

Downregulation of the DST Transcription Factor Using Artificial microRNA to Increase Yield, Salt and Drought Tolerance in Rice

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Abstract

Abiotic stresses like salinity and drought directly affect plant growth and water availability, resulting in lower yield in rice. So, a combination of stress tolerance along with enhanced grain yield is a major focus of rice breeding. It was reported earlier that loss in function of the drought and salt tolerance (*DST*) gene results in increase in grain production through downregulating *Gn1a/OsCKX2* expression. Moreover, *dst* mutants also showed enhanced drought and salt tolerance in rice by regulating genes involved in ROS homeostasis. In the present study, we proceeded to test these reports by downregulating *DST* using artificial microRNA technology in the commercial but salt sensitive, high-yielding, BRRI dhan 28 (BR28). This cultivar was transformed with *DST* artificial microRNA (*DST* amiRNA) driven by the constitutive *CaMV35S* promoter using tissue culture independent *Agrobacterium* mediated *in planta* transformation. *DST* amiRNA transgenic plants were confirmed by artificial microRNA specific PCR. Transformed plants at T₀ generation showed vigorous growth with significantly longer panicle length and higher primary branching resulting in higher yield, compared to the wild type (WT) BR28. Semi-quantitative RT PCR confirmed the decrease in *DST* expression in the BR28 transgenic plants compared to WT. T₁ transgenic plants also showed improvement in a number of physiological parameters and greater growth compared to WT after 14 days of 120 mM salt (NaCl) stress at seedling stage. Therefore, *DST* downregulated transgenic plants showed both higher stress tolerance as well as better yields. Furthermore, stable inheritance of the improved phenotype of the *DST* amiRNA transgenics will be tested in advanced generations.

*Equal contribution.

Keywords

Drought and Salt Tolerance (DST) Transcription Factor, Artificial microRNA Technology, *In Planta* Transformation, Yield, Stress Tolerance

1. Introduction

Feeding a growing population that is expected to reach roughly 9 billion by the middle of this century is among the major challenges of our time [1]. Modern agriculture has greatly improved food production, but progress towards mitigating the negative effects of climate change has been insufficient [2]. Most worryingly, many of the plants upon which we depend for food production, like rice, are particularly sensitive to environmental stresses like drought and salinity [3]. Drought is one of the most common environmental factors that limit crop productivity, especially in Asia where at least 23 million hectares of rice (20% of the total world rice area) are drought prone [4]. It affects rice at morphological, physiological and molecular levels and thereby affects its yield [5]. Moreover, production of rice is under pressure due to increase in soil salinity stress as more cultivable areas are becoming saline due to added anthropogenic contribution and global warming [6] [7].

Plant adaptation to environmental stresses such as salinity and drought is dependent on the activation of cascades of molecular networks. As a result, engineering of multiple genes or regulatory genes (such as gene for transcription regulators, like SNAC1, HARDY) instead of a single stress related gene can augment better tolerance to environmental stresses [8] [9]. Nevertheless, stress tolerant transgenic lines generally show some loss in grain yield production under stress [10]. So, development of rice which is simultaneously high yielding as well as stress tolerant is crucial to ensure food security.

Furthermore, agriculture currently uses over 70% of available freshwater [11]. One of the approaches that may be adopted to conserve water in agriculture is the development of plants that use less water yet maintain high yields in conditions of water scarcity. As plants lose over 95% of their water via transpiration through stomata, the engineering of stomatal activity is a promising approach to reduce the water requirement of crops and to enhance productivity under stress conditions [12]. To fulfill all these demands, engineering of transcription factors is expected to be the ideal candidates as they regulate a cascade of downstream molecular targets important for fighting stress [13] [14] [15].

Among various transcription factors, DST has drawn particular attention because of its role in conferring drought and salinity tolerance as well as increased yield in plants. Mutation in the *DST* gene enhances grain production through downregulating *Gn1a/OsCKX2* expression [16]. Rice *Gn1a/OsCKX2* (*Grain number 1a/Cytokinin oxidase 2*) encodes a cytokinin oxidase that catalyzes the degradation of active cytokinin which positively regulates shoot apical meristem

(SAM), a major parameter determining grain yield production [17] [18] [19]. Moreover, loss of *DST* function increases stomatal closure and reduces stomatal density via regulation of genes involved in ROS (Reactive oxygen species) homeostasis. This in turn leads to enhanced drought and salt tolerance in rice [20].

So, in the current study, we tried to obtain the combination of stress tolerance as well as increase in yield by targeting a single gene, *DST*. Therefore, to down-regulate *DST*, we selected the commercial but salt sensitive, early maturing, high-yielding rice, BRRIdhan 28 (BR28) having distinctive plant architecture. Artificial microRNA (amiRNA) technology was adopted to downregulate *DST* because of its exquisite specificity, uniqueness and effectiveness compared to other gene silencing approaches like RNA interference, RNA silencing, mutation, etc. [21]. Like endogenous micro RNA, amiRNA also bind to the *DST* transcript as a result inhibits *DST* transcription and hence downregulate its expression. Moreover, it is easy to optimize amiRNA sequences for targeting specific gene expression with minimal off-targets [22]. This technology has already been successfully used in downregulating genes in different plants [23]. However quantitative measures of the level of enhancement in tolerance as well as gain in yield are missing from previous publications. For introducing *DST*_artificial microRNA (*DST*_amiRNA) into BR28, a tissue culture-independent *Agrobacterium*-mediated *in planta* transformation was used because BR28 belongs to *indica* sub-species and is recalcitrant to tissue culture [24]. So, the objective of this study was to determine the level of tolerance achieved concomitantly with the amount of grain yield production by downregulating *DST*.

2. Materials and Methods

2.1. Plant Material

BR28 documented as BRRIdhan 28 was produced by Bangladesh Rice Research Institute (BRRI) in 1994. It is a commercial, high yielding, salt sensitive variety derived from the crossing of IR28 (from IRRI) and Purbachi. This early Boro (winter season) variety is extremely popular all around Bangladesh due to its high-yielding characteristics. This variety is cultivated in relatively high ground and suitable for flood prone areas due to its early harvesting (May) [25].

2.2. Construction of *DST*_amiRNA for Downregulating *DST* Gene

For designing an amiRNA specific for the *DST* gene, WMD3 (Web MicroRNA Designer 3, website with appropriate software) was used to generate 21 nucleotides of mature amiRNA sequences that resemble natural miRNAs while minimizing possible off-target effects to other transcripts. At first, the target gene sequence (*DST*) was incorporated in the WMD3 target search tool. A list of probable amiRNA sequences was provided by WMD3 against the particular target gene of interest. To facilitate the selection of amiRNA, specific hybridization energy (–35 and –40 kcal/mole) and a minimal target annealing site was chosen. WMD3 primer designer tool was then used to generate oligos for synthesizing

the amiRNA. Four different oligos for each gene were suggested by the program. For amplifying full length *DST*_amiRNA, overlapping PCR was set up using these four different oligos (amiRNA-I, amiRNA-II, amiRNA-III, amiRNA-IV, amiRNA_full_F, amiRNA_full_R; **Table 1, Figure 1**) with the plasmid, *pNW55*. *pNW55* was the template containing Osa-MIR528 precursor backbone sequence of *Oryza sativa* (Osa-MIR528) (Addgene, Cambridge, UK). The *DST* specific amiRNA and its complementary (amiRNA*) sequences (with *AflIII* cutting site) was introduced into *pNW55* by site-directed overlapping PCR amplification replacing the Osa-MIR528 amiRNA and its complementary (amiRNA*) sequence of *Oryza sativa* (**Figure 1(A)**). Overlapping PCR analyses were carried out in a 25 µl reaction mixture containing 10 ng of *pNW55* vector, 100 µM of each dNTP, 2.4 ng each of primers, 1 unit of Taq DNA polymerase (Invitrogen, USA), 1.5 mM MgCl₂, DMSO 2.4%, and 1 × PCR Buffer-MgCl₂ (Invitrogen, USA). The reaction was performed in a thermocycler (GeneAtlas of Astec) at 95°C for 2 min followed by 35 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 52°C and 40 sec extension at 72°C, then a final extension of 7 min at 72°C. Then a fusion PCR was performed to amplify full length of *DST*_amiRNA along with sequences for restriction cutting sites at the 5' and the 3' ends (**Figure 1(B)** and **Figure 1(C)**). Fusion PCR condition was similar to the condition of overlapping PCR.

2.3. Cloning *DST*_amiRNA into Destination Vector *pCAMBIA1305.2* and Transformed into *E. coli* and *Agrobacterium*

Both the insert (*DST*_amiRNA) and the destination vector (*pCAMBIA1305.2*) were digested with *SpeI* and *AflIII* restriction enzymes and extracted from the gel (**Figure 1(D)**). Ligation reaction was done with the ratio 5:1 (vector: insert) at 16°C overnight and the mix transformed into *E. coli* by heat shock method following the protocol by Sambrook *et al.* (1989) [26]. Specifically a 1893 bp size, *GUSPlus*TM (*β-glucuronidase*) sequence was removed from *pCAMBIA1305.2* vector with *SpeI* and *AflIII* and a 296 bp size *DST*_amiRNA backbone was ligated in its place producing *pCAMBIA1305.2_DST_amiRNA* (*pDST_amiRNA*) vector (**Figure 1(E)**). Positive clones were first screened by PCR using *DST*_amiRNA specific primers and then by restriction digestion with *SpeI* and *AflIII* restriction enzymes (NEB, Ipswich, MA, USA). The insert was also confirmed by sequencing by sending the plasmid to 1st Base, Malaysia. Finally, *pDST_amiRNA* construct was transformed into *Agrobacterium tumefaciens* (LBA4404 strain) by electroporation using standard protocols [26]. Positive clones were selected based on PCR reactions with *DST*_amiRNA specific primers.

2.4. Transformation of the Construct into Rice Varieties

Transformed *Agrobacterium* strain (LBA4404) containing *pDST_amiRNA* construct was cultured in solid YM media containing kanamycin (20 mg/l) antibiotic and prepared for plant transformation following the standard protocol discussed in Parvin *et al.* (2015) [27]. According to the protocol, densely grown bacterial cells were inoculated into liquid YM medium (Yeast extract mannitol

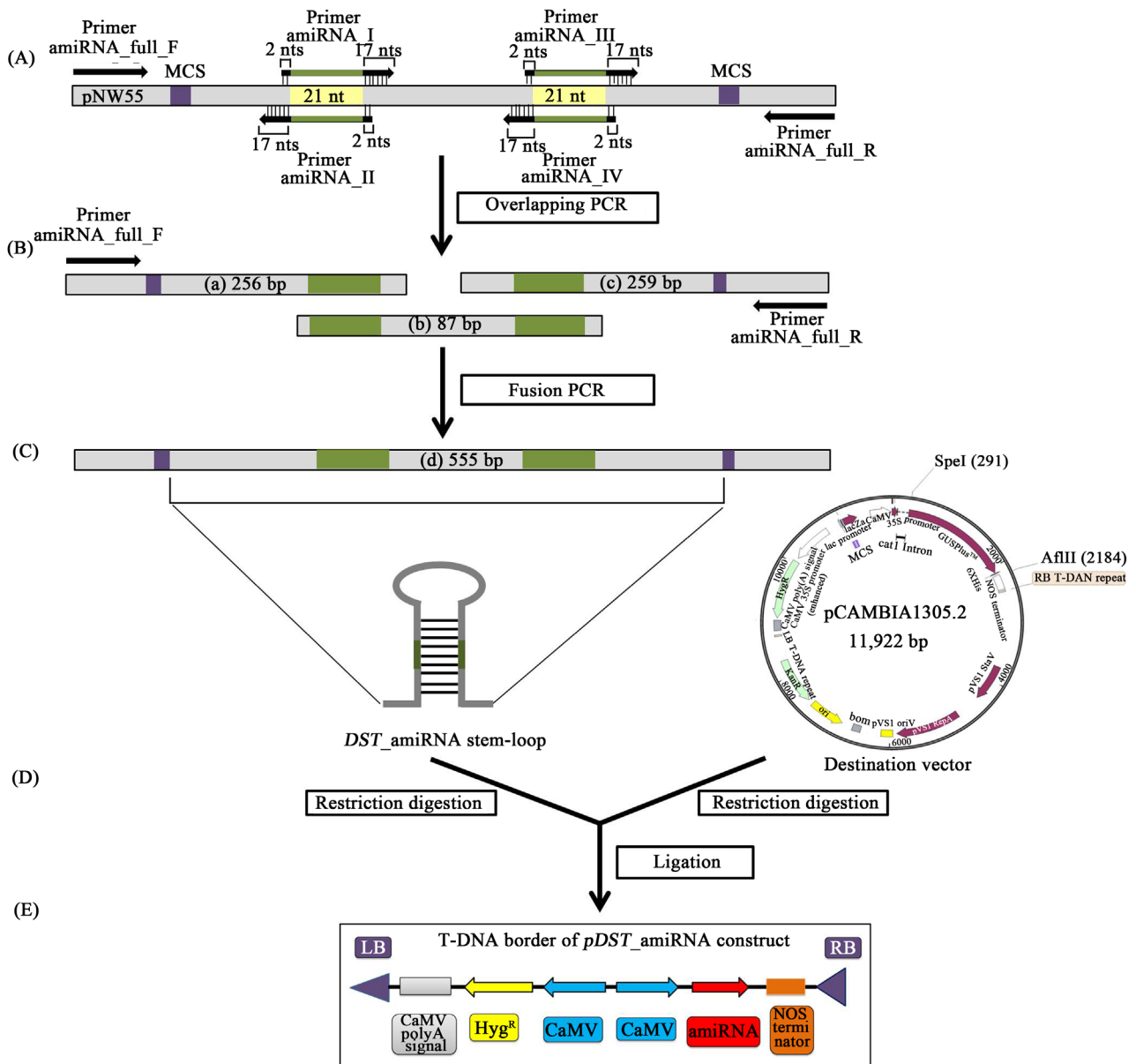


Figure 1. Schematic representation of the generation of the *pDST_amiRNA* construct. (A) Illustration of the template plasmid, *pNW55* with oligonucleotide binding sites indicated; (B) PCR amplicons (a), (b), and (c) generated using oligonucleotides (Table 1); fragment-A generated using *amiRNA_full_F* and *amiRNA-II* primers, fragment-B generated using *amiRNA-I* and *amiRNA-IV* primers, fragment-C generated using *amiRNA-III* and *amiRNA_full_R* primers; (C) Three fragments were fused to form full length *DST_amiRNA* by fusion PCR; (D) Both the insert (*DST_amiRNA*) and the destination vector (*pCambia1305.2*) were digested with *SpeI* and *AflII* restriction enzyme (E) T-DNA border of *pDST_amiRNA* construct; both the *DST_amiRNA* and *hygromycin phosphotransferase* genes are driven by the separately constitutive promoter, *CaMV35S*.

broth) [28] and incubated at 28°C overnight, then centrifuged and re-suspended into bacterial re-suspension medium (Sucrose, glucose, AB buffer and AB salt). For improving the transformation efficiency acetosyringone was added to both liquid YM medium and bacterial re-suspension medium. Finally, at OD₆₀₀ bacterial density was measured and kept fixed at 0.6. For *Agrobacterium* inoculation two-day-old germinated BR28 seeds were used. During the transformation

events, each time embryonic apical meristem of approximately 50 germinated seeds of BR28 were infected with *A. tumefaciens* containing *pDST_amiRNA* construct. Infected seeds were transferred onto petri dishes containing wet filter paper followed by incubated in the dark at 28°C for 6 - 7 days. Seedlings were then treated with carbenicillin solution (250 mg/l) for 1 hour to kill and remove the remnants of *Agrobacterium*. After that, seedlings were washed well with distilled deionized H₂O and transferred to new petri dishes containing wet filter paper and kept in light for 16 hours and in dark for 8 hours at 28°C. They were subsequently transferred to hydroponic solution [29] when they appeared green and healthy. After 2 - 3 days, the hydroponic pots were transferred to the net house [30]. Matured seedlings (18 - 21 days old) were transferred to soil and allowed to pollinate naturally to set seeds (T₁).

2.5. DNA Isolation and PCR Analysis

Genomic DNA was isolated from the upper leaves (flag leaves) of transformants which gave positive result in leaf disk senescence (LDS) assay. Leaves were cut finely, crushed to powder in liquid nitrogen and DNA was isolated using CTAB (Cetyltrimethylammonium bromide) method following the protocol mentioned by Doyle (1991) [31]. PCR was performed with different sets of *DST_amiRNA* and *CaMV35S* specific primers to confirm the *pDST_amiRNA* construct as well as the *DST_amiRNA* transformed BR28 at T₀ generation. PCR analyses were carried out in a 25 µl reaction mixture containing 100 ng of plant DNA, 100 µM of each dNTP, 2.4 ng each of primers, 1 unit of Taq DNA polymerase (Invitrogen, USA), 1.5 mM MgCl₂, DMSO 2.4%, and 1× PCR Buffer-MgCl₂ (Invitrogen, USA). The reaction was performed in a thermocycler (GeneAtlas of Astec) at 95°C for 5 min followed by 35 cycles of 1 min denaturation at 95°C, 1 min annealing at the annealing temperature depending on specific primer and 1.5 min extension at 72°C, then a final extension of 10 min at 72°C.

2.6. RNA Isolation and Semi-Quantitative RT-PCR Analysis

Total RNA was extracted from the shoot of matured T₁ BR28 *DST_amiRNA* transgenics and WT BR28 rice under both 0 and 100 mM NaCl stress (24 hours) using Trizol reagent (Ambion, Invitrogen) following the manufacturer's protocol. First-strand cDNA was synthesized from 1.5 µg of total RNA using the Invitrogen Superscript III reverse transcription (RT)-PCR following the manufacturer's protocol (Invitrogen, USA). For the optimization of cDNA for both WT and transgenic lines, PCR reactions were conducted using primers specific for house-keeping gene, *eEF-1α* (eukaryotic elongation factor-1α). After normalization, same level of *eEF-1α* expression was found in both the transformed and the WT. Then, semi-quantitative RT-PCR was done using *DST* specific primers with same concentration of cDNAs (normalized with respect to the concentration level of *eEF-1α*) to see the expression of *DST* (Table 1). The 1D-Multi tool of Alpha Ease FC imaging system and R programming language were used for the graphical representation of the intensity of the bands in the gel.

Table 1. Description of the primers used in this study.

Primer name	Sequence
amiRNA-I	AGTATTAACGTCCCTAAGAGCTGCAGGAGATTTCAGTTTGA
amiRNA-II	TGCAGCTCTTAGGGACGTTAATACTGCTGCTGCTACAGCC
amiRNA-III	CTCAGCTGTTACGGACGTTAATATTCTGCTGCTAGGCTG
amiRNA-IV	AATATTAACGTCCGTAACAGCTGAGAGAGGCCAAAAGTGAA
amiRNA_full_F	CTGCAAGGCGATTAAGTTGGGTAAC
amiRNA_full_R	GCGGATAACAATTTACACAGGAAACAG
amiRNA_AflII_R	CATACTTAAGATTCTGCAGCCCTC
<i>hpt</i> _F	CGTCTGCTGCTCCATACAAG
<i>hpt</i> _R	GCGAAGAATCTCGTGCTTTC
<i>CaMV35S</i> _F	GAACTCGCCGTAAGACTGG
amiRNA_R2	CATAGGTNACCATTCTGCAGCCCTC
<i>DST</i> _amiRNA_F3	TGGTTTGGGATAGGTAGGTG
<i>DST</i> _amiRNA_R3	GCGCGCTATATTTTGTTC
<i>eEF-1α</i> _F	TTTCACTCTTGGTGTGAAGCAGAT
<i>eEF-1α</i> _R	GACTTCCTTCACGATTTTCATCGTAA
<i>DST</i> _F	CACCGAGCGAGAGCGGCGTGCGAG
<i>DST</i> _R	GGTGGTGGTGCTTGCCGGGG

2.7. Leaf Disk Senescence (LDS) Assay

Leaf squares of ~1.0-cm dimension were excised from healthy and fully expanded rice flag leaves of similar age from both the transgenic lines and WT plants. The disks were floated in a 20 ml solution of 150 mM NaCl for T₀ transgenic lines for 4 days at 25°C. The analysis was carried out with three biological replicates in three independent experiments [10].

2.8. Salt Stress Screening at Seedling Stage

Seedling stage phenotypic screening of WT BR28 and *DST*_amiRNA transformed plants at T₁ generation was done under salt stress condition by the method described by Amin *et al.* (2012) [32]. 60 mM salt (NaCl) stress was applied to the fourteen days old T₁ seedlings with a gradual increment of 20 mM per day to 120 mM salt stress. Then the seedlings were subjected to 120 mM salt for 14 days. After that, the level of salinity tolerance was evaluated based on various parameters like change in root length, shoot length, electrolyte leakage, chlorophyll content in the stress condition compared to the control.

2.9. Measurement of Relative Electrolyte Leakage

0.1 g weighed plant leaf from the seedlings of both WT and transgenics were placed in a falcon tube containing 25 ml deionized water and kept in shaker for 2 hours. The initial electrical conductivity (C1) of the solution was measured using

a conductivity detector. Then the leaf segments in deionized water were autoclaved. After being thoroughly cooled to room temperature, the conductivities (C2) of the resulting solutions were determined. The values of C1 to C2 (C1/C2) were calculated and used to evaluate the relative electrolyte leakage [10]. Percentage increase in relative electrolyte leakage was determined using following formula:

$$\text{Increase in relative electrolyte leakage} = \left(\frac{\text{Stress} - \text{Control}}{\text{Control}} \right) \times 100\%$$

2.10. Measurement of the Chlorophyll Concentration

0.1 g weighed plant leaf from the seedlings of both WT and transgenics were kept in a bottle containing 12 ml of 80% acetone for 48 hours in dark. Absorbance of leaf tissues extract was measured at wavelength 663 and 645 nm after 48 hours [10]. The chlorophyll content was calculated using the following equation:

$$A = E C d; A \text{ is proportional to } C \text{ (because } E \text{ and } d \text{ is constant)}$$

where,

A = observed absorbance; C = chlorophyll concentration (mg/ml); d = distance of the light path (= 1 cm); E = a proportionality constant (extinction Co-efficient) (=36 ml/cm).

Reduction of chlorophyll content was determined using following formula:

$$\text{Chlorophyll reduction} = \left(\frac{\text{Control} - \text{Stress}}{\text{Control}} \right) \times 100\%$$

2.11. Measurement of Hydrogen Peroxide (H₂O₂) Level

0.3 g weighed plant leaf from the seedlings of both WT and transgenics were ground to a fine powder in the presence of liquid nitrogen and 5 ml of 0.1% (w/v) TCA was added to it. The homogenate was centrifuged at 12,000 g for 15 min at room temperature. Then 3 ml of supernatant was collected in a fresh screw capped tube. 0.5 ml of 1 M potassium phosphate buffer (pH 7.0) and 1 ml of potassium iodide (1 M) were added in the tube. Then the absorbance of the mixture was taken at 390 nm using 1 ml of 0.1% (w/v) TCA and 1 ml of potassium iodide in the absence of leaf extract as the blank control [33].

The amount of H₂O₂ was calculated using the equation:

$$\text{H}_2\text{O}_2 \left(\mu\text{mol} \cdot \text{g}^{-1} \text{FW} \right) = 1 + (227.8 \times \text{OD}_{390})$$

Percentage increase in hydrogen peroxide level was determined using following formula:

$$\text{Increase in hydrogen peroxide level} = \left(\frac{\text{Stress} - \text{Control}}{\text{Control}} \right) \times 100\%$$

2.12. Measurement of Root Relative Water Content

Roots were detached and weighed immediately to record the fresh weight (FW), followed by dipping them in distilled water for 24 hours. The roots were then blotted dry, weighed to record the turgid weight (TW), and subjected to oven drying at 70°C for 24 hours to determine the dry weight (DW) [33]. The root

relative water content was calculated using the equation:

$$\text{Relative water content} = \frac{((\text{FW} - \text{DW}) \times 100)}{(\text{TW} - \text{DW})}$$

Percentage increase in root relative water content was determined using following formula:

$$\text{Increase in root relative water content} = \frac{((\text{Stress} - \text{Control}) / \text{Control}) \times 100\%}{}$$

2.13. Agronomic Trait Analysis

Agronomic traits like, total tiller number, leaf width, plant height were measured when the plants became mature. After harvesting, parameters like primary branching number, panicle lengths, number of filled grains and numbers of unfilled grains were measured.

2.14. Data Analysis

All statistical analyses were done in R Studio version 1.0.143 and R programming language version 2.14.1. The ANOVA and the Duncan test were performed to compare significant differences ($P < 0.05$) between the transgenic lines and the WT.

3. Results and Discussion

3.1. Construction and Cloning of *DST_amiRNA* for Downregulating *DST* Gene

Using oligos for synthesizing the full length *DST_amiRNA* (555 bp), first three PCR reactions were performed to amplify three overlapping fragments (256 bp, 87 bp and 259 bp) (**Figure 2(A)**). Then these individual fragments were gel purified and another fusion PCR was performed to join the three fragments (**Figure 2(B)**). And finally one PCR was done where only reverse primer at the 5' end was designed with *AflIII* cutting site (**Figure 2(C)**) and the desired size band (555 bp) was extracted from the gel. Then, the extracted *DST_amiRNA* and the *pCAMBIA1305.2*, destination vector were digested using *SpeI* and *AflIII* restriction enzymes. The *pDST_amiRNA* construct was developed through the ligation reaction and transformed into *E. coli* by heat shock method.

3.2. Confirmation of *pDST_amiRNA* Vector into *E. coli*

After transformation, the positive bands (211 bp for *amiRNA* and 809 bp for *hpt* gene) were confirmed through lysate PCR of different colonies using *DST_amiRNA* and *hpt* specific primers (**Figure 2(D)** and **Figure 2(E)**; **Table 1**). Then, plasmids were isolated from lysate positive clones; desired band for *DST_amiRNA* plasmid was also found (**Figure 2(F)**). This positive *pDST_amiRNA* plasmid was further confirmed by restriction digestion with *SpeI* and *AflIII* restriction enzymes (**Figure 2(G)**). By digestion with *SpeI* and *AflIII* restriction enzymes the expected product size of 296 bp and 10,029 bp were found which confirmed the presence of *DST_amiRNA* in the *pCAMBIA1305.2* expression vector. Finally, the insert was also confirmed by sequencing.

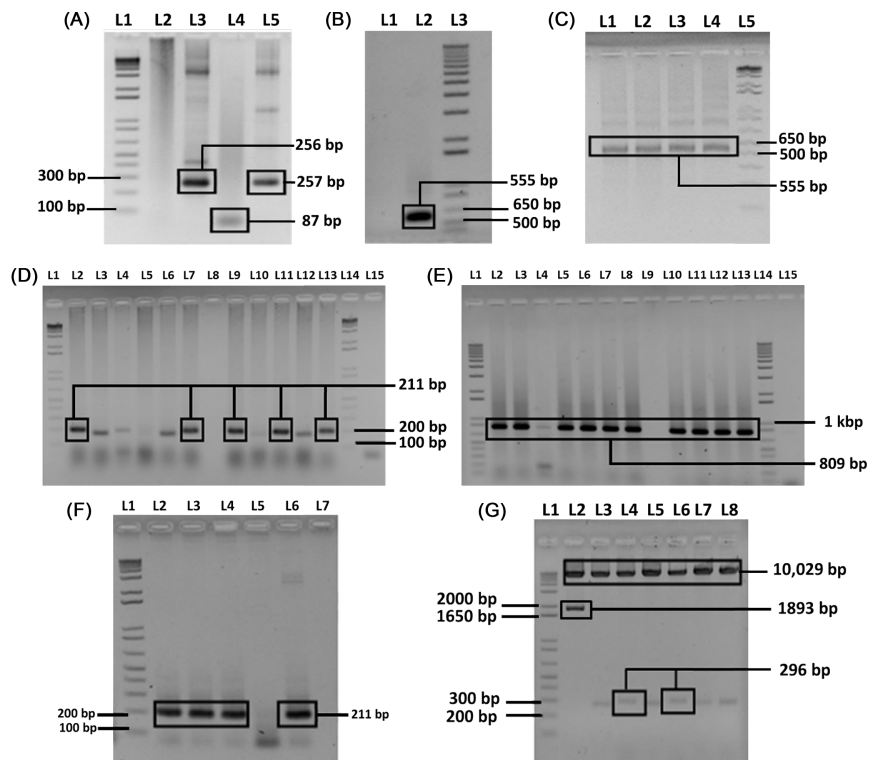


Figure 2. Cloning of *DST_amiRNA* into *pCAMBIA1305.2* and transformation into *E. coli*. (A) Amplification of the three fragments of *DST_amiRNA* from *pNW55* vector; L1:1kb+ ladder, L2: negative control, L3: fragment-A with desired product size 256 bp using *amiRNA_full_F* and *amiRNA-II* (Table 1), L4: fragment-B with desired product size 87 bp using *amiRNA-I* and *amiRNA-IV* (Table 1), L5: fragment-C with desired product size 259 bp using *amiRNA-III* and *amiRNA_full_R* (Table 1); (B) Amplification of full length of *DST_amiRNA*; L1: negative control, L2: full length of *DST_amiRNA* with desired product size 555 bp using *amiRNA_full_F* and *amiRNA_full_R* (Table 1), L3:1 kb + ladder; (C) Amplification of full length of *DST_amiRNA* with restriction cutting site; L1 - L4: full length of *DST_amiRNA* with restriction cutting site using *amiRNA_full_F* and *amiRNA_AflII_R* (Table 1), L5:1 kb + ladder; (D) Confirmation of positive colony containing *pDST_amiRNA* by lysate PCR after transformation into *E. coli* using *DST_amiRNA* specific primers; L1 and L14: 1 kb + ladder, L8 and L15: negative control, L2, L7, L9, L11 and L13: positive colony containing *pDST_amiRNA*; (E) Confirmation of positive colony containing *pDST_amiRNA* by lysate PCR with *hpt* specific primers (Table 1) after transformation into *E. coli*; L1 and L14: 1 kb + ladder, L9 and L15: negative control, L2 - L8 and L10 - L13: positive colony containing *pDST_amiRNA*. (F) Confirmation of positive *pDST_amiRNA* plasmid after isolation from lysate positive colonies. L1: 1 kb + ladder; L2 - L4: positive *pDST_amiRNA* construct with desired band size 211 bp, L5 and L7: negative control, L6: positive control; (G) Confirmation of positive *pDST_amiRNA* plasmid by restriction digestion with *SpeI* and *AflII* restriction enzyme. L1: 1 kb + DNA ladder, L2: blank vector (*pCAMBIA1305.2*), L4, L6: positive *pDST_amiRNA* plasmid with desired band size of 296 bp and 10,029 bp.

3.3. Transformation and Confirmation of *pDST_amiRNA* Vector into *Agrobacterium*

Finally, the vector *pCAMBIA1305.2* containing *DST_amiRNA* was electroporated into *Agrobacterium* and positive transformed *Agrobacterium* was con-

firmed by PCR using two sets of primers (Set 1: *amiRNA_full_F* and *amiRNA_AflII_R*; Set 2: *CaMV35S_F* and *amiRNA_R2*; **Table 1**). Desired band size of 211 bp and 1.1kbp were found (**Figure 3**).

3.4. *Agrobacterium* Mediated Transformation and Selection of Transformed Plants

In this study, germinating seeds of BR28 were subjected to *in planta* transformation where they were directly infected with *Agrobacterium* containing the *pDST_amiRNA* construct. Then they were allowed to grow and set T₁ seeds as described in methods. When the plants were matured and grains were set, leaf disk senescence (LDS) assay on flag leaves was done.

3.5. Leaf Disk Senescence (LDS) Assay at T₀ Generation

Positive transformed *DST_amiRNA* BR28 was identified by leaf disk senescence (LDS) assay under 150 mM salt stress. As downregulation of *DST* results in gain in salt tolerance characteristics, the transformed plants should perform better under salt stress. During the assay, flag leaf pieces of non-inoculated BR28 (control) showed dark brown stripes and necrosis after 4 days (**Figure 4(A)**) whereas

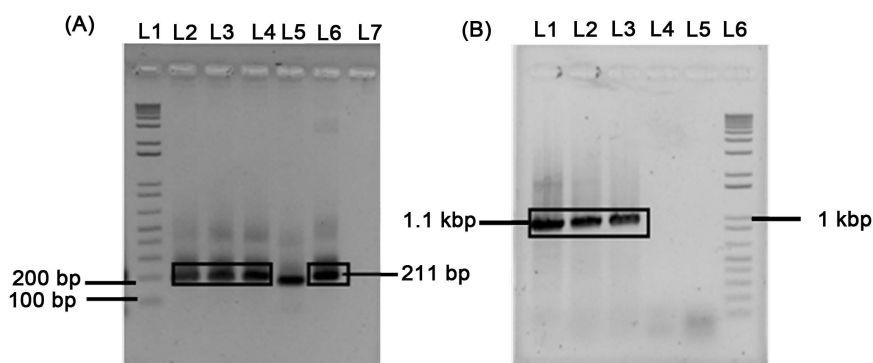


Figure 3. PCR confirmation of *pDST_amiRNA* plasmid into *Agrobacterium*. (A) L1: 1 kb + DNA ladder, L2 - L4: *pDST_amiRNA* plasmid, L6: positive control, L7: negative control; (B) L1 and L2: *pDST_amiRNA* plasmid, L3: positive control; L4, L5: negative control; L6: 1 kb + DNA ladder.

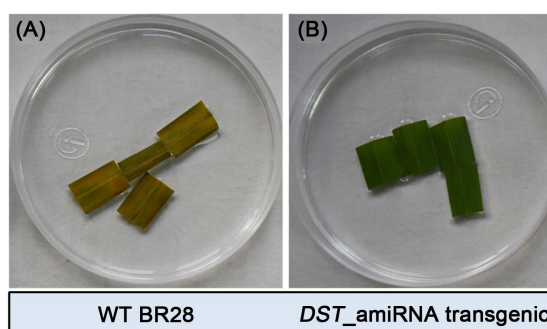


Figure 4. Leaf disk senescence assay of flag leaves from T₀ transformants. Transformed plant's leaf showed healthier and greener appearance whereas WT leaves showed necrosis and dark-brown stripes after 4 days in 150 mM NaCl solution.

flag leaf of 12 out of 41 plants of infected BR28 using *DST_amiRNA* construct remained largely green and healthy (**Figure 4(B)**). These plants were assumed to be putatively transformed and used for further analysis.

3.6. Transformation Efficiency of the *in Planta* Method

The transformation efficiency for *indica* rice varieties as reported earlier was about 6.0% [24]. Due to some modifications of this published method as discussed in Parvin *et al.* (2015) [27] our transformation efficiency was enhanced from the reported 6 to 29% (**Table 2**).

3.7. Selection of the Transgenic Lines through Molecular Analysis

For confirmation of the T₀ transformants at molecular level, DNA was isolated from the plants that showed better result in LDS assay (putatively transformed plants) and PCR analysis was done for the presence of the *DST_amiRNA*. The desired band (430 bp) for *DST_amiRNA* specific primer was found only in the transformed plants that confirmed the presence of desired transgene (**Figure 5**).

3.8. Agronomic Trait Analysis at T₀ Generation

In rice breeding programs, enhanced grain yield is a major focus which is determined by various components. Among them, grain number per panicle is highly variable and contributes the most to yield formation [16].

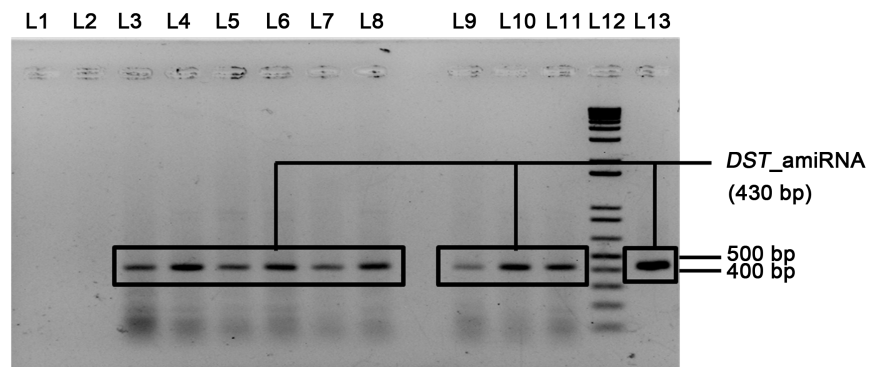


Figure 5. PCR analysis for the detection of *DST_amiRNA* at T₀ BR28 transformed plants. L1: negative control; L2: WT BR28; L3 - L11: transformed BR28; L12: 1 kb + DNA ladder; L13: positive control. The transformed samples showed the correct band size of 430 bp.

Table 2. Transformation efficiency of *in planta* transformation at T₀ generation (based on LDS assay).

Gene construct used for infection	Variety	No. of the germinated seedlings after infection (a)	No. of salt stress positive plants with positive flag leaves (b)	Transformation efficiency (b/a × 100)
<i>pDST_amiRNA</i>	BR28	41	12	29.27%

Results from an average of three independent experiments.

In the study, BR28 transformed plants showed significantly strong phenology with broader leaves, greater height, higher total tiller number, higher primary branching, and higher spikelet numbers and hence higher yield, compared to WT (**Figure 6(A)**). The number of filled grains per panicle was also significantly increased in the transformed plants (**Figure 6(B)**). Among all the transformed plants, four plants (P2, P6, P11 and P17) showed significantly higher panicle length as well as higher primary branching and hence higher yield compared to the WT BR28 (**Figure 7**). Among these four plants, average grain yield production in P11 plant was increased more than 2.5 folds compared to the average grain yield production of WT (0.65 g yield per panicle in P11 compared to 0.24 g in WT BR28). So, BR28 transformed plants showed significantly higher yield compared to the WT and demonstrated the positive effect of downregulation of the *DST* on increasing yield parameters.

3.9. Effect of Salt Stress at Seedling Stage

Transformed lines at T_1 generation that showed positive results in leaf disk senescence (LDS) assay, PCR analysis and better agronomic traits were selected for the salt stress screening at the seedling stage. Among the selected eight lines, three lines (line P11-1, P11-3 and P6-2) performed extremely well with strong vigor along with significantly higher percentage increase in root length and low-

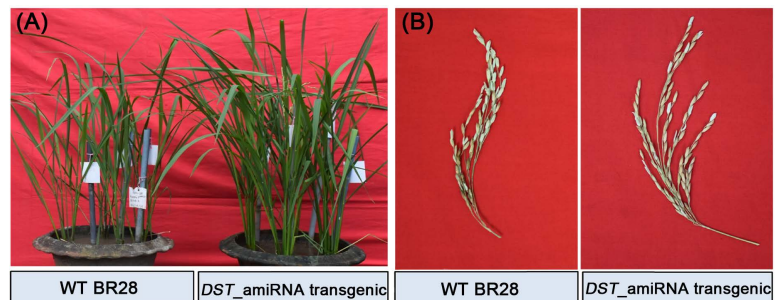


Figure 6. Pictorial view of BR28 transformed plant (A) and their panicle (B) along with the WT (Non-stressed). Transformed plants show better phenology with significantly higher filled grains and primary branches compared to the WT.

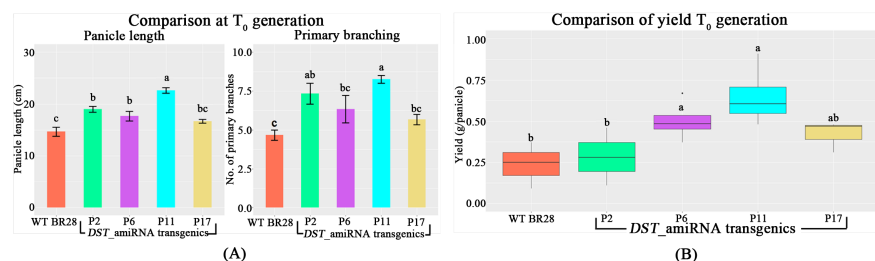


Figure 7. Comparison of agronomic traits of BR28 transgenics with the WT at T_0 generation. (A) Comparison of panicle length and primary branches of the transformed plants with WT; (B) Comparison of yield of the transformed plants with WT. Each bar represents the mean \pm SE ($n = 5$). Different letters in each graph ((a)-(c)) indicate significant differences ($P < 0.05$, ANOVA and Duncan test).

er percentage reduction in shoot length even after 14 days of 120 mM NaCl treatment whereas non-transformed WT plants turned yellow and then brown (Figure 8).

After 14 days of 120 mM salt stress, it was observed that the transgenic lines showed better performance with significantly lower electrolyte leakage, lower reduction of chlorophyll content, higher increase in root relative water content and in hydrogen peroxide (H_2O_2) level indicating the significantly higher stress tolerance of transgenics compared to the WT (Figure 9).

However, hydrogen peroxide level at the seedling stage showed that transgenic line P11-1 contained significantly higher level of H_2O_2 compared to WT in control condition (Figure 10). Again, higher accumulation of H_2O_2 results in increased stomatal closure that plays crucial role in preventing water loss and maintaining water content and hence provide drought tolerance in plants [20]. As *DST*_amiRNA transgenic showed higher level of H_2O_2 in control condition, therefore, the result of this experiment is a proof of concept that *DST*_amiRNA transgenics would provide drought tolerance as well.

So, in all the tests performed to compare the stress tolerance in transgenic lines with respect to WT, transgenic lines showed significant better performance validating the gain in the salt tolerance characteristics in *DST*_amiRNA transgenics.

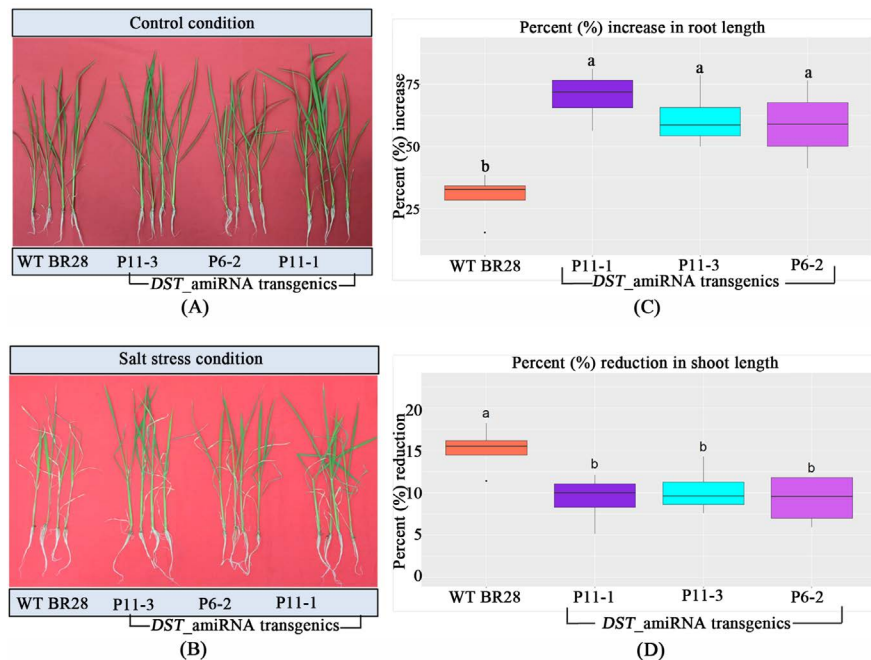


Figure 8. Seedling stage salt stress screening at T₁ generation. (A) in control condition (in hydroponic solution without salt treatment); and (B) after 14 days of 120 mM salt (NaCl) stress in hydroponics. The transgenic lines performed better compared to WT with significantly higher percentage increase in root length (C) and lower percentage reduction in shoot length (D) even after 14 days of 120 mM NaCl treatment. Different letters in each graph ((a) and (b)) indicate significant differences ($P < 0.05$, ANOVA and Duncan test).

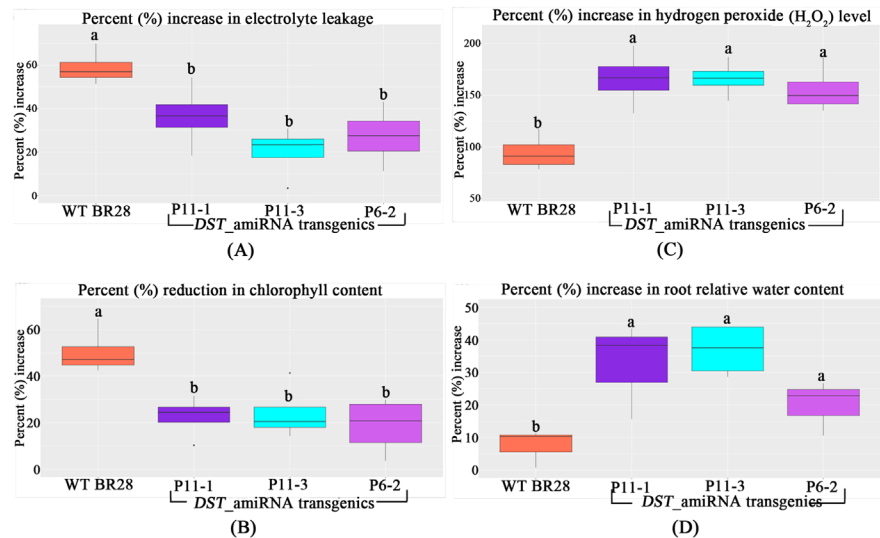


Figure 9. (A) Percentage increase in electrolyte leakage; (B) Percentage reduction in chlorophyll content; (C) Percentage increase in hydrogen peroxide (H_2O_2) level and (D) root relative water content of transgenic lines and WT BR28 after 14 days of 120 mM salt (NaCl) stress. In all the cases the transgenic lines showed significantly better result compared to the WT. Different letters in each graph ((a) and (b)) indicate significant differences ($P < 0.05$, ANOVA and Duncan test).

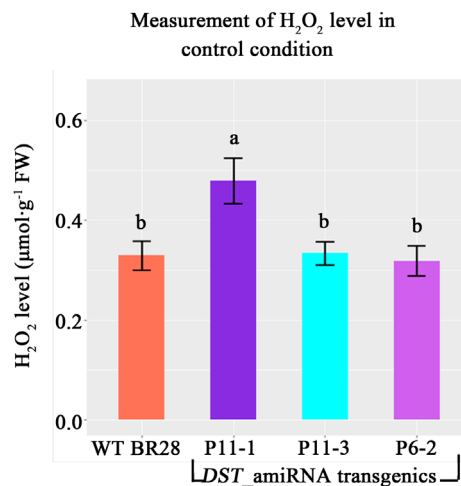


Figure 10. Measurement of hydrogen peroxide (H_2O_2) level at the seedling stage in control condition at T_1 . Line P11-1 showed significantly higher H_2O_2 level compared to WT BR28. Different letters in each graph ((a) and (b)) indicate significant differences ($P < 0.05$, ANOVA and Duncan test).

3.10. Expression Analysis of *DST* Gene in T_1 Plants by Semi-Quantitative RT-PCR

Based on the salt stress screening, agronomic trait analysis and other physiological screening experiments, we selected best two lines (line P11-1 and line P6-2) to check the expression level of *DST* gene and to confirm that these stress tolerance and higher grain yield production characters were obtained due to down-regulation of *DST*. Semi quantitative RT (reverse transcriptase) PCR was used to

compare the expression of *DST* gene of T₁ transformants of BR28 with WT plants under both control and stress condition (100 mM salt stress). For the optimization of cDNA for both WT and the selected lines (P11-1, P6-2), PCR reactions were conducted using primers specific for house-keeping gene, *eEF-1 α* (eukaryotic elongation factor –1 α). After normalization, both the transformed lines (P11-1, P6-2) and the WT showed almost same level of expression in case of *eEF-1 α* in both control and stress condition (Figure 11(A) and Figure 11(C)). Semi-quantitative RT (reverse transcriptase) PCR at 26 cycles was done using *DST* specific primers with same concentration of cDNAs (normalized with respect to the concentration level of *eEF-1 α*). The desired size of 210 bp bands was found (Figure 11(B) and Figure 11(D)). Intensity of the bands revealed that both the transformed lines (P11-1, P6-2) showed a lower level of expression of *DST* gene in both the conditions (control and salt stress) compared to the WT indicating the constitutive downregulation of the *DST*. Therefore, it can be clearly demonstrated that *DST*_amiRNA successfully and constitutively down-regulated the *DST* gene. Concomitantly it was also shown that the stress tolerance characteristics and increase in grain yield production of the transgenic lines occurred due to the downregulation of the *DST*.

4. Conclusion

Most of the studies involving the ectopic insertion of transgenes show some reduction in yield in abiotic stress conditions [10]. Previous studies on *DST* separately showed abiotic stress tolerance and enhanced yield in rice [16] [20]. In our

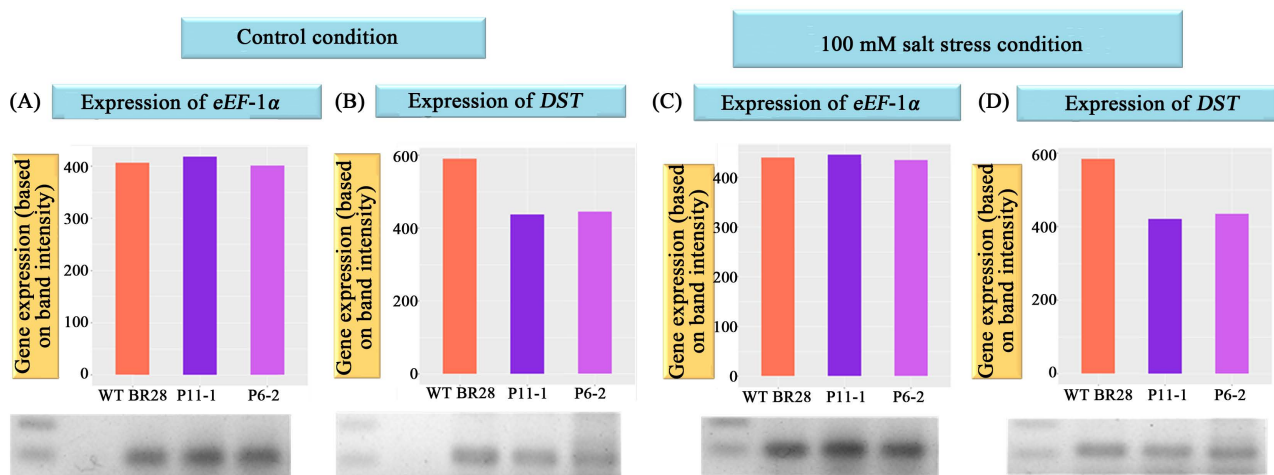


Figure 11. Expression analysis of *DST* gene in control and salt stress condition, in both wild type and T₁ BR28 transformed plants by semi-quantitative RT (reverse transcriptase) PCR. PCR amplification of housekeeping gene *eEF-1 α* (A) in control condition and (C) in salt stress condition, in wild type and T₁ transformed BR28. PCR amplification of *DST* gene (B) in control condition and (D) in salt stress condition, in wild type and T₁ transformed BR28 (26 cycles). In ((A) and (B)), L1: 1 Kb + ladder, L3: WT BR28; L4: line P11-1; L5: line P6-2. In ((C) and (D)), L1: 1 Kb + ladder, L2: WT BR28; L3: line P11-1; L4: line P6-2. Results showed that the expression level was almost same in both WT and two transgenic lines in case of *eEF-1 α* and lower level of expression of *DST* gene were found in both the transgenic lines (in both the conditions) compared to WT. 1D-Multi tool of Alpha Ease FC imaging system and R programming language were used for the graphical representation of the intensity of the bands in the gel.

studies, by downregulating *DST* using amiRNA we showed the combination of simultaneous stress tolerance and improved yield in rice. Moreover, results obtained from the studies at T₀ generation are immensely promising. So, we will now need to examine whether there is any loss in yield in the lines shown to be stress tolerant above after subjecting them to stress at the reproductive stage and analyzing the any gains in yield compared to WT.

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