P] dCTP using Megaprime Labelling System (Amersham Biosciences), according to the manufacturer's instructions. Hybridization and washings were performed at 65°C before autoradiography with X-ray films (Kodak).

Molecular characterization was also carried out for confirmation of *P5CSF129A* gene in T_1 plants using PCR products obtained from DNA amplified by primers specific to *hptII* gene in the construct. For this purpose, seedlings which showed the sign of hygromycin resistance and for one seedling which started turning brown (hygromycin sensitive) after 5 days of inoculation were used for genomic DNA extraction, as described below to be used for PCR analysis. Two convergent primers complementary to the *hptII* gene were used for PCR amplification as F: GCT GGG GCG TCG GTT TCC ACT ATC CG and R: CGC ATA ACA GCG CTC ATT GAC TGG AG leading to a 340-bp product. The PCR amplification was done using *P5CS* gene-specific primers with the annealing temperature of 55°C.

Proline content in T_0 plants

Free proline content was estimated from leaves of all five PCR positive T_0 plants independently along with the in vitro raised non-transformed (NT) plant of KJT-3 by following Bates et al. (1973). The proline content was estimated from fresh leaves of each plant independently and converted to dry matter, on the basis of water content of the sample and proline content was expressed as μg g^{-1} dry weight (DW).

Segregation analysis of progeny (T_1) plants

The inheritance of transgene in KJT-3 progenies was tested using hygromycin selection. Segregation analysis was done as described by Mohanty et al. (2002). T_1 seeds, obtained from PCR positive T_0 plants were used for selection. Twenty dehusked sterilised seeds from each transgenic line with one seed per test tube were inoculated for germination on MS medium for 2 days; after that the cultures were transferred to MS medium supplemented with hygromycin. The seedlings obtained from seeds of each of five transgenic lines were maintained independently. The germinated seedlings were allowed to grow for 7 days under hygromycin selection, and the survival rate was assayed on the 7th day after inoculation. In addition, 10 dehusked, surface sterilised seeds of non-transgenic control plants, with 1 seed per test tube, were also inoculated on MS medium for 1 week.

Salt stress tolerance evaluation of transgenic (T_1) plants

Hygromycin resistant transgenic seedlings along with 10 non-transgenic in vitro germinated seedlings were hardened to soil conditions, with 1 plant per magenta box and assayed for their responses under NaCl stress. The hardened plants were irrigated with 50 ml of Yoshida's nutrient solution as per their evapotranspiration demand for the first 2 weeks in greenhouse conditions. After this, the plants were irrigated every other day with 50 ml of Yoshida's nutrient solution containing 150 mM NaCl for 7 days. The plant growth as well as biochemical analyses including proline content and lipid peroxidation was done on the 7th day after NaCl treatment, both in NT as well as transformed (T_1) events.

Estimation of proline content

Free proline was estimated from fresh leaves of each transgenic (T_1) and non-transgenic plant independently by following Bates et al. (1973).

Estimation of lipid peroxidation

The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) contents as given by Heath and Packer (1968). Fresh samples of shoots and roots (500 mg each) were homogenised separately in 10 ml of 0.1% trichloro acetic acid (TCA). The homogenate was centrifuged at 15,000*g* for 5 min, then 2 ml aliquot of supernatant was taken and 4 ml of 0.5% thiobarbituric acid (TBA) in 20% TCA was added into it. The mixture was heated at 95°C for 30 min, and then quickly cooled in an ice bath. After centrifugation at 10,000*g* for 10 min to remove suspended turbidity, the absorbance of supernatant was recorded at 532 nm absorbance. The value for non-specific absorption at 600 nm was subtracted. The MDA content was calculated using its absorption coefficient of 155 $mmol^{-1}$ cm^{-1}.

Statistical analyses

The physiological data were subjected to Duncan's multiple range test (DMRT) at $P \leq 0.05$ for the comparison of different transgenic events. All the statistical analyses were done by using MSTATC statistical software package.

Results

Plant transformation and selection of transformants

Mature embryo-derived callus produced on CIM (Fig. 1a) and multiple shoots regenerated from shoot apex region (Fig. 1e) were successfully used as targeting material for co-cultivation with *A. tumefaciens.* Various parameters were optimized for efficient *Agrobacterium*-mediated transformation in *indica* rice cv KJT-3 using *P5CSF129A* gene under the control of CaMV35S. An amount of 20 mg l^{-1} of hygromycin B was found suitable for selecting the putative transformants obtained through

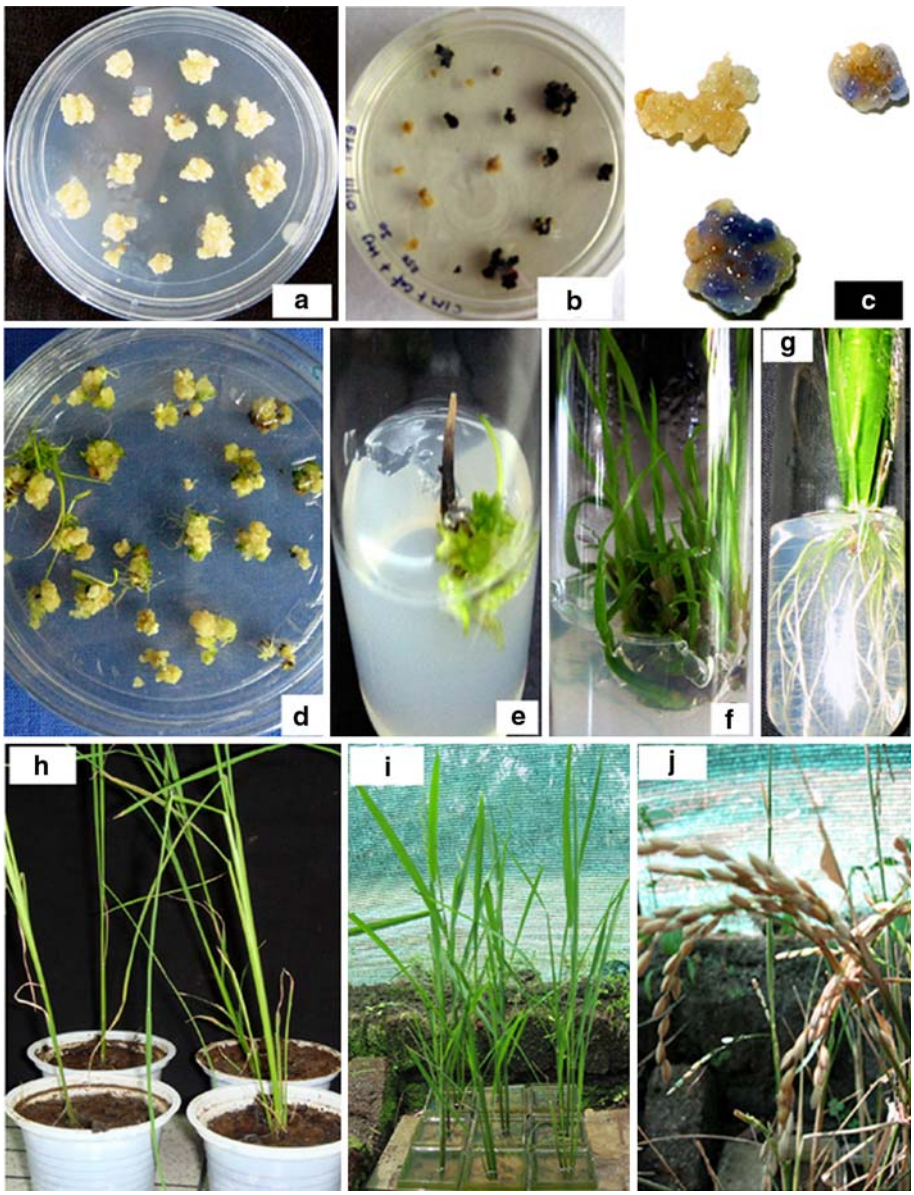

Fig. 1 Different stages of *Agrobacterium*-mediated transformation of *indica* rice cv KJT-3 using mature embryo derived callus: **a** embryogenic-like callus placed on co-cultivation media; **b** selection of calli after cocultivation on CIM containing 20 mg l^{-1} hygromycin and 250 mg l^{-1} cefotaxime; **c** GUS expression in callus tissues (indigenous *blue color*) after first cycle of antibiotic selection; **d** indirect shoot regeneration from antibiotic resistant putative transformant calli on regeneration media in presence of 20 mg l^{-1} hygromycin and 250 mg l^{-1} cefotaxime (albino plants can be seen); **e** shoot initiation from shoot apex on antibiotic selection medium; **f** multiple shoot regeneration on antibiotic selection medium containing 30 mg l^{-1} hygromycin and 250 mg l^{-1} cefotaxime; **g** rooting of putative transformed shootlet on ½ MS; **h** acclimatized putative transformants growing in plastic cups in tissue culture laboratory; **i** hardened putative transformed plants of T_0 growing in the greenhouse at the Botanical Garden, Department of Botany, University of Pune; **j** primary transformants (T_0) at the grain-filling stage in the greenhouse

callus, while for shoot apexes, 30 mg l^{-1} concentration was used. Similarly, the optimal concentration of *Agrobacterium* culture for transformation of rice tissues was found to be at 0.6 OD. AS proved to be essential for transformation of *indica* rice cv KJT-3 for both the targeting tissues used in this investigation and 100 µM was found to be optimal concentration, as suggested by the GUS activities in calluses and shoots following *Agrobacterium*-mediated transformation with *pCAMBIA1301-P5CSF129A*. In addition, it was observed that the addition of AS to pre-culture medium (bacterial suspension 1 h prior to infection) is important for efficient gene transfer.

Out of 300 callus pieces used for transformation, 20 calli were tested for the expression of GUS reporter gene (*uidA*) after two cycles of selection on CIM containing 250 mg l^{-1} cefotaxime and 20 mg l^{-1} hygromycin B. About 40% calli showed GUS expression in the tissues transformed by *Agrobacterium* indicating the integration and expression of transgene into the host genome (Fig. 1c). From the remaining 280 calli, 40 callus pieces were found to be hygromycin resistant (hyg^R) (Fig. 1b), and following the transfer of these calluses, 30 could regenerate into shoots (Fig. 1d). Five shoots were albino and were transferred to rooting medium separately; however, even though they rooted successfully, they died during acclimatization process. The normal shoots (excluding albino) grown on hygromycin selection medium were transferred to ½ MS for rooting. Well-developed roots were observed in 20 out of 25 shootlets (Fig. 1g). The shoots that developed roots were grown for 1 week and subcultured again for another

Table 1 Frequency of shoot regeneration, rooting and survival of plants after *Agrobacterium* mediated gene transfer

No. of calli infected	No. of hyg^R calli after two selection cycles on CIM	No. of regenerated shoots on RM-III	No. of shoots developed rooting	No. of regenerated plants transferred to pots for hardening	No. of plants surviving after hardening and transfer to greenhouse	Transformation efficiency (%)
300	40	30[a]	20	12	3	1.07

Out of 300 calli, 20 infected calli were separately used for checking the GUS activity; therefore transformation efficiency (%) is calculated on the basis of total 280 calluses used. All the media (columns 2, 3, 4) contained 250 mg l^{-1} cefotaxime and 20 mg l^{-1} hygromycin B

hyg^R Hygromycin resistant

[a] Out of 30 regenerated shoots, 5 were albino

Table 2 Percent frequency of shoot regeneration, rooting and survival of plants after *Agrobacterium* mediated gene transfer

No. of calli infected	No. of hyg^R apical meristems	No. of multiple shoots regenerated	No. of shoots developed rooting	No. of regenerated Plants transferred to pots	No. of plants survived after hardening	Transformation efficiency (%)
300	56	200[a]	85	15	5	1.78[a]

Out of 300 explants, 20 were separately used for checking the GUS activity; therefore, transformation efficiency (%) is calculated on the basis of total 280 calluses used. All the media (columns 2, 3, 4) contained 250 mg l^{-1} cefotaxime and 30 mg l^{-1} hygromycin B

hyg^R Hygromycin resistant

[a] Around 20 shoots were albino

1 week period on the same medium for good root differentiation and growth before transferring to the pots. Finally, 12 putative transgenic plants were transferred to plastic pots in laboratory conditions (Fig. 1h) for gradual acclimatization, and in the end only 5 plants were able to become successfully acclimatized to the greenhouse conditions (Fig. 1i), and out of these 5 plants, only 3 survived to the flowering and harvesting stages and bore seeds (Fig. 1j), with 1.07% transformation efficiency (Table 1).

Similarly, a total of 300 shoot apices were used (Fig. 1e), out of which 20 were tested for GUS assay after two cycles of selection on selection medium containing MS plus 6 mg l^{-1} TDZ, 250 mg l^{-1} cefotaxime and 30 mg l^{-1} hygromycin B. About 42% calluses showed GUS expression in the tissues transformed by *Agrobacterium* indicating the integration and expression of transgene into the host genome. From the remaining 280 tissues, 56 survived and regenerated into multiple shoots (3–12 shoots per explant; Fig. 1f). Eighty-five hyg^R shoots were transferred to ½ MS medium for rooting. Finally, only 15 plants survived and showed well-developed roots. These plantlets gradually acclimatized to greenhouse conditions, where five plants grown to maturity with 1.78% transformation efficiency (Table 2). All the five plants were found to be fertile (Fig. 1j) and seeds were collected along with seeds raised from tissue culture-grown non-transformed (NT) plants.

Molecular characterisation of transformants

The PCR analysis of T_0 plants carried out using gene-specific primers revealed that out of eight T_0 plants (three and five from callus and apical shoots mediated transformants, respectively) tested, only five samples—two from the former and three from the latter—were PCR positive while the remaining were negative (Fig. 2a).

Southern analysis revealed that *indica* rice cv KJT-3 genomic DNA digested with *Eco*RI, followed by hybridization with the *P5CS* probe, gave the expected 3.8-kb fragment in each transgenic line analyzed. As expected, no hybridization signal was detected in the non-transformed control plants of *indica* rice cv KJT-3 (Fig. 2b). These results clearly indicated the integration of *Vigna P5CSF129A* gene in *Oryza sativa* L. subsp. *indica* cv KJT-3.

Differential proline content in putative T_0 and non-transformed (NT) plants

Proline content was measured from tissue culture grown NT as well as from T_0 plants 15 days after their transfer to the greenhouse. A significant difference in terms of proline content was seen between the NT- and T_0-transformed KJT-3 plants; however, with variations amongst the transgenics (Fig. 3). In general, all the T_0 lines showed higher proline content than NT plant; however, no considerable difference was evident in terms of proline level between the transgenics produced via callus and apical shoot meristems. Amongst all the five transgenic plants, T_0 line 5 showed maximum proline content (4,320 μg g^{-1} DW), which is around four times more than non-transformed line (1,150 μg g^{-1} DW) followed by line 2 with 4,225 μg proline g^{-1} DW of leaf. These results confirmed

the functional expression of *P5CSF129A* gene into KJT-3 genome.

Segregation analysis of progeny plants (T_1)

Twenty seeds of each T_0 line were germinated and grown for 7 days on MS media containing hygromycin for selection of stable inheritance of *P5CS*-transgene to T_1 progenies. Both, Mendelian as well as non-Mendelian segregation ratios were obtained (Table 3). Most of the lines exhibited hygromycin resistance and sensitivity ratio as 3:1 (except line T_{1-3}).

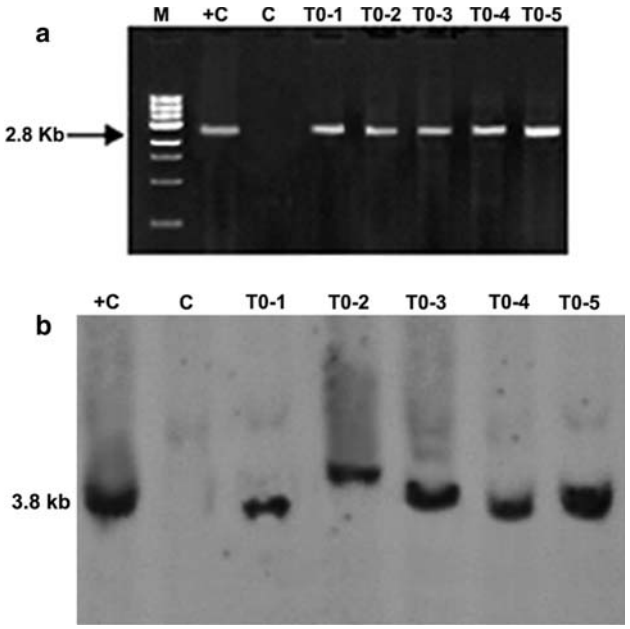

Fig. 2 Confirmation of the presence of *P5CSF129A* transgene in hygromycin resistant T_0 plants of *indica* rice cv KJT-3: **a** PCR amplification of *P5CSF129A* gene using gene specific primers in T_0 transformed plants, *lane C* non-transformed control plants, *lane +C* amplification of vector DNA, *lane M* 1 kb molecular weight marker and *lanes* T_{0-1} to T_{0-5} putative transformed T_0 lines; **b** Southern blot of *Eco*RI-digested genomic DNA obtained from T_0 plants, *lane C* non-transformed control plants, *lane +C* positive control, showing 3.8-kb gene insert obtained from plasmid DNA digested with *Eco*RI, separated and hybridized to a *P5CSF129A* probe and *lanes* T_{0-1} to T_{0-5} shows transformation events of primary transgenics (T_0)

Detection of *hptII* gene by PCR in T_1 plants

PCR amplification of *hptII* gene was also performed to confirm the stable establishment of the gene into the genome of KJT-3 cv. The PCR products, electrophoresed on agarose gel, clearly showed the stable insertion of the gene (Fig. 4). As expected, the DNA extracted from the leaf tissues of the seedlings, which showed signs of hygromycin sensitivity, did not show any gene insertion (lane 3 in Fig. 4).

Salt stress tolerance evaluation

Growth performance under salt stress

Striking differences were evidenced between non-transgenic and *P5CSF129A*-KJT-3 plants under 7 days of 150 mM NaCl-driven salt stress. The increase in plant height and biomass production was greatly reduced in non-transgenic plants; however, the magnitude of growth reduction was very low in transgenic lines (Table 4; Fig. 5). The growth of leaves was effected very badly in NT lines, and leaves started browning after 2 days of stress. On the other hand, the leaves of all the transgenic lines were comparably greener and healthier. On the 7th day of salt stress, all the transgenic lines showed better height under 150 mM NaCl stress condition, and out of these lines, T_{1-1} showed best performance followed by T_{1-5}, while T_{1-2} did not show much difference from the non-transgenic plants. These results are well supported by fresh weight (FW) and dry weight (DW) of transgenic plants under salt stress; amongst transgenic events, T_{1-1} showed highest FW and DW.

Proline assay under salt stress

The results of free proline content in non-transgenic and transgenic lines under saline conditions supported the

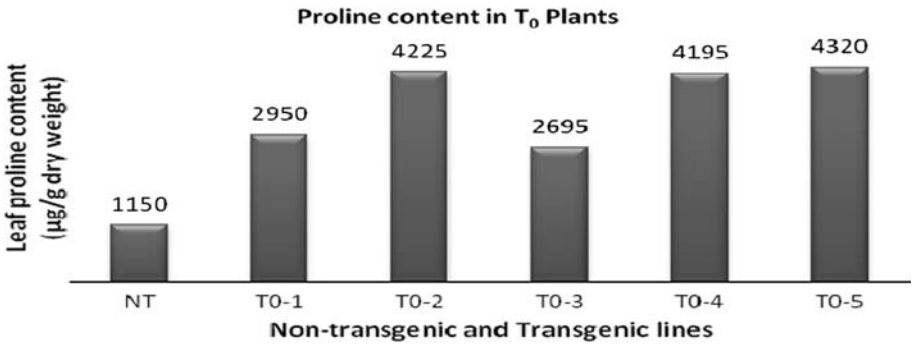

Fig. 3 Comparison between proline content in leaves of non-transgenic (NT) and T_0 transgenic line generated via *Agrobacterium*-mediated transformation of *indica* rice cv KJT-3. Rice lines, *NT*: in vitro grown control (non-transgenic) KJT-3 line and lines T_{0-1} and T_{0-2} are T_0 lines transformed via callus, while lines T_{0-3} to T_{0-5} are transformed KJT-3 T_0 lines via shoot apical meristems

Table 3 Segregation analysis of hygromycin resistance in T_1 progeny of *P5CS F129A* transgenic rice plants

T_1 lines	No. of seeds inoculated	No. of germinated seeds transferred to hygromycin	No. of seedlings resistant to hygromycin	No. of seedlings sensitive to hygromycin	Segregation ratio
T_{1-1}	20	20	15	5	3:1
T_{1-2}	20	20	12	8	3:1
T_{1-3}	20	20	12	8	1:1
T_{1-4}	20	20	16	4	3:1
T_{1-5}	20	20	15	5	3:1

T_{1-1} and T_{1-2} plants were obtained from seeds of T_{0-1} and T_{0-2} (via callus) respectively; T_{1-3} to T_{1-5} plants were obtained from seeds of T_{0-3} to T_{0-5} (via shoot apex),respectively. The data were recorded on the 7th day after inoculation

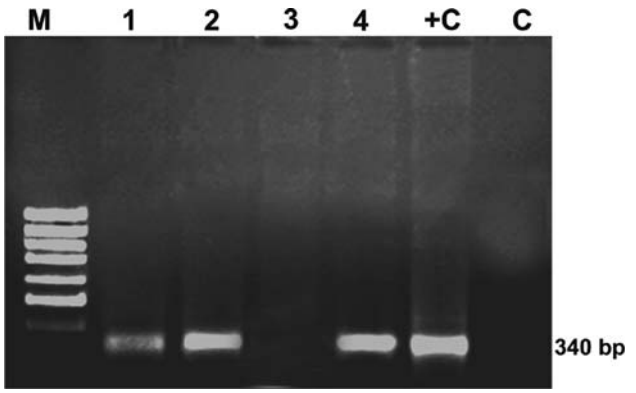

Fig. 4 PCR amplification of *hptII* gene using specific primers in transgenic T_1 plants: *lanes 1, 2 and 4* hygromycin-resistant T_1 plants, *lane 3* hygromycin-sensitive T_1 plant, *lane C* non-transformed control, *lane +C* amplification of vector DNA and *lane M* 1-kb molecular weight marker

Table 4 Comparative plant growth and biomass production of non-transgenic (*NT*) and *P5CSF129A*-transgenic T_1 plants of rice cv KJT-3 under salt stress

T_1 lines	After 7 days of continuos 150 mM NaCl treatment		
	Plant height (cm)	Fresh weight (g per plantlet)	Dry weight (g per plantlet)
NT	28 ± 1.7	0.670 ± 0.015[a]	0.147 ± 0.011[a]
T_{1-1}	50 ± 3.5	1.198 ± 0.023[f]	0.263 ± 0.021[e]
T_{1-2}	30 ± 2.6	0.719 ± 0.020[b]	0.158 ± 0.019[b]
T_{1-3}	39 ± 3.3	0.934 ± 0.031[c]	0.205 ± 0.027[c]
T_{1-4}	45 ± 4.9	1.078 ± 0.037[d]	0.236 ± 0.031[d]
T_{1-5}	48 ± 5.0	1.150 ± 0.031[e]	0.252 ± 0.024[de]

NT Non-transgenic KJT-3 plants; T_{1-1} and T_{1-2} plants were obtained from seeds of T_{0-1} and T_{0-2} (via callus) respectively; T_{1-3} to T_{1-5} plants were obtained from seeds of T_{0-3} to T_{0-5} (via shoot apex) respectively. The values represent average of 12–16 plants of transgenic lines and average of 10 plants in case of NT ± SE

Means within a column followed by different letters were significantly different from each other according to Duncan's multiple range test (DMRT) at $P \leq 0.05$

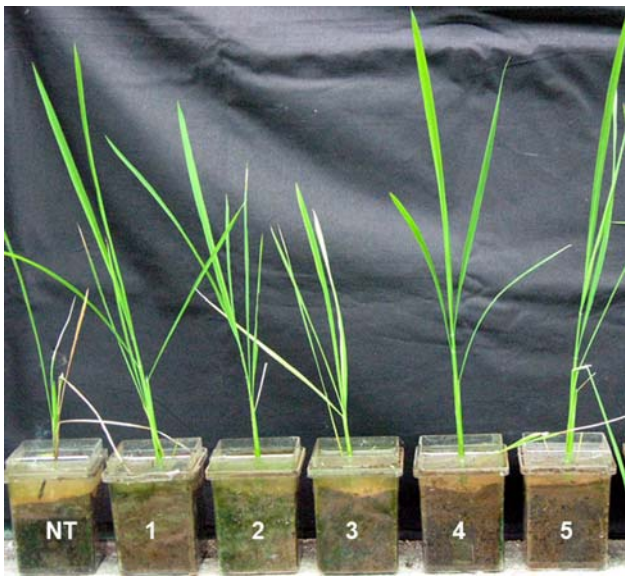

Fig. 5 Effect of NaCl stress on non-transformed and transformed (T_1) plants: hygromycin resistance T_1 plants after 1 week of selection in vitro were acclimatized to pots filled with soil in the greenhouse, and after 2 weeks, the salt stress was introduced by 150 mM NaCl for 7 days. *NT* Non-transformed plants without NaCl stress; T_{1-1} to T_{1-5} independent T_1 KJT-3 transformed lines, under 150 mM NaCl stress

hypothesis of positive correlation between the proline accumulation and salt stress tolerance of plants. In general, all the transgenic lines showed proline accumulation higher by four or more times than the NT plants under salt stress (Table 5). Transgenic events of T_{1-4} showed highest average proline level amongst all the five lines. The results clearly indicated the functional expression of *P5CSF129A* gene into the genome of *indica*-type rice cv KJT-3.

Lipid peroxidation (MDA content) under salt stress

Free radical formation and membrane damage levels were analyzed by observing the MDA content in transgenic and non-transgenic plants under 150 mM NaCl stress to see the

Table 5 Comparative proline accumulation and lipid peroxidation in non-transgenic and transgenic T_1 plants of rice cv KJT-3 under salt stress

T_1 lines	Proline content (μg g^{-1} dry weight)	Lipid peroxidation (MDA content) (nmol g^{-1} fresh weight)
NT	1055 ± 8.9^{a} (100)	32.9 ± 1.2^{f} (100)
T_{1-1}	5075 ± 18.7^{cd} (481)	19.4 ± 1.4^{d} (59)
T_{1-2}	4615 ± 15.6^{c} (437)	20.5 ± 1.1^{e} (62)
T_{1-3}	5205 ± 19.9^{de} (493)	16.4 ± 0.9^{c} (50)
T_{1-4}	5725 ± 21.4^{g} (543)	15.7 ± 1.3^{b} (48)
T_{1-5}	5430 ± 23.7^{f} (515)	16.8 ± 0.8^{c} (50)

NT Non-transgenic KJT-3 lines grown under greenhouse conditions; T_{1-1} and T_{1-2} plants were obtained from seeds of T_{0-1} and T_{0-2} (via callus) respectively; T_{1-3} to T_{1-5} plants were obtained from seeds of T_{0-3} to T_{0-5} (via shoot apex) respectively

Means within a column followed by different letters were significantly different from each other according to Duncan's multiple range test (DMRT) at $P \leq 0.05$

The values in parentheses shows the increase in proline content and decrease in MDA content by considering proline and MDA content in control non-transgenic plants as 100%, respectively. The plants were subjected to 150 mM NaCl stress for 7 days

comparison between the two (Table 5). When compared to the non-transgenic plants under salt stress, all the transgenic plants showed significantly lower lipid peroxidation, thereby indicating lesser membrane damage in *P5CS-F129A* transgenic lines as compared to non-transgenic lines.

The above results clearly showed that *P5CSF129A* transgene expression confers increased tolerance to transgenic plants for salinity stress. It was evident from these results that *P5CS*-transgenic plants produced significantly more proline and protected the transgenic plants from damages due to stress treatments. On the other hand, the control plants could not tolerate the same extent of stress due to the lower level of proline. The results of the present investigation clearly confirmed that proline accumulation can be positively correlated with the salt stress tolerance nature of the transgenic plants obtained. The over-expression of a mutated proline biosynthetic pathway gene *P5CSF129A* into the *indica* rice cv KJT-3 resulted in better biomass production and growth performance associated with higher proline accumulation and lower lipid peroxidation.

Discussion

Efficient transformation and subsequent regeneration using *Agrobacterium*-mediated methods are dependent on several factors including choice of explant, hormonal composition of the medium used, nutritional supplements, culture conditions prior to and during inoculation, duration of co-cultivation, virulence of *Agrobacterium* strain, concentration and composition of the bacteriostatic agent used, duration of selection and concentration of antibiotic selection marker, cultivar of plants and various conditions of tissue culture, including a robust system of plant regeneration; all are of critical importance (Mohanty et al. 2002; Yookongkaew et al. 2007). The genotypic influence is often overcome by modifying the nutrient medium or transformation conditions, since the same nutrient medium is not ideal for all the varieties (Ge et al. 2006).

Various researchers, however, have used 50 mg l^{-1} hygromycin B for selecting the putative transformants of different *indica*-type rice genotypes (Sridevi et al. 2005; Kant et al. 2007), while in this study 20 and 30 mg l^{-1} hygromycin B was used for selecting the putative transformants obtained via callus and shoot apices, respectively. The addition of AS in pre-culture as well as in co-cultivation medium has been reported to induce *Vir* genes, extend host range of some *Agrobacterium* strains and found essential for rice transformation (Saharan et al. 2004). Our observations are in conformity with these findings, and the inclusion of AS (100 μM) was found to be inevitable for transformation; this observation is consistent with a number of previous reports of rice transformation (Kumar et al. 2005). However, there are some successful transformation reports without adding phenolic compounds such as AS (Yookongkaew et al. 2007), indicating the varying requirements for transformation from plant to plant.

Generally, co-cultivation times for *indica* rice transformation have been reported to vary from 2 to 5 days. However, in this study, it was found that 3 days was optimal for rice transformation. Although calluses, which were co-cultivated for more than 3 days, showed GUS activity, they were adversely affected by over-growth of *Agrobacterium* and subsequently died. The same co-cultivation time has been reported by a number of researchers for efficient *indica* rice transformation using callus (Nandakumar et al. 2007) as well as apical shoot meristem (Yookongkaew et al. 2007) as target materials.

An efficient and reproducible method for transformation using mature embryo-derived embryogenic-like callus was standardized with optimization of various parameters for efficient transformation of *indica* rice cv KJT-3. We could achieve around 1% transformation efficiency by using callus of KJT-3. Varying transformation frequencies have been reported in *indica* rice cvs—Khanna and Raina (2002): 9%, and Nandakumar et al. (2007): 0.9–5.2%. The low efficiency of *indica* rice transformation in these studies is possibly attributed to toxicity of antibiotics to callus growth (Khanna and Raina 2002) and the authors have suggested withholding use of antibiotics on regeneration media. However, in the present study, pressure of hygromycin was maintained up to regeneration medium,

resulting in the selective proliferation of resistant calli with transgene.

Among various explants used, scutellum-derived embryogenic calli are the material of choice for efficient transformation of rice (Kant et al. 2007). However, in the present study, in addition to embryogenic-like calli, we have also used apical shoot meristems for transformation to reduce the time period required to get transgenic plants. TDZ, a phenylurea-type cytokinin, has been reported to facilitate multiple shoot proliferation in many plants (Srivatanakul et al. 2000). Our results are in agreement of these reports, and TDZ was observed to be a sole hormone to induce multiple shoots from apical shoot meristems and resulted in $\sim$1.8% rate of transformation in KJT-3. In conclusion, we have effectively accomplished multiple shoot regeneration from shoot apical meristem in *indica* rice, and no somaclonal variation was observed in transgenic plants.

The comparison between callus and shoot apices as targeting materials clearly indicated that the latter requires much less time than the former, as it does not require a long callus production cycle. The total time required for obtaining transformed plants (in greenhouse conditions) regenerated through apical shoot meristems takes at least 5–6 weeks less than regeneration of transgenics via mature embryo-derived callus. Further, use of 20 g l^{-1} sorbitol was observed as inevitable for shoot regeneration through calluses; however, in apical shoot meristems, no such supplementation was required. The results clearly indicated that the rate of transformation was about two times higher in the case of apical shoot meristems used as target tissues for transformation than calli.

T-DNA was shown to be stably maintained in transformed (T_0) rice plants. PCR analysis was consistent with genomic integration of *P5CS-F129A*. The stable gene insertion and establishment was further confirmed by southern hybridization, which showed stable transformation of the gene at T_0 level. *P5CS* stable insertion was also confirmed by following the histochemical GUS analysis in T_1 generation plant tissues. The insertion was further confirmed by PCR products obtained from DNA amplification of T_1 plants using *hptII* specific primers and electrophoresed on agarose gel electrophoresis.

All the primary PCR positive transformants showed considerably higher proline content than their NT counterpart, which clearly confirmed the insertion and functional expression of *P5CSF129A* gene into the KJT-3 genome. Similar to our results, the primary *P5CS*-transgenic wheat plants showed much higher (more than ten times) proline content than their wild-type counterparts under non-stress conditions (Sawahel and Hassan 2002). More recently, Yamchi et al. (2007) observed that there was 26 times more proline production in tobacco plants transformed with *P5CS* gene as compared to non-transgenic tobacco plants. Results of the present work are in harmony with these reports and confirmed the integration of moth bean *P5CSF129A* gene into the genome of *indica* rice cv KJT-3 and its functional expression. The variation among the transgenic lines in terms of proline content may be attributed to the integration position of this gene and transcription level (Yamchi et al. 2007).

Stable integration and inheritance of introduced gene to the next generation was evident through hygromycin selection for T_1 progeny. The T_1 progenies exhibited both Mendelian as well as non-Mendelian segregation ratios in terms of hygromycin resistance of the transgenics. Most of the lines exhibited the the hygromycin resistance and sensitivity ratio as 3:1 (except line T_{1-3}). Similar results have been reported by Anoop and Gupta (2003) in transformed progenies of *indica* rice cv IR50 with moth bean *P5CS* gene. PCR amplification of *hptII* gene using gene-specific primers also confirmed the stable establishment of the gene into the genome of rice cv KJT-3.

In the context of the recommendation of the 'Task Force on Agricultural Biotechnology', committee chaired by Prof. M.S. Swaminathan that genetic engineering of rice should be confined to non-basmati-type rice varieties (Task Force on Agricultural Biotechnology 2004), it has become important to identify elite, non-basmati *indica* varieties to select for genetic engineering (Sridevi et al. 2005). Though the transformation frequency for *indica* rice cvs remains low, the present investigation has shown that KJT-3 is amenable for *Agrobacterium*-mediated transformation using both callus as well as shoot apex as target material for co-cultivation.

Our results clearly showed that *P5CSF129A*-transgene expression confers increased tolerance to transgenic plants for salt stress. Generally, all the transgenic lines tested of KJT-3 at T_1 level showed better plant growth and biomass production than the NT control plants under salt stress driven by 150 mM NaCl. The leaves were much greener in the transgenics, whereas the leaves of NT lines were brown and dry under salt stress. It was noticed that *P5CS*-transgenic plants produced significantly more proline (four- to five-fold more than non-transgenics) and protected the transgenic plants from damage due to salt stress treatments; on the other hand, the control plants could not tolerate the same extent of stress.

Results presented in Table 4 showed that the line T_{1-2} did not show considerably better growth performance and biomass production as compared to the NT line under salt stress. Even though it accumulated around four times higher proline content than NT, it was the lowest amongst all T1 lines accompanied by the highest level of lipid peroxidation. This indicates that this level of proline accumulation seems to be insufficient for counteracting the stress-inducing effects including lipid peroxidation. In

addition, there may be a necessity of involvement of other endogenous mechanisms for stress tolerance in addition to proline accumulation, and these mechanisms vary from plant to plant.

The enhanced salt tolerance of *P5CS*-transgenic KJT-3 plants was associated with higher proline accumulation and lower lipid peroxidation levels, while the inability of NT plants to tolerate NaCl stress may be due to a lower level of proline and a higher magnitude of free radical production as suggested by the higher MDA content under salt stress conditions. These results have proved the successful insertion and functional expression of the *P5CS-F129A* gene into the genome of rice cv KJT-3.

Earlier, various researchers have reported higher proline accumulation and subsequent abiotic stress tolerance of transgenic plants over-expressing *P5CS* genes. Enhanced proline accumulation and better growth performances of rice cvs, both *indica* and *japonica*, are attributed to the over-expression of *P5CS* transgene into their genome (Anoop and Gupta 2003; Su and Wu 2004). Anoop and Gupta (2003) reported almost two times more proline content in P5CS-transgenic rice lines under 200 mM NaCl stress. The authors have credited proline accumulation in transgenic plants for higher germination frequency and biomass production. Similar to our findings, Su and Wu (2004) also observed significantly higher tolerance of P5CS containing rice transgenics showing higher proline accumulation to stress produced by NaCl or water deficiency, as judged by faster growth of shoots and roots in comparison with NT plants. Further, they reported that stress-inducible synthesis of proline in transgenic rice resulted in faster growth under stress conditions than that with constitutive accumulation of proline.

Coming to the over-expression of mutagenic version *P5CSF129A*, transgenic tobacco plants expressing this gene accumulated about twofold more proline than the plants expressing *V. acontifolia* wild-type *P5CS*. This difference was further increased in plants treated with 200 mM NaCl (Hong et al. 2000). The same mutagenized *P5CS* have been used by Pileggi (2002) for transformation of lettuce, and observed that this gene conferred osmotic tolerance induced by freezing, high temperature and high saline conditions to the transgenics. Further, Molinari et al. (2004) used this gene under the control of constitutive promoter 35S and reported enhanced drought tolerance of the resulting transgenic plants of Carrizo citrange by over-producing proline. The findings of the present investigation are in harmony with these reports as we observed a steep increase in proline levels under salt stress conditions in transgenic plants obtained by introduction of *V. acontifolia* mutated gene *P5CSF129A*. Proline content was increased under salt stress conditions both in non-transgenic as well as *P5CS*-transgenic plants; however, with a significant variation in the extent of increase with 152% increase in the earlier while there was a 302–352% increase in the case of the latter. Such a response to salt stress in transgenic plants indicated that T-DNA is integrated in the chromosome, which leads to its efficient transcription.

In addition to proline enhancement, another important parameter to check the level of stress-induced damage at the cellular level is lipid peroxidation measured as the MDA content (Parvanova et al. 2004). As a consequence of ROS, lipid peroxidation can lead to cellular membrane rupture in plants submitted to stress. As expected, there were significant differences between the non-transgenic and transgenic plants of KJT-3 in terms of MDA content under 7 days of 150 mM NaCl stress. It has been reported that higher proline accumulation in *P5CS*-transformed tobacco plants reduced free radical levels measured by MDA content in response to osmotic stress (Parvanova et al. 2004; Vendruscolo et al. 2007). The transformed plants presented low MDA values that could be translated into a higher maintenance of cellular integrity and basic physiological processes.

In conclusion, we achieved successful insertion of the *P5CSF129A* gene in the primary generation and its inheritance to the progeny plants as revealed by PCR products and Southern analysis. The gene was functionally expressed in T_0 as well as T_1 plants as indicated by higher proline accumulation in transgenics as compared with non-transgenic plants. The salt stress evaluation of transgenic plants of T_1 generation revealed better growth performance, biomass production, higher proline accumulation and lower rate of lipid peroxidation in comparison with the non-transgenic plants under 150 mM NaCl stress.

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References

Ali G, Srivastava PS, Iqbal M (1999) Proline accumulation, protein pattern and photosynthesis in regenerants grown under NaCl stress. Biol Plant 42:89–95

Anoop N, Gupta AK (2003) Transgenic indica rice cv IR-50 over-expressing *Vigna aconitifolia* δ(1)-pyrroline-5-carboxylate synthetase cDNA shows tolerance to high salt. J Plant Biochem Biotechnol 12:109–116

Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water studies. Plant Soil 39:205–208

Bhatnagar-Mathur P, Vincent V, Devi MJ, Lavanya M, Vani G, Sharma KK (2009) Genetic engineering of chickpea (*Cicer arietinum* L.) with the *P5CSF129A* gene for osmoregulation with implications on drought tolerance. Mol Breeding 23:591–606

Ge X, Chu Z, Lin Y, Wang S (2006) A tissue culture system for different germplasms of indica rice. Plant Cell Rep 25:392–402

Heath RL, Packer I (1968) Photoperoxidation in isolated chloroplast I. kinetics and stochiometry of fatty acid peroxidation. Arch Biochem Biophys 125:189–198

Hmida-Sayari A, Gargouri-Bouzid R, Bidani A, Jaoua L, Savoure A, Jaoua S (2005) Overexpression of Δ^1-pyrroline-5-carboxylate synthetase increases proline production and confers salt tolerance in transgenic potato plants. Plant Sci 169:746–752

Hong Z, Lakkineni K, Zhang Z, Verma DPS (2000) Removal of feedback inhibition of pyrroline-5-carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. Plant Physiol 122:1129–1136

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-Glucuronidase as sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Jogeswar G, Pallela R, Jakka NM, Reddy PS, Rao JV, Sreeniwasulu N, Kavi Kishor PB (2006) Antioxidative response in different sorghum species under short term salinity stress. Acta Physiol Plant 28:465–475

Kant P, Kant S, Jain RK, Chaudhury VK (2007) *Agrobacterium*-mediated high frequency transformation in dwarf recalcitrant rice cultivars. Biol Plant 51:61–68

Kavi Kishor PB, Sangam S, Amrutha RN, Laxmi PS, Naidu NR, Rao KRSS, Rao S, Reddy KJ, Theriappan P, Sreenivasulu N (2005) Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: its implications in plant growth and abiotic stress tolerance. Curr Sci 88:424–438

Khanna HK, Raina SK (2002) Elite indica transgenic rice plants expressing modified Cry1Ac endotoxin of *Bacillus thuringiensis* show enhanced resistance to yellow stem borer (*Scirpophaga incertulas*). Transgenic Res 11:411–423

Kumar SG, Reddy AM, Sudhakar C (2003) NaCl effects on proline metabolism in two high yielding genotypes of mulberry (*Morus alba* L.) with contrasting salt tolerance. Plant Sci 165:1245–1251

Kumar KK, Muruthasalam S, Loganathan M, Sudhakar D, Balasubramanian P (2005) An improved *Agrobacterium*-mediated transformation protocol for recalcitrant elite indica rice cultivars. Plant Mol Biol Rep 23:67–73

Kumar V, Shriram V, Nikam TD, Kavi Kishor PB, Jawali N, Shitole MG (2008) Assessment of tissue culture and antibiotic selection parameters useful for transformation of an important indica rice genotype Karjat-3. Asian Australas J Plant Sci Biotechnol 2:84–87

Kumar V, Shriram V, Nikam TD, Jawali N, Shitole MG (2009) Antioxidant enzyme activities and protein profiling under salt stress in indica rice genotypes differing in salt tolerance. Arch Agron Soil Sci 55:379–394

Mansour MMF (2000) Nitrogen containing compounds and adaptation of plants to salinity stress. Biol Plant 43:491–500

Matysik J, Bhalu AB, Mohanty P (2002) Molecular mechanisms of quenching of reactive oxygen species by proline under stress in plants. Curr Sci 82:525–532

Misra N, Gupta AK (2005) Effect of salt stress on proline metabolism in two high yielding genotypes of green gram. Plant Sci 169:331–339

Mohanty A, Kathuria H, Ferjani A, Sakamoto A, Mohanty P, Murata N, Tyagi AK (2002) Transgenic plants of an elite *indica* rice variety Pusa Basmati 1 harbouring the *codA* gene are highly tolerant to salt stress. Theor Appl Genet 106:51–57

Molinari HBC, Marur CJ, Filho JCB, Kobayashi AK, Pileggi M, Junior RPL, Pereira LFP, Vieira LGE (2004) Osmotic adjustment in transgenic citrus rootstock *Carrizo citrange* (*Citrus sinensis* Osb. X *Poncirus trifoliata* L. Raf.) overproducing proline. Plant Sci 167:1375–1381

Munns R, Tester M (2008) Mechanisms of salinity tolerance. Annu Rev Plant Biol 59:651–681

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol Plant 15:473–497

Nandakumar R, Babu S, Kalpana K, Raguchander T, Balasubramanian P, Samiyappan R (2007) *Agrobacterium*-mediated transformation of indica rice with chitinase gene for enhanced sheath blight resistance. Biol Plant 51:142–148

Parida AK, Dagaonkar VS, Phalak MS, Aurangabadkar LP (2008) Differential responses of the enzymes involved in proline biosynthesis and degradation in drought tolerant and sensitive cotton genotypes during drought stress and recovery. Acta Physiol Plant 30:619–627

Parvanova D, Ivanov S, Konstantinova T, Karanov E, Atanassov A, Tsvetkov T, Alexieva V, Djilianov D (2004) Transgenic tobacco plants accumulating osmolytes show reduced oxidative damage under freezing stress. Plant Physiol Biochem 42:57–63

Pileggi M (2002) Genetic transformation of the lettuce cultivar Grand Rapids (*Lectuca sativa* L.) by *Agrobacterium tumefaciens* to improve osmotic stress tolerance. Gen Mol Res 1:176

Pujni D, Chaudhary A, Rajam MV (2007) Increased tolerance to salinity and drought in transgenic indica rice by mannitol accumulation. J Plant Biochem Biotechnol 16:1–8

Saharan V, Yadav RC, Yadav NR, Ram K (2004) Studies on improved *Agrobacterium*-mediated transformation in two indica rice (*O. sativa* L.). Afr J Biotechnol 3:572–575

Sairam RK, Tyagi A (2004) Physiology and molecular biology of salinity stress tolerance in plants. Curr Sci 86:407–421

Sairam RK, Srivastava GC, Agarwal S, Meena RC (2005) Differences in response to salinity stress in tolerant and susceptible wheat genotypes. Biol Plant 49:85–91

Sambrook J, Russell RW (2001) Molecular cloning: A laboratory manual. Cold Spring Harbour Laboratory Press, Cold Spring Harbour

Sawahel WA, Hassan AH (2002) Generation of transgenic wheat plants producing high levels of the osmoprotectant proline. Biotech Lett 24:721–725

Sridevi G, Dhandapani M, Veluthambi K (2005) *Agrobacterium*-mediated transformation of White Ponni, a non-basmati variety of indica rice (*Oryza sativa* L.). Curr Sci 88:128–132

Srivatanakul M, Park S, Sanders J, Salas M, Smith R (2000) Multiple shoot regeneration of kenaf (*Hibicus cannabinus*) from a shoot apex culture system. Plant Cell Rep 19:1165–1170

Su J, Wu R (2004) Stress-inducible synthesis of proline in transgenic rice confers faster growth under stress conditions than that with constitutive synthesis. Plant Sci 166:941–948

Task Force on Agricultural Biotechnology (2004) Agricultural biotechnology: safe and responsible use. Executive summary of the Task Force on Agricultural Biotechnology chaired by MS Swaminathan, submitted to the Ministry of Agriculture, Government of India. Curr Sci 87:425–426

Vendruscolo ECG, Schuster I, Pileggi M, Scapim CA, Molinari HBC, Marur CJ, Vieira LGE (2007) Stress-induced synthesis of proline confers tolerance to water deficit in transgenic wheat. J Plant Physiol 164:1367–1376

Verbruggen N, Hermans C (2008) Proline accumulation in plants: a review. Amino Acids 35:753–759

Yamchi A, Jazii FR, Mousav A, Karkhane AA, Renu (2007) Proline accumulation in transgenic tobacco as a result of expression of arabidopsis Δ^1-pyrroline-5-carboxylate synthetase (p5cs) during osmotic stress. J Plant Biochem Biotechnol 16:9–15

Yookongkaew N, Srivatanakul M, Narangajavana J (2007) Development of genotype-independent regeneration system for transformation of rice (*Oryza sativa* ssp. *indica*). J Plant Res 120:237–245

Zhao F, Zhang H (2006) Expression of *Suaeda salsa* glutathione S-transferase in transgenic rice resulted in a different level of abiotic stress resistance. J Agric Sci 144:547–554