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Overexpression of RNA-binding bacterial chaperones in rice leads to stay-green phenotype, improved yield and tolerance to salt and drought stresses

Rajasheker Guddimalli[1] | Anil Kumar Somanaboina[2] | Surender Reddy Palle[1] | Sujatha Edupuganti[3] | Divya Kummari[4] | Sudhakar Reddy Palakolanu[4] | Jalaja Naravula[2] | Jawahar Gandra[5] | Insaf A. Qureshi[6] | Nagaraju Marka[7] | Rathnagiri Polavarapu[8] | Polavarapu Bilhan Kavi Kishor[2]

[1]Department of Genetics, Osmania University, Hyderabad, India

[2]Department of Biotechnology, Vignan's Foundation for Science, Technology and Research, Guntur, India

[3]Department of Botany, Osmania University, Hyderabad, India

[4]Cell, Molecular & Genetic Engineering Lab, International Crops Research Institute for the Semi-Arid Tropics, Hyderabad, India

[5]Department of Life Sciences, School of Sciences B-II, Jain University, Bengaluru, India

[6]Department of Biotechnology and Bioinformatics, School of Life Sciences, University of Hyderabad, Hyderabad, India

[7]Biochemistry Division, ICMR-National Institute of Nutrition, Hyderabad, India

[8]Research & Development Division, Genomix CARL, Pulivendula, India

Correspondence
Polavarapu Bilhan Kavi Kishor, Department of Genetics, Osmania University, Hyderabad 500 007, India.
Email: pbkavi@yahoo.com

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Abstract

Genes encoding bacterial cold shock proteins A (*CspA*, 213 bp) and B (*CspB*, 216 bp) were isolated from *Escherichia coli* strain K12, which showed 100% homology with gene sequences isolated from other bacterial species. In silico domain, analysis showed eukaryotic conserved cold shock domain (CSD) and ribonuclease-binding domain (RBD) indicating that they bind to RNA and are involved in temperature stress tolerance. Overexpression of these two genes in *E. coli* resulted in higher growth in presence of 200 mM NaCl and 300 mM mannitol. Western blot confirmed the translational products of the two genes. Seedlings of indica rice were transformed with *Agrobacterium tumefaciens* containing pCAMBIA1301 *CspA* and *CspB* genes. Transgene integration was confirmed by β-glucuronidase (GUS) histochemical assay, polymerase chain reaction (PCR) amplification, and gene copy number by Southern blotting. Chlorophyll, proline, Na^+, and K^+ contents were higher in transgenics exposed to 150 mM NaCl and drought (imposed by withholding water) stresses during floral initiation stage. Catalase (CAT), superoxide dismutase (SOD), and guaiacol peroxidase (GPX) activities increased, while malondialdehyde (MDA) content was low in transgenics. Transgenics displayed increased root, shoot, and panicle lengths, root dry mass, and a distinct stay-green (SGR) phenotype. Higher transcript levels of *CspA*, *CspB*, *SGR*, *chlorophyllase*, isopentenyl adenine transferase 1 (*IPT1*), 9-*cis*-epoxycarotenoid dioxygenase (*NCED*), *SOD*, and sirtuin 1 (*SIRT1*) genes were observed in transgenics compared to wild type plants (WT) under multiple stresses. Present work indicates that bacterial chaperone proteins are capable of imparting SGR phenotype, salt and drought stress tolerance alongside grain improvement.

1 | INTRODUCTION

Salt and drought stresses are the most devastating environmental factors that result in severe crop losses throughout the world. While salt imposes ionic stress, drought rapidly depletes underground water tables. Often salt and drought stresses are coupled with high temperature stress. Combined effects of major abiotic stresses cause over 50% losses in agricultural crop production (Bray et al., 2020).

Consequently, this entails the generation of crop plants that use water with higher efficiencies (Morison et al., 2008). Breeding methods helped to improve several crop plants for salt and drought stress tolerance, but potential exists for further improvement of crops with better yields and grain quality under different degrees of stress conditions or their combined effects (Fukai & Cooper, 1995; Sreenivasulu et al., 2015). Rice is sensitive to salt and drought stresses (Ismail & Thomson, 2011; Sreenivasulu et al., 2015). Salt stress leads to ion (Na^+ and Cl^-) toxicity, alters cellular metabolism, lowers osmotic potential and photosynthetic efficiency resulting in reduced plant growth rate and yield (Acosta-Motos et al., 2017). Response to drought stress depends upon timing of the stress during early seedling growth, vegetative stage, at the time of flowering or during grain maturity. Terminal drought stress severely impairs the number of filled grains and grain quality (Sreenivasulu et al., 2015). Genetic engineering approaches, such as incorporation of microbial and plant genes associated with biosynthetic pathways of compatible solutes like mannitol, proline, and glycine betaine into crop plants, resulted in the development of abiotic stress tolerant plants (Huang et al., 2000; Kishor et al., 1995; Kishor et al., 2005; Tarczynski et al., 1993; Xu et al., 1996). Further, overexpression of yeast trehalose-6-phosphate synthase gene in tobacco ensued in less dehydration and necrosis (Romero et al., 1997). Similarly, modulation of the polyamine biosynthetic pathway genes in transgenic rice conferred drought tolerance (Capell et al., 2004). Sato and Yokoya (2008) noticed increased drought stress tolerance in rice by overexpressing a small heat-shock protein sHSP17.7. Gu et al. (2013) observed enhanced drought tolerance in transgenic rice overexpressing genes that encode C_4 photosynthetic pathway enzymes. Maheshwari et al. (2017) reported combined drought and high temperature stress tolerance in rice by overexpressing APETALA-type of transcription factor *SbAP37*. Amara et al. (2013) reported enhanced water stress tolerance of transgenic maize overexpressing *LEA Rab28* gene. However, such transgenics did not exhibit greater tolerance to multiple stresses and final productivity under terminal drought stress conditions. Therefore, it is necessary to explore and exploit the extensive genetic diversity that exist in bacteria, fungi, and plants for improving plant abiotic stress tolerance especially during flowering and grain development stages of plant growth. Since, rice is highly susceptible to salt and water stresses, imparting stress tolerance is vital for stabilizing its yields.

In *Escherichia coli*, nine members of the cold shock protein (*CSP*) gene family *cold shock protein A - cold shock protein I* (*CspA-CspI*) have been identified, which function as RNA chaperons and help the cells to facilitate proper transcription as well as translation under low temperature stress (Graumann & Marahiel, 1998; Jiang et al., 1997). It has been observed that *CspD* inhibits DNA replication (Uppal et al. 2014), though Csps in general have been found to play important roles during osmotic, starvation, ethanol, and pH stresses (Keto-Timonen et al., 2016). *CspB* and *CspC* have been found to play critical roles in NaCl, pH, and ethanol stresses, but not *CspA* (Derman et al., 2015). Schmid et al. (2009) found that *CspA*, *CspB*, and *CspD* genes are dispensable for cold and NaCl stress adaptations in *Listeria monocytogenes*. Thus, conflicting roles for CspA have been noticed in literature. CspD protein of *E. coli* has been found to modulate growth-phase-dependent nutrient-stress adaptation (Yamanaka & Inouye, 1997). On the other hand, in *B. subtilis*, CspA, CspB, and CspD have been found to be associated with normal as well as growth-phase-dependent stress adaptation responses (Weber & Marahiel, 2002). Csps appear to be essential for growth of bacteria under cold stress conditions and also bind to single stranded RNA and DNA molecules. Of the several Csps, CspA has been shown to unwind the secondary structures of partially double stranded RNA molecules generated during low temperature stress conditions (Jiang et al., 1997). Premature transcription termination occurs due to RNA secondary structure formation in several prokaryotic systems, but *CspA*, *CspC*, and *CspE* have the transcription anti-termination activity (Bae et al., 2000). Jiang et al. (1997) demonstrated increased translation by *CspA* under cold stress conditions through the removal of stabilized RNA secondary structures. Castiglioni et al. (2008) expressed *CspA* and *CspB* genes in maize, which conferred abiotic stress tolerance with improved grain yield. Likewise, improved drought stress tolerance in wheat was noticed with the overexpression of synthetic bacterial cold shock protein gene *SeCspA* (Yu et al., 2017). Transgenic Arabidopsis overexpressing *SeCspA* and *SeCspB* genes displayed better seed germination and enhanced root length under water-deficit conditions when compared to wild-type (WT) plants (Yu et al., 2017). They also noticed important desirable characters like low levels of MDA, Na^+, higher chlorophyll, and proline levels under salt and water stress conditions. But they did not notice any change in the phenotype under abiotic stress conditions. Field experiments with transgenic wheat containing SeCspA gene (but not SeCspB transgenics) revealed increased 1000-grain weight as well as grain yield under drought stress (Yu et al., 2017). Accordingly, in the present study, two *Csp* genes (*CspA* and *CspB*) were cloned and overexpressed in rice for testing against salt and drought stresses during the flowering stage. We report the generation of transgenic rice tolerant to drought and salt stresses (exposed during the floral initiation stage) with significant increase in panicle length and improved yield. Further, for the first time, we report the occurrence of a stay-green (SGR) phenotype as a result of the overexpression of both *CspA* and *CspB* genes. Since a SGR phenotype was noticed, we looked at the expression levels of some of the associated genes like *STAY GREEN* (*SGR*, linked to drought stress), *CHLOROPHYLLASE* (involved in chlorophyll catabolism), *ISOPENTENYLTRANSFERASE 1* (*IPT1*, associated with cytokinin biosynthesis and control of senescence), *9-CIS-EPOXYCAROTENOID DIOXYGENASE* (*NCED*, involved in ABA biosynthesis), copper-zinc chloroplastic *SUPEROXIDE DISMUTASE* (*SOD*, associated with scavenging superoxide radicals), *sirtuin 1* (*SIRT1*) gene (implicated in leaf senescence and regulation of photosynthetic activity), besides *CspA* and *CspB* (Cucurachi et al., 2012) and linked their expressions with abiotic stresses.

2 | MATERIALS AND METHODS

2.1 | Isolation and vector construction of *CspA* and *CspB* genes

Full length primers were designed using IDT OligoAnalyzer online tool (https://eu.idtdna.com/calc/analyzer) for gene sequences available at the NCBI GenBank. The following forward 5′ATGTCCGGTAAAATG

ACTGGTATCG3′, 5′ATGTCAAATAAAATGACTGGTTTAGTA3′ and reverse 5′TTACAGGCTGGTTACGTTACCAG3′, 5′TTAATCAGTAATG ATGACATTTGCT3′ primers were used for *CspA* and *CspB* gene amplifications, respectively, from the *E. coli* K12 strain. PCR conditions used were initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, annealing at 57°C for *CspA* gene and 54°C for *CspB* for 30 s and extension at 72°C for 30 s; final extension step at 72°C for 5 min using the Biorad C1000 Touch™ Thermal Cycler PCR System. The amplicons were cloned into TA cloning vector pTZ57R/T (Thermo Fischer Fermentas #K0691) separately using T_4 DNA ligase (Fermentas), then transferred into the *E. coli* Top10 strain using 0.1 M $CaCl_2$ and heat shock at 42°C. The transformed colonies were grown on Luria-Bertani (LB) medium (Bertani, 1951) containing 100 mg L^{-1} ampicillin, and blue/white colonies were screened with isopropyl β-D-1-thiogalactopyranoside (IPTG)/5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Transformed white colonies were picked; the plasmid was isolated by alkaline lysis method, confirmed by polymerase chain reaction (PCR), and the genes were sequenced (Eurofins). The DNA sequences were blasted against NCBI GenBank reference genes and submitted to the National Center for Biotechnology Information (NCBI). Both *CspA* and *CspB* genes were ligated into the intermediate PRT101 vector (3.3 Kb) containing the cauliflower mosaic virus 35S (CaMV35S) promoter and CaMV polyA terminator and transferred into *E. coli* using the *KpnI* and *BamHI* restriction sites. The construct was confirmed with *HindIII* restriction enzyme digestion. The released construct was transferred into the plant binary vector pCAMBIA1301 (13.1 Kb) containing *β-glucuronidase* (*GUS*) as reporter gene and *hygromycin phosphotransferase II* (*hptII*) as selection marker. The pCAMBIA1301 plasmid harboring *CspA* and *CspB* genes were separately mobilized into *Agrobacterium tumefaciens* LBA4404 strain. The recombinant colonies were selected on yeast extract peptone (YEP) medium containing 50 mg L^{-1} kanamycin, and 20 mg L^{-1} rifampicin.

2.2 | Rice genetic transformation by in planta method

Seeds of indica rice variety BPT5204 were dehusked and sterilized with 0.1% mercuric chloride for 5 min. Seeds were washed thoroughly with sterile distilled water for 3 times. For germination, sterile seeds were soaked in autoclaved water for 2 days in dark at 26–28°C. For each construct (*CspA* and *CspB*), 2000 seedlings were infected with *Agrobacterium*. Once shoots and roots emerged, they were pricked with 0.5 × 25 mm sterile needles. Immediately, wounded seedlings were incubated in half-strength Murashige and Skoog's (MS) basal liquid medium (Murashige & Skoog, 1962) containing 100 μM acetosyringone and *Agrobacterium* containing *CspA* and *CspB* genes separately. Seedlings were agitated for 20 min at 100*g* and inoculated onto MS medium containing 100 μM acetosyringone, and incubated for 2–3 days at 28°C. For elimination of bacterial infection, seedlings were treated with 250 mg L^{-1} cefotaxime. Seedlings growing on 50 mg L^{-1} hygromycin containing medium were finally transferred into pots filled with garden soil. After one-month, putative transformants were transplanted to new pots. Transgenic lines developed from *CspA* and *CspB* genes were labeled as A and B lines, respectively.

2.3 | Screening of transgenics for GUS assay and molecular analysis

Leaf material from 45-day-old putative transgenic plants was taken, rinsed twice with sterile distilled water, and incubated at 37°C in an incubator in presence of *GUS* staining buffer [2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc)], 2 mM potassium ferricyanide and 2 mM potassium ferrocyanide in 0.1 M sodium phosphate buffer, pH 7.0. The samples were incubated for 16–20 h and destained by incubating in 90% ethanol. The procedure was repeated several times to visualize the blue color. Genomic DNA was isolated from *CspA*, *CspB* transgenics and WT plants by cetyltrimethyl ammonium bromide (CTAB) method (Dellaporta et al., 1983). All the in planta transformed plants along with WT were screened by PCR using gene-specific primers for the presence of transgenes (*CspA* and *CspB*) and *hptII* marker. Gene copy number analysis was performed by following the procedure laid down by Sambrook and Russell (2001). *CspA* and *CspB* cassettes were released using *HindIII* restriction enzyme and probed with *hptII* gene. Nitrocellulose membrane was developed by chemiluminescence method (Roche DIG DNA labeling kit).

2.4 | Bacterial growth study by pET expression

CspA and *CspB* genes were cloned into bacterial expression vector pET32a(+) using *EcoRI* and *HindIII* restriction enzymes and transformed into *E. coli* expression compatible cells BL21 (DE3). For functional analysis, recombinant *CspA* and *CspB* along with plain vector cells were grown in 200 ml LB medium with and without 200 mM NaCl and 300 mM mannitol. Corresponding controls were also maintained. The bacterial growth was measured by UV–Visible spectrophotometer (Thermo Scientific). Cells were induced with 1 mM IPTG at OD_{600} nm at 30°C. Whole protein was isolated from 4-h-induced culture, using 6 M urea, 1% sodium dodecyl sulfate (SDS), and 0.1 M dithiothreitol (DTT), the cell lysate was heat denaturated (90°C) for 5 min with 1× SDS sample buffer. Samples were resolved in 12% SDS-PAGE gel with prestained protein ladder (Genei), blotted on nitrocellulose membrane, AntiHis tag primary antibody (Invitrogen 100 mg per 200 μl, 1:1000 dilution) was added. Membrane was washed twice with washing buffer, secondary antibody (Invitrogen, 1 mg) conjugated with horse radish peroxidase (HRP) (1 mg ml^{-1}, 1:10 000 dilution) was added and the blot developed by adding 3,3′, 5,5″-tetramethylbenzidine (TMB) solution (Thermo Scientific).

2.5 | Segregation analysis of *hptII* in T_1 and T_2 generations

The T_1 seeds obtained from single copy selfed T_0 seeds were sterilized and transferred onto Petri dishes containing MS medium with

50 mg L^{-1} hygromycin. After 10 days of growth, transgenics were screened for segregation analysis by studying hygromycin resistant and sensitive seedlings. Hemizygous seedlings were later transferred to the pots containing garden soil. Similarly, T_2 transgenic lines obtained from selfing of hemizygous lines were grown on MS medium with 50 mg L^{-1} hygromycin along with WT plants and screened for their homozygous nature. Homozygous T_2 lines were used for subsequent analysis.

2.6 | Salt and drought stress treatments

Before floral initiation stage, homozygous $A_{15\text{-}1\text{-}1}$, $A_{59\text{-}1\text{-}1}$, $A_{62\text{-}1\text{-}3}$, $B_{2\text{-}1\text{-}4}$, $B_{16\text{-}1\text{-}2}$, and $B_{42\text{-}1\text{-}3}$ transgenic lines alongside WT plants growing in the pot conditions were used to assess salt and drought stress tolerance. Salt treatment was given by adding 250 ml of 150 mM NaCl solution to each pot on the first day. The drought stress was imposed by withholding water for 8 days. The corresponding controls were maintained for all the treatments. After 8 days of stress treatment, all plants were watered with normal tap water. The same treatment was used for all experiments unless otherwise mentioned. After stress treatment, transgenic lines and WT plants were evaluated for salt and drought stress respectively.

2.7 | Estimation of chlorophyll, proline, malondialdehyde (MDA), relative water content (RWC), and antioxidant enzyme activities

Salt and drought-treated transgenic lines and WT plants were used for the estimation of chlorophyll, proline, MDA content, and antioxidant enzyme activities. Chlorophyll *a* and *b* were estimated following the method of Arnon et al. (1974). Quantities of chlorophyll *a* and *b* were calculated and represented as mg g^{-1} fresh weight. Proline was estimated following the method of Bates et al. (1973) and expressed as μmol proline g^{-1} fresh weight of leaf tissue. Lipid peroxidation was determined by measuring the amount of MDA produced by thiobarbituric acid (TBA) reaction as described by Hodges et al. (1999). Lipid peroxidation is expressed in MDA μmol g^{-1} fresh weight of leaf tissue. The RWC was measured by taking fresh weight, turgid weight, and dry weight of leaves (Van Heerden & de Villiers, 1996). Activity of catalase (CAT) enzyme was assessed by following the procedure of Aebi (1984). One unit of enzyme activity is defined as 1 μmol of H_2O_2decomposed to water min^{-1} mg^{-1} of protein. The reaction of superoxide dismutase (SOD) was carried out by the method of Beauchamp and Fridovich (1971). SOD activity is defined as that amount of enzyme required to inhibit the reduction of nitrobluetetrazolium by 50% under the specified conditions. Activity is expressed as units mg^{-1} protein min^{-1}. Guaiacol peroxidase (GPX) was estimated according to Egley et al. (1983). Enzyme activity is expressed as μmol H_2O_2 reduced mg^{-1} of protein min^{-1}. Protein was quantified by Bradford assay (Bradford, 1976) using bovine serum albumin as standard.

2.8 | Estimation of ions in transgenics and WT plants

Transgenic lines $A_{62\text{-}1\text{-}3}$ and $B_{42\text{-}1\text{-}3}$ (selected based on the performance under stress) and WT plants aftersalt and drought treatments were used for measuring ions. Na^+ and K^+ ions were quantified using atomic absorption spectrometer. Samples were dried in an oven at 104°C, powdered, digested with 8 ml of nitric acid and peroxide, filtered and diluted with 50 μl deionized water.

2.9 | Anatomy of WT and transgenic rice roots

One-month-old seedlings of rice WT, CspA (transgenic line $A_{62\text{-}1\text{-}3}$), and CspB (transgenic line $B_{42\text{-}1\text{-}3}$) were subjected to 150 mM NaCl and drought stress (withholding water) stresses for 48 h. Anatomical sections of the roots were taken with a sharp blade, stained with toluidine blue (5 mg ml^{-1}) for 15 min, destained with water and observed under brightfield microscope (Olympus model CH20iBIMF).

2.10 | Root morphology, shoot, and panicle lengths, seed number per panicle in transgenics and WT plants

Before seed setting, salt treatment was given by adding 250 ml of 150 mM NaCl solution to each pot on the first day. The drought stress was imposed by withholding water for 8 days. After 8 days of stress treatment, all plants were watered with normal tap water. The corresponding controls were maintained for all the treatments. Transgenic lines and WT plants were analyzed for root length, root dry mass, shoot, panicle lengths, and number of seeds per panicle.

2.11 | Gene expressions using quantitative real-time PCR (qRT-PCR) under multiple stress conditions

The relative quantification of gene expressions was studied in 1-month-old seedlings of T_2 transgenic lines $A_{62\text{-}1\text{-}3}$ and $B_{42\text{-}1\text{-}3}$. Seedlings were treated with salt (150 mM), drought (200 mM mannitol), cold (4°C), and high temperature (42°C) stresses. After 4 h of treatment, total RNA was isolated from root and leaf samples using guanidine thiocyanate method. Concentration of RNA was measured by Nano Drop and diluted to the final RNA concentration of 1 μg. It was converted to cDNA using Thermo Revert Aid cDNA Kit (Thermo Scientific). The cDNA was used as template for the gene expression studies (Applied Biosystem Fast PCR 7500) using SYBR green method. Actin gene served as an internal control. In 96 well plates, reaction volume was adjusted to final volume of 20 μl with Tm 60°C. The $2^{-\Delta\Delta Ct}$ values calculated are represented in the form of a graph. The relative expression levels of the genes *CspA*, *CspB*, *SGR*, *chlorophyllase*,

IPT1, *NCED*, *SOD*, and *SIRT1* were studied under diverse abiotic stress conditions in the transgenic lines and WT plants.

2.12 | Statistical analysis of data

All experiments were repeated with biological triplicates and also technical triplicates. For gene expression studies, data from biological triplicates along with technical replicates were collected. Average values along with standard deviations are represented in graphs/tables. All the data were subjected to one-way analysis of variance (ANOVA), since *CspA* and *CspB* lines were developed separately. *P*-value <0.5 was determined and the level of significance indicated in the graphs/tables.

3 | RESULTS

3.1 | *CspA* and *CspB* gene cloning and *in planta* transformation

In the present study, both *CspA* and *CspB* genes were cloned from the *E. coli* strain K12. The length of the *CspA* (MH029279) and *CspB* (MH029280) gene sequences were 213 and 216 bp, respectively (Figure 1A). *In silico*, studies of domain analysis showed eukaryotic conserved cold shock domain (CSD) and ribonuclease-binding domain (RBD) in CspA (Figure S1) and CspB (Figure S2) indicating that they are involved in temperature stress. Presence of the gene cassettes were confirmed by restriction digestion (Figure 1B). *A. tumefaciens* LBA4404 pCAMBIA1301 *CspA* (Figure 1C) and pCAMBIA1301 *CspB* (Figure 1D) gene constructs driven by CaMV35S promoter and CaMV polyA terminator were used for in planta transformation. The in planta transformed seedlings were grown to maturity in pot conditions.

3.2 | Characterization of transgenics for transgene integration

All the T_0 generation, plants were tested for GUS histochemical activity alongside WT plants. GUS histochemical activity was noticed in transgenics, but not in WT plants (Figure 2). A total of six *CspA* and six *CspB* transgenic lines were found PCR positive. Thus, the transformation frequency was only 0.33%. Putative transgenic lines were named as A_{12}, A_{15}, A_{45}, A_{50}, A_{59}, A_{62} and B_2, B_{16}, B_{35}, B_{42}, B_{82}, B_{98} for *CspA* and *CspB* genes, respectively. PCR analysis showed an amplification

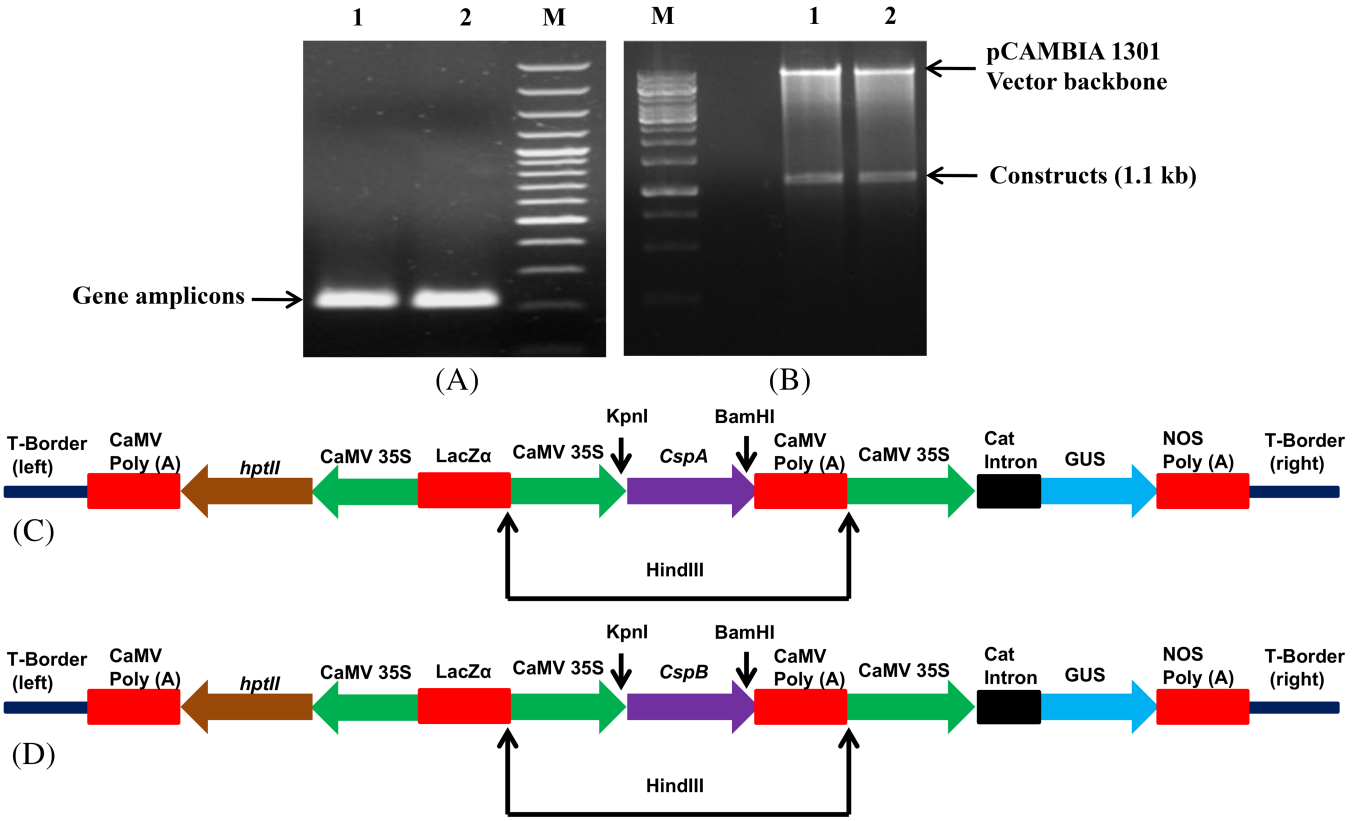

FIGURE 1 Gene amplification, vector construction, and recombinant vector map. (A) Amplification of bacterial gene 1 = *CspA* (213 bp), 2 = *CspB* (216 bp), M = 100 bp DNA marker, (B) recombinant construct harboring binary vector pCAMBIA1301 with *HindIII* restriction enzyme digestion, (C) recombinant vector map of pCAMBIA1301 containing hygromycin phosphotransferase (*hptII*), CaMV35S, cauliflower mosaic virus 35S (*CaMV35S*) promoter; *CspA* gene, *CaMV* poly A terminator, (D) recombinant vector map of pCAMBIA1301 containing *hptII*. *CaMV35S*, *CspB*gene, *CaMV* poly A terminator. M = 1Kb DNA marker, 1 = pCAMBIA 1301 + *CspA* construct (11 kb), 2 = pCAMBIA 1301 + *CspB* construct, CaMV35S promoter, *CspA* gene, *CspB*gene, *CaMV* poly A terminator

size of 213 bp for *CspA* (Figure 3A), and 216 bp for *CspB* (Figure 3B). Similarly, amplification of 750 bp for *hptII* gene was observed in both the transgenic lines (Figure 3C,D). Gene amplification by PCR was not detected in the WT plants (Figures 3A–D). Gene copy number by Southern blotting confirmed the integration of *CspA* and *CspB* genes in the transgenic lines (Figure 3E,F). Out of 12 transgenics, four lines from *CspA* (A_{15}, A_{45}, A_{59}, A_{62}) and four from *CspB* (B_2, B_{16}, B_{42}, B_{98}) were confirmed to have a single gene copy insertions (Figure 3E, F). On the other hand, in WT plants, no band was noticed, indicating that there was no transgene integration (Figure 3E,F). Subsequently, transgenics with single gene copy insertions were used for salt and drought stress tolerance experiments.

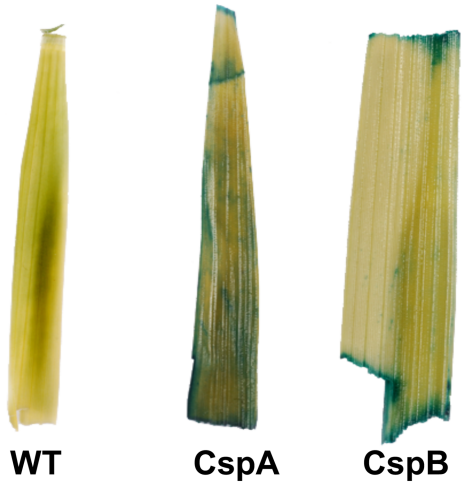

FIGURE 2 Histochemical GUS assay in T_0 transgenic plants. *CspA*, *CspA* putative transgenic plant; *CspB*, *CspB* putative transgenic plant; WT, wild type plant

3.3 | Bacterial expression of *CspA* and *CspB* under NaCl and mannitol stresses

CspA and *CspB* genes were transferred into pET-32a(+) expression vector (Figure 4A). The presence of approximately 25.4 KD recombinant proteins along with the tags was noticed (Figure 4B), and protein expressions confirmed by blot with Anti-6× His Tag antibodies (Figure 4C). Once the expression of the recombinant proteins was confirmed, the growth of the recombinants was measured with and without salt and mannitol stresses. Growth of the untransformed cells decreased slightly in 200 mM NaCl and drastically under 300 mM mannitol (Figure 5A,B). Contrarily, *CspB* cells showed more resistance to mannitol than *CspA* (Figure 5B) but growth of *CspA* was better under salt stress treatment (Figure 5A).

3.4 | Mendelian inheritance pattern of *hptII* in T_1 and T_2 generations

T_1 transgenic line progenies segregated in a ratio of 3 tolerant:1 susceptible (Table S1). All T_2 progenies obtained from selfed hemizygous lines ($A_{15\text{-}1\text{-}1}$, $A_{59\text{-}1\text{-}1}$, $A_{62\text{-}1\text{-}3}$, $B_{2\text{-}1\text{-}4}$, $B_{16\text{-}1\text{-}2}$, and $B_{42\text{-}1\text{-}3}$) segregated in

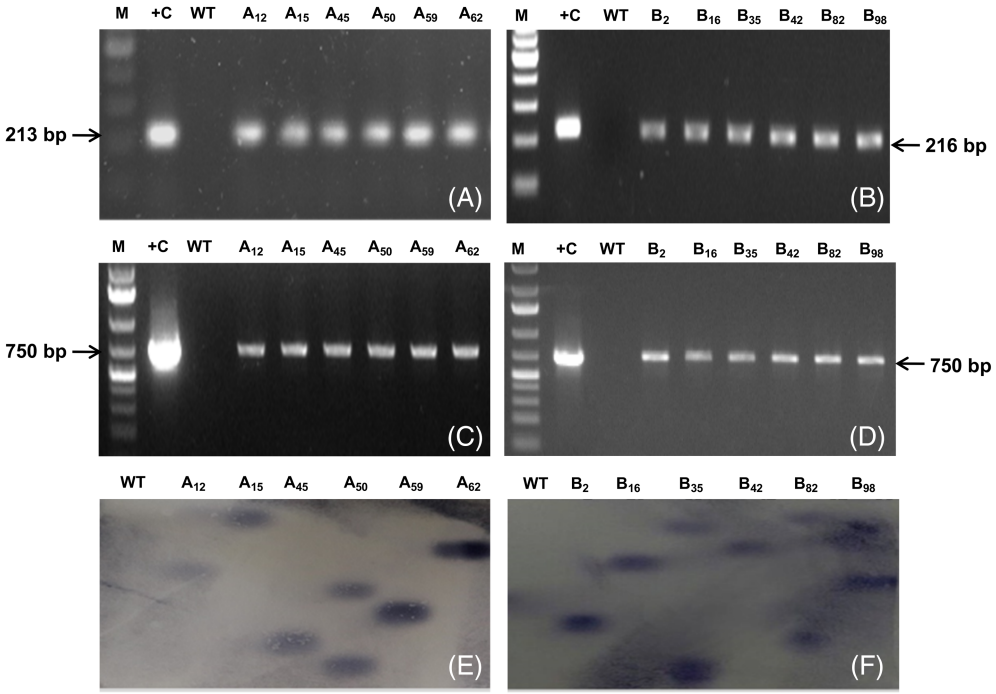

FIGURE 3 Molecular characterization of transgenics. (A) Amplification of *CspA* gene in T_0 transgenics. (B) Amplification of *CspB* gene in T_0 transgenics. (C) *hptII* selection marker gene (750 bp) amplification from T_0*CspA* transgenics. (D) *hptII* selection marker gene (750 bp) amplification from T_0 *CspB* transgenics. (E) *CspA* gene copy number as analyzed by Southern blot (genomic DNA was digested with *HindIII* and probed by *hptII* marker). (F) *CspB* gene copy number as analyzed by Southern (genomic DNA was digested with *HindIII* and probed by *hptII* marker). A_{12}, A_{15}, A_{45}, A_{50}, A_{59}, A_{62}, T_0*CspA* transgenics; B_2, B_{16}, B_{35}, B_{42}, B_{82}, B_{98}, *CspB* transgenics; +C, positive control; M, 100 bp DNA marker; WT, wild type

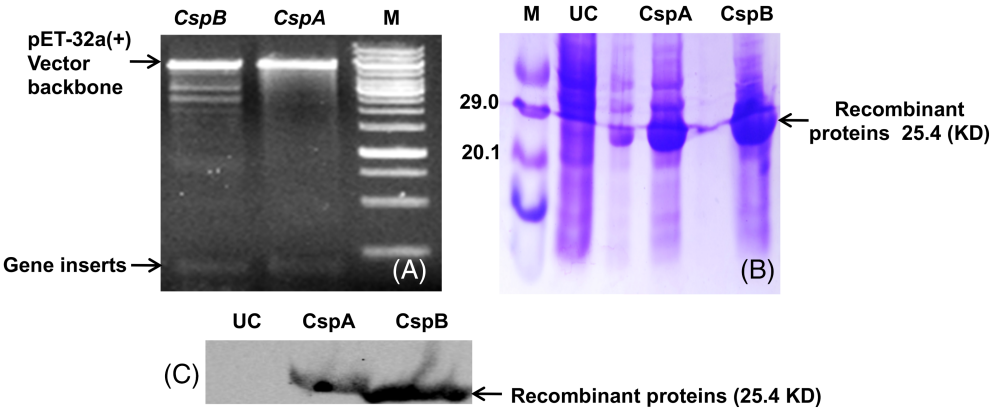

FIGURE 4 Expression of *CspA* and *CspB* genes in pET. (A) *CspA* and *CspB*, pET-32a (+) vector was double digested with *EcoRI* and *HindIII* enzymes. (B) SDS-PAGE, 1 mM IPTG was used for induction of recombinant protein in *E coli* BL21. (C) Western blot analysis of recombinant CspA and CspB protein expression with pET-32a(±) vector. *CspA*, CspA + pET-32a(±); *CspB*, *CspB*+ pET-32a(±); M, protein marker; UC, untransformed control

a 1:2:1 ratio (Table S2). Homozygous dominant lines displayed 100% germination on the selection medium. On the other hand, WT seeds failed to germinate at all on the hygromycin medium.

3.5 | Salt and drought stress tolerance in T_2 generation

Transgenic lines exhibited enhanced tolerance to salt and drought stresses in comparison with WT plants. Transgenic leaves remained relatively green in color with less yellowing, less leaf rolling, delayed senescence and higher tillering. Conversely, WT leaves were found sensitive to salt and drought stresses, exhibited wilting, increased leaf rolling, early senescence, and lower tillering (Figure 6). Transgenic lines overexpressing *CspA* and *CspB* exhibited SGR phenotype and delayed senescence compared to WT plants (Figure 6).

3.6 | Chlorophyll, proline, MDA contents, and antioxidative enzyme activities

No significant decrease in chlorophyll *a* content was observed in transgenic lines. However, chlorophyll *b* decreased slightly under the treatments. Content of chlorophyll *a* declined in WT plants under salt and drought stress conditions from 2.1 to 1.41 mg g^{-1} and 2.1 to 1.45 mg g^{-1}, respectively. Similarly, chlorophyll *b* in WT declined from 0.75 to 0.46 mg g^{-1} and 0.75 to 0.53 mg g^{-1} under salt and drought stresses, respectively (Table 1). Proline content significantly increased in all transgenic lines compared to WT when treated with drought and salt stresses. Proline content increased to 2.1- and 2.7-folds in *CspA* and *CspB* transgenics treated with salt and drought stresses respectively (Figure 7A). Contrarily, levels of MDA were higher in WT than the transgenics under stress conditions. Similarly, under salt stress, MDA content was enhanced from 0.481 to 0.707 μmol g^{-1} fresh weight in *CspA* and 0.482 to 0.690 μmol g^{-1} fresh weight in *CspB* transgenics (Figure 7B). Under drought stress, MDA content increased from 0.481 to 0.570 μmol g^{-1} fresh weight in *CspA* and 0.482 to 0.536 μmol g^{-1} fresh weight in *CspB* transgenic lines (Figure 7B). *CspA* and *CspB* transgenics exhibited significant increase in RWC compared to WT under salt and drought stresses (Figure 7C). After treatment with salt and drought, CAT specific activity was increased in both the transgenic lines and WT plants (Figure 7D). Under salt stress, specific CAT activity increased from 58.6 to 69 units in *CspA* and 65.6 to 66.3 units mg^{-1} protein min^{-1} in *CspB* transgenic lines (Figure 7D). Similarly, under drought stress, CAT activity leaped from 58.6 to 82 units mg^{-1} protein min^{-1} in *CspA* and 65.6 to 94.3 units mg^{-1} protein min^{-1} in *CspB* (Figure 7D). Under salt stress, the SOD activity was elevated from 10.85 to 26.6 units mg^{-1} protein min^{-1} in *CspA* and from 12.3 to 26.5 units mg^{-1} protein min^{-1} in *CspB* transgenics (Figure 7E). Under drought stress, SOD activity increased from 10.85 to 26.7 units mg^{-1} protein min^{-1} in *CspA* and 12.3 to 26.5 units mg^{-1} protein min^{-1} in *CspB* (Figure 7E). Specific activity of GPX increased in both the transgenic lines and WT plants, respectively (Figure 7F). Under salt stress, GPX activity increased from 297.3 to 312 units mg^{-1} protein min^{-1} in *CspA* and 298 to 343 units mg^{-1} protein min^{-1} in *CspB* transgenics (Figure 7F). Under drought stress, activity of GPX increased from 297.3 to 358 units in *CspA* and 298 to 374 units mg^{-1} protein min^{-1} in *CspB* (Figure 7F).

3.7 | Estimation of Na^+ and K^+ under stress conditions

No significant accumulation of Na^+ and K^+ was noticed in WT root and leaf tissues (Figure 8). On the contrary, significant accumulation of Na^+ was recorded in transgenic lines under salt and drought stresses. Under salt stress, Na^+ levels in root tissues increased 3.6- and 2.2-folds in *CspA* and *CspB* lines, respectively. Likewise, under drought stress, 2.4- and 1.7-folds higher Na^+ levels were noticed in leaf tissues of *CspA* and *CspB* lines, respectively (Figure 8A). Similarly,

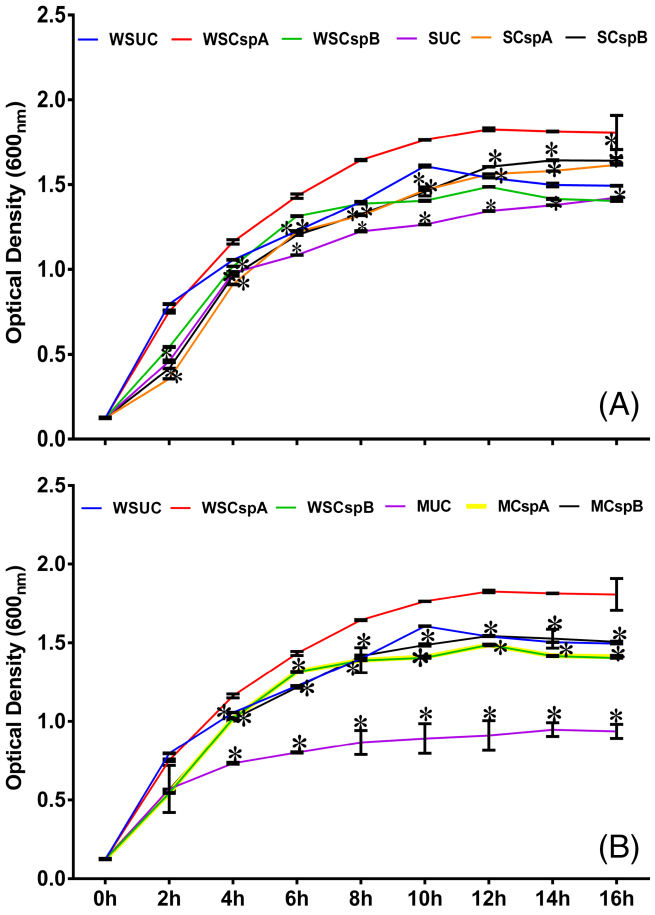

FIGURE 5 Recombinant BL21 strain with pET-32a(±) vector containing *CspA* and *CspB* genes and the growth of the strain with and without stress. (A) Effect of salt stress on bacterial growth, (B) effect of mannitol stress on bacterial growth. MNCspA, recombinant CspA strain grown in presence of 300 mM mannitol; MNCspB, recombinant strain CspB grown in presence of 300 mM mannitol; MNUC, UC grown in presence of 300 mM mannitol; STCspA, recombinant *CspA* strain grown in 200 mM NaCl; STCspB, recombinant *CspB* strain grown in 200 mM NaCl; STUC, UC grown in presence of 200 mM NaCl; WSCspA, *CspA* recombinant strain grown without stress; WSCspB, *CspB* recombinant strain grown without stress; WSUC, untransformed cells (UC) grown without stress (WS), . OD was measured at 600_{nm} after growing for a duration of 16 h. Statistical significance is indicated as star (*) (*P*-value <0.05)

significant increase in Na^+ was noticed in transgenic root tissues after the imposition of salt and drought stresses (Figure 8B). Under stress conditions, enhanced accumulation of K^+ content was recorded in transgenics and WT leaf and root tissues compared to their corresponding controls. Accumulation of K^+ was higher in transgenics compared to WT (Figure 8C,D).

3.8 | Root, shoot, panicle lengths, root biomass, and number of seeds per panicle

After stress treatments, the root traits varied in transgenics and WT plants (Figure 9A). Devoid of stress, transgenic lines showed better root length (32.5 cm) than WT (26.02 cm) plants (Figure 9A). Root lengths decreased under salt stress conditions (Figure 9B). The decrease in root lengths was more pronounced in salt-treated transgenics compared to roots grown under drought (Figure 9B). With the imposition of drought stress, root length decreased significantly in both the transgenic lines compared to WT (Figure 9B). Root dry mass decreased under salt and drought stresses in WT in comparison with transgenics (Figure 9C). After treatment with salt and drought stresses, significant decrease in shoot length was observed in WT and transgenic lines. The decrease in shoot length was higher under salt stress in comparison with drought stress-treated plants (Figure 10A). Devoid of stresses, the average length of the panicle in WT and transgenic lines was 14.21 ± 0.61 cm, and 18.54 ± 0.44 cm, respectively (Figure 10B). Panicle length decreased in WT plants (13.50 ± 0.83 cm and 10.03 ± 2.13 cm) as well as in transgenics under salt and drought stresses (15.39 ± 0.42 cm and 15.83 ± 0.67 cm), respectively (Figure 10B). Thus, the decrease in panicle length was more drastic under drought compared to salt stress. After stress imposition, a higher decrease in the number of seeds per panicle was noticed in WT compared to transgenics. A number of seeds per panicle in WT plants were reduced from 115.3 (devoid of stress) to 100.6 and 92.3 under salt and drought stresses, respectively. However, in CspA transgenic line, the reduction in seed number was less. In the line $A_{15\text{-}1\text{-}1}$, seed number decreased from 140 to 120.5 and 132.6, and in $A_{59\text{-}1\text{-}1}$, from 129.3 to 125.5 and 124.6 under salt and drought stresses, respectively. In the lines $B_{2\text{-}1\text{-}4}$ and $B_{42\text{-}1\text{-}3}$, number of seeds decreased from 140.16 and 147.3 to 124.3, 107.1, 115.5 and 129.3 under salt and drought stress conditions, respectively (Table 2).

3.9 | Anatomy of WT and transgenic roots

One-month-old seedlings of WT, CspA, and CspB were exposed to 150 mM NaCl and also to drought stress (by withholding water) for 48 h along with appropriate controls. Anatomical sections of the WT roots revealed thin-walled sclerenchyma cells and loosely organized aerenchyma (Figure S3). In contrast, transgenic plants (*CspA* and *CspB* lines) were characterized by thick-walled sclerenchyma cells. Further, compactly arranged cortical cells with narrow intercellular spaces were the characteristic feature of the transgenic roots (Figure S3).

3.10 | *CspA* and *CspB* transcript levels in T_2 transgenics and expression of *SGR* associated genes under salt, drought, cold, and heat stresses

Transcript levels were higher in *CspA* (RTA) leaves and roots than in *CspB* (RTB) leaves and roots. Induction of the *chlorophyllase* gene in both *CspA* and *CspB* was markedly higher under the four abiotic stress conditions, than without stress. All the four stresses influenced the *IPT1* gene, more so under cold and drought compared to salt and high temperature stresses (Figure 11). *NCED* was

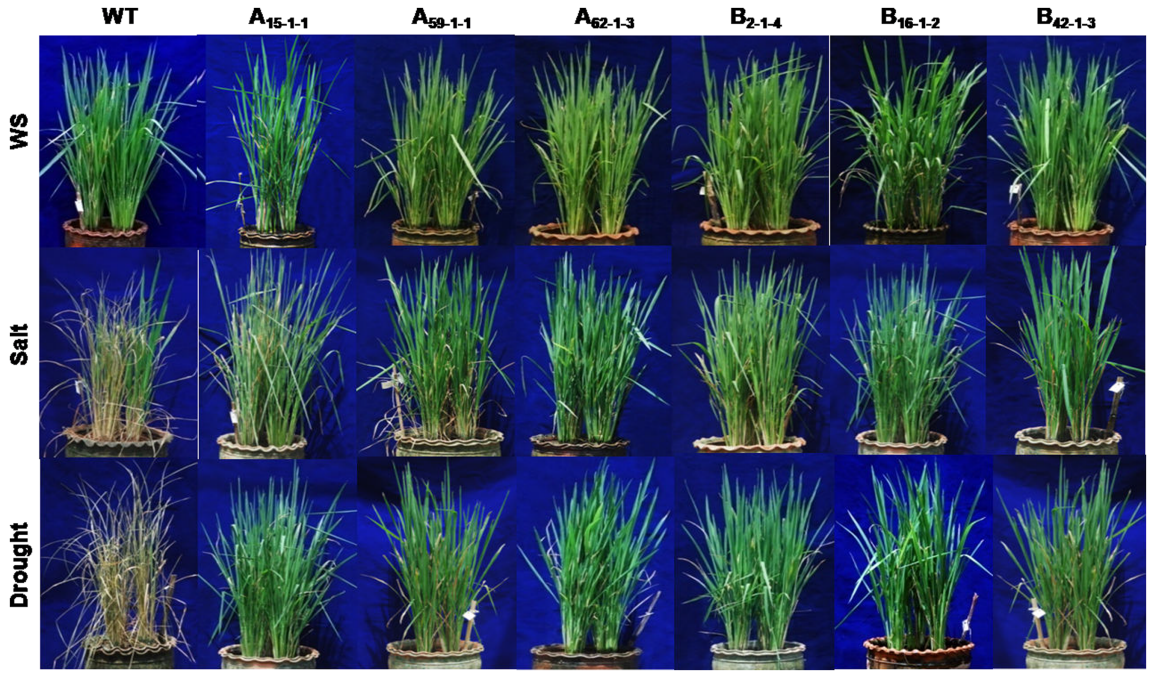

FIGURE 6 Evaluation of T_2 transgenic lines subjected to salt and drought stresses. $A_{15\text{-}1\text{-}1}$, $A_{59\text{-}1\text{-}1}$, $A_{62\text{-}1\text{-}3}$, T_0 *CspA* transgenic lines; $B_{2\text{-}1\text{-}4}$, $B_{16\text{-}1\text{-}2}$, $B_{42\text{-}1\text{-}3}$, T_0 *CspB* transgenic lines; drought, drought stress imposed by water withholding; salt, salt stress imposed by 150 mM NaCl; WS, without stress; WT, wild type

TABLE 1 Chlorophyll *a* and *b* contents in WT and transgenic lines grown under salt and drought stress conditions

Stress	WT	$A_{15\text{-}1\text{-}1}$	$A_{59\text{-}1\text{-}1}$	$A_{62\text{-}1\text{-}3}$	$B_{2\text{-}1\text{-}4}$	$B_{16\text{-}1\text{-}2}$	$B_{42\text{-}1\text{-}3}$
Chlorophyll *a*							
WS	2.10 (±0.001)	2.11(±0.001)	2.15 (±0.001)	2.14 (±0.005)	2.03 (±0.002)	2.16 (±0.001)	2.15 (±0.001)
Salt	1.41 (±0.47)	1.88 (±0.093)[a]	1.84 (±0.10)[a]	1.88 (±0.05)[a]	1.95 (±0.22)	1.93 (±0.13)	2.01 (±0.04)[a]
Drought	1.45 (±0.54)	1.94 (±0.12)	1.93 (±0.11)	1.89 (±0.12)	1.94 (±0.13)	1.94 (±0.12)	2.02 (±0.05)
Chlorophyll *b*							
WS	0.75 (±0.001)	0.88 (±0.001)	0.80 (±0.002)	0.80 (±0.008)	0.75 (±0.006)	0.79 (±0.004)	0.81 (±0.01)
Salt	0.46 (±0.47)	0.58 (±0.09)[a]	0.501 (±0.10)	0.505 (±0.05)	0.75 (±0.17)	0.775 (±0.09)[a]	0.687 (±0.032)
Drought	0.53 (±0.54)	0.71 (±0.12)	0.60 (±0.11)[a]	0.64 (±0.12)	0.88 (±0.10)	0.89 (±0.06)	0.75 (±0.07)

Note: Data represent means ± SD ($n = 9$; three biological replicates).
Abbreviations: $A_{15\text{-}1\text{-}1}$, $A_{59\text{-}1\text{-}1}$, $A_{62\text{-}1\text{-}3}$, $B_{2\text{-}1\text{-}4}$, $B_{16\text{-}1\text{-}2}$, $B_{42\text{-}1\text{-}3}$, transgenic lines; drought, drought by withholding water; salt, 150 mM NaCl; WS, without stress, WT, wild-type plants.
[a]Indicates significant at $P < 0.05$ as analyzed by one-way ANOVA.

also induced by the abiotic stresses and the expression was higher under salt, cold, and high temperatures than under drought stress. Both *CspA* and *CspB* were strongly upregulated by cold (but not in *CspB* roots), followed by salt and drought. Expressions were low under high temperature stress, especially in *CspB* roots. *SGR* gene expression was enhanced steeply by drought and cold in comparison with salt and high temperature stresses (Figure 11). *SOD* expression was prompted by salt and high temperature stresses in comparison with cold and drought. Comparatively, cold and drought stresses promoted *SIRT1* gene expressions more than salt and high temperatures (Figure 11).

4 | DISCUSSION

4.1 | Gene cloning, validation of *CspA* and *CspB* genes under salt and drought (mannitol) stresses

Csps have been reported to contain a CSD, which is generally composed of 65 to 70 amino acid residues in bacteria, as well as in higher organisms including plants (Horn et al., 2007). Csp proteins are 7 to 10 kDa in size and contain the nucleic acid-binding activities. Jiang et al. (1997) pointed out that in *E. coli*, CSD acts as an RNA chaperone and converts double-stranded RNA into single-stranded RNA. Several

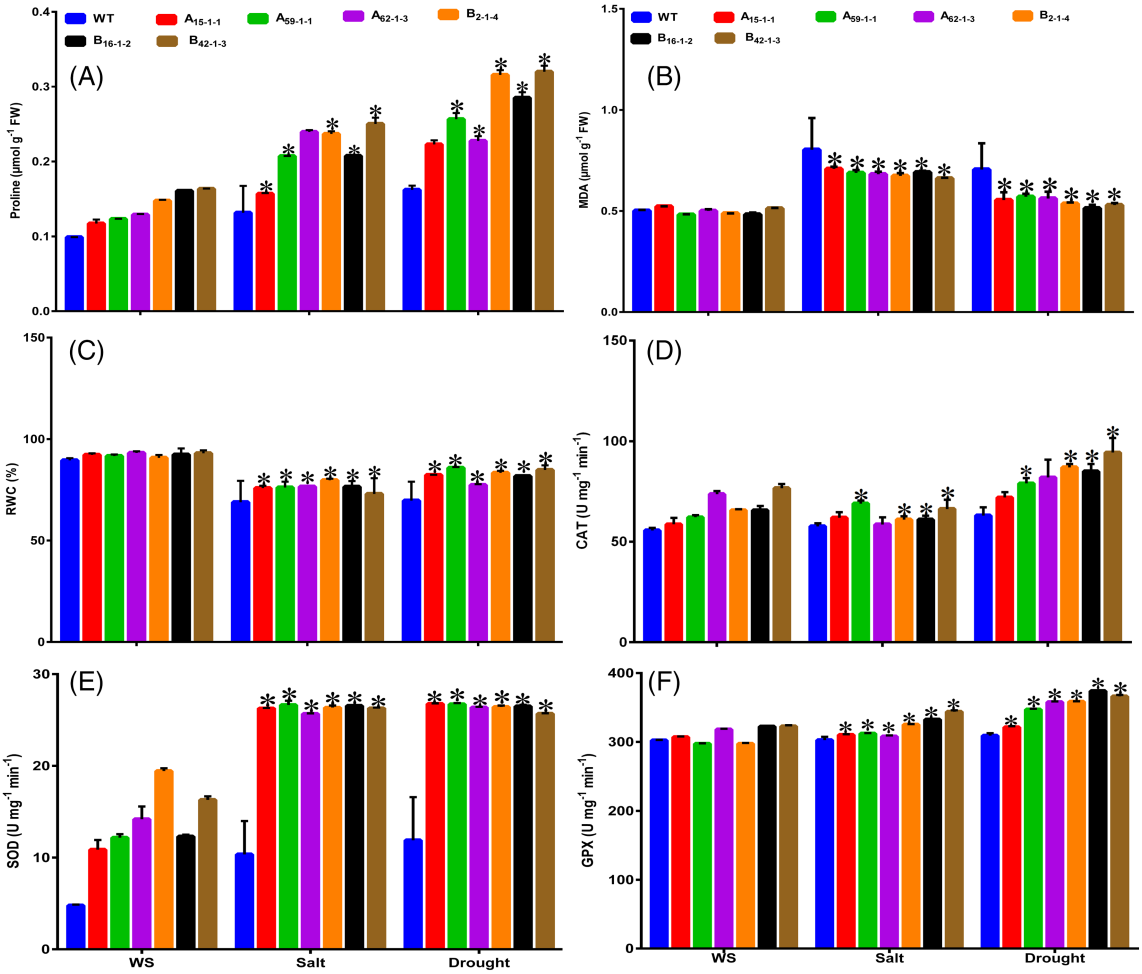

FIGURE 7 Estimation of proline, malondialdehyde (MDA), relative water content (RWC), activities of catalase (CAT), superoxide dismutase (SOD), and guaiacol peroxidase (GPX) in transgenic rice exposed to salt stress and drought conditions (A) Proline, (B) MDA, (C) RWC, (D) CAT (units mg^{-1} protein min^{-1}), (E) SOD (units mg^{-1} protein min^{-1}), (F) GPX (units mg^{-1} protein min^{-1}). Salt and drought stresses were imposed by adding 150 mM NaCl water and withholding respectively. $A_{15\text{-}1\text{-}1}$, $A_{59\text{-}1\text{-}1}$, $A_{62\text{-}1\text{-}3}$, $B_{2\text{-}1\text{-}4}$, $B_{16\text{-}1\text{-}2}$, $B_{42\text{-}1\text{-}3}$, transgenic lines; FW, leaf fresh weight; WS, without stress; WT, wild type. Statistical significance is indicated as star (*) (*P*-value < 0.05)

of the Csp proteins may stimulate growth during stress acclimation. Hunger et al. (2006) found out that Csps work in concert with a DEAD box helicase to rescue misfolded mRNA and help in transcription (El-Sharoud & Graumann, 2007). Bae et al. (2000) showed that *CspA*, *CspC*, and *CspE* genes act as antiterminators and regulate the expression of cold-inducible genes. In the present study, bacterial *CspA* and *CspB* genes were cloned which shared 100% homology with the *E. coli* sequences and displayed both CSD as well as RBD. Plants also have CSD proteins, which differ from that of Csps known to occur in prokaryotes (Sasaki & Imai, 2012). Several of the bacterial *Csps* and plant CSDs were found to be induced under cold stress (Jung et al., 2010; Sasaki et al., 2007). Interestingly, though *E. coli Csps* are responsive to cold stress and function as RNA chaperones (Graumann & Marahiel, 1998), they share a domain with AtCSP3, which plays a pivotal role in low temperature tolerance (Kim et al., 2009). In the present study, both *CspA* and *CspB* genes were validated for the growth profile of *E. coli* under mannitol (drought) and salt stress conditions. Growth of untransformed *E. coli* cells decreased drastically in presence of mannitol unlike that of *CspA* and *CspB* containing bacterial cells indicating that these genes are associated with mannitol/drought stress besides cold stress tolerance. *E. coli* containing both *CspA* and *CspB* displayed almost similar responses in terms of growth. Genetically altered *E. coli* also displayed a similar growth pattern under salt stress indicating that both *CspA* and *CspB* protect bacteria against salt stress. Western blot results confirmed the presence of translational product in transformed *E. coli* inferring functional expression of the genes. Nakaminami et al. (2006) determined the importance of C-terminal region of a plant cold shock domain protein (CSDP) and showed that deletion of all C-terminal zinc fingers in wheat cold shock protein 1 (WCSP1) abolished the growth stimulatory activity in *E. coli* during cold stress indicating that the CCHC-type zinc fingers in CSDPs are highly vital for growth. These experiments were not performed in the present study, but such a possibility cannot be ruled out.

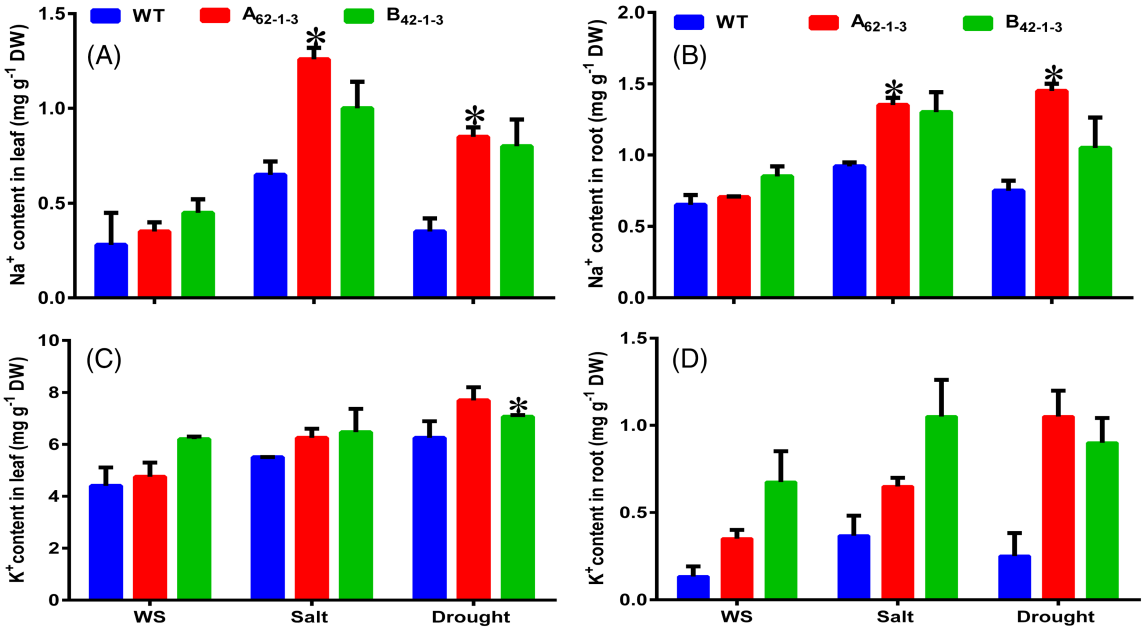

FIGURE 8 Ion analysis of leaf and root tissues under salt and drought stresses. (A) Na^+ content in leaf mg g^{-1} DW, (B) Na^+ content in root mg g^{-1} DW, (C) K^+ content in leaf mg g^{-1} DW, (D) K^+ content in root mg g^{-1} DW. $A_{42\text{-}1\text{-}3}$and $B_{62\text{-}1\text{-}3}$, transgenic lines; DW, dry weight; WT, wild type. Statistical significance is indicated as star (*) (*P*-value < 0.05)

4.2 | Generation and characterization of transgenics

Characterization of both *CspA* and *CspB* genes by PCR amplifications and Southern blot analysis indicated that these genes were incorporated stably into the host plant. qRT-PCR analysis showed that both *CspA* and *CspB* genes are expressed in leaf, root, and internodal tissues at the transcriptional level under drought stress. Fang et al. (1997) found that *E. coli CspA* does not increase the transcription but increases the stability of mRNA during cold stress. Protein folding becomes highly inefficient and the function of ribosomes is impeded under stress conditions (Keto-Timonen et al., 2016). However, the Csp family of proteins counteracts and prevents the formation of secondary structures in mRNA and help in the initiation of translation.

4.3 | Chlorophyll, proline, MDA, ion analysis, and antioxidative enzyme activities under stresses

Chlorophyll (both *a* and *b*) levels did not decline much under stress conditions in *CspA* and *CspB* lines in comparison with WT plants. A higher chlorophyll content in wheat transgenics (in comparison with WT plants) containing *SeCspA* was reported by Yu et al. (2017). These results indicate that *CspA* and *CspB* genes prevent faster degradation of chlorophyll or slow down the process of senescence under stress by an unknown mechanism. Both the transgenic lines exhibited a SGR phenotype in rice, but have not been reported earlier in maize and wheat (Castiglioni et al., 2008; Yu et al., 2017). The higher chlorophyll content in the transgenics (*CspA* and *CspB*) supports the SGR phenotype that generally maintains higher chlorophyll content than the non-SGR genotype. SGR is an important agronomic trait that permits plants to maintain active photosynthesis and subsequently improve the grain-filling even under adverse conditions (Borrell et al., 2014; Jaegglia et al., 2017). Proline levels were higher in both the transgenics in comparison with WT plants under stress. Accumulation of compatible osmolytes such as proline under stress conditions has been registered in many transgenics (Anjaneyulu et al., 2014; Reddy et al., 2015). Proline helps in the conversion of O_2^- to H_2O_2 and O_2 in the chloroplasts, mitochondria, and peroxisomes (Fridovich, 1989; Yiu & Tseng, 2005). Further, it has been noticed that proline protects plants against the oxidative damage caused by abiotic stress conditions (Molinari et al., 2007). Studies on transcriptomic analysis of gene expressions between SGR and senescing lines of sorghum revealed enrichment of genes associated with the "response to osmotic stress" (Jhonson et al., 2015). They noticed high expression of the delta-pyrroline-5-carboxylate synthase 2 (*P5CS2*) gene (involved in proline biosynthesis) in SGR compared to senescent line. Further, the expression of *P5CS2* was correlated with high levels of proline accumulation. Surprisingly, *P5CS2* has been found to lie within the Stg1 (stay-green) quantitative trait loci. Also, polymorphisms have been identified in known *cis*-elements in the promoter regions of *P5CS2* (Jhonson et al., 2015). Thus, these elements could be responsible perhaps for the differences in the expression of *P5CS2* gene between SGR and senescent lines. Such a finding has a bearing in our understanding of drought tolerance mechanism in crop plants. Lipid peroxidation levels (MDA) were reduced in *CspA* and *CspB* transgenics compared to WT plants, an indication of the consequence of *CspA* and *CspB* genes in transgenics (Semchuk et al., 2012). Significantly higher ionic levels

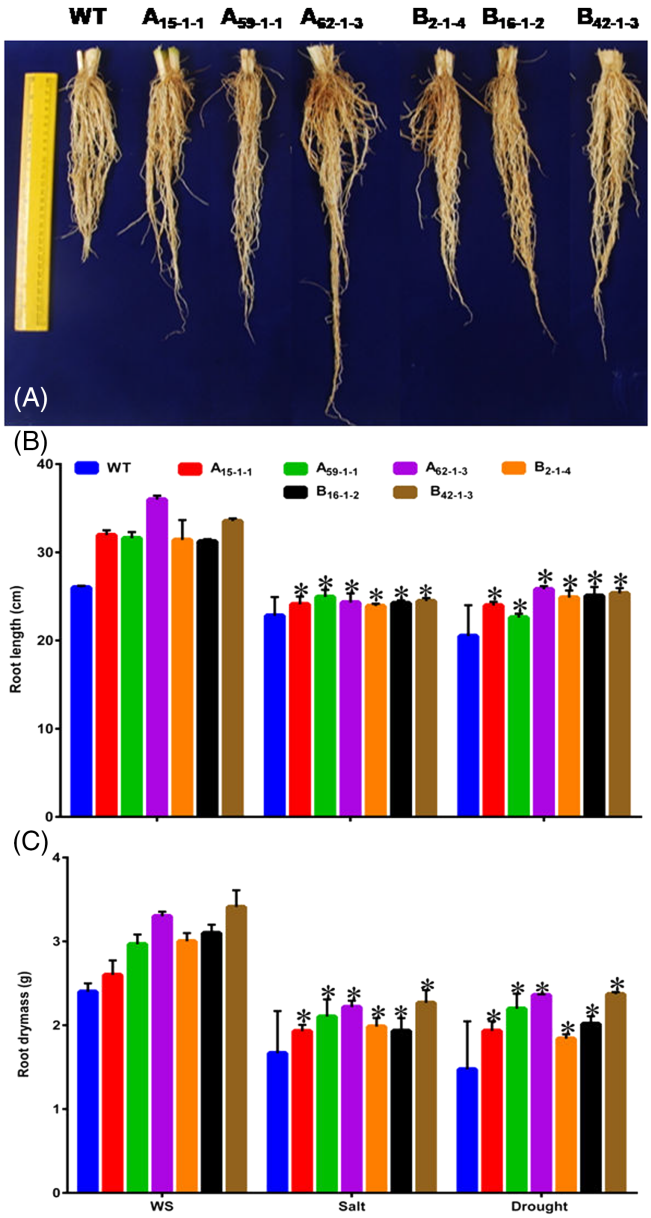

FIGURE 9 Root phenotype, root length, and root biomass in transgenics and WT. (A) Root morphology, (B) root length (cm), (C) root dry mass (g) in transgenic lines under salt and drought stresses. $A_{15\text{-}1\text{-}1}$, $A_{59\text{-}1\text{-}1}$, $A_{62\text{-}1\text{-}3}$, *CspA* transgenic lines; $B_{2\text{-}1\text{-}4}$, $B_{16\text{-}1\text{-}2}$, $B_{42\text{-}1\text{-}3}$, *CspB* transgenic lines; drought, withholding water;salt, 150 mM NaCl; WS, without stress; WT, wild type. Statistical significance is indicated as star (*) (*P*-value < 0.05)

(Na^+ and K^+) were observed in the transgenics in comparison with WT plants upon exposure to water-deficit and salt stress conditions. Na^+ inhibits the influx of K^+ into cytosol under high salt stress conditions leading to the accumulation high levels of Na^+ inside the cells (Hanin et al., 2016). Higher Na^+ levels inside the cytoplasm is generally accompanied by loss of K^+. So, enhanced uptake of K^+ under NaCl stress is difficult because of competition from Na^+ for K^+-binding sites on transport systems (Chen et al., 2007). But, being a macronutrient, K^+ plays a crucial role in protein synthesis and phloem sugar loading in plants (De Schepper et al., 2013). In line with this, Chen et al. (2007) demonstrated that cytosolic K^+ to Na^+ ratio is an important determinant of plant salinity tolerance. So, preventing the loss of K^+ and acquisition of K^+ under salt stress is crucial for maintaining a proper cytosolic K^+ concentration (Assaha et al., 2017; Chen et al., 2007). In transgenic *CspA* and *CspB*, accumulation of K^+ was higher in comparison with Na^+, which is vital for stress tolerance. Though uptake of K^+ is through activation of HAK transporters, how Csps help to maintain K^+ acquisition is obscure at the moment. Overall, the specific activities of CAT, SOD, and GPX were enhanced in transgenics compared to WT plants, perhaps an outcome of the transgenes. SOD catalyzes superoxide radicals into H_2O_2, which can be converted subsequently by CAT and GPX into water and O_2. A large body of information exists about the involvement and effective purging of superoxide radical and in stress protection in transgenics (Reddy et al., 2015; Tseng et al., 2007; Yiu & Tseng, 2005). Both CAT and GPX help in the conversion of H_2O_2 and therefore prevent oxidative damage (Esfandiari & Shekari, 2007; Willekens et al., 1995). Increased CAT activity assists in cell membrane stability by decreasing H_2O_2 levels under stress. Increased levels of CAT were also recorded under abiotic stress conditions in maize and *Sesamum* (Koca et al., 2007; Neto et al., 2006). Enhanced GPX was noticed in transgenic finger millet and sorghum under salt stress (Anjaneyulu et al., 2014, Reddy et al., 2015). Antioxidative enzyme activities are vital since they can modulate redox homeostasis of cells (Bhavanath et al., 2011; Huang et al., 2013). Thus, a clear correlation between increased proline (implicated in SGR phenotype), decreased lipid peroxidation and higher activities of antioxidative enzyme activities in *CspA* and *CspB* rice transgenics support that bacterial chaperones help to protect the activities and prevail over salt and drought stress-caused damages.

4.4 | Anatomy of WT differs from transgenic rice plants

Root morphology in transgenics differed from that of the WT plants, which led us to investigate the root anatomy. Roots in WT plants when exposed to abiotic stresses, displayed thin-walled sclerenchymatous cells and loosely organized aerenchyma, an indication of poor defense against stress. This may allow transport of ions such as Na^+ and Cl^- under salt stress or dehydration to occur under drought. Transport of ions into cortex and phloem tissues might damage the cells in all root zones before initiating the process of any structural defense. Contrary to this, transgenic roots (*CspA* and *CspB* lines) under stress conditions exhibited thick-walled sclerenchyma cells indicating a strong defense against salinity and water-limited conditions. Compactly arranged cortical cells with narrow intercellular spaces may reduce the transport of ions into inner phloem cells. Overall, it appears that cell wall integrity features (unlike that of WT) in transgenic roots help the cell viability, which then trigger the biosynthesis and accumulation of polyphenolic substances or deposition of lignin in the epidermal, hypodermal and intercellular spaces of outer cortical cells (Zagorchev et al., 2014, Gall et al., 2015, Kishor et al., 2015). Mourasobczak et al. (2011) and Chun et al. (2019) have provided

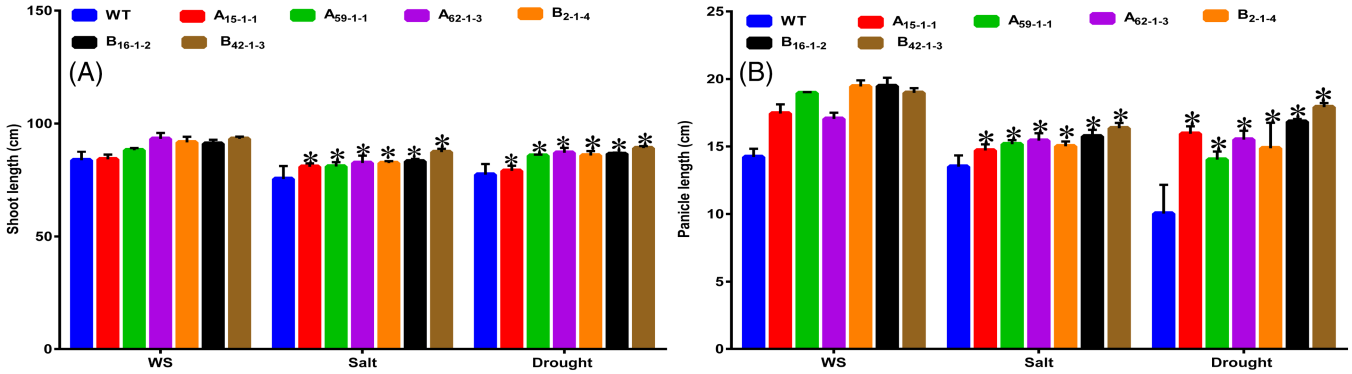

FIGURE 10 Analysis of shoot length and panicle length. (A) Shoot length (cm), (B) panicle length (cm) in transgenic lines under salt and drought stresses. Salt and drought stresses were imposed by 150 mM NaCl and water withholding water before booting stage. $A_{15\text{-}1\text{-}1}$, $A_{59\text{-}1\text{-}1}$, $A_{62\text{-}1\text{-}3}$, *CspA* transgenic lines; $B_{2\text{-}1\text{-}4}$, $B_{16\text{-}1\text{-}2}$, $B_{42\text{-}1\text{-}3}$, *CspB* transgenic lines; WS, without stress; WT, wild type. Statistical significance is indicated as star (*) (*P*-value < 0.05)

TABLE 2 Number of seeds/panicle in WT and transgenics under salt and drought stress conditions

Stress	WT	$A_{15\text{-}1\text{-}1}$	$A_{59\text{-}1\text{-}1}$	$A_{62\text{-}1\text{-}3}$	$B_{2\text{-}1\text{-}4}$	$B_{16\text{-}1\text{-}2}$	$B_{42\text{-}1\text{-}3}$
WS	115.3 (±4.92)	140 (±1.63)	129.3 (±0.68)	141 (±0.81)	140.16 (±0.95)	137.8 (±2.21)	147.3 (±2.72)
Salt	100.6 (±6.59)	120.5 (±2.85)[a]	125.5 (±0.4)[a]	125.6 (±4.64)[a]	124.3 (±0.27)[a]	116.3 (±1.24)[a]	115.5 (±4.49)[a]
Drought	92.3 (±9.39)	132.6 (±0.54)[a]	124.6 (±1.8)[a]	121.3 (±0.54)[a]	107.1 (±8.47)[a]	121.6 (±2.68)[a]	129.3 (±0.54)[a]

Note: Data represent means ± SD (n = 9 for each line).
Abbreviations: $A_{15\text{-}1\text{-}1}$, $A_{59\text{-}1\text{-}1}$, $A_{62\text{-}1\text{-}3}$, $B_{2\text{-}1\text{-}4}$, $B_{16\text{-}1\text{-}2}$, $B_{42\text{-}1\text{-}3}$, transgenic lines; drought, drought by withholding water; salt, 150 mM NaCl; WS, without stress; WT, wild-type plants.
[a]Indicates significant at $P < 0.05$.

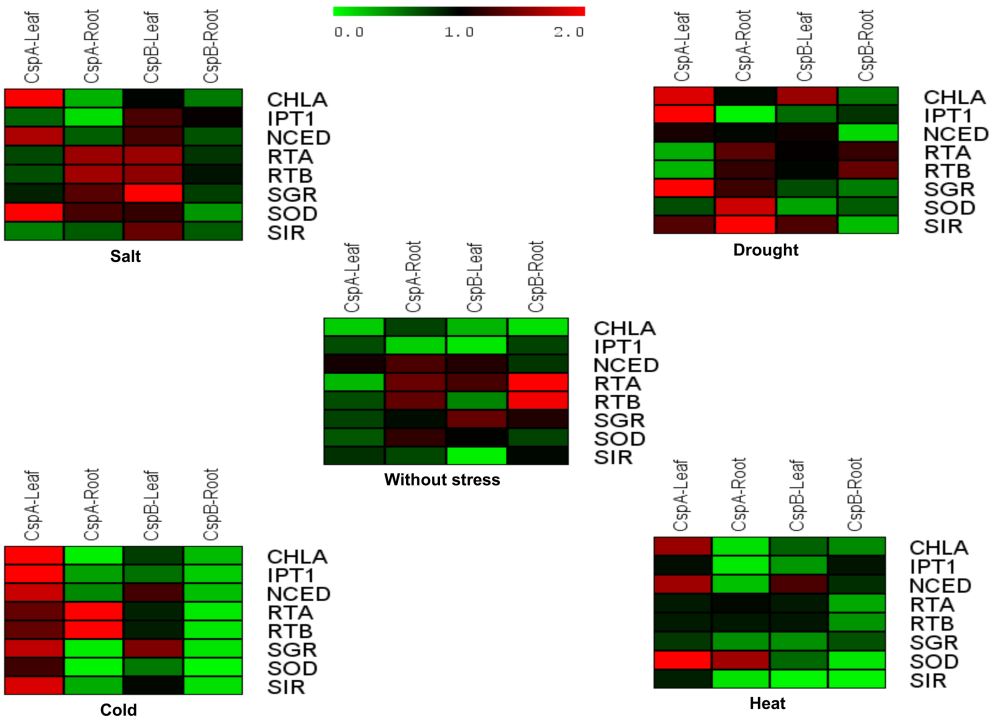

FIGURE 11 Transcript analysis of transgenes and stay-green (SGR) associated gene expression of transgenics against different abiotic stresses using qRT-PCR. Relative gene expressions were measured under abiotic stress (48 h) conditions in transgenic rice seedlings ($CspA_{62\text{-}1\text{-}3}$) ($CspB_{42\text{-}1\text{-}3}$) under salt (150 mM NaCl), drought (200 mM mannitol), cold (4°C), and high temperature (42°C) stresses. Expression (qRT-PCR) of *chlorophyllase*, *isopentenyltransferase* 1 (*IPT1*), *9-cis-epoxycarotenoid dioxygenase* (*NCED*), bacterial *CspA* transgene (RTA), bacterial *CspB* transgene (RTB), *SGR*, mitochondrial *SOD* and *SIRT* genes. Actin gene was used as an internal control

molecular and genetic evidence indicating the importance of enhanced lignin accumulation in the plant cell wall during the responses to salt stress. Also, expression of genes of the lignin biosynthetic pathway was positively correlated with drought stress tolerance (Hu et al., 2009). Associated with this phenomenon, increased lignin biosynthesis has been noticed under abiotic stress conditions (Mourasobczak et al., 2011),

which can reduce penetration and transpiration of cell wall water leading to osmotic balance during abiotic stress conditions (Monties & Fukushima, 2001). Lignins are the cross-linked phenolic polymers, and the enzyme peroxidase enhances the content of phenolics (Kim et al., 2008). In the present study, an increase in peroxidase activity (glutathione peroxidase) has been found in transgenics, which may lead to lignin deposition in the cell walls. Anatomical sections of roots in deed show increased lignin deposition. In line with this, double mutants (*atprx2/atprx25*, *atprx2/atprx71* and *atprx25/atprx71*) with reduced peroxidase activity have been shown to contain lower levels of lignin than single mutants (Shigeto et al., 2015). Taken together, it appears that lignin deposition in cell walls is critical under salt stress conditions.

4.5 | *CspA* and *CspB* overexpression increased root length, shoot length, panicle length, and seed number per panicle

Roots in transgenic plants were longer compared to WT plants indicating that bacterial chaperones by some mechanism improve root length. Such a morphological variation in root length was noticed earlier in tobacco and sorghum transgenics (Kishor et al., 1995; Reddy et al., 2015). Average lengths of transgenic (*CspA* and *CspB*) rice shoots were found to be slightly higher than in WT plants. Since *Csp*s are involved in many cellular processes to promote normal growth, protect RNA structure and stress adaptation responses (Keto-Timonen et al., 2016), it is possible that they might play a role on the increased lengths of both roots and shoots in the transgenic plants. WT plants matured 20 days earlier than the transgenics which displayed SGR phenotype indicating that CspA and CspB proteins delay chlorophyll degradation or slow down the process of senescence, though the mechanism is unclear. Higher chlorophyll content in transgenics is an indication for drawing such an inference. It has been suggested by Sasaki and Imai (2012) that CSDPs regulate embryo development, flowering time and fruit development indicating their diverse roles. On the other hand, *SeCspA* and *SeCspB* did not improve cold stress in transgenic wheat, but synthetic *CspA* gene improved drought stress under the field conditions. In the present study, transgenic rice exhibited better drought and salt (150 mM NaCl) stress tolerance under pot conditions compared to the WT plants. In contrast, Sasaki et al. (2015) showed that *Arabidopsis AtCSDP2* negatively regulates freezing tolerance. Further, they demonstrated that overexpression of *AtCSP2* resulted in reduced salt stress tolerance in *Arabidopsis*, indicating that it is a negative regulator of salt stress. Park et al. (2009) demonstrated that CSDPs affect seed germination and growth of *Arabidopsis* plants under abiotic stress, thus inferring the implication of CSDPs during seed germination. *AtCSP3* overexpression in *Arabidopsis* resulted in improved salt and drought stress tolerance by upregulating the expression of stress-related proteins (Kim et al., 2013). Yu et al. (2017) showed that overexpression of *CspA* and *CspB* genes caused the upregulation of *TaCDPK3* transcription factor in wheat. It is known that CDPKs play crucial roles in stress signal transduction and regulate the downstream genes that can be activated in turn by ABA (Sanders et al., 2002). Bacterial chaperones (*CspA* and *CspB*) upon expression conferred abiotic stress tolerance in maize in the field conditions (Castiglioni et al., 2008). These experiments indicate that bacterial chaperones have the potential to combat abiotic stresses in higher plants and hence need to be exploited further, especially a combination of *Csp* genes (*CspA* through *CspI*) for obtaining superior transgenics with higher levels of abiotic stress tolerance. Melencion et al. (2017) showed that an RNA chaperone functioned as a universal stress protein in *Arabidopsis* and displayed increased cold stress tolerance. *E. coli CspA* and *CspB* synthetic genes also enhanced the cold tolerance when overexpressed in *Arabidopsis thaliana* (Yu et al., 2017), suggesting that synthetic genes display identical functions in *Arabidopsis* and impart cold stress tolerance (Yu et al., 2017). The above experiments prove that bacterial Csps play crucial roles under abiotic stresses in plants also. But, so far, the SGR phenotype has not been reported upon the expression of bacterial *Csp* genes in crop plants. Transgenic rice plants expressing *CspA* and *CspB* genes revealed enhanced drought tolerance as seen by better panicle lengths. Both salt and drought stresses reduced the number of seeds per panicle in transgenics in comparison with the plants not exposed to stress conditions. The yield loss under salt and drought stresses ranged from 13% to 18% when compared with non-stressed conditions in WT plants. In contrast, only 3% loss was recorded in the line $A_{59\text{-}1\text{-}1}$ irrespective of salt and drought stress imposition. In the transgenic line $B_{2\text{-}1\text{-}4}$, 10% and 25% loss in yield were noticed in salt and drought stress conditions, respectively, in comparison with unstressed environments. Thus, yield penalty was noticed in transgenics in comparison with stressed conditions, but not devoid of stress. Yield was higher in transgenics when compared to WT plants under both salt and water-limited conditions. Similarly, transgenic maize displayed yield benefits of up to 15% under drought stress in the field, with no yield penalty even under non-stressed conditions (Castiglioni et al., 2008). These data suggest that effective RNA chaperone activity is indispensable to plants growing under water deficit conditions since they improve panicle length and seed weight as pointed out by Castiglioni et al. (2008). They noticed that a functional RNA-binding site is critical to confer improved yields under drought stress in maize.

4.6 | Enhanced *CspA*, *CspB*, and SGR-associated transcript levels in T_2 transgenics

Upregulation of both *CspA* and *CspB* genes was noticed under normal as well as abiotic stress conditions. Breakdown of chlorophyll is common during leaf senescence, seed maturation and fruit ripening. Consistent with this observation, expression of the gene *chlorophyllase* was high under all the four abiotic stresses. Chlorophyll is broken down to products via the pheophorbide A oxygenase pathway (Hortensteiner, 2013). However, in the present study, breakdown of chlorophyll during seed maturation stage is delayed and *CspA* and *CspB* transgenics displayed SGR phenotype consistently. This is quite surprising, unexpected and has not been reported earlier in *Csp* expressed crop plants like maize and wheat (Castiglioni et al., 2008;

Yu et al., 2017). It has been suggested that the *SGR* gene discovery is an association between stress-induced leaf senescence and stability of chlorophyll metabolism (Sakuraba et al., 2014; Thomas & Ougham, 2014). Further, the *SGR* phenotype is a result of alterations in chlorophyll metabolism. It could be either due to delayed degradation or over-production of chlorophyll under stress conditions (Hortensteiner & Kräutler, 2011). *SGRs* can play a role in stress tolerance as pointed out by Jagadish et al. (2015). It has been reported that some *Csps* are inducible by abiotic stresses other than cold (Keto-Timonen et al., 2016). Cytokinin is an important phytohormone and is associated with alterations of source/sink relationships (Leopold & Kawase, 1964), counteraction of high temperature stress (Caers et al., 1985), and also stimulation of chlorophyll synthesis (Arnold & Fletcher, 1986). In line with this, expression of the *IPT1* gene (associated with cytokinin biosynthesis) was found higher in transgenics especially under cold and drought stresses. *IPT1* may stimulate chlorophyll biosynthesis and thus help to maintain the SGR phenotype. Expression of *NCED* was also higher in transgenics under different abiotic stresses when compared to non-stress conditions. This is a crucial gene associated with ABA biosynthesis. Its expression was induced by NaCl, cold, high temperature as well as drought, suggesting the critical role that it plays in response to the multiple abiotic stress tolerance in rice. Earlier, Huang et al. (2018) also noticed induction of *NCED* by NaCl, PEG, and H_2O_2 in rice. PYL9, a member of the ABA receptors, increases leaf senescence, but promotes drought tolerance in *Arabidopsis* (Zhao et al., 2016). This is in contrast to the other studies, which show that delayed leaf senescence or the SGR type increases tolerance to drought stress by regulating the *IPT* gene (Rivero et al., 2007). PYL9 is an ABA-dependent pathway and hence plants get adapted to drought. On the other hand, *IPT* involves ABA-independent signal transduction. *IPT* overexpressed plants increase cytokinin synthesis, become greener, and thus adapt to drought stress (Clark et al., 2004; Merewitz et al., 2010; Rivero et al., 2007). Phytohormones like jasmonic acid, salicylic acid, and ABA promote senescence (Liang et al., 2014; Qi et al., 2015). Contrarily, cytokinin has an antipodal function and is known to delay leaf senescence. *IPT* overexpression in *Agrostis stolonifera* alleviated drought stress-induced inhibition of root growth by activating ROS-scavenging systems (Xu et al., 2016). In the present study, cold and drought stresses induced higher expression of the plant *SIRT1* gene (a human homolog) than salt and high temperature, indicting its association with these stresses. In plants, *SIRT* genes have been implicated against genome instability and cell oxidative damage (Huang et al., 2007). *SIRTs* are also associated with leaf senescence, regulation of photosynthetic activity, transcription, metabolism, and DNA damage repair (Cucurachi et al. 2012, Lagunas-Rangel, 2019, Zheng, 2020). Thus, several of these genes might be associated with the SGR phenotype to protect the plants against abiotic stress conditions. Also, *Csps* have been shown to contribute to osmotic and oxidative stress tolerance as pointed out by Keto-Timonen et al. (2016). Our results reveal that transgenic rice plants containing both *CspA* and *CspB* genes display stronger abiotic stress tolerance and improved yields compared to WT plants.

5 | CONCLUSIONS

We demonstrate here for the first time that overexpression of bacterial chaperone genes (*CspA* and *CspB*) in rice results in a SGR phenotype, late maturity, and improved yields under salt and drought stress conditions in comparison with that of WT plants. Higher expression of *IPT1*, *NCED*, *SGR*, and *SOD* genes under diverse abiotic stress conditions in transgenics illustrates that these genes play a pivotal role in imparting SGR character in rice. Thus, there is an urgent need to explore diverse bacterial *Csp* genes for generating abiotic stress-tolerant crop plants.

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AUTHOR CONTRIBUTIONS

P. B. K. Kishor conceived and designed the experiments and analyzed the data. Guddimalli Rajasheker cloned the genes, carried out the experiments and analyzed the data. Guddimalli Rajasheker, Somanaboina A. Kumar, Palle S. Reddy, Divya Kummari, Sudhakar R. Palakolanu, Gandra Jawahar, Rathnagiri Polavarapu, and P. B. K. Kishor analyzed and interpreted the data and wrote the manuscript. Dr. Insaf A. Qureshi helped in carrying out the western blot. Guddimalli Rajasheker, Palle S. Reddy, Somanaboina A. Kumar, Divya Kummari, Sujatha Edupuganti, Nagaraju Marka, Sudhakar R. Palakolanu, Gandra Jawahar, Rathnagiri Polavarapu, Jalaja Naravula, Insaf A. Qureshi, and P. B. K. Kishor critically analyzed the manuscript. All authors read and approved the manuscript.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article

ORCID

Rathnagiri Polavarapu https://orcid.org/0000-0002-9587-7547

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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