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ORIGINAL ARTICLE

Pea p68, a DEAD-box helicase, enhances salt tolerance in marker-free transgenic plants of soybean [*Glycine max* (L.) Merrill]

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Abstract

Protein p68 is a prototype constituent of DEAD-box protein family, which is involved in RNA metabolism, induced during abiotic stress conditions. In order to address the salinity stress faced by economically important soybean crop, we have transformed soybean cv. PUSA 9712 via direct organogenesis with marker free construct of *p68* gene by *Agrobacterium*-mediated genetic transformation. The putative transgenic plants were screened by Polymerase chain reaction (PCR), Dot blot analysis and Southern blot hybridization. Reverse transcriptase-PCR (RT-PCR) and Quantitative real-time PCR (qRT-PCR) established that the *p68* gene expressed in three out of five southern positive (T_1) plants. The transformed (T_1) soybean plants survived irrigation upto 200 mM of NaCl whereas the non-transformed (NT) plants could not survive even 150 mM NaCl. The transgenic soybean (T_1) plants showed a higher accumulation of chlorophyll, proline, CAT, APX, SOD, RWC, DHAR and MDHAR than the NT plants under salinity stress conditions. The transformed (T_1) soybean plants also retained a higher net photosynthetic rate, stomatal conductance and CO_2 assimilation as compared to NT plants. Further analysis revealed that (T_1) soybean plants accumulated higher K^+ and lower Na^+ levels than NT plants. Yield performance of transformed soybean plants was estimated in the transgenic green house under salinity stress conditions. The transformed (T_1) soybean plants expressing the *p68* gene were morphologically similar to non-transformed plants and produced 22–24 soybean pods/plant containing 8–9 g (dry weight) of seeds at 200 mM NaCl concentration. The present investigation evidenced the role of the *p68* gene against salinity, by enhancing the tolerance towards salinity stress in soybean plants.

Keywords Soybean · *Agrobacterium tumefaciens* · EHA105 · *p68* gene · Salinity · CaMV 35S promoter · Binary vector · qRT-PCR

Abbreviations

NaCl	Sodium chloride
CaMV 35S	Cauliflower mosaic virus 35S promoter
APX	Ascorbate peroxidase
CAT	Catalase
SOD	Superoxide dismutase
DHAR	Dehydroascorbate reductase
MDHAR	Monodehydroascorbate reductase
MDA	Malondialdehyde
RWC	Relative water content

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Introduction

Soybean [*Glycine max* (L.) Merrill] is an annual food crop native to East Asia, Japan and China that belongs to the family *Leguminosae* or *Fabaceae*. It is widely cultivated as a source of edible oil and protein in tropical and subtropical regions. USA is the leading producer of soybean (82.11 million metric tonnes) followed by Brazil, Argentina and China. India holds the fifth position for soybean production in the world and produces 8.6 million metric tonnes per year (FAOSTAT 2016). It has a high nutrient content and mature dry soybean seeds consist of 36.49% of protein, 19.94% of fat, 4.87% of ash, 30.16% of carbohydrate, 9.3% of fiber, 7.33% of sugars, minerals, vitamins, amino acids, isoflavones and lipids (USDA 2018). The protein and

fiber content in soybean can be of help in preventing high blood sugar levels (Birt et al. 2004). Soybean seeds are also rich in isoflavones such as genistein and daidzein, which were used in the treatment of pancreatic cancer (McCue and Shetty 2004). Soybean oil is used for culinary purposes in margarine, bread, salad dressing and a few other food products like soy milk, soy meal and soy flour. Apart from food products, it has also been used in numerous industrial applications such as paints, hydraulic fluids, printing inks, building materials, pharmaceuticals, cosmetics and plastics (Soy Stats 2011).

Abiotic stresses such as salinity, drought, high or low temperature, cold, radiation, and heavy metal contamination of soil limit agricultural productivity. Amongst them, salinity stress adversely affects plant growth, reducing both crop productivity and quality. Around the world, soil salinity occurs generally in semi-arid and arid regions, due to natural build up or by irrigation with saline water (Meloni et al. 2004). The incidence of excess salts in soil interferes with nutrient uptake and water required by the plant in the crop root zone, thereby decreasing crop development and yield (Bauder and Brock 2001; Hanson et al. 1999). The higher levels of sodium and chloride ions, in saline soil exert adverse conditions in plants such as nutrition disorders, ion toxicity, oxidative stress, membrane degradation and alteration of metabolic processes (Hasegawa et al. 2000; Munns 2002). Globally, high salinity occurrence in soil has damaged more than 45 million hectares of irrigated land, and 1.5 million hectares are becoming uncultivable every year (Munns and Tester 2008).

Soybean is classified as a moderately tolerant crop to salinity stress (Munns and Tester 2008; Guan et al. 2014), with a threshold of 0.5 S m^{-1}, beyond which growth is markedly reduced (Maas and Hoffman 1977). Guan et al. (2014) have identified that *GmSALT3* confers the observed moderate tolerance to salinity in soybean. Salinity affects the root nodulation and reduces the number and biomass of roots, which in turn reduces the nitrogen-fixing ability of soybean (Elsheikh and Wood 1995). However, under increased salinity stress conditions, various biochemical and metabolic alterations are evidenced, which includes ionic imbalance, alterations in the synthesis of specific proteins, accumulation of osmotically active compounds and induction of a series of biochemical and physiological responses in plants, such as repression of cell growth, stomatal closure, loss in photosynthesis, activation of respiration, and reduced yield (Subramanyam et al. 2012). In such adverse conditions, free-radical scavengers, osmoprotectants, heat shock proteins, late embryogenesis abundant (LEA) proteins and chaperones activate important stress response mechanisms in alleviating plant stress (Khan et al. 2015).

The *p68* helicase is one of the prototype members of 'DEAD-box' protein (Linder et al. 1989). Helicase enzymes are known to unwind energetically stable duplex DNA and RNA secondary structures (DNA and RNA helicases), respectively. The *p68* enzyme is an RNA helicase that efficiently unwinds dsRNA in both 3′–5′ and 5′–3′ directions (Tuteja and Tuteja 2004a, b; Tuteja and Pradhan 2006; Huang and Liu 2002). RNA helicases play essential roles in RNA metabolism, including mRNA repair, transcription, pre-mRNA and pre-rRNA processing, gene splicing, editing, regulation of RNA stability and translation, which regulates plant growth and development (Tuteja 2003; Hoi and Tuteja 2012; Guan et al. 2013; Tuteja et al. 2014). It belongs to the largest family of RNA helicases with a molecular mass of 68 kDa protein (Hoi and Tuteja 2012). Pradhan et al. (2005b) reported that the helicase and the ATPase activity of *p68* RNA helicase is stimulated following stimulation by protein kinase C in *Plasmodium falciparum*, a human malaria parasite.

The *p68* DEAD-box helicase confers tolerance to salinity stress by reducing oxidative stress, controlling the reactive oxygen species (ROS) and improving photosynthesis machinery in tobacco (Tuteja et al. 2014). Pea DNA helicases (PDH47 and PDH45) are reportedly induced by a range of abiotic stress, including salinity stress in shoots and roots, ABA treatment in roots, dehydration and wounding (Vashisht et al. 2005; Hoi and Tuteja 2012). Expression of *p68* DNA helicase (PDH45) reportedly enhanced salinity stress tolerance in tobacco and rice, respectively (Sanan-Mishra et al. 2005; Gill et al. 2013). A DEAD-box RNA helicase (OsBIRH1) showed increased tolerance against pathogens and oxidative stresses in rice (Li et al. 2008). DEAD-box RNA helicase family member, OsSUV3, was found to play a key role in the enhancement of salinity stress tolerance (Tuteja et al. 2013). A *p68* DEAD-box RNA helicase (AtDRH1) transcript from *Arabibopsis thaliana* was described to be stored at an immense level, nearly in all the parts of the plant (Okanami et al. 1998). Proteins ZmDRH1 (*Z. mays* DEAD-box RNA helicase 1) and MA16 (maize RNA-binding protein) were found to be involved in ribosomal RNA (rRNA) metabolism and are speculated to be a part of the ribonucleoprotein complex (Gendra et al. 2004).

Hence, for the first time, in the present study, *p68* gene (marker free construct) from Pea *(Pisum sativum)*, has been transferred into the soybean with the help of *Agrobacterium tumefaciens* strain EHA105, to confer enhanced tolerance to salinity stress, following which the plant characteristics and yield analysis have been carried out for transformed and non-transformed plants comparatively.

Materials and methods

Plant material and seed source

Seeds of soybean cultivar cv. Pusa 9712 (Fig. 1a) were used in the present investigation. The cv. Pusa 9712 is

appropriate for growing under normally sown irrigated conditions and has an average yield of 20.5 q/ha. It matures in 116 days and shows significant resistance to soybean mosaic virus, yellow mosaic virus, charcoal rot, bacterial pustule, stem fly and myrothecium leaf spot. The seeds were procured from the Indian Agricultural Research Institute (IARI), Pusa Campus, New Delhi, India. The seeds were germinated and maintained in the greenhouse facility, Department of Biotechnology, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India during the suitable season.

Seed surface sterilization and preparation of explants

The seeds of cv. Pusa 9712 were surface sterilized by exposing them to chlorine gas for 16 h (Di et al. 1996), which was achieved by combining 3.5 ml of 12N HCl (Qualigens, Mumbai, India) and 100 ml of 5.25% sodium hypochlorite (Qualigens, Mumbai, India) in a tightly sealed vacuum desiccator along with seeds (Tarsons Product Pvt. Ltd, Kolkata, India). The surface sterilized seeds were then imbibed in an Erlenmeyer flask containing sterile distilled water (Fig. 1b), incubated in total darkness at 25 ± 2 °C on an orbital shaker (Orbitek-Scigenics Biotech Pvt.Ltd, Chennai, India) at 120 rpm for one day. Following, one-half of the imbibed seed cotyledons with attached embryonic axis (Fig. 1c) were used as explants for genetic transformation mediated by *Agrobacterium tumefaciens*, and the transgenic plants were regenerated through direct organogenesis (Arun et al. 2016).

Agrobacterium strain and construction of binary vector

Agrobacterium strain EHA105 (kindly provided by Rafael Perl Treves, Bar-Ilan University, Israel) accommodating binary vector pCAMBIA 1300-*p68* was used in the present study. The binary vector (Supplementary Fig. 1) contains *p68* gene expression cassette composed of *p68* gene under the regulation of CaMV 35S promoter and the nopaline synthase (*nos*) terminator within the T-DNA, and *neomycin phosphotransferase* gene (*npt* II) cassette for bacterial selection in the backbone. The *hygromycin phosphotransferase* gene (*hpt* II) fragment of T-DNA, was removed from the transformation vector (Supplementary Fig. 1) by restricting with *XhoI* so as to achieve marker free transformation. The EHA105 strain harboring pCAMBIA 1300-*p68* was maintained on solid AB agar medium (*Agrobacterium* minimal medium) supplemented with 50 mg l^{-1} kanamycin (SRL, Mumbai, India) and 50 mg l^{-1} rifampicin (SRL, Mumbai, India).

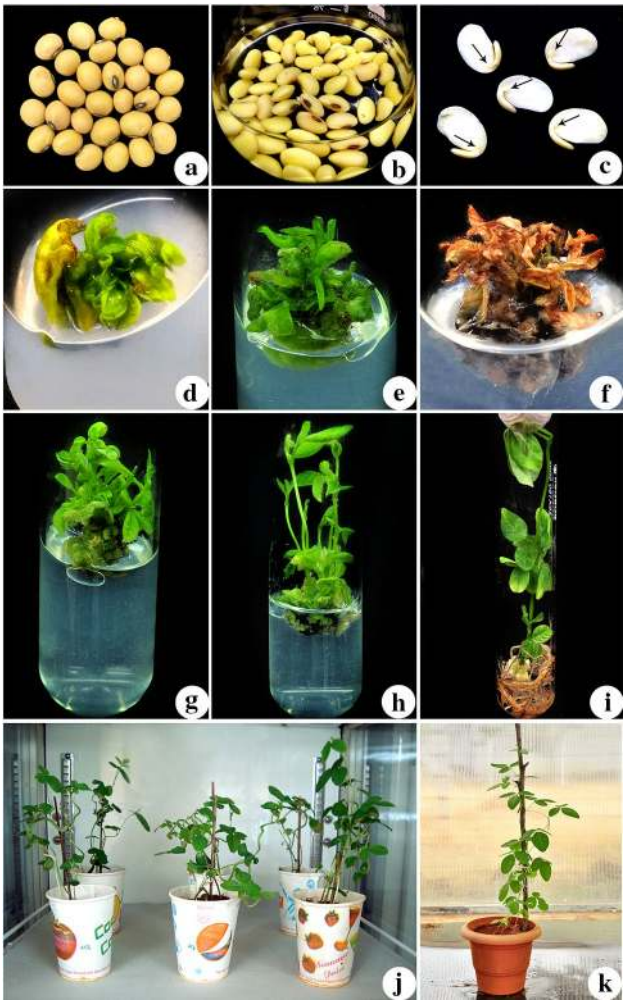

Fig. 1 Transformation of cv.Pusa 9712 half seed explants by *Agrobacterium tumefaciens* strain EHA105 harboring binary vector pCAMBIA1300-*p68* to enhance salinity stress tolerance. **a** mature dry seeds of soybean cv. Pusa 9712 used for preparing the half seed explants; **b** 1-day old imbibed seeds; **c** half seed explants made from imbibed seeds (black arrows direct the embryonic area); **d** explant after infection with *A. tumefaciens* EHA105 carrying pCAMBIA1300-*p68* plasmid and co-cultivated for 5 days in the dark; **e** shoot induction from explant in SIM supplemented with cefotaxime (200 mg l^{-1}) without NaCl (after 15 days of culture); **f**, **g** selection of regenerated shoots in SIM containing cefotaxime (200 mg l^{-1}) and NaCl (75 mM) [after 30 days of culture]; **h** elongated shoots in SEM supplemented with cefotaxime (100 mg l^{-1}) and NaCl (75 mM) [after 30 days of culture]; **i** rooted shoots in RIM supplemented with cefotaxime (100 mg l^{-1}) and NaCl (50 mM) [after 30 days of culture]; **j** putatively transformed (T_o) plants maintained in growth chamber; **k** acclimatization of survived (T_o) plants under green house condition

Sensitivity of half seed explants and seed germination to NaCl

Half seed explants were inoculated on shoot induction medium [SIM: MS salts, MSIII iron, B5 vitamins (Murashige and Skoogs 1962; Gamborg et al. 1968),

3 mM 2-(*N*-morpholino) ethanesulfonic acid (MES), 87.65 mM sucrose, 4.44 µM N^6-benzylaminopurine (BA) and 0.2% phytagel (pH 5.8)] and incubated for 15 days at 25 ± 2 °C with 16-h photoperiod at a light intensity of 50 µmol m^{-2} s^{-1}. After 15 days the half seed explants with microshoots were inoculated into SIM containing different concentrations (0, 25, 50, 75, 100 and 125 mM) of NaCl. Half seed explants were sub cultured into the same medium twice at 15 days interval. After 45 days the multiple shoots were inoculated into shoot elongation medium [SEM: MS salts, MSIII iron, B5 vitamins, 3 mM MES, 87.65 mM sucrose, 1.45 µM gibberellic acid (GA_3) and 0.2% phytagel (pH 5.8)] with different concentrations of NaCl (0, 25, 50, 75, 100 and 125 mM) and sub-cultured twice at 15 days interval. Elongated shoots were inoculated into root induction medium [RIM: MS salts, MSIII iron, B5 vitamins, 3 mM MES, 87.65 mM sucrose, 4.93 µM indole-3-butyric acid (IBA) and 0.2% phytagel (pH 5.8)] supplemented with different concentration of NaCl (0, 25, 50, 75, 100 and 125 mM). The sensitivity concentration of NaCl was determined at the stage of shoot induction, elongation and rooting period to lessen the number of escapes. The control explants were simultaneously maintained in the respective medium devoid of NaCl.

Matured and dried soybean seeds were inoculated on MS basal medium containing different concentration of NaCl (0, 25, 50, 75, 100 and 125 mM). The particular concentration of NaCl, wherein the seeds failed to develop into well rooted plantlets was considered as minimum inhibitory concentration (MIC), and this concentration of NaCl was used for screening the seeds harvested from transformed plants. All the cultures with respective medium were incubated at 25 ± 2 °C with 16-h photoperiod at a light intensity of 50 µmol m^{-2} s^{-1} provided by cool white fluorescent lamps (Philips, Delhi, India).

Overexpression of *p68* gene for production of transformed soybean plants

The polyamines-assisted plant transformation was performed by *Agrobacterium*-mediated genetic transformation methodology developed by Arun et al. (2016), with minor modifications. Half-seed explants following agroinfection were cultured in co-cultivation medium [CCM: MS salts, MSIII iron, B5 vitamins, 3 mM MES, 87.65 mM sucrose, 200 µM acetosyringone and 0.2% phytagel (pH 5.4)]. After 5 days of co-cultivation with EHA105 carrying pCAMBIA 1300-*p68*, the explants were washed, blot dried and inoculated on SIM supplemented with 200 mg l^{-1} cefotaxime (Duchefa, Haarlem, Netherlands), for initiation of shoot buds. To eliminate chimeric transformants the explants were sub-cultured twice at 15 days interval onto selection medium (75 mM NaCl). The explants with surviving shoots were transferred into SEM supplemented with 100 mg l^{-1} cefotaxime and 75 mM NaCl, and subcultured for 30 days at 15 days interval. The elongated shoots from explants were subcultured to RIM supplemented with 100 mg l^{-1} cefotaxime and 50 mM NaCl incubated for 30 days at 25 ± 2 °C with a 16-h photoperiod at a light intensity of 50 µmol m^{-2} s^{-1} delivered by cool white fluorescent lamps (Philips, Delhi, India). The surviving plants with adequate roots were transplanted to paper cups filled with sand and peat (1:1 v/v) and allowed to harden in the growth chamber. The hardened plants after 12 days were transferred to earthen pots and grown to maturity in the greenhouse under controlled conditions. Similarly, non-transformed plants were maintained in the respective medium. Transformed (T_0) and non-transformed (NT) plants were allowed to undergo self-pollination and set T_1 seeds. Following the seeds were harvested, sterilized and inoculated on MS basal medium containing 100 mM NaCl. After 20 days, the seedlings with well-developed rooted plantlets were hardened and acclimatized in the greenhouse for further analysis.

Molecular confirmation of *p68* gene

One-month-old transformed (T_1) and NT plants were transplanted to paper cups filled with sand and peat (1:1 v/v). The plants in cups were covered with plastic covers, so as to maintain high humidity. Two weeks later, the plants were transferred to green house, wherein they were transplanted to plastic pots containing sand and peat (1:1 v/v). The established plants in the green house, were subjected to molecular analysis.

Polymerase chain reaction (PCR)

Genomic DNA was isolated from the young leaf tissues collected from seventeen transformed lines (T_1) and non-transformed (NT) plant using the CTAB method as earlier reported by Dellaporta et al. (1983). The primer pairs (FP-*p68*: 5′-GGATCCATGTCGTATGTTCCTCCACAC-3′; RP-*p68*: 5′-GGATCCCCATTACCTACAAACATGACT GAT-3′) was used to amplify 1.8 kb fragment of *p68* gene. The NT plant genomic DNA and binary vector pCAMBIA 1300-*p68* were used as negative and positive controls, respectively. PCR was performed in the PTC-100™ thermal cycler (MJ research Inc, Waltham, Mass, USA) using the following conditions: Initial denaturation of DNA template at 95 °C for 5 min, followed by 30 cycles of 94 °C (denaturation) for 40 s, 58 °C (annealing) for 40 s and 72 °C (extension) for 2 min, followed by a final extension at 72 °C for 10 min. The amplified products were analyzed by electrophoresis on a 1.0% agarose (Sigma, St. Louis, USA) gel stained with 0.1% ethidium bromide (Sigma, St. Louis,

USA) and photographed using the Gel Documentation system (UVITEC, Cambridge, UK).

Dot blot analysis

Five µg of genomic DNA extracted from the transformed plants (T_1), non-transformed (NT) plant and 5 µg of binary vector pCAMBIA 1300-*p68* were dotted into 1 cm square grids on a nylon membrane (Hybond N^+, GE Health care Limted, Buckinghamshire, UK). The membrane was then dried for 5 min and rinsed with Neutralization buffer (0.5 M Tris–HCl (pH 7.4) and 1.5 M NaCl) for 3 min followed by a wash with 2× SSC (saline sodium citrate buffer). The membrane was then UV cross linked and hybridized with probe prepared by labeling the PCR purified fragment of *p68* gene, according to the kit manufacturer's instructions (AlkPhos Direct Labeling and Detection System with CDP Star, GE Healthcare Limited, Buckinghamshire, UK).

Southern hybridization

Southern hybridization analysis was carried out to confirm the transgene integration and determine the copy number of *p68* gene in transformed soybean plants. Ten µg of genomic DNA from Dot-positive (T_1) plants (named as S1, S2, S12, S13 and S15), NT plant and 5 µg of pCAMBIA 1300-*p68* plasmid were digested with *Xho*I, which recognizes a single site within the T-DNA. The digested DNA samples and plasmid DNA were resolved on a 1% agarose gel and transferred to a nylon membrane (Hybond N^+, GE Health care Limted, Buckinghamshire, UK). The membrane was hybridized at 55 °C with ALP-labeled 1.8 kb PCR purified fragment of *p68* gene for 8 h and the probe was detected according to manufacturer's instructions (AlkPhos Direct Labeling and Detection System with CDP Star, GE Healthcare Limited, Buckinghamshire, UK).

Reverse transcriptase-PCR (RT-PCR) and Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from Southern hybridization positive (T_1) and NT plants using a RNAqueous kit (Ambion Inc., Austin, USA) and the DNA contamination were eliminated by treatment with *DNase* I. RT-PCR was performed by a one-step RT-PCR kit (Qiagen, USA) as instructed by the manufacturer. The *p68* gene was amplified using the above mentioned gene-specific primers. According to Hu et al. (2009) the *GmUKN1* gene (FP: 5′-TGGTGCTGCCGC TATTTACTG-3′ and RP: 5′-GGTGGAAGGAACTGCTAA CAATC-3′) was used as an internal control. The PCR amplification profile consisted of an initial denaturation of cDNA at 95 °C for 5 min and followed by 28 cycles for 40 s at 94 °C, 40 s at 58 °C and 20 s at 72 °C, followed by a final extension at 72 °C for 5 min. The amplified products were determined on a 1.0% agarose gel (Sigma, St. Louis, USA) stained with 0.1% ethidium bromide (Sigma, St. Louis, USA) and photographed using the Gel Documentation system (UVITEC, Cambridge, UK).

qRT-PCR analysis was performed in LightCycler® 480 Real-time PCR system (Roche, Germany). The expression analysis of the RT-PCR positive (T_1) and NT plants were conducted using Prime Script™ RT Reagent Kit (Takara Bio Inc, Japan) and *p68* gene-specific primer set (FP: 5′-CCT CGCATTCTCTTCCTCGTA-3′ and RP: 5′-CGACGAGAA CCATTGGCTAGA-3′) (Banu et al. 2015). The *GmUKN1* gene was used as the housekeeping gene (NCBI Accession No: BU578186; Unigene ID: Gma.32694) for normalizing the expression values. The experiments were carried out in triplicates. The expression level of *p68* was normalized against *GmUKN1*gene expression level, calculated from cycle threshold (Ct) values, using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Physiological and biochemical analysis of the transgenic soybean plants

One-month-old qRT-PCR positive (T_1) and NT plants, irrigated with different concentration of NaCl (0, 25, 50, 100, 150 and 200 mM) for 2 weeks were subjected to physiological and biochemical analysis.

Total chlorophyll content was measured as described by Arnon (1949). Proline was estimated, as reported by Bates et al. (1973). Catalase activity (CAT) and Ascorbate peroxidase (APX) activity was determined as previously detailed by Aebi (1984) and Chen and Asada (1989) respectively. Monodehydroascorbate reductase (MDHAR) and Dehydroascorbate reductase (DHAR) activities were calculated as previously described by Doulis et al. (1997) and Miyake and Asada (1992), respectively. Superoxide dismutase (SOD) activity was calculated as previously reported by McCord and Fridovich (1969). Malondialdehyde (MDA) content was calculated as previously detailed by Heath and Packer (1968). Relative water content (RWC) was evaluated as described by Turner (1981). Hydrogen peroxide (H_2O_2) level was evaluated as earlier described by Velikova et al. (2000). All the above mentioned analyses were carried out in triplicates.

Measurement of photosynthetic characteristics

Fully expanded leaves from qRT-PCR positive (T_1) and NT plants were analyzed for gas exchange parameters using infra-red gas analyzer (CIRAS-1-PP systems, Hitchin, Herts, UK). The net photosynthetic rate (*Pn*), stomatal conductance (*Gs*) and CO_2 assimilation rate (*A*) were measured under the following conditions. The relative humidity, air temperature,

photosynthetic photon flux density (PPFD) and CO_2 concentration were sustained at 80–90%, 25 °C, 1000 µmol m^{-2} s^{-1} and 400 µl l^{-1}, respectively, and the analysis was carried out between 09:00 and 11:00 h. The experiments were performed in triplicates.

Measurement of Na^+ and K^+ ion content

Na^+ and K^+ ion content was determined as described earlier by Munns et al. (2010). Fresh leaves were harvested from 1 month old qRT-PCR positive (T_1) and NT plants which were treated with different concentrations of NaCl (0, 25, 50, 100, 150 and 200 mM). Harvested leaf material was washed thoroughly with distilled water and then dried at 70 °C for 48 h. Dried leaf material was made into fine powder and used for Na^+ and K^+ ion estimation. A known weight of finely grounded leaf material was digested overnight in HNO_3/H_2O_2 solutions. After digestion the extract was filtered through a 0.22-µM filter membrane. The Na^+ and K^+ ion content in the digested sample was determined by inductively coupled plasma emission spectrometry (ICP trace analyzer, Labtam, Braeside, Australia). The analysis was carried out in triplicates.

Yield performance

Three plants of each qRT-PCR positive (T_1) transgenic lines (S1, S12 and S15) and NT plants were transplanted into plastic pots in green house containing sand and peat (1:1 v/v). The freshly potted plants were irrigated with water for 1 week, following which they were irrigated with water containing varying concentrations of NaCl (0, 100, 150 and 200 mM) once every 2 days till the pods matured. The matured pods were harvested and the number of seeds per plant, as well as the dry weight of the seeds, was evaluated and noted. The analysis was carried out in triplicates.

Statistical analysis

One-way ANOVA was used to analyze the data, and Duncan's multiple range test (DMRT) was used to contrast the differences. SPSS 16.0 (SPSS Inc.USA) has been used to perform the statistical analysis at the level of $P < 0.05$.

Results and discussion

Sensitivity analysis of matured and half-seed explants to NaCl

The proper selection pressure is a vital step in plant genetic transformation to select the transformed cells/tissues/plants from non-transformed counterparts. Hence, to minimize the number of non-transformed plant escapes following transformation and regeneration of half-seed explant, an efficient selection pressure was determined by inoculating and incubating the half-seed explants and seeds on MS basal medium containing varying concentrations of NaCl (0, 25, 50, 75, 100 and 125 mM) for 15 days. Among the various NaCl concentrations evaluated, shoot induction medium supplemented with 75 mM NaCl barred the development of shoot and 50 mM NaCl arrested root formation with dried leaves from the elongated shoots of half seed explants, whereas the MS basal medium supplemented with 100 mM NaCl prohibited seed germination. Hence, the 75, 50 and 100 mM of NaCl was considered as a minimum inhibitory concentration (MIC) in selecting the transformed multiple shoot induction, elongation, rooting and germinating seeds, respectively. Similarly, Zhang et al. (2009) reported that 200 mM NaCl was able to eliminate the non-transformants and thereby was helpful in selecting transformed tobacco plants carrying the *rst*B gene. *Arabidopsis* seeds transformed with *OsMSR*2 gene were successfully screened on 100 mM NaCl supplemented medium (Xu et al. 2011). Transformed somatic embryos and soybean seeds were selected in a medium containing 75 and 100 mM of NaCl, respectively (Subramanyam et al. 2012).

Development of transformed soybean plants by overexpressing *p68* gene

Half-seed explants with the embryonic axis were infected with *Agrobacterium tumefaciens* strain EHA105 harboring the binary vector pCAMBIA1300-*p68* and co-cultivated for 5 days in the dark (Fig. 1d). After co-cultivation, the half-seed explants were cultured on SIM incorporated with 200 mg l^{-1} cefotaxime for 15 days (Fig. 1e). After 15 days, the half seed explants with initiated shoots were sub-cultured in the same medium incorporated with 75 mM NaCl, for multiplication and selection of putatively transformed shoots (Fig. 1f, g). The actively multiplying and surviving shoots were subcultured to SEM containing 100 mg l^{-1} cefotaxime and 75 mM NaCl, wherein they elongated successfully (Fig. 1h). The elongated shoots were subcultured to RIM containing 100 mg l^{-1} cefotaxime and 50 mM NaCl developed roots (Fig. 1i). Hardening and acclimatization of transformed plantlets were accomplished in the environmental growth chamber (Fig. 1j) and in the greenhouse, respectively (Fig. 1k). The putative transgenic seeds, collected and labeled as T_1 seeds, were screened on MS basal medium incorporated with 100 mM NaCl.

Molecular and expression analysis of transformed plants to confirm *p68* gene

The transformed (T_1) and NT plants were analyzed for the presence of *p68* gene using PCR. The PCR analysis revealed

a 1.8-kb amplicon of *p68* gene fragment from the genomic DNA of (T_1) soybean plants (Fig. 2a, lanes 3–19) which is similar to the amplicon from the pCAMBIA 1300-*p68* (Fig. 2a, lane 2). No amplification was observed from the genomic DNA isolated from NT soybean plants (Fig. 2a, lane 20).

Dot blot analysis was performed to screen a large number of PCR-positive lines. Samples which were exhibiting strong signal in dot blot (Fig. 2b) were further analyzed by southern blotting to validate the integration of the transgene (*p68*) and its copy number in (T_1) soybean plants. The Genomic DNA was digested with *XhoI* and hybridized with the probe prepared using the PCR-amplified product of *p68* gene fragment as there is a single *Xho* I site between left TDNA border and the *p68* gene cassette with in the T-DNA region of the pCAMBIA1300-*p68*. Therefore, probing with *p68* gene fragment would give us an indication of the number copies of *p68* gene integrated into the plant genome. The positive control (pCAMBIA1300-*p68)* generated hybridization signal (Fig. 2c, lane 1) while the negative control (DNA from NT plant) did not exhibit hybridization signal (Fig. 2c, lane 7). The transformed plants were found to have up to four copies of *p68* gene integration (Fig. 2c, lanes 2, 5 and 6), with non-identical hybridization patterns evidencing their individuality.

In RT-PCR, a 1.8-kb amplified fragment, specifically amplified by the gene-specific primer, confirmed the expression of *p68* gene in three (S1, S12 and S15) of the five Southern-positive (T_1) plants (Fig. 3a, lanes 2, 4, 6), and no amplification was observed in remaining two Southern-positive (S2 and S13) (Fig. 3a, lanes 3, 5) and NT plants (Fig. 3a, lane 1). This might be due to gene silencing by the position effect on *p68* gene. Similary, Maghuly et al. (2006) observed no expression of *GFLV* gene in few lines of transformed grapevine plants, suggesting that there is no correlation between the T-DNA copy number and mRNA expression level of transgene.

The qRT-PCR analysis confirmed the expression of *p68* in three lines of RT-PCR-positive (T_1) plants (Fig. 3b) and no expression was observed in non-transformed (NT) plants (Fig. 3b). In the present study, highest level of *p68* transcript expression was recorded in S15 and the lowest was observed in line S12 (Fig. 3b). Similarly, Banu et al. (2015) employed qRT-PCR assay to evaluate the expression of *p68* gene in transformed rice which conferred salt tolerance.

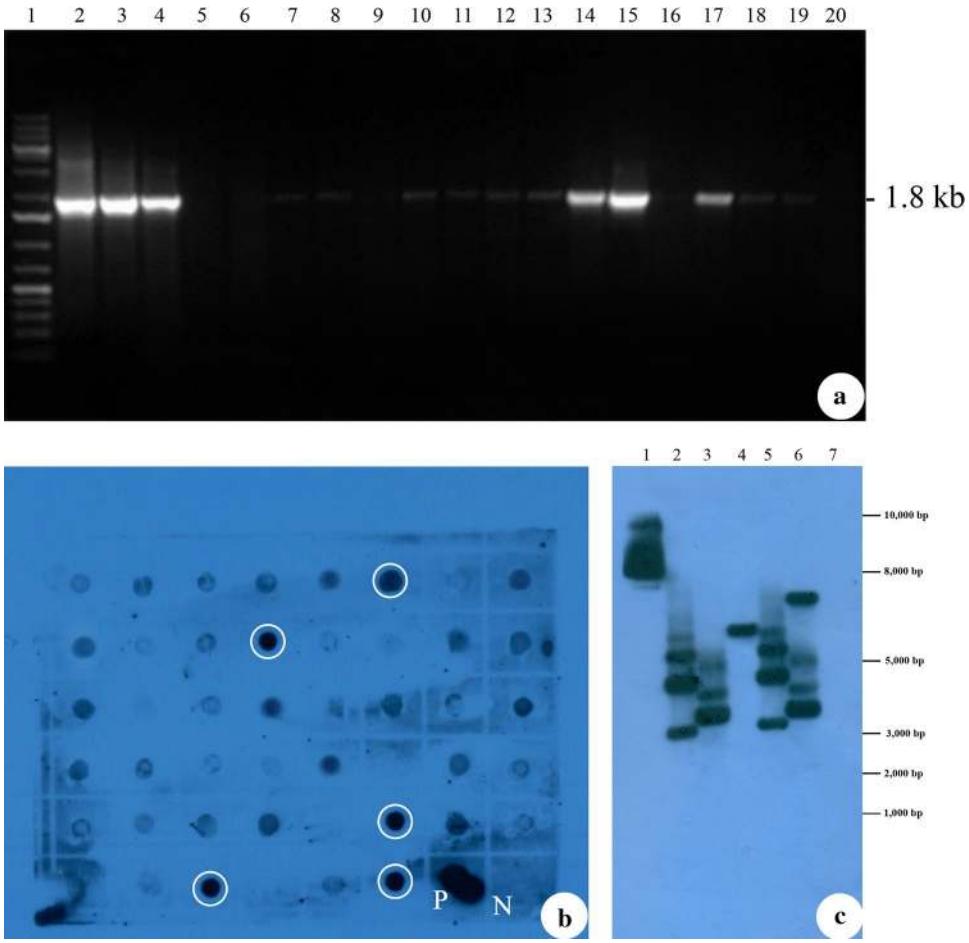

Fig. 2 Detection of *p68* gene integration in transformed soybean plants. **a** PCR analysis of transformed soybean plants (T_1) for the presence of *p68* gene. Lane 1, Gene Ruler 1 kb DNA ladder marker, ready to use (#SM0313); lane 2, pCAMBIA1300-*p68* as a positive control; lanes 3, 4,14, 15 and 17, transformed soybean plants (S1, S2, S12, S13 and S15) lane 20, non-transformed (NT) soybean plants genomic DNA as a negative control. **b** Dot blot analysis of transformed soybean plants (T_1). Five transgenic lines showing strong positive signal marked in white circles (S1, S2, S12, S13 and S15) genomic DNA; P-pCAMBIA1300-*p68* as a positive control; N-non-transformed (NT) soybean genomic DNA. **c** Southern blot analysis of transformed soybean plants (T_1). lane 1, pCAMBIA1300-*p68* gene as a positive control; lanes 2–6, transformed soybean plants (S1, S2, S12, S13 and S15) genomic DNA; lane 7, non-transformed (NT) soybean genomic DNA

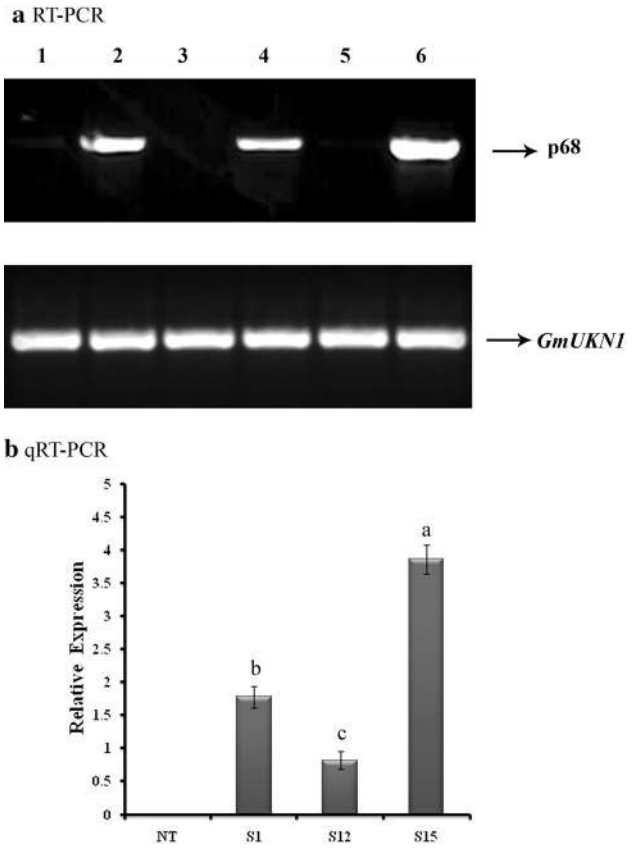

Fig. 3 Expression analysis of transformed soybean plants. **a** RT-PCR expression analysis of *p68* gene in transformed soybean plants. Lane 1, non-transformed (NT) soybean plant RNA as a negative control; lanes 2, 4 and 6, transformed (T_1) soybean (S1, S12 and S15) plant RNA samples; lanes 3 and 5, failed to amplify *p68* gene from transformed (T_1) soybean (S2 and S13) plant RNA samples. **b** qRT-PCR analysis to analyze the expression level of *p68* gene in transformed soybean plants. Relative expression of three transgenic lines (S1, S12 and S15) and non-transformed (NT) soybean plant; data were analyzed according to the $2^{-\Delta\Delta Ct}$ method. Mean of three individual experiments with standard errors. Different letters denote significantly different values according to Duncan's multiple range test (DMRT) at a 5% level

Assessment of transgenic soybean plants under salt stress

One-month-old qRT-PCR positive (T_1) plants were irrigated with 200 mM of NaCl along with non-transformed (NT) control plants. Both (T_1) and NT plants exhibited no phenotypic differences under normal growth conditions (0 mM NaCl) (Fig. 4a). However, under increased salinity stress the NT plants exhibited chlorosis, necrosis, leaf burning, defoliation, and completely withered at 200 mM of NaCl (Fig. 4b). Moreover, three (S1, S12 and S15) out of five southern blot positive (T_1) plants showed healthy growth at 200 mM NaCl and produced soybean pods (Fig. 4c). This clearly indicates that the expression of *p68* gene in (T_1) plants played an important role in the salinity tolerance.

It has been confirmed that *p68* has potentiality towards cell injury recovery. *p68* gene in particular stressed condition plays a vital role in preventing aggregation and stabilizing non-native proteins. Thus, considering the aforesaid statements, we presume that *p68* expression in soybean played an important role in salinity tolerance. Similarly, earlier reports also support the role of *p68* in salinity stress alleviation in rice and tobacco (Banu et al. 2015; Tuteja et al. 2014).

Biochemical analysis of transformed soybean plants under salt stress

The NT plants, showed tolerance upto 100 mM NaCl, beyond which they perished, whereas the transformed plants showed tolerance towards salinity stress of upto 200 mM. Hence, the NT and transformed (T_1) plants exposed to salinity stress of 100 mM and 200 mM, respectively, were subjected to physiological and biochemical analysis.

Effect of salinity stress on chlorophyll content

The salinity stress induces photo-oxidative reaction which in turn results in degradation of cell organelle membranes, especially of chloroplast thylakoids which ultimately leads to chlorophyll degradation (Husaini and Abdin 2008). Change in chlorophyll content is fundamental to understand the plant response to the salinity stress. In the present study, the leaf chlorophyll content gradually decreased with an increased concentration of NaCl. However, under same concentrations of NaCl, the chlorophyll loss in (T_1) plants was significantly less noticeable than that of the NT plant. At 100 mM of NaCl, the chlorophyll loss was (52.24%) in NT plant, whereas (11.03%) in 'S12' (T_1) plant (Fig. 5a). A possible explanation is that, overexpression of *p68* gene in (T_1) plants, induced the expression of downstream genes that inhibited chlorophyll disintegration, thereby increasing the chlorophyll concentration (Zhang et al. 2004; Gao et al. 2009). In accordance with the current study, the expression of *p68* gene in tobacco plant resulted in a higher chlorophyll content (Tuteja et al. 2014) which surpassed the WT plants. Similarly, the expression of stress responsive genes such as alfalfa *WRKY11* gene in soybean (Wang et al. 2018), *Tbosm* gene in chilli pepper (Subramanyam et al. 2011), *AhBADH* gene in trifoliate orange (Fu et al. 2011), *Rab 16A* gene in tobacco plants (Roychowdhury et al. 2007) and *ATHK1* gene in *Lyceum barbarum* (Chen et al. 2009) resulted in higher chlorophyll content than NT plants.

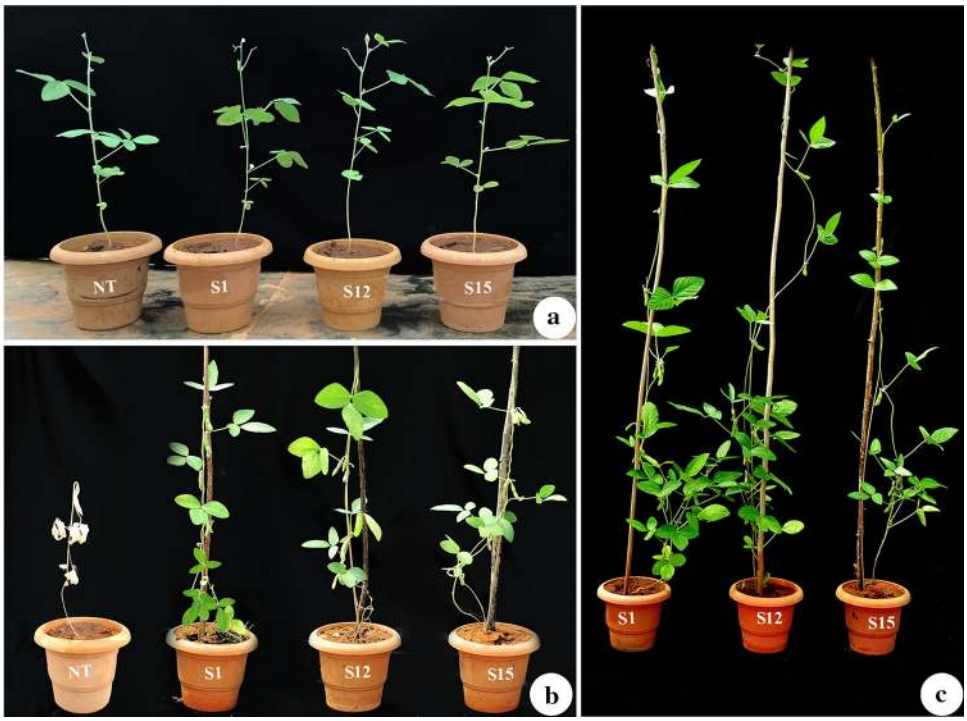

Fig. 4 Phenotypic appearance of transformed (T_1) and non-transformed (NT) soybean plants under salinity stress condition (200 mM NaCl). **a** Phenotype before salt treatment; **b** phenotype after two weeks of salt treatment; **c** green house grown (T_1) generation transgenic soybean plants produced soybean pods

Effect of salinity stress on proline

Increase in the osmotic pressure is caused due to the excessive loss of intracellular water in plants which directs them to accumulate compatible solutes to tolerate the osmotic pressure (Turkan and Demiral 2009). As like all other abiotic stresses, salt stress also affects the flow of metabolic pathways, resulting in accumulation of ROS (Gill and Tuteja 2010). Salinity affected plants require free proline for osmotic adjustment, cellular macromolecules protection, and scavenging hydroxyl radicals (Singh et al. 2000; Chen et al. 2011). Hence, the accumulation of proline in plants during abiotic stress is vital, compared to other compatible solutes (Gao et al. 2009). In the present investigation, under normal growth conditions, the concentration of free proline in leaves of (T_1) plants was similar to that of NT plants. When the (T_1) and NT plants were treated with increased concentrations of NaCl, the (T_1) plants accumulated higher proline than NT plant and (T_1) plant 'S12' showed highest proline content at all tested NaCl concentrations (Fig. 5b). It indicates that the expression of *p68* gene might activate the key enzymes of the proline biosynthetic pathway, which in turn enhance the tolerance to salinity. Similarly, the expression of *Tbosm* gene in cotton, soybean (Parkhi et al. 2009; Subramanyam et al. 2012) *Hva1* gene in mulberry (Checker et al. 2012), *ATHK1* gene in *Lycium barbarum* (Chen et al. 2009) and *CgDREBa* gene in *Chrysanthemum* (Chen et al. 2011), enhanced the level of proline content in transgenic plants than that of its counterparts. In addition, overexpression of *p68* gene in rice (Banu et al. 2015) enhanced the proline content in transgenic plant than WT plant under salinity stress condition.

Effect of salinity stress on activities of APX, CAT, and SOD

ROS are continuously produced in plants, during salinity and drought stress conditions as by-products of various metabolic pathways, which interact with the macromolecules making them malfunction (Gill and Tuteja 2010). The chloroplasts and mitochondria are prime locations for the production of ROS owing to their active electron transport activities (Tambussi et al. 2000; Bartoli et al. 2004). To overcome oxidative stress induced by drought and salinity, plants up-regulate antioxidative enzymes such as APX, CAT, and SOD to detoxify their system (Turkan and Demiral 2009). In the present investigation, under standard growth conditions, the (T_1) and NT soybean plants accumulated APX, CAT, and SOD similarly. The antioxidative enzyme activities gradually increased with the increased concentration of NaCl in both (T_1) and NT soybean plants (Fig. 5c–e). However, the (T_1) plants maintained a higher APX, CAT, and SOD activity than their counterparts (Fig. 5c–e). Among the three (T_1) plants analyzed, plant 'S12' showed the highest APX, CAT, and SOD activities than 'S15' at all tested NaCl concentrations (Fig. 5c–e). The results obtained from the current study are relatable to earlier reports where soybean plants expressing *Tbosm* showed elevated levels of SOD, CAT, and APX

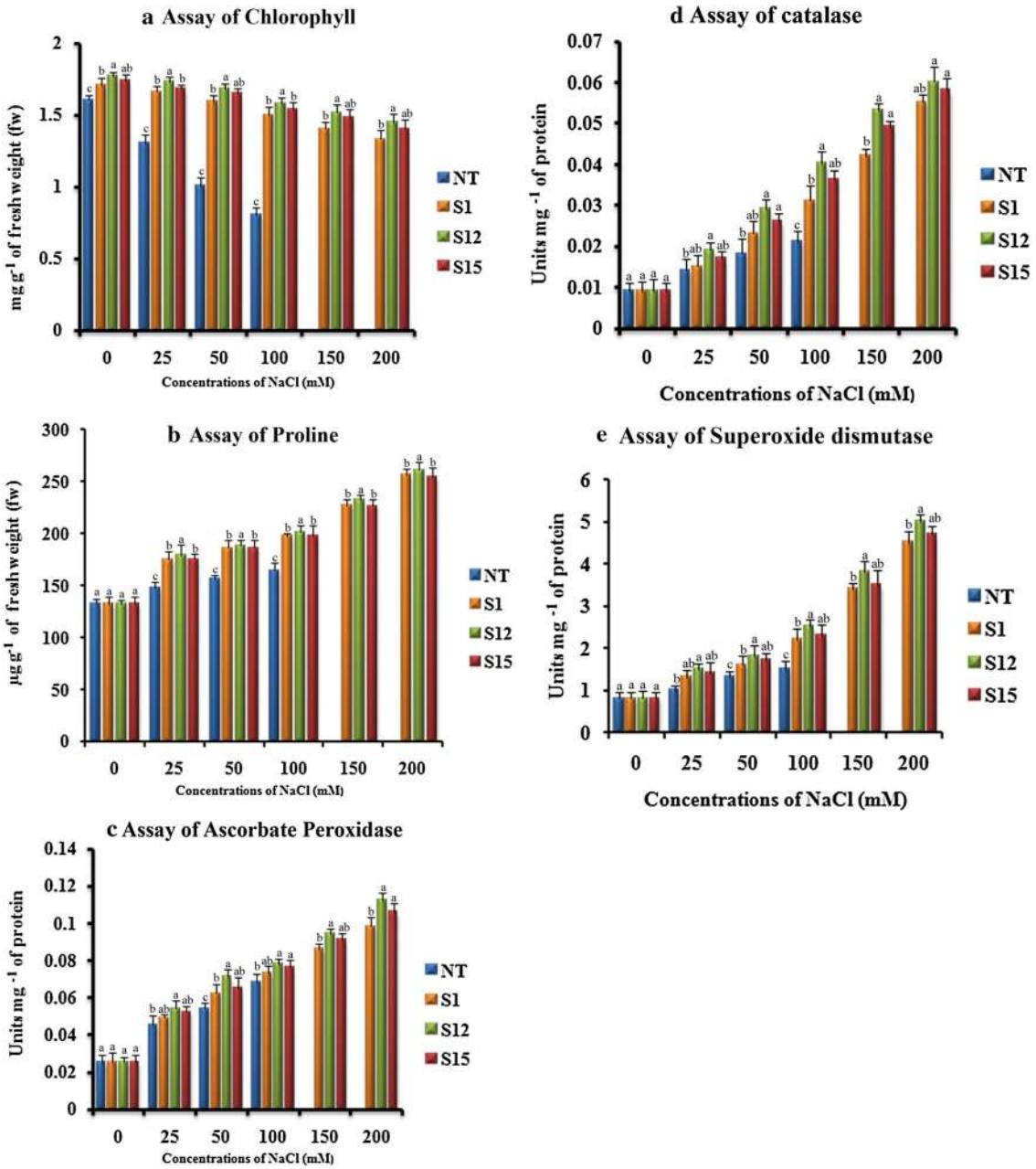

Fig. 5 Response of transformed (T_1) and non-transformed (NT) soybean plants subjected to different concentrations of NaCl for 12 days under greenhouse conditions. **a** Chlorophyll concentration (mg g^{-1} of fw); **b** proline concentration (µg $g^{-1}$of fw); **c** APX concentration (Units mg^{-1} of protein); **d** CAT activity (Units mg^{-1} of protein); **e** SOD activity (Units mg^{-1} of protein). Mean of three individual experiments with standard errors. Different letters denote significantly different values according to Duncan's multiple range test (DMRT) at a 5% level

(Subramanyam et al. 2012). Similarly, SOD, CAT, and APX levels were elevated in the transformed chilli and tomato expressing *Tbosm* and *BcZAT12* respectively, under stressed salinity and drought conditions (Subramanyam et al. 2011; Shah et al. 2013). Tobacco plant expressing *p68* gene showed an improved level of APX, CAT and SOD activity under salinity stress conditions (Tuteja et al. 2014). Transgenic rice plants expressing *p68* gene also displayed a higher APX and CAT activity upon exposure to salt stress as compared to NT plants (Banu et al. 2015).

Effect of salinity stress on activities of DHAR and MDHAR

Noctor and Foyer (1998) reported that the two enzymes DHAR and MDHAR involved in the production of reducing

agents such as ascorbate and glutathione during several abiotic and biotic stress conditions, helps in foraging the increased levels of H_2O_2. In our studies, the activities of DHAR and MDHAR were similar in (T_1) and NT plants under normal growth conditions (Fig. 6a, b). When the (T_1) and NT soybean plants were irrigated with different concentrations of NaCl, the activities of DHAR and MDHAR increased up to 150 mM and thereafter decreased. However, the (T_1) soybean plants accumulated DHAR and MDHAR higher than NT soybean plants (Fig. 6a, b). Among the three (T_1) soybean plants, 'S12' showed higher activity than the rest at all tested NaCl concentrations. The results obtained were in accordance with the earlier reports where *glutathione S-transferase* expression in cotton (Light et al. 2005) and *Tbosm* gene expression in chilli pepper and soybean (Subramanyam et al. 2011, 2012) showed enhanced levels of DHAR and MDHAR for effective elimination of H_2O_2 under the salinity conditions.

Estimation of H_2O_2 concentration

It is a well-known fact that excessive accumulation of H_2O_2 leads to the prevalence of oxidative stress in plants. This is a cause for apoptosis, DNA fragmentation, chromatin condensation as well as cell shrinkage (Houot et al. 2001). A variety of enzymatic and non-enzymatic anti-oxidants play crucial role in eliminating H_2O_2 (Gill and Tuteja 2010). Foyer et al. (1994) reported that CAT or APX, which converts H_2O_2 to water molecules, prevents the accumulation of H_2O_2. In our studies, there were no prominent differences in H_2O_2 content between the (T_1) and NT soybean plants under normal growth conditions (Fig. 7a). However, when (T_1) and NT soybean plants were irrigated with different concentrations of NaCl, the (T_1) soybean plants accumulated less amount of H_2O_2 than NT plant (Fig. 7a). As we noticed earlier, higher accumulation of antioxidative enzymes in the transgenic soybean plants might have lowered the amount of H_2O_2 under stressed conditions. In a similar fashion, *p68* gene expressed in tobacco and rice, respectively, maintained a decent level of H_2O_2 content, even under stressed condition (Tuteja et al. 2014; Banu et al. 2015).

Effect of salinity stress on MDA

Membranes are the main targets of lipid peroxidation degradative process due to the accumulation of ROS induced by salinity. In plant cells, MDA content is an indicator of lipid peroxidation levels (Shalata and Tal 1998). In the present study, under normal growth conditions, the (T_1) and NT soybean plants displayed similar concentrations of MDA (Fig. 7b). The MDA concentration gradually increased in both (T_1) and NT plants with increased concentrations of NaCl (Fig. 7b). However, under all tested

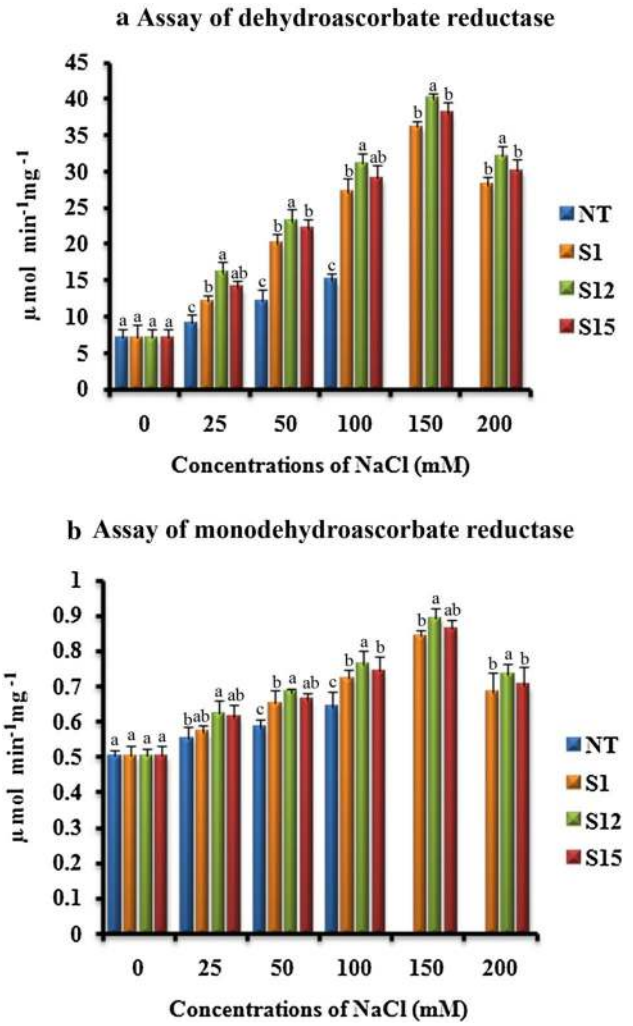

Fig. 6 Response of transformed (T_1) and non-transformed (NT) soybean plants subjected to different concentrations of NaCl for 12 days under greenhouse conditions. **a** DHAR activity (µ mol min^{-1} mg^{-1}); **b** MDHAR activity (µ mol min^{-1} mg^{-1}). Mean of three individual experiments with standard errors. Different letters denote significantly different values according to Duncan's multiple range test (DMRT) at a 5% level

NaCl concentrations, the NT plant showed higher MDA content than (T_1) plants (Fig. 7b). This implies that the degree of lipid peroxidation in (T_1) plants is lower than that in the NT plant. Therefore, suggesting that the expression of *p68* gene in (T_1) soybean plants protected the cell membrane from damage caused by salinity stress. In accordance with the present study, *p68* gene expressed in rice recorded a less amount of MDA under salinity condition (Banu et al. 2015). Similar results were also observed during the expression of *BcZAT12* gene in tomato plants under heat stress (Shah et al. 2013) and osmotin gene in strawberry plants under salt stress (Husaini and Abdin 2008).

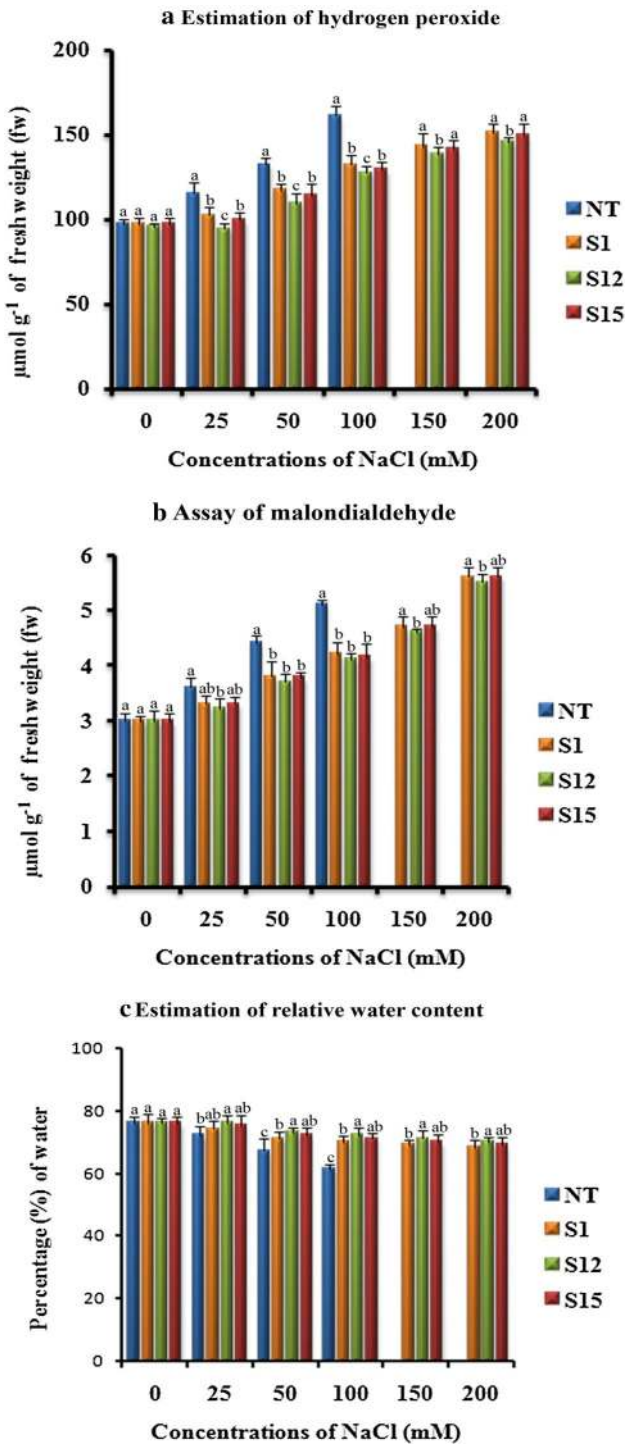

Fig. 7 Response of transformed (T_1) and non-transformed (NT) soybean plants subjected to different concentrations of NaCl for 12 days under greenhouse conditions. **a** H_2O_2 concentration (µmol g^{-1} of fw); **b** MDA content (µmol g^{-1} of fw); **c** relative water content (%). Mean of three individual experiments with standard errors. Different letters denote significantly different values according to Duncan's multiple range test (DMRT) at a 5% level

Effect of salinity stress on RWC

Flower and Ludlow (1986) reported that maintenance of plants in high water level helped them to survive under salt stress. Hence, RWC is considered as an important parameter for salinity tolerance (Malatrasi et al. 2002; Rampino et al. 2006; Talame et al. 2007). We noticed a gradual decrease in the RWC in both NT and (T_1) plants with an increase in NaCl concentration, however, the (T_1) plants maintained a higher RWC than that of its counterparts (Fig. 7c). In a similar fashion, Banu et al. (2015) reported that *p68* gene expressed in *rice* revealed higher amount of RWC under salinity condition when compared to WT plants. RWC was observed to be similar in the NT and (T_1) plants under normal growth conditions (Fig. 7c). Likewise, expression of *BcZAT12* and *Tbosm* genes in transgenic tomato and soybean, respectively (Shah et al. 2013; Subramanyam et al. 2012), exhibited high RWC than that of its non-transformed counterparts.

Effect of salinity stress on photosynthetic characteristics

Net photosynthetic rate (*Pn*), stomatal conductance (*Gs*) and CO_2 assimilation rate (*A*) are the important parameters to evaluate the plant performance under salinity stress (Subramanyam et al. 2012). The difference in *Pn, Gs* and *A* was not significant between the (T_1) and NT plants when grown under control conditions (Fig. 8a–c). However, *Pn, Gs* and *A* was retained at a higher level in (T_1) plants as compared to NT plants under all tested NaCl concentrations (Fig. 8a–c). The observed results suggested that (T_1) plants subjected to salinity stress utilized internal carbon dioxide efficiently. Similarly, a higher retention of *Pn, Gs* and *A* was observed in *mtlD* expressing *Populus tomentosa* (Hu et al. 2005), *AVP1* expressing cotton (Pasapula et al. 2011), *tbosm* expressing soybean (Subramanyam et al. 2012) and *p68* expressing tobacco (Tuteja et al. 2014) which displayed higher tolerance to salinity stress.

Effect of salinity stress on Na^+ and K^+ ion content

Ionic balance is necessary for cellular metabolism, plant development, growth and productivity that provides tolerance to salinity stress (Cuin et al. 2008; Conde et al. 2011; Huda et al. 2013a). There was no significant variation observed in the accumulation of Na^+ ions between (T_1) and NT plants grown under control conditions. However, in the plants subjected to salinity stress the Na^+ ion content gradually increased to a significant level in NT plant as compared to the (T_1) plants (Fig. 9a). Further analysis of K^+ ion content revealed that (T_1) and NT plants displayed similar K^+ ion content under normal growth conditions, however, when subjected to salinity stress the transgenic plants retained higher K^+ ions compared to the NT plant (Fig. 9b). Thereby, we finally observed that Na^+/K^+

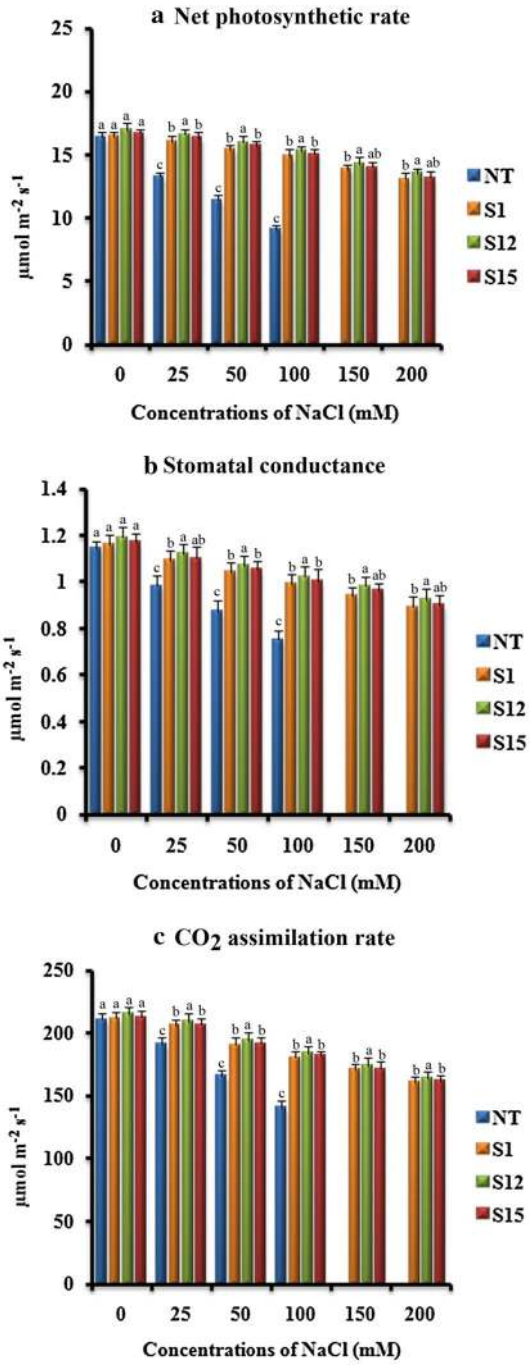

Fig. 8 Response of transformed (T_1) and non-transformed (NT) soybean plants subjected to different concentrations of NaCl for 12 days under greenhouse conditions. **a** Net photosynthetic rate (*Pn*) (µmol m^{-2} s^{-1}); **b** stomatal conductance (*Gs*) (µmol m^{-2} s^{-1}); **c** CO_2 assimilation (*A*) (µmol m^{-2} s^{-1}). Mean of three individual experiments with standard errors. Different letters denote significantly different values according to Duncan's multiple range test (DMRT) at a 5% level

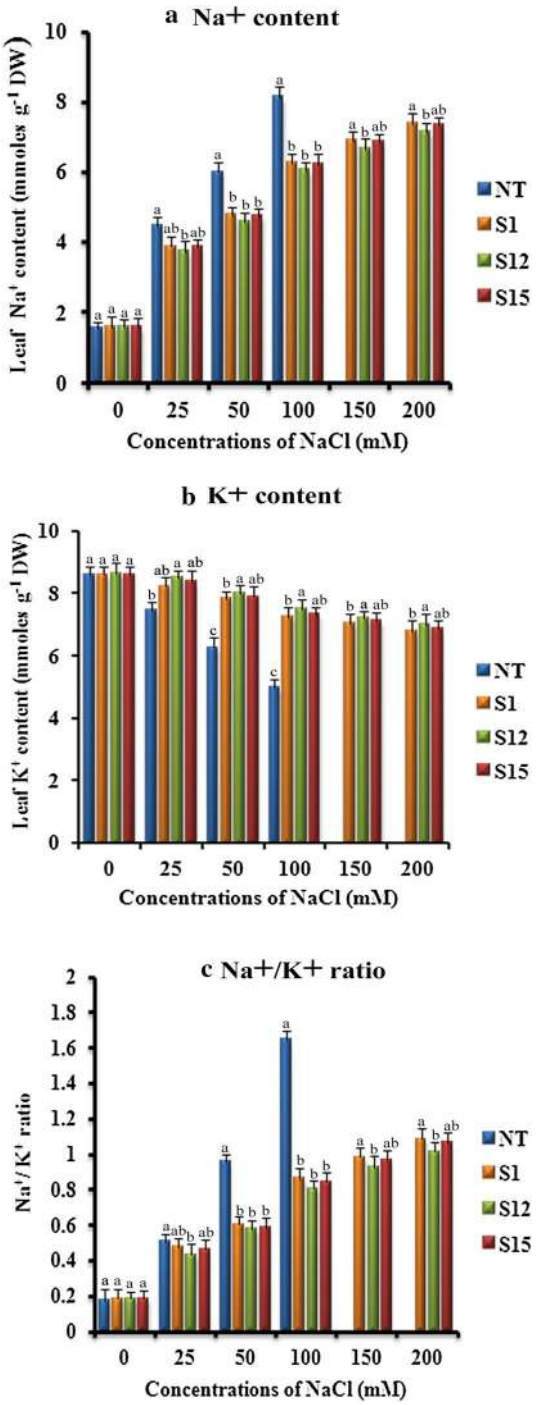

Fig. 9 Response of transformed (T_1) and non-transformed (NT) soybean plants subjected to different concentrations of NaCl for 12 days under greenhouse conditions. **a** Na^+ content (mmoles g^{-1} DW); **b** K^+content (mmoles g^{-1} DW); **c** Na^+/K^+ ratio. Mean of three individual experiments with standard errors. Different letters denote significantly different values according to Duncan's multiple range test (DMRT) at a 5% level

Table 1 Yield assay of transformed (T_1) and non-transformed (NT) soybean plants subjected to 0, 100, 150 and 200 mM NaCl induced salinity stress conditions

S. no	Soybean plant	Yield in the absence of NaCl		Yield (100 mM of NaCl)		Yield (150 mM of NaCl)		Yield (200 mM of NaCl)	
		Number of matured (pods/plant)	Dry weight of seeds (g/plant)	Number of matured (pods/plant)	Dry weight of seeds (g/plant)	Number of matured (pods/plant)	Dry weight of seeds (g/plant)	Number of matured (pods/plant)	Dry weight of seeds (g/plant)
1	NT	$31.3 \pm 0.27^{a,b}$	11.3 ± 0.28^{b}	19.6 ± 0.20^{c}	6.3 ± 0.34^{c}	–	–	–	–
2	S1	30.6 ± 0.20^{b}	10.0 ± 0.25^{c}	$27.3 \pm 0.24^{b,a}$	$12.6 \pm 0.24^{a,b}$	25.6 ± 0.20^{b}	10.3 ± 0.28^{c}	22.0 ± 0.25^{b}	$8.6 \pm 0.15^{a,b}$
3	S12	32.0 ± 0.25^{a}	12.3 ± 0.23^{a}	28.6 ± 0.20^{a}	13.3 ± 0.14^{a}	26.6 ± 0.28^{a}	12.6 ± 0.24^{a}	24.3 ± 0.26^{a}	9.0 ± 0.25^{a}
4	S15	29.3 ± 0.19^{c}	$11.6 \pm 0.21^{a,b}$	27.6 ± 0.27^{b}	12.0 ± 0.21^{b}	24.0 ± 0.21^{c}	11.3 ± 0.28^{b}	$23.6 \pm 0.18^{a,b}$	8.3 ± 0.24^{b}

Mean of three individual experiments (±) with standard errors. Different letters inside the same column denote significantly different values according to Duncan's multiple range test (DMRT) at a 5% level

ratio was less in (T_1) plants as compared to NT plant (Fig. 9c), which concluded that the transgenic plants have higher potential to tolerate salinity stress. Similarly, Tuteja et al. (2014) reported that *p68* expressing tobacco plants accumulated higher K^+ and lower Na^+ ions as compared to the wild-type plants when subjected to salinity stress. The higher K^+ ion content has been reported to delay leaf senescence, whereas lower K^+ ion content reportedly regulates caspase-like protease and endonuclease activity resulting in leaf senescence (Huda et al. 2013a; Shabala 2009). Munns et al. (2006) also reported increase of K^+ ions and decrease of Na^+ ions in wheat as a response to salinity stress. The expression of *p68* in tobacco plants reduced Na^+/K^+ ratio in transgenic plants as compared to control plants, and the restricted entry of Na^+ ions into the cells protected the photosynthetic machinery from abiotic stress (Tuteja et al. 2014).

Yield performance of transformed soybean plants

Under the greenhouse conditions, (T_1) and NT soybean plants were phenotypically similar (Fig. 4a), but NT plants were unable to survive beyond 100 mM NaCl, which indicates that beyond 100 mM NaCl is lethal and prevented further development of plants. Whereas (T_1) soybean plants grew fairly even at 200 mM NaCl and produced upto 22–24 pods containing 8–9 g dry weight of seeds (Table 1). On the other hand, NT soybean plants produced 19 matured soybean pods containing 6 g of dry weight of seeds at 100 mM NaCl (Table 1).

Conclusion

In conclusion, the *p68* gene was transferred and expressed in soybean plants. The expression of the *p68* gene in three transgenic lines of soybean exhibited enhanced salinity tolerance. The morphological, physiological and biochemical evidence revealed that three transgenic lines are more tolerant to salinity stress as compared to NT plants. These results suggest the role of *p68* in conferring tolerance to salinity stress without affecting yield, photosynthesis, and by controlling the reactive oxygen species (ROS) through modulating antioxidative defence machinery. The obtained results pioneered the efficacy of the *p68* gene against salinity and paved way to understand its role under abiotic stress conditions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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