Plant Physiology and Biochemistry 94 (2015) 104–113

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Proline over-accumulation alleviates salt stress and protects photosynthetic and antioxidant enzyme activities in transgenic sorghum [*Sorghum bicolor* (L.) Moench]

P. Surender Reddy [a], Gadi Jogeswar [a, 1], Girish K. Rasineni [b], M. Maheswari [c], Attipalli R. Reddy [b], Rajeev K. Varshney [d], P.B. Kavi Kishor [a, *]

[a] Department of Genetics, Osmania University, Hyderabad 500 007, India
[b] Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad 500 047, India
[c] Central Research Institute for Dryland Agriculture, Santoshnagar, Hyderabad 500 059, India
[d] International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru 502 324, India

ARTICLE INFO

Article history:
Received 27 January 2015
Received in revised form
17 May 2015
Accepted 28 May 2015
Available online 30 May 2015

Keywords:
Proline
Pyrroline-5-carboxylate synthetase gene
Salt stress
Sorghum bicolor (L.) Moench
Antioxidant enzymes

ABSTRACT

Shoot-tip derived callus cultures of *Sorghum bicolor* were transformed by *Agrobacterium tumefaciens* as well as by bombardment methods with the mutated *pyrroline-5-carboxylate synthetase* (*P5CSF129A*) gene encoding the key enzyme for proline biosynthesis from glutamate. The transgenics were selfed for three generations and T_4 plants were examined for 100 mM NaCl stress tolerance in pot conditions. The effect of salt stress on chlorophyll and carotenoid contents, photosynthetic rate, stomatal conductance, internal carbon dioxide concentration, transpiration rates, intrinsic transpiration and water use efficiencies, proline content, MDA levels, and antioxidant enzyme activities were evaluated in 40-day-old transgenic lines and the results were compared with untransformed control plants. The results show that chlorophyll content declines by 65% in untransformed controls compared to 30–38% loss (significant at $P < 0.05$) in transgenics but not carotenoid levels. Photosynthetic rate (PSII activity) was reduced in untransformed controls almost completely, while it declined by 62–88% in different transgenic lines. Salinity induced ca 100% stomatal closure in untransformed plants, while stomatal conductance was decreased only by 64–81% in transgenics after 4 days. The intercellular CO_2 decreased by ca 30% in individual transgenic lines. Malondialdehyde (MDA) content was lower in transgenics compared to untransformed controls. The activities of superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6) and glutathione reductase (GR; EC1.8.1.7) were quantified in leaves exposed to 100 mM NaCl stress and found higher in transgenics. The results suggest that transgenic lines were able to cope better with salt stress than untransformed controls by protecting photosynthetic and antioxidant enzyme activities.

1. Introduction

Salt stress is one of the major abiotic stresses and it severely affects growth and development of sorghum, an important crop grown in arid and semiarid regions. Saline soils cause ionic (Na^+ and Cl^-) toxicity, low osmotic potential, and changes in nutrient uptake leading to major disturbances such as photosynthetic carbon gain and leaf growth rate. Plants respond to low water potential (ψ_w) by decreasing stomatal conductance and by changes in the growth of roots in order to take up water from deeper layers of soil (Kramer and Boyer, 1995). Closure of stomata may lead to reduction in CO_2 uptake, photosynthetic activity and photosynthetic electron transport chain becomes over-reduced resulting in the generation of ROS (Apel and Hirt, 2004). Plants respond to the external salinity by expression of genes like *SOS1*, *NHX* and *P5CS* and by accumulating osmoprotectants. Yamaguchi-Shinozaki (2001) showed that both glycinebetaine and proline are responsible for osmotic adjustment and they protect subcellular structures in stressed plants. Proline increases

Abbreviations: APX, ascorbate peroxidase; CAT, catalase; EDTA, ethylenediaminetetraacetic acid; GR, glutathione reductase; MDA, malondialdehyde; MS, Murashige and Skoog's medium; NADPH, nicotinamide adenine dinucleotide phosphate reduced; NBT, nitroblue tetrazolium; P5CS, pyrroline-5-carboxylate synthetase; ROS, reactive oxygen species; SOD, superoxide dismutase; TCA, trichloroacetic acid.

* Corresponding author.
E-mail address: pbkavi@yahoo.com (P.B. Kavi Kishor).
[1] Present address: A. U. College of Pharmaceutical Sciences, Andhra University, Visakhapatnam 530 003, India.

http://dx.doi.org/10.1016/j.plaphy.2015.05.014
0981-9428/

cellular osmolarity (turgor pressure) that provides the turgor necessary for cell expansion under stress conditions (Matysik et al., 2002). Several studies have demonstrated that over-expression of *P5CS* gene increases proline production and confers salt tolerance in transgenic plants (Kishor and Sreenivasulu, 2014). Hong et al. (2000) removed the feedback inhibition by using site-directed mutagenesis to replace the Phe residue at position 129 in *Vigna aconitifolia P5CS* 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). Proline is considered as the only osmolyte which has been shown to scavenge singlet oxygen (Alia et al., 2001), and free radicals including hydroxyl ions, and hence stabilize proteins, DNA, as well as membrane (Matysik et al., 2002). The effective detoxification enzymatic machinery includes SOD, APX and CAT. Exogenous application of proline or genetic manipulation of its biosynthesis or degradation has amply demonstrated its role in plant responses to water stress (Ozden et al., 2009). Proline was also reported to reduce the enzyme denaturation caused due to heat, NaCl and other stresses. Exogenous application of proline alleviates the salt stress induced reduction in the activities of antioxidant enzymes in grapevine cells (Ozden et al., 2009). Over-expression of *P5CSF129A* in 'Swingle' citrumelo plants improved the drought tolerance and antioxidant enzymatic activity (de Campos et al., 2011). Recently, Signorelli et al. (2013) demonstrated that proline does not quench singlet oxygen. Thus, confusion and controversies exist for the role of proline in quenching singlet oxygen and hydroxyl radicals under stress conditions. Though Sarvesh et al. (1996) were the first to show that high proline producing genotypes exhibit higher specific activities of antioxidant enzymes as compared to low proline producing genotypes under salt stress; this has not been shown in transgenics overproducing proline. Also, higher photosynthetic activity and the yield performance of the transgenics exhibiting higher antioxidant enzyme activities were not demonstrated. The present study was therefore undertaken to generate the transgenic sorghum plants over-expressing *P5CSF129A* gene and to evaluate the activities of antioxidant enzymes such as SOD, CAT and GR under salt stress. The transgenic *Sorghum* plants were subjected to saline stress and various physiological parameters like photosynthesis, PSII performance, lipid peroxidation, and antioxidant enzyme activities were studied along with the proline levels since these physiological parameters affiliated with growth parameters will ultimately determine the yield capacity under salt stress.

2. Materials and methods

2.1. Preparation of shoot tip explants and in vitro regeneration

Seeds of *Sorghum bicolor* variety SPV-462, a salt susceptible one, were collected from the National Research Center for Sorghum, Hyderabad, India. Seeds were washed with 0.1% Tween-20 for 1 min with vigorous shaking, washed with sterile distilled water and then surface sterilized using 0.1% (w/v) $HgCl_2$ for 10 min followed by three washes with sterile distilled water for 2–3 min each to remove traces of $HgCl_2$. Surface sterilized seeds were transferred into sterile bottles containing moistened cotton and kept in dark at 26 ± 2 °C until germination. Shoot tips measuring 0.5–1.0 cm were excised from 3-day-old seedlings with a sterile scalpel and an incision was made at the base of each shoot tip. Shoot tip explants were cultured on Murashige and Skoog's (MS) medium (Murashige and Skoog, 1962) containing 1.5 mg l^{-1} 2,4-D and 0.5 mg l^{-1} kinetin for callus initiation. The callus obtained on this medium was excised and used for regeneration and for genetic transformation. Both *Agrobacterium* and particle-inflow-gun-mediated methods were followed for genetic transformation.

2.2. Plant transformation using Agrobacterium tumefaciens

Transformation experiments were conducted by using *A. tumefaciens* strain LBA 4404 harboring the binary vector pCAMBIA carrying *V. aconitifolia P5CSF129A* cDNA under the control of CaMV35S promoter. *A. tumefaciens* was grown at 28 °C in liquid yeast extract, mannitol medium for 24 h to mid-log phase so as to reach an optical density of 1.0 (at $O.D_{600}$ nm). Calli were infected by immersing in *Agrobacterium* culture suspension for about 15–30 min. The excess culture adhered to callus masses was removed through blotting on sterilized Whatman filter paper and cultured on MS basal agar medium containing 100 μM acetosyringone and incubated for 48 h at 24 ± 2 °C. After co-cultivation, calli were transferred onto shoot induction medium (MS + 1.5 mg l^{-1} BAP) containing 300 mg l^{-1} cefotaxime and 6 mg l^{-1} hygromycin as a selection agent.

2.3. Transformation by particle-inflow-gun

Shoot tip derived calli (30-day-old) of sorghum were placed on an osmotic medium (MS basal + 36.4 g l^{-1} mannitol + 36.4 g l^{-1} sorbitol + 30 g l^{-1} sucrose) for 4 h before bombardment. The tungsten suspension (10 mg of tungsten particles dissolved in 200 μl of 50% glycerol) was prepared by washing the particles in absolute ethanol followed by sterile water while vortexing in each step in microfuge tubes and stored at 4 °C. For 50 μl of tungsten suspension, 10 μl of plasmid pCAMBIA-*P5CSF129A* DNA (10 μg), 50 μl of 2.5 M $CaCl_2$ and 20 μl of 100 mM spermidine were added, vortexed for 3 min, allowed to settle for 1 min and centrifuged for 15 s. The supernatant was removed and pellet was washed three times with 200 μl of 100% ethanol. Pellet was dissolved in 30 μl of 100% ethanol, vortexed for 1 min and 6 μl of the mixture was used for each bombardment. All parameters were optimized before experimentation. Thirty callus pieces were arranged at the center of each Petri dish and placed on the adjustable shelf at a distance of 18.5 cm in a particle-inflow-gun. Calli were bombarded with a helium gas pressure of 8 kg cm^{-2} under partial vacuum (550 mm Hg). The time was set to 0.05 milli seconds for each shot. After bombardment, calli were incubated for 48 h in dark on the medium containing osmotic agents (mannitol + sorbitol + sucrose) as mentioned above.

2.4. Plant regeneration from transformed calli

Shoot tip derived calli after transformation with particle-inflow-gun were placed on shoot induction medium. They were allowed to differentiate into shoots for 3–4 weeks with weekly subcultures onto the same media. Shoots that survived in presence of hygromycin (6 mg l^{-1}) were subsequently transferred to half-strength MS medium fortified with 0.5 mg l^{-1} NAA for root initiation for 3 weeks. Shoots with well-formed roots that attained 5–10 cm height were washed gently under running tap water to remove adhered agar and planted into plastic cups containing garden soil, coco peat, sand and vermiculate in a ratio of 2:2:1:2. The cups were covered with a plastic bag for three weeks to maintain humidity in the greenhouse and were slowly exposed to the ambient environment. The per cent frequency of transformation was 5–6% irrespective of the method used. The per cent frequency was calculated using the following formula:

$$f = \frac{\text{Total number of calli differentiating plantlets} \times 100}{\text{Total number of calli inoculated into the regenerating medium}}$$

Plants were transferred to a containment greenhouse with 28/20 °C day/night temperatures where they were allowed to grow until maturity and subsequent progression of generations (T_1, T_2, and T_3). T_4 transgenic lines confirmed for gene integration using Southern analysis were tested for salt stress tolerance. All transgenics looked morphologically similar when compared to that of seed raised plants.

2.5. Molecular analysis of transformed plants and NaCl stress treatment

Eleven independent transgenic lines (8 generated through particle-inflow-gun and 3 obtained through *Agrobacterium* method) were analyzed for the presence and expression of the *hptII* and *P5CSF129A* genes. Out of the eight transgenic lines, two lines obtained through *Agrobacterium* and three obtained through microprojectile bombardment methods were analyzed. Genomic DNA was isolated from young leaves of the putative transformants using the CTAB method described by Doyle and Doyle (1990). Initial screening of the transformants was carried out by PCR amplification of *hptII* and *P5CSF129A* genes. The 750 bp region of *hptII* gene was amplified by using 22-mer oligonucleotide primers (FP 5′-GCT GGG GCG TCG GTT TCC ACT A-3′ and RP 5′-CGA GGT CAG TTA CTC GCG ACA A-3′) with a PCR profile of 30 cycles at (94 °C for 5 min, 94 °C for 45 s, 56 °C for 1 min, and 72 °C for 90 s) and a final extension at 72 °C for 5 min. Presence of the introduced *P5CSF129A* gene was detected by using 22-mer primers (FP 5′-ACA TTA TAC TCG TCT CCT CTG G-3′ and RP 5′-AAT TTT GTT TCC TTT CCT CTG A-3′) designed to obtain a 800 bp amplicon with a PCR profile of 30 cycles at 94 °C for 45 s, 56 °C for 1 min, and 72 °C for 90 s and a final extension at 72 °C for 5 min. Amplified products were assayed by electrophoresis on 1.2% agarose gels after staining with ethidium bromide. Southern blot analysis was carried out according to Sambrook et al. (1989). Genomic DNA (20–30 μg) from each of transformants along with untransformed plant leaves were digested with *Eco*RI and *P5CSF129A* gene was liberated. Digested DNA was separated on 0.8% agarose gel and the DNA fragments were transferred onto nylon membranes (Hybond N^+, Amersham Biosciences, UK). PCR amplified fragments of respective coding sequences of *hptII* and *P5CSF129A* genes were used as probes labeled with non-radioactive Alk-Phos direct system (Amersham). Labeling, hybridization and detection methods were performed according to the manufacturer's instructions. Copy number of the inserted gene was determined by digesting the genomic DNA samples with *kpnI* and probed with *P5CSF129A*. Total RNA was isolated from transgenic plants along with untransformed control plants using TRIzol® reagent from 100 mg leaf tissue. RT-PCR analysis was carried out for the selected transgenic plants using Thermoscript RT-PCR system® (Invitrogen) according to the manufacturer's instructions. The synthesized cDNA was used for PCR amplification of the coding sequences of *hptII* and *P5CSF129A* genes using respective gene specific primers as described above. Twelve plants from each of transgenic line and untransformed control plants were grown in the greenhouse with 28/20 °C day/night temperatures under well watered conditions until 40 days after sowing the seeds. The pots were placed in similar positions in relation to incidence of solar radiation. Thereafter, plants were divided into two groups: one set kept as control without salt stress and to the other set, 100 mM NaCl was added (to each pot).

2.6. Chlorophyll fluorescence and photosynthetic leaf gas exchange measurements

Chlorophyll and carotenoid contents in the leaf discs were determined as per the procedure of Arnon et al. (1974). Chlorophyll *a* and *b* were measured at 663 and 645 nm, while carotenoids were measured at 480 nm. The PSII activity in the intact leaves was determined by measuring chlorophyll *a* fluorescence transient using Handy Plant Efficiency Analyzer (Hansatech instruments Ltd., King's Lynn, Norfolk, England). The transients were induced by 1 s illumination with an array of three light emitting diodes providing a maximum light intensity of 3000 μmol m^{-1} s^{-1} and a homogenous irradiation over a 4 mm diameter leaf area. The leaf exposed to salt stress treatments (one liter solution of 100 mM NaCl to each pot) were dark adapted (in order to ensure that all the components of PSII are in oxidized state i.e. PSII reaction centers are fully open) for 30 min prior to the measurement. Initial (F_0) and maximal (F_m) fluorescence yields were measured. Variable fluorescence yield (F_v) was defined as $F_m - F_0$. Variation in PSII activity was measured as the ratio of variable to maximum fluorescence (F_v/F_m). Plant responses to salinity treatments were quantified by measuring photosynthetic leaf gas exchange. The rate of leaf gas exchange was measured using a portable infrared CO_2/H_2O gas analyzer (IRGA) (LC Pro+, ADC Bioscientific Ltd., U.K.) equipped with a broad leaf chamber. Gas analyzer was used to measure instantaneous net photosynthetic rates (A; micromol m^{-2} s^{-1}), stomatal conductance to CO_2 (g_s; mol m^{-2} s^{-1}) and transpiration rates (E; mmol m^{-2} s^{-1}), periodically during each growing season between 10 and 11 h solar time. Instantaneous water use efficiency ($WUEi = A/E$ μmol CO_2 mol^{-1} H_2O) was calculated. Plants were analyzed for internal CO_2 concentrations (C_i). Both A and g_s were expressed on a projected leaf area basis which was measured with an automatic image analyzer. All photosynthetic measurements were performed *in situ* on young, fully expanded and light-exposed leaves randomly chosen from the upper half of the plant. The approach was adopted to avoid variation in photosynthetic characteristics between leaves of different age. The leaves were tagged to allow repeated measurements.

2.7. Proline determination

Leaf samples were ground in liquid nitrogen, and free proline was extracted with 3% (w/v) sulfosalicylic acid. After centrifugation, the supernatant was used for determination of the proline content at 520 nm, as described by Bates et al. (1973). Values are expressed in μmol proline g^{-1} fresh weight.

2.8. Lipid peroxidation

Lipid peroxidation was determined according to Fu and Huang (2001). Fresh leaves were homogenized in 5 ml of 0.1% TCA. The homogenate was centrifuged at 5000 g for 10 min. Five hundred μl of the supernatant was added to 4 ml of 20% TCA which contained 0.5% thiobarbituric acid. The mixture was heated at 95°C for 30 min, quickly cooled on ice, and centrifuged at 5000 g for 15 min. The absorbance of the supernatant was read at 532 and 600 nm. After subtracting the non-specific absorbance at 600 nm, MDA concentration was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}. Lipid peroxidation is expressed in nmol MDA g^{-1} fresh weight of leaf tissue.

2.9. Antioxidant enzyme activities

Activity of SOD was determined in controls and 100 mM NaCl treated transgenic plants by the method of Beauchamp and Fridovich (1971) following the photo reduction of NBT. The reaction mixture contained 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 75 mM NBT, 2 mM riboflavin and 100 μl of supernatant. Riboflavin was added as the last component and the

reaction was incubated by placing the tubes under two 15 W fluorescent lamps. The reaction was terminated after 10 min by removing the reaction tubes from the light source. Non-illuminated and illuminated reactions without supernatant served as calibrated standards. Reaction products were measured at 560 nm. Volume of the supernatant corresponding to 50% inhibition of the reaction was assigned a value of one enzyme unit. Enzyme activity was expressed as units mg^{-1} protein. Catalase activity was determined according to the method described by Luck (1974). Leaf material was homogenized with 50 mM phosphate buffer (pH 7.0). The homogenate was centrifuged at 8000 g for 20 min at 4 °C. Twenty µl of enzyme extract was added to 3 ml of hydrogen peroxide-phosphate buffer (pH 7.0). Time required for the decrease in the absorbance at 240 nm from 0.45 to 0.40 was noted. Enzyme solution containing hydrogen peroxide-free phosphate buffer was used as control. GR activity was measured according to Foyer and Halliwell (1976) by following the rate of NADPH oxidation as measured by the decrease in absorbance at 340 nm. One ml of assay mixture contained 100 mM Tris–HCl buffer (pH 7.8), 2 mM EDTA, 50 mM NADPH, 0.5 mM GSSG and 20 µl of the enzyme extract. Assay was initiated by the addition of 50 mM NADPH at 25 °C and was performed at 340 nm for 5 min. Enzyme activity was expressed in terms of µmols of NADPH oxidized mg^{-1} of protein min^{-1}. Leaf protein content was determined according to modified Lowry's procedure (Lowry et al., 1951).

2.10. Statistical analysis

All experiments were repeated and the data represent average values taken from 6 replicates unless otherwise mentioned. The physiological data were analyzed by two-way ANOVA, taking $P < 0.05$ as significant according to Tukey's *post hoc* test.

3. Results

3.1. Plant regeneration from callus cultures and genetic transformation

Parameters that influence callus initiation from shoot tips, subsequent plant regeneration and genetic transformation have been standardized initially using sorghum variety SPV-462. Mature embryos, inflorescences and shoot tips were tried for callus initiation and subsequent plant regeneration. Since shoot tip derived calli were found better for plant regeneration, they were used in the present study (data not shown). Devoid of 100 µM acetosyringone to the co-cultivation medium, genetic transformation was not noticed. Age of callus, concentration of acetosyringone, helium pressure, target distance, concentration of DNA/mg of tungsten particles, and duration of osmotic treatment influenced the transformation frequency (data not shown). After co-cultivation of calli with *Agrobacterium* for 48 h, the tissues were transferred onto selection medium containing 6 mg l^{-1} hygromycin and 300 mg l^{-1} cefotaxime. For particle bombardment, optimized conditions were followed with 8 kg cm^{-2} helium pressure, a target distance of 18.5 cm, 2 µg of DNA mg^{-1} tungsten particles, 0.25 M sorbitol plus 0.25 M mannitol as osmoticum, 4 h of pre-bombardment and 16 h of post-bombardment osmotic treatment and vacuum maintenance at 550 mm Hg and 0.05 milli seconds for each shot. After 28 h of microprojectile bombardment, callus tissues were transferred from medium containing osmotic agents to the selection medium supplemented with 6 mg l^{-1} hygromycin. Shoots grown to about 4–5 cm on hygromycin (6 mg l^{-1}) selection medium were transferred to half-strength MS medium supplemented with 0.5 mg l^{-1} NAA for rooting. Shoots with well-developed roots were transferred into pots containing gareden soil and sand in a ratio of 1:1 for subsequent growth and analysis.

3.2. Stable integration of the transgene and evaluation of transgenics

Oligonucleotide primers specific to the coding regions of *hptII* and *P5CSF129A* genes amplified the expected size of the gene fragments from all the analyzed transformants, but not from untransformed controls. The results based on PCR analysis were confirmed by Southern hybridization using *P5CSF129A* gene. Southern blot analysis of transgenics indicated that the number of copies of the transgene integration into the genome of the host plants varied from one to three (Fig. 1A). RT-PCR analysis of cDNA from the transgenic plants showed positive amplification of *hptII* and *P5CSF129A* transcripts in all the selected transgenic events (Fig. 1B). Transgenic events accumulating high proline levels in presence of 100 mM NaCl sress conditions (compared to untransformed controls) were selected for subsequent gene expression and phenotypic studies. Greenhouse experiments were conducted for the physiological and biochemical characterization of the 5 selected transgenic events namely $A_{1\text{-}3\text{-}2}$, $A_{2\text{-}2}$, $B_{4\text{-}4\text{-}3\text{-}2}$, $B_{10\text{-}3\text{-}2}$, $B_{11\text{-}2\text{-}1}$, (A and B representing transgenic events generated through *Agrobacterium* and bombardment respectively) in T_4 generation (control and salt stress treatments). These transgenic lines were selected for this study based on proline and gene expression levels. Transgenics as well as untransformed sorghum plants were subjected to 100 mM NaCl stress in the pot conditions. Leaves in untransformed plants turned brown after 2-days of salt stress, but in the transgenics, the younger leaves were green and retained chlorophyll even after one-week of stress (Fig. 2A). Plants were uprooted 5-weeks after salt stress and the root biomass (fresh weights) was measured. No significant differences in the biomass of roots were observed in untransformed controls between stress and non-stress conditions, while the biomass was significantly different ($P < 0.05$) in the transgenics (Fig. 2B).

3.3. Proline content

The amount of proline in T_3 independent transgenic lines was estimated from fresh leaf tissues and was found several-folds higher compared to untransformed plants (Table 1). After 2-days of 100 mM NaCl stress, the concentration of free proline in the transgenic events raised up to 84–98 µg g^{-1} FW from 20 µg g^{-1} FW on day 0. In transgenic lines, proline content was considerably higher after 2-days of salt treatment when compared to

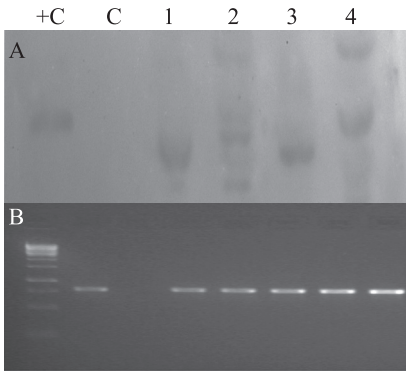

Fig. 1. (A) Southern hybridization of genomic DNA samples of T_4 transgenic plants using *P5CSF129A* gene specific probe. +C = vector DNA; C = untransformed control showing no insert of the *P5CS* gene. Lanes 1 to 4 = transgenic sorghum plants (A1-3-2, A2-2, B4-4-2-3, B10-3-2 respectively). (B) RT-PCR analysis of transgenic plants, +C = vector DNA; C = untransformed control and lanes 1 to 4 = transgenic lines A1-3-2, A2-2, B4-4-2-3, B10-3-2, B11-2-1 respectively.

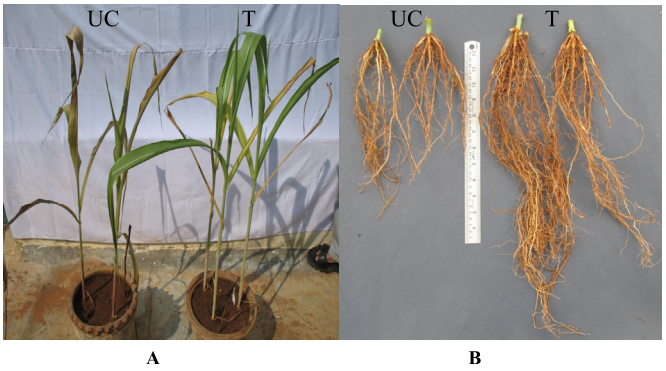

Fig. 2. (A) Effect of 100 mM NaCl stress on the leaves in untransformed control (UC) which turned brown and transgenics (T) that retained chlorophyll (transgenic line A2-2), and (B) Root biomass of untransformed control (UC) and transgenic line A2-2 in presence of 100 mM NaCl. Similar results were obtained with other transgenic plants. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

untransformed controls, and these values were maintained until the end of the stress treatment (Table 1).

3.4. Photosynthetic leaf gas exchange

Leaf gas exchange in *P5CSF129A* transgenic sorghum lines and control was measured daily for 4 days, starting at the onset of salt treatment. Under control conditions without stress, no significant difference was observed in leaf gas exchange both in transgenic lines and untransformed plants. The photosynthetic rate (*A*) or net CO_2 assimilation rate of transgenic and untransformed plants showed no significant differences and the average *A* was 24 μmol mol^{-2} S^{-1}. Photosynthetic rates decreased in both transgenic and untransformed control plants under salt stress conditions. The extent of decline in photosynthetic rate increased with the time of exposure to salt stress and *A* was almost completely inhibited in untransformed control plants. However, by the end of the experiment, 91% decrease in photosynthetic rate was noticed in untransformed control plants, whereas in transgenic lines, the decrease was 61–80% under salt stress (Fig. 3). The salinity caused almost total inhibition of stomatal conductance and transpiration rate of untransformed controls (Figs. 4 and 5). In transgenic lines, stomatal aperture decreased by 64–81%. The effect of salinity on intercellular CO_2 concentration was less severe in transgenic lines when compared to wild-type plants. In untransformed plants, C_i concentration decreased significantly with the exposure of salt stress (Fig. 6). Water use efficiency (*A*/*E*) was higher in transgenic plants in contrast to untransformed controls (Fig. 7). With the exposure of plants to the stress, *A*/*E* decreased to 0 in untransformed plants by day 4. On the other hand, in transgenic lines, only slight decrease was noticed.

Table 1
Effect of 100 mM NaCl stress on proline content (expressed as μg/g fresh weight of tissue) in untransformed and transgenic lines.

Plants	Day 0	Day 1	Day 2	Day 3	Day 4
Untransformed control	20 (±2)	34 (±3)*	39 (±4)*	36 (±2)*	34 (±1)*
$A_{1\text{-}3\text{-}2}$	23 (±3)	62 (±5)**	98 (±4)**	95 (±2)**	93 (±3)**
$A_{2\text{-}2}$	21 (±2)	54 (±2)**	90 (±4)**	88 (±5)**	81 (±3)**
$B_{4\text{-}4\text{-}2\text{-}3}$	20 (±3)	58 (±4)**	92 (±2)**	91 (±5)**	90 (±6)**
$B_{10\text{-}3\text{-}2}$	21 (±2)	52 (±2)**	86 (±4)**	87 (±5)**	86 (±3)**
$B_{11\text{-}2\text{-}1}$	21 (±3)	50 (±2)**	84 (±5)**	82 (±4)**	80 (±3)**

*indicates significant difference at $p < 0.05$ and **indicates significant difference at $p < 0.01$ by Tukey's *post-hoc* test (ANOVA) from the mean values ± standard error (n = 6).

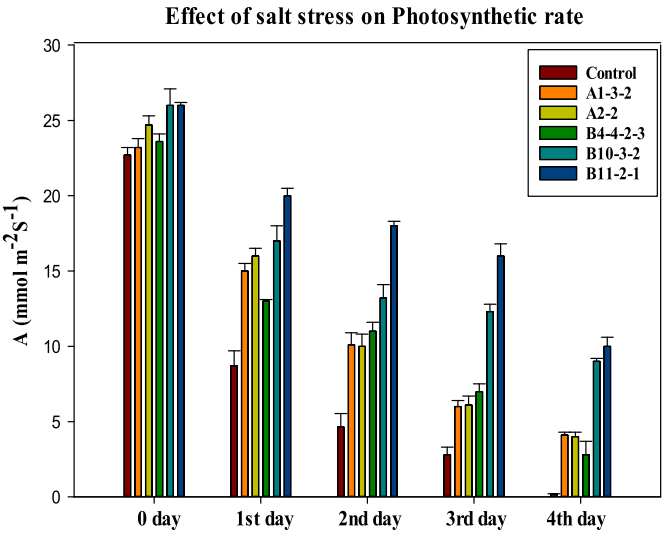

Fig. 3. Photosynthetic rates of untransformed control and different transgenic lines A1-3-2, A2-2, B4-4-2-3, B10-3-2, B11-2-1 under 100 mM salt stress. Vertical bars represent *SE* (n = 4). Significant difference between means at the ($P < 0.05$) level determined by Tukey's *post-hoc* test (ANOVA).

3.5. Chlorophyll a fluorescence analysis

The quantum yield of photosynthetic electron transport (Fv/Fm) is a parameter that allows the detection of any damages to the photosystem II (PSII) and the possible existence of photoinhibition. Fv/Fm is considered to be an important parameter for evaluation of the response of plants to oxidative stresses generated by salt and drought. During the first two days of 100 mM salt stress, no significant difference was observed in Fv/Fm ratio in both the transgenic lines and untransformed controls. After day 2, the photochemical efficiency of PSII decreased by 8–16% in the transformed lines, but by 56% in untransformed plants. Transgenic lines recorded approximately 45% higher Fv/Fm than the untransformed controls (Fig. 8). In general, chlorophyll content in the leaf discs of

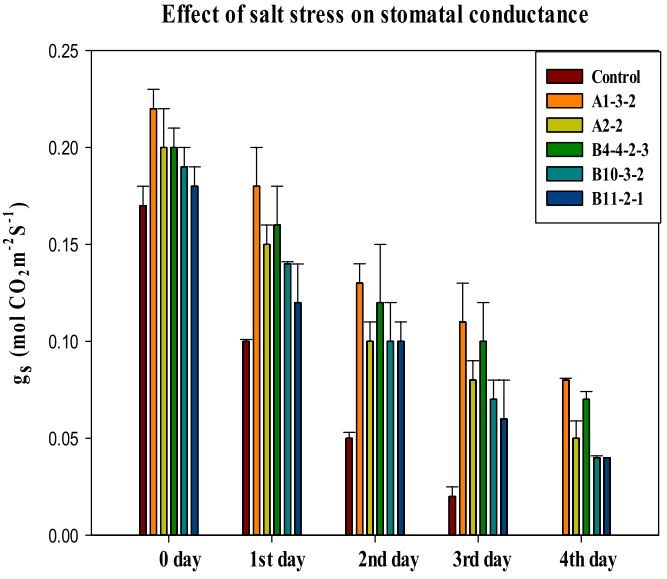

Fig. 4. Stomatal conductance of untransformed control and different transgenic lines A1-3-2, A2-2, B4-4-2-3, B10-3-2, B11-2-1 under 100 mM salt stress. Vertical bars represent *SE* (n = 4). Significant difference between means at the ($P < 0.05$) level determined by Tukey's *post-hoc* test (ANOVA).

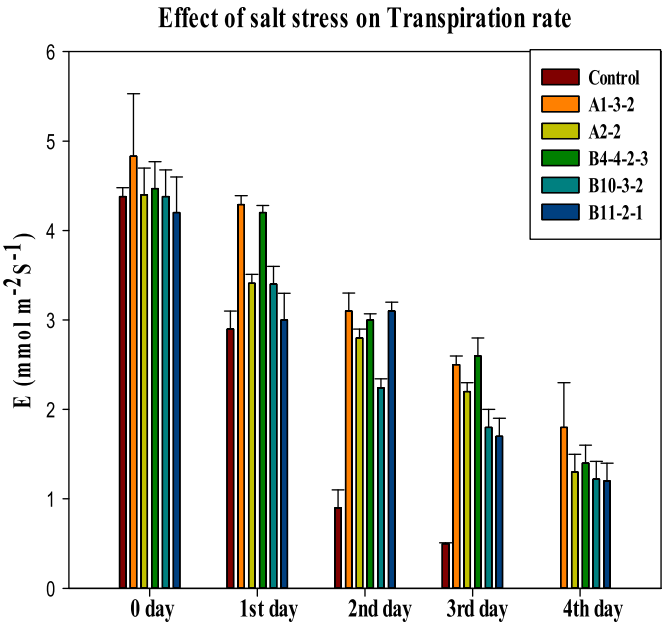

Fig. 5. Transpiration rates of untransformed control and different transgenic lines A1-3-2, A2-2, B4-4-2-3, B10-3-2, B11-2-1 under 100 mM salt stress. Vertical bars represent *SE* (n = 4). Significant difference between means at the (P < 0.05) level determined by Tukey's *post-hoc* test (ANOVA).

both untransformed and the transformed lines decreased with the time of exposure to the salt stress treatments. However, chlorophyll degradation was significantly faster in case of untransformed plants as compared to the transgenic lines (Table 2). After 4 days of exposure to stress, leaf discs of untransformed plants showed 65% decline in chlorophyll content in contrast to 33% loss in transgenics under salt stress. The impact of salt stress on carotenoids was also similar to that of total chlorophyll content both in untransformed and transgenic lines (Table 3).

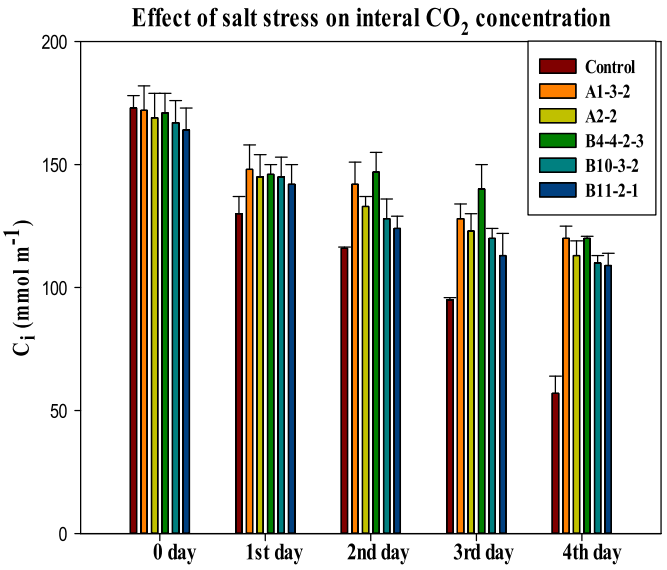

Fig. 6. Internal CO_2 concentration of untransformed control and different transgenic lines A1-3-2, A2-2, B4-4-2-3, B10-3-2, B11-2-1 under 100 mM salt stress. Vertical bars represent *SE* (n = 4). Significant difference between means at the (P < 0.05) level determined by Tukey's *post-hoc* test.

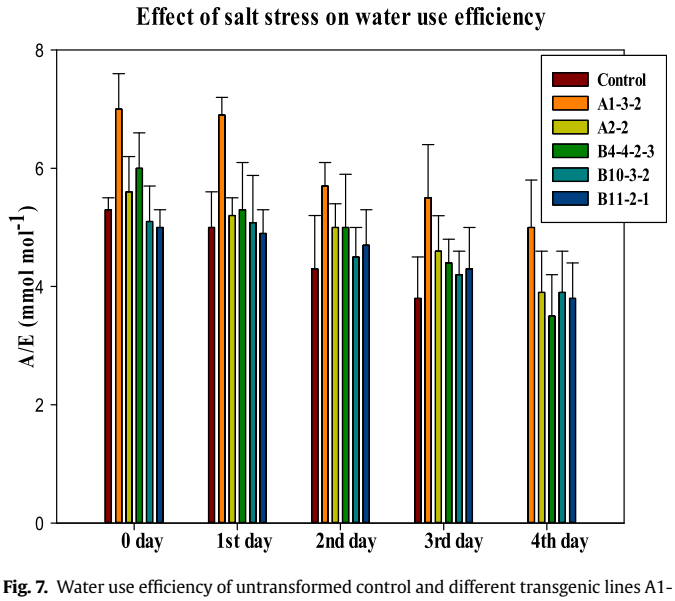

Fig. 7. Water use efficiency of untransformed control and different transgenic lines A1-3-2, A2-2, B4-4-2-3, B10-3-2, B11-2-1 under 100 mM salt stress. Vertical bars represent *SE* (n = 4). Significant difference between means at the (P < 0.05) level determined by Tukey's *post-hoc* test (ANOVA).

3.6. Lipid peroxidation

Lipid peroxidation (MDA content) is used as an indicator of oxidative stress. Free radical formation and membrane damage levels were analyzed by measuring the MDA content in transgenic and untransformed plants before and during salt stress. The results showed that there were no significant differences in MDA content between the two before imposing stress (Fig. 9). One day after salt stress, lipid peroxidation levels increased in both transgenic and untransformed plants, but the content of MDA in each transgenic line was significantly lower than in the untransformed plants even after 4 days. Analysis of variance showed that there was no great difference in MDA content among different transgenic lines till 4th day (Fig. 9).

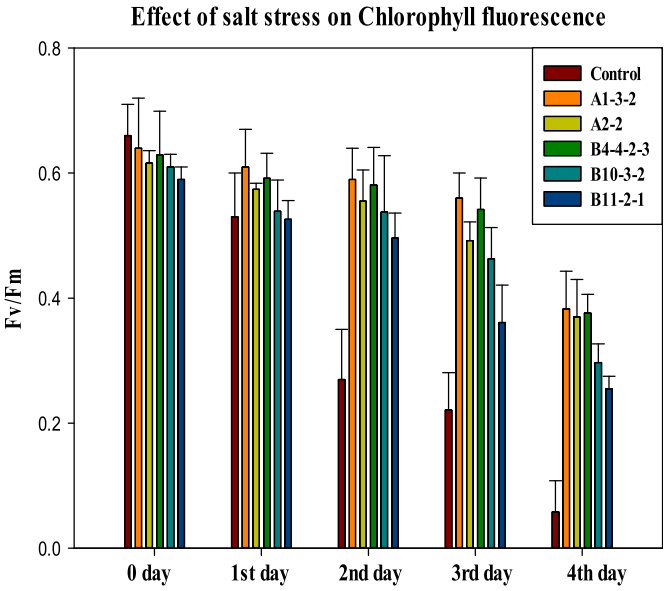

Fig. 8. Chlorophyll fluorescence of untransformed control and different transgenic lines A1-3-2, A2-2, B4-4-2-3, B10-3-2, B11-2-1 under 100 mM salt stress. Vertical bars represent *SE* (n = 4). Significant difference between means at the (P < 0.05) level determined by Tukey's *post-hoc* test (ANOVA).

Table 2
Effect of 100 mM NaCl stress on total chlorophyll content (expressed as μg/g fresh weight of tissue) in untransformed and transgenic lines.

Plants	Day 0	Day 1	Day 2	Day 3	Day 4
Untransformed control	6 (+0.2)	5.8 (+0.1)	4.2 (+0.5)*	3.8 (+0.3)*	2.1 (+0.6)*
$A_{1\text{-}3\text{-}2}$	6.5 (+0.4)	6.4 (+0.6)	5.6 (+0.3)**	5.3 (+0.5)**	4.7 (+0.2)**
$A_{2\text{-}2}$	6.5 (+0.5)	6.2 (+0.4)	5.5 (+0.6)**	4.6 (+0.4)**	4.4 (+0.3)**
$B_{4\text{-}4\text{-}2\text{-}3}$	6.5 (+0.2)	6.3 (+0.4)	5.5 (+0.5)**	4.9 (+0.3)**	4.5 (+0.4)**
$B_{10\text{-}3\text{-}2}$	6.1 (+0.3)	5.9 (+0.5)	5.3 (+0.2)**	4.6 (+0.6)**	4.3 (+0.9)**
$B_{11\text{-}2\text{-}1}$	6.1 (+0.2)	5.9 (+0.4)	4.9 (+0.5)**	4.5 (+0.7)**	4.1 (+0.5)**

*indicates significant difference at $p < 0.05$ and **indicates significant difference at $p < 0.01$ by Tukey's *post-hoc* test (ANOVA) from the mean values ± standard error ($n = 6$).

3.7. Antioxidant enzyme activities

No differences in the activity of SOD were observed in transgenics when compared to untransformed plants devoid of stress. Under salt stress, slight increase in SOD activity was noticed in transgenic lines after day 1. However, the activity of SOD decreased in transgenics and untransformed plants after 2 days of salt stress. But the amplitude of decrease is significantly higher in untransformed plants than the transformants (Fig. 10). Thereafter, on progression of the stress, SOD activity decreased by 58% in salt stressed untransformed plants. In contrast, SOD activity in transgenic lines decreased by only 24%. Catalase activity in untransformed plants declined significantly under salt stress right from day 1 in comparison to their corresponding controls and reached the lowest levels (0.22–0.012 units mg^{-1} protein) by 4th day of stress (Fig. 11). Catalase activity increased for 3 days (0.25–0.38 units mg^{-1} protein) during the course of salt stress but declined to 0.023 units in transgenic lines by day 4. Glutathione reductase (GR) that catalyses the NADPH-dependent reduction of oxidized glutathione did not show any difference in its activity both in transgenic lines and untransformed plants under normal conditions. The decrease in GR activity was significantly more in untransformed plants throughout the experiments compared to *P5CS*-transgenic lines (Fig. 12). The effect of salinity impacted the GR activity both in transgenic lines and untransformed plants. After 4 days of stress treatment, GR activity decreased by 84% in salt stress conditions in untransformed plants whereas transgenic lines showed only 52% decrease.

4. Discussion

We report an efficient and reproducible genetic transformation for sorghum through *Agrobacterium*-mediated and microprojectile bombardment methods using shoot apices-derived callus. Genotype, type of explant and age of callus greatly influence plant regeneration frequency in sorghum and also genetic transformation as reported by Gao et al. (2006). Our experiments indicated that genotypic differences exist for shoot regeneration in sorghum. Also, shoot apices-derived callus was found better for regeneration of shoots compared to immature inflorescences and mature embryos (Jogeswar, 2005). Sorghum in general has been regarded as recalcitrant both for plant regeneration and genetic transformation efficiency. In the present study, however, shoot apices were preferred since the regeneration frequency from shoot apex derived callus was higher and also the transformation frequency. In this study, transgenic nature of plants was confirmed by PCR amplification of *hptII* and *P5CSF129A* genes in the host genome. Further, Southern blot hybridizations confirmed not only integration of *P5CSF129A* gene but also its copy number (2–3 copies) and RT-PCR, the expression of introduced genes at mRNA level. Out of 11 transgenics lines containing the *P5CS* transgene, five plants were selected for salt stress treatment based on free proline content.

4.1. Salt stress tolerance of transgenic plants

The key metabolic event, photosynthesis, which is essential for growth and development of plants, is sensitive to salt stress. The loss in the chlorophyll content accompanied by inactivation of photochemical reactions especially those mediated by PSII is known in plants exposed to salt, drought and low temperature stresses (Fedina et al., 1993). Investigations were carried out to determine the photosynthetic efficiency, in terms of chlorophyll content and PSII activity of leaves of untransformed and *P5CSF129A*-transgenic lines exposed to salt stress. After 4-days of exposure to 100 mM NaCl, the leaves of untransformed plants showed a decline in chlorophyll content by 65%, in contrast to 33% loss in chlorophyll content exhibited by transgenic lines. Increased photosynthetic pigment content might be due to high proline levels in the transgenic lines. The protective effect of proline to the damage caused by salt stress sustains the hypothesis of its role in scavenging ROS especially •OH radical.

The decline in PSII activity in untransformed plants with the time of exposure to salt stress was significantly faster in comparison to that noted in transgenics. The decrease in PSII activity in presence of salt stress can primarily be accounted to destabilization of the pigment protein complexes as pointed out by Fedina et al. (1993) and dissociation of extrinsic proteins from water oxidation complex (Papageorgiou and Murata, 1995). Because of stomatal closure in salt stress conditions, photosynthetic activity decreased considerably as has been shown earlier by Lawlor and Cornic (2002). But in proline over-producing transgenic sugarcane lines, lesser damage in photosynthetic apparatus was noticed compared with untransformed

Table 3
Effect of 100 mM NaCl stress on carotenoid content (expressed as μg/g fresh weight of tissue) in untransformed controls and transgenic lines (the values are not significantly different).[a]

Plants	Day 0	Day 1	Day 2	Day 3	Day 4
Untransformed control	2.1 (±0.2)	1.8 (±0.7)	1.6 (±0.5)	1.5 (±0.6)	1 (±0.4)
A_1	2.4 (±0.7)	2.3 (±0.4)	1.9 (±0.5)	1.8 (±0.5)	1.7 (±0.2)
$A_{1\text{-}3\text{-}2}$	2.3 (±0.5)	2.0 (±0.7)	1.8 (±0.4)	1.7 (±0.5)	1.6 (±0.3)
$B_{4\text{-}4\text{-}2\text{-}3}$	2.3 (±0.4)	2.1 (±0.8)	1.9 (±0.5)	1.8 (±0.2)	1.6 (±0.6)
$B_{10\text{-}3\text{-}2}$	2.2 (±0.8)	2.0 (±0.5)	1.8 (±0.6)	1.7 (±0.4)	1.4 (±0.2)
$B_{11\text{-}2\text{-}1}$	2.3 (±0.5)	1.9 (±0.6)	1.7 (±0.1)	1.6 (±0.3)	1.4 (±0.4)

[a] Values represent data taken from 6 replicates.

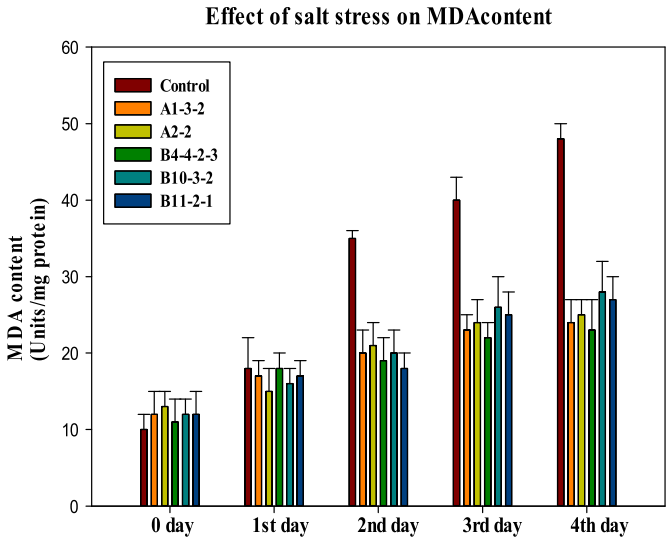

Fig. 9. MDA content of untransformed control and different transgenic lines A1-3-2, A2-2, B4-4-2-3, B10-3-2, B11-2-1 under 100 mM salt stress. Vertical bars represent *SE* (n = 4). Significant difference between means at the (P < 0.05) level determined by Tukey's *post-hoc* test (ANOVA).

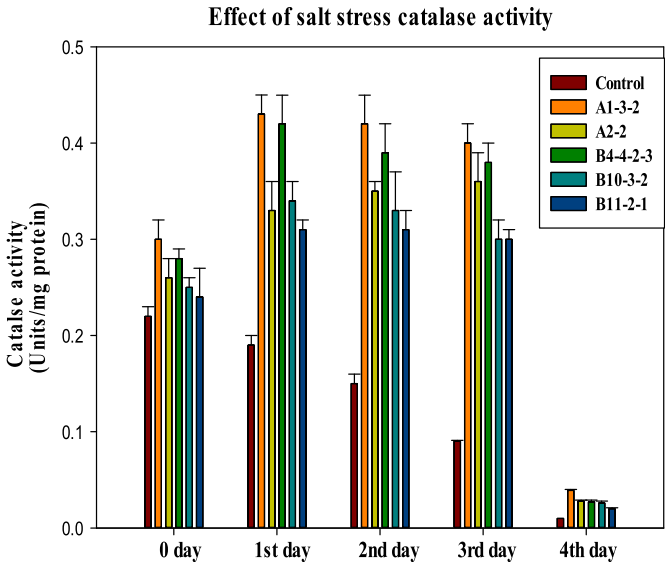

Fig. 11. Catalase activity of untransformed control and different transgenic lines A1-3-2, A2-2, B4-4-2-3, B10-3-2, B11-2-1 under 100 mM salt stress. Vertical bars represent *SE* (n = 4). Significant difference between means at the (P < 0.05) level determined by Tukey's *post-hoc* test (ANOVA).

control plants. The photochemical efficiency of PSII was higher by 65% in transgenic sugarcane at the end of water deficit experiments compared to untransformed controls (Molinari et al., 2007). They also opined that proline protected the plants against the oxidative stress caused by water deficit. Sivakumar et al. (2000) showed the involvement of proline in the protection of thylakoid membranes against free radical induced photodamage. This lends support to our earlier observation that proline may protect chlorophyll *a* as well as chlorophyll *b* against salt induced damage by scavenging reactive oxygen species (Sarvesh et al., 1996). In the present study, total chlorophyll content (Table 2) and chlorophyll fluorescence measurements (Fig. 8) in proline overproducing transgenic sorghum lines indicated lower damage in photosynthetic apparatus when compared with untransformed control plants. The maintenance of Fv/Fm ratio may be due to high chlorophyll, as well as decreased lipid peroxidation (Cui et al., 2006). No significant difference was observed in the PSII quantum yield in transgenic events during salt stress. These findings clearly demonstrated that the introduction of *P5CSF129A* gene lead to an increase in the tolerance of transgenic sorghum plants to salt stress.

4.2. Leaf gas exchange in the transgenics under stress

The decline in the transpiration rate in untransformed plants was higher than in the transgenic lines across salt stress experiment, indicating the capacity of transgenic plants to maintain a high rate of transpiration. This suggests a possible causal relation between a high proline level and the capacity to maintain stomata

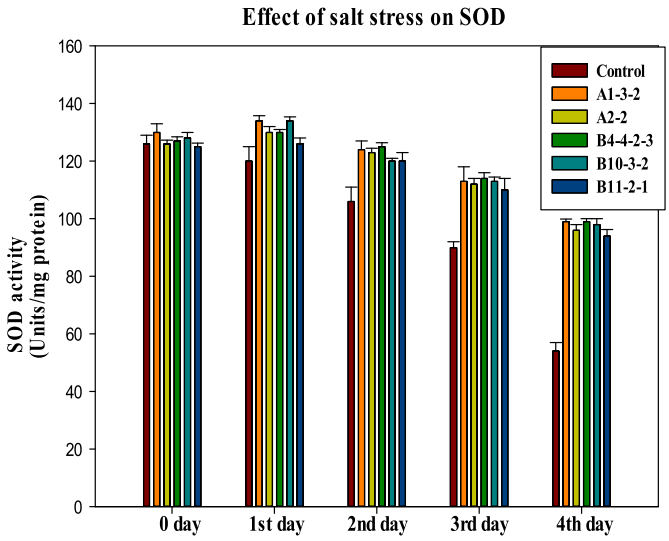

Fig. 10. SOD activity of untransformed control and different transgenic lines A1-3-2, A2-2, B4-4-2-3, B10-3-2, B11-2-1 under 100 mM salt stress. Vertical bars represent *SE* (n = 4). Significant difference between means at the (P < 0.05) level determined by Tukey's *post-hoc* test (ANOVA).

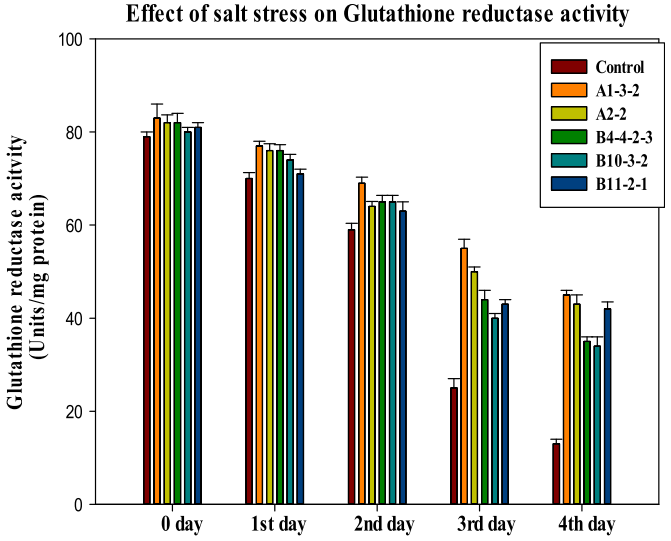

Fig. 12. GR activity of untransformed control and different transgenic lines A1-3-2, A2-2, B4-4-2-3, B10-3-2, B11-2-1 under 100 mM salt stress. Vertical bars represent *SE* (n = 4). Significant difference between means at the (P < 0.05) level determined by Tukey's *post-hoc* test (ANOVA).

in open condition in dryer soils that very well fits the expected role of proline in maintaining turgidity of the leaf cells including stomatal guard cells. Our results are in line with a few other reports where the overproduction of proline led to increased tolerance to water stress based on one or more parameters like total water potential, osmotic and membrane potentials, membrane stability and MDA content, root length and weight, seed and capsule numbers etc. (Kishor et al., 1995; Vendruscolo et al., 2007). On an average, *A*, *TE* and g_s were reduced by about 61%, 64% and 79% respectively, in the salt stressed transgenics. By contrast, all these gas exchange parameters were significantly inhibited in untransformed plants under salt stress conditions towards the termination of the experiment. The lowered leaf chlorophyll content in untransformed control plants might have contributed to the decrease of net photosynthesis. Furthermore, the similarity between the patterns of *A*, *TE* rates and g_s revealed the correlation between all these parameters expressed *via* the high relationships determined between them. The effect of salinity stress on intercellular CO_2 concentration in transgenic lines was not high. But in untransformed plants, C_i concentration decreased with exposure to salt stress. Koyro (2006) reported that gas exchange properties such as net photosynthesis and water use efficiency were strongly affected by salt stress and has also attributed the reduction in net photosynthesis in salt stressed plants to stomatal limitations. It is important to assess how *TE* was affected by the overproduction of proline in these transformants.

4.3. Antioxidant enzymes and abiotic stress

Alleviation of oxidative damage by scavenging ROS, with the use of antioxidant enzymes, is an important strategy of plants for increasing stress tolerance. Hoque et al. (2007) verified positive correlation between proline accumulation and enhancement of antioxidant enzyme activity in tobacco cells submitted to salinity stress. In the present study, negative influence of salinity stress was observed on photosystem II exhibiting variation between transgenic lines and untransformed control plants. Proline has been considered not only as a compatible solute and osmoprotectant but also a hydroxyl radical scavenger. Under osmotic stress, proline stabilizes proteins, membranes, and subcellular structures and protects cellular functions by scavenging ROS. It appears from our study that proline may play the same role and in the improved growth of sorghum under stress. Transgenic plants overexpressing the *P5CS* gene produced a high level of the antioxidant enzymes and synthesized more proline than the controls. The transformants tolerated salt stress and showed enhanced root biomass under greenhouse conditions. Enhanced proline levels in transgenic plants suggests that *P5CS* gene activity is the rate-limiting step for its accumulation (Kishor et al., 2005). In addition to synthesis, proline catabolism and transport are thought to control endogenous proline accumulation and energy levels in plants (Kishor et al., 2005; Kishor and Sreenivasulu, 2014). Transport of proline to root mitochondria and its degradation there results in higher release of NAD(P)H which might help in improving root biomass. Salt tolerance due to overexpression of the *P5CS* gene has also been reported in transgenic rice and pigeonpea earlier (Zhu et al., 1998; Surekha et al., 2014).

In the present study, decreased activities of SOD in salt-treated plants indicate that SOD could not play a crucial role in scavenging superoxide radicals during salinity stress. However, in transgenics, the dismutation of $O_2^{-\cdot}$ was high compared to wild-type plants. Exogenous application of proline protected cell membranes from salt-induced oxidative stress by enhancing the activities of various antioxidant enzymes (Yan et al., 2000). Our results show that SOD activity is well protected in transgenics by high proline levels unlike that of controls. In the present study, untransformed control and transgenic lines showed differential CAT activity in response to salt stress. In *Pancratium maritimum* L., salt stress resulted in the inhibition of antioxidant enzymes CAT and peroxidase, but activities of these enzymes were also maintained significantly higher in the presence of proline. It is concluded that proline improves the salt-tolerance of *P. maritimum* L. Exogenous proline increased the catalase activity in olive tree leaves (Ahmed et al., 2010). Their results indicate that high proline levels may protect antioxidant enzyme machinery and thus protect the plants from oxidative damage under stress conditions. The decline in GR activity was more in untransformed plants than the transgenic lines. Our data show that activities of SOD, CAT and GR enzymes in leaves of transgenic plants were higher compared to untransformed plants under salt stress. Since a major role of these antioxidant enzymes is scavenging of ROS that are produced due to salt stress, it can be concluded that proline accumulation might play a critical role in protecting antioxidant activities under salt stress. In plants, proline may also scavenge singlet oxygen and free radicals that induce damages, and performs an important role in the protection of proteins against denaturation (Alia et al., 1997). Increased proline content and improved tolerance to abiotic stresses, in salt-stressed calli of *Suaeda nudiflora*, a halophytic plant, suggested that proline protects the callus from membrane damage caused by free radicals during salt stress (Cherian and Reddy, 2003). Our data strongly suggest that salt stress can induce membrane lipid peroxidation resulting in membrane fluidity, leading to enhanced electrolytic leakage in wild type and transgenic lines. However, transgenic lines exhibited relatively less membrane injury and lipid peroxidation levels compared to the untransformed control plants. Higher proline levels in transgenics might result in less accumulation of MDA in transgenics as seen in the present study compared to higher MDA levels in untransformed control plants. This confirms the earlier observations on the production of free radicals under salinity stress where a significant negative correlation was observed between the proline levels and MDA production (Alia et al., 1993). It has been reported that higher proline accumulation in *P5CS*-transformed plants (Vendruscolo et al., 2007) might have reduced the free radical levels as measured by MDA content in response to osmotic stress. These observations suggest that the elevated proline reduces the levels of free radicals in response to osmotic stress, thereby, significantly improving the ability of the transgenic plants to better survive under stress. .

5. Conclusion

Our results support the hypothesis that proline accumulated in sorghum transgenic events might have contributed for better chlorophyll protection, increased water use efficiency and protection of antioxidant activities.

Author contribution

Conceived and designed experiments: RKV, PBK.
Performed the experiments: PSR, GKR, GJ, ARR, MM.
Analyzed the data: PSR, GJ, PBK.
Wrote the manuscript: PSR, RKV, ARR, GJ, PBK.

Acknowledgments

The authors are grateful for financial assistance from Netherlands through AP-Netherlands Project; UGC, New Delhi, for sanctioning funds through Center for Advanced Studies in Genetics, and to DBT, New Delhi, for sanctioning ISLARE program. PBK is also thankful to the CSIR, New Delhi [21 (0934)/12/EMR-II], for sanctioning Emeritus fellowship.

References

Ahmed, C.B., Rouina, B.B., Sensoy, S., Boukhriss, M., Abdullah, F.B., 2010. Exogenous proline effects on photosynthetic performance and antioxidant defense system of young olive tree. J. Agric. Food Chem. 58, 4216–4222.

Alia, Mohanty, P., Matysik, J., 2001. Effect of proline on the production of singlet oxygen. Amino Acids 21, 195–200.

Alia, Saradhi, P.P., Mohanty, P., 1993. Proline in relation to free radical production in seedlings of *Brassica juncea* raised under sodium chloride stress. Plant Soil 155, 497–500.

Alia, Saradhi, P.P., Mohanty, P., 1997. Involvement of proline in protecting thylakoid membranes against free radical-induced photo damage. J. Photochem. Photobiol. 38, 253–257.

Apel, K., Hirt, H., 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. 55, 373–399.

Arnon, D.I., McSwain, B.D., Tsujimoto, H.Y., Wada, K., 1974. 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.

Bates, L.S., waldren, R.P., Teare, I.D., 1973. Rapid determination of free proline for water studies. Plant Soil 39, 205–208.

Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. Rev. 44, 276–287.

Cherian, S., Reddy, M.P., 2003. Evaluation of NaCl tolerance in the callus cultures of *Suaeda nudiflora*. Moq. Biol. Plant 46, 193–198.

Cui, L., Li, J., Fan, Y., Xu, S., Zhang, Z., 2006. High temperature effects on photosynthesis, PS II functionality and antioxidant activity of two *Festuca arundinacea* cultivars with different heat susceptibility. Bot. Stud. 47, 61–69.

de Campos, M.K.F., de Carvalho, K., de Souza, F.S., Marur, C.J., Pereira, L.F.P., Filho, J.C.B., Vieira, L.G.E., 2011. Drought tolerance and antioxidant enzymatic activity in transgenic 'Swingle' citrumelo plants over-accumulating proline. Env. Exp. Bot. 72, 242–250.

Doyle, J.J., Doyle, J.L., 1990. Isolation of plant DNA from fresh tissue. Focus 12, 12–14.

Fedina, I.S., Tsonev, T.S., Guleva, E.I., 1993. The effect of pre-treatment with proline on the responses of *Pisum sativum* to salt stress. Photosynthetica 29, 521–527.

Foyer, C.H., Halliwell, B., 1976. The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta 133, 21–25.

Fu, J., Huang, B., 2001. Involvement of antioxidants and lipid peroxidation in the adaptation of two cool-season grasses to localized drought stress. Environ. Exp. Bot. 45, 105–114.

Gao, C., Jiang, L., Folling, M., Han, L., Nielsen, K.K., 2006. Generation of large numbers of transgenic Kentucy bluegrass (*Poa pratensis* L.) plants following biolistic gene transfer. Plant Cell. Rep. 25, 19–25.

Hong, Z., Lakkineni, K., Zhang, Z., Verma, D.P.S., 2000. Removal of feedback inhibition of delta-1-pyrroline-5-carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. Plant Physiol. 122, 1129–1136.

Hoque, M.N., Okuma, E., Banu, M.N.A., Nakamura, Y., Shimoishi, Y., Murata, Y., 2007. Exogenous proline mitigates the detrimental effects of salt stress more than exogenous betaine by increasing antioxidant enzyme activities. J. Plant Physiol. 164, 553–561.

Jogeswar, G., 2005. *In vitro* Plant Regeneration and Genetic Transformation for Abiotic Stress Tolerance in *Sorghum bicolor* L. Moench (Ph.D. thesis submitted to the Osmania University, Hyderabad, India).

Kishor, P.B.K., Hong, Z., Miao, G., Hu, C., Verma, D.P.S., 1995. Overexpression of Δ^1-pyrroline 5-carboxylate synthetase increases proline overproduction and confers osmotolerance in transgenic plants. Plant Physiol. 108, 1387–1394.

Kishor, P.B.K., Sangam, S., Amruta, R.N., Sri laxmi, P., Naidu, K.R., Rao, K.R.S.S., Rao, S., Reddy, K.J., 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.

Kishor, P.B.K., Sreenivasulu, N., 2014. Is proline accumulation *per se* correlated with stress tolerance or is proline homeostasis a more critical issue? Plant. Cell. Environ. 37, 300–311.

Koyro, H.W., 2006. Effect of salinity on growth, photosynthesis, water relations and solute composition of the potential cash crop halophyte *Plantago coronopus* (L.). Env. Exp. Bot. 56, 136–146.

Kramer, P.J., Boyer, J.S., 1995. Water Relations of Plants and Soils. Published by Academic Press, San Diego.

Lawlor, D.W., Cornic, G., 2002. Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. Plant Cell. Environ. 89, 871–885.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Luck, H., 1974. Catalase. In: Bergmeyer, H.U. (Ed.), Methods in Enzymatic Analysis. Academic Press, New York, pp. 885–894.

Matysik, J., Alia, A., Bhalu, B., Mohanty, P., 2002. Molecular mechanisms of quenching of reactive oxygen species by proline under stress in plants. Curr. Sci. 82, 525–532.

Molinari, H.B.C., Marur, C.J., Daros, E., Campos, M.K.F., Carvalho, J.F.R.P., Filho, J.C.B., Pereira, L.F.P., Vieira, L.G.E., 2007. Evaluation of the stress-inducible production of proline in transgenic sugarcane: osmotic adjustment, chlorophyll fluorescence and oxidative stress. Physiol. Plant 130, 218–229.

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

Ozden, M., Demirel, U., Kahraman, A., 2009. Effects of proline on antioxidant system in leaves of grapevine (Vitis vinifera L.) exposed to oxidative stress by H_2O_2. Sci. Hortic. 119, 163–168.

Papageorgiou, G.C., Murata, N., 1995. The unusually strong stabilizing effects of glycine betaine on the structure and function of the oxygen-evolving photosystem II complex. Photosyn. Res. 44, 243–252.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sarvesh, A., Anuradha, M., Pullaiah, T., Reddy, T.P., Kavi Kishor, P.B., 1996. Salt stress and antioxidant response in high and low proline producing cultivars of niger, *Guizotia abyssinica* (L.F. Cass. Ind. J. Exp. Biol. 34, 252–256.

Signorelli, S., Arellano, J.B., Melo, T.B., Borsani, O., Monza, J., 2013. Proline does not quench singlet oxygen: evidence to reconsider its protective role in plants. Plant Physiol. Biochem. 64, 80–83.

Sivakumar, P., Sharmila, P., Saradgi, P.P., 2000. Proline accumulation alleviates salt-stress induced enhancement in ribulose-1,5-bisphosphate oxygenase activity. Biochem. Biophys. Res. Commun. 279, 512–515.

Surekha, C., Nirmala Kumari, K., Aruna, L.V., Suneetha, G., Arundhati, A., Kishor, P.B.K., 2014. Expression of the *Vigna aconitifolia P5CSF129A* gene in transgenic pigeonpea enhances proline accumulation and salt tolerance. Plant Cell. Tiss. Organ Cult. 116, 27–36.

Vendruscolo, E.C.G., Schuster, I., Pileggi, M., Scapim, C.A., Molinari, H.B.C., Marur, C.J., Vieira, L.G.E., 2007. Stress-induced synthesis of proline confers tolerance to water deficit in transgenic wheat. J. Plant Physiol. 164, 1367–1376.

Yamaguchi-Shinozaki, K., 2001. Biological Functions of Proline Osmotolerance Revealed in Antisense Transgenic Plants. JIRCAS Newslett. 27.

Yan, H., Gang, L.Z., Zhao, C.Y., Cuo, W.Y., 2000. Effects of exogenous proline on the physiology of soybean plantlets regenerated from embryos *in vitro* and on the ultrastructure of their mitochondria under NaCl stress. Soybean Sci. 19, 314–319.

Zhu, B., Su, J., Chang, M., Verma, D.P.S., Fan, Y.L., Wu, R., 1998. 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.