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Enhancement of Drought Tolerance in Rice through Introgression of Arabidopsis *DREB1A* through Transgenic Approach

Chaitanya Kumar Geda, Sai Krishna Repalli*, Goutam Kumar Dash, Padmini Swain and GJN Rao

ICAR-National Rice Research Institute, Cuttack, India-753006

*Corresponding author: Sai Krishna Repalli, ICAR-National Rice Research Institute, Cuttack, India, Tel: +919938996514; E-mail: saikrishnarepalli@gmail.com

Abstract

With global temperatures on the raise, drought stress is turning out to be the major impediment to increase in rice production all over the world. In India, where 60% of the rice lands are rain-fed, rice production is severely hampered by drought as the crop depends heavily on monsoon rains. In this context, we attempted to incorporate tolerance against drought in *Indica* rice through introgression of *AtDREB1A* gene from Arabidopsis employing *Agrobacterium* mediated transgenic approach. Putative transgenics were generated and the presence of the incorporated gene was confirmed through Polymerase Chain Reaction (PCR) and Southern blotting assays. The inheritance pattern of the incorporated gene was Mendelian. The physiological studies conducted on eight transgenic lines revealed that introduced gene could confer significantly higher levels of tolerance to drought stress. This promising result suggests that transgenic approach can be a viable option for genetic enhancement of rice against abiotic stresses like drought.

Keywords: Transgenic; *Indica* rice; Pusa Basmati 1; Drought tolerance

Introduction

Global temperatures increased drastically in the last decade and continuous global warming prevailed across the globe. In India, highest temperatures were recorded according to the World Meteorological Organization report. Cereal production in Asian continent is severely affected by drought, predominantly during dry season. According to a recent report from Intergovernmental Panel on Climate Change, about one fifth area which corresponds to 23 million hectares of rice ecosystem is prone to drought. In eastern India, about 11% of the total agricultural GDP is affected by drought [1].

Indian agriculture is mainly monsoon based, with over 60% of agricultural land being rain-fed, a failed monsoon causes a crippling impact on food production and farmers often encounter a complete loss of crops thus threatening their livelihoods and food security. As there is no control over the monsoon, it is essential to develop rice varieties that can grow in adverse situations like drought. Rice is mostly grown in hydrological conditions and limited success for drought tolerance was achieved through conventional approaches due to the lack of available donors in the rice gene pool to improve drought tolerance, which is a complex polygenic trait. Drought is fast becoming the most serious abiotic stress due to increase in temperature and decrease in ground water level.

Recent progress on targeting abiotic stress tolerance focused mainly on structural and regulatory genes through genetic engineering approach. This study focused on the applications of regulatory genes including: i) Transcription factor genes and ii) Dehydration Responsive Element Binding Factors (DREBs). It was reported that there was an increase in level of transcription of several downstream target genes at a single stretch by over expressing certain transcription factors [2]. Yamaguchi and Shinozaki [3] first reported the presence of

transcription factor termed as Dehydration-Responsive Element (DRE), in the promoter region of RD29A gene in Arabidopsis. DRE binds to special signature sequence and targets the expressions of several downstream genes. Specific signature sequence of C-repeats in DRE transcription factor, are termed as CRT elements or CRT-binding factors. Moreover, these Dehydration Responsive Element-Binding (DREB) factors were found to be targeting several genes involved in drought, and cold tolerance and they function independent of leaf senescence hormone abscisic acid.

Experiments carried out in the model plant Arabidopsis have shown that the constitutive over expression of either *DREB1A* from rice (*OsDREB1A*) or *DREB1A* from maize (*ZmDREB1A*) have resulted in the regulation of downstream targeted genes imparting dehydration tolerance [4], cold and salinity tolerance [5]. Successful results of desiccation tolerance in Arabidopsis had encouraged various research groups to target *AtDREB1A* gene into various crops but the results varied under the influence of constitutive promoters (e.g. 35S, ZmUbi, OsActin1) and stress inducible promoters (e.g. RD29A, OsHVA22p). Desiccation tolerance was more profound in the case of stress inducible promoters [6] than the constitutive promoters [7].

Considering the success with *DREB* genes, the present work, aimed to generate transgenic rice through incorporation of *AtDREB1A* transcription factor under the control of *RD29A* promoter into Pusa Basmati 1 (PB1), an elite Basmati rice variety, which is extensively grown in the Basmati region of northern India with an yield potential of 4.5 t/ha and is amenable to tissue culture [8].

Materials and Methods

Generation of transgenic plants

Mature kernels of elite *Indica* rice genotype Pusa Basmati 1 were surface sterilized [9] and inoculated on MS media [10] supplemented with 2,4-Dichlorophenoxy acetic acid (2,4-D @2.0 mg L^{-1}) and

incubated at 26 ± 1°C in dark for three weeks. Scutellum derived embryogenic callus was used as the target tissue for *Agrobacterium* transformation using a super virulent *Agrobacterium tumefaciens* strain *LBA4404* (pSB1) carrying the binary vector pC1200-RD29A-*AtDREB1A* with a hygromycin-resistant gene (*HPT*) as plant selection marker and chloramphenicol as the bacterial selection marker. The target gene was driven by the stress-inducible *RD29A* promoter and a nos terminator derived from *Arabidopsis thaliana* (Figure 1).
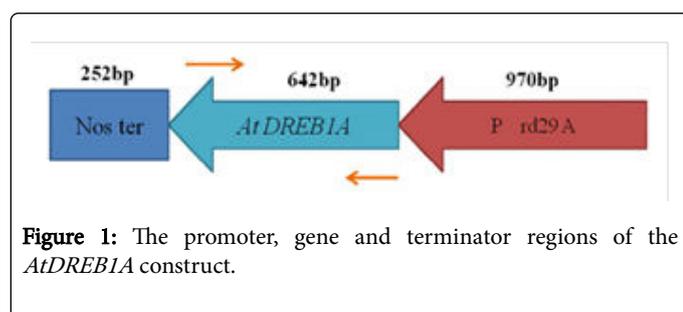
Figure 1: The promoter, gene and terminator regions of the *AtDREB1A* construct.

Agrobacterium mediated transformation, co-cultivation, washing, selection (four cycles of 15 days each) were carried out as per Saikrishna et al. [11] and the resistant calli were transferred onto regeneration media as per Chaitanya et al. [8]. Shoots were transferred into rooting media (MS media supplemented with maltose (50 g l-1), naphthalene acetic-acid (1.0 mg L-1), and kinetin (0.1 mg L-1)). Putative transgenic plants with well-established roots were transferred into plastic pots having sterile soil in the institutional biosafety glass house.

Molecular analyses (polymerase chain reaction (PCR) and Southern blot analysis)

DNA was extracted from the fresh leaves employing CTAB method as proposed by Murray and Thompson [12]. Isolated DNA was examined for the presence of transgene (*DREB1A*) and antibiotic marker gene (*HPT*) with the help of specific primers through Polymerase Chain Reaction (PCR) assays in a total reaction volume of 10 μL per reaction in a programmable thermal cycler (Eppendorf Vapo protect). The constituents of the PCR reaction include: 1 μL of plant DNA (20 ng), 0.8 μL of 2.5 mM dNTPs (Fermentas), 1.0 μL of 10X PCR buffer (Sigma), 0.2 μL of Taq DNA Polymerase (5 U/μL Sigma), 1 μL each of both forward & reverse primers (5 pico moles/μL Sigma) and 5 μL of autoclaved sterile distilled water. The PCR reaction conditions followed for both the genes include; an initial denaturation of template DNA at 94°C for 5 min followed by 40 repeated cycles of denaturation at 94°C for 1 min, primer annealing at 58°C for 1 min, and primer extension at 72°C for 1 min followed by a final extension for 5 min. PCR amplicons were separated through electrophoresis on 1.2% agarose gel containing 0.5 μg/mL ethidium bromide (in 1X TBE electrophoresis buffer). Separated PCR products were photographed in a gel documentation system to examine the size of the product compared with one-kb molecular weight marker.

For Southern analysis, DNA isolated from all the test samples and control were subjected to overnight restriction digestion at 37°C with Hind III (Promega) restriction enzyme for gene of interest *DREB1A* in a total reaction volume of 40 μL. Restriction digestion was checked by loading one microliter each of digested sample on to 0.8% (w/v) agarose gel, pre-stained with ethidium bromide (0.5 μg/mL) and observed under Ultra Violet light. Rest of the digested samples were fractionated on 0.8% (w/v) agarose gel. The gel was depurinated and

equilibrated for 30 min each subsequently with respective buffers and the DNA fragments were transferred from the gel to Hybond-N+ nylon membrane under alkaline denaturation conditions according to manufacturer's (Amersham, Arlington Heights, IL) instructions. The membrane was pre-hybridized with 50 mL of hybridization buffer for at least 1 h according to Zoller and Smith [13] in to which sheared single stranded salmon sperm DNA (10 μg/mL) was added for blocking. *DREB1A* fragment (0.56 kb) to be used as hybridization probe was radio labelled with (α-P^{32}) dCTP using the Rediprime Labeling Kit (Promega). After the overnight hybridization, the membranes were washed thrice for 30 min each subsequently with wash buffers 1,2,3 as proposed by Sambrook and Russell [14]. Excess wash solution was removed and the membrane was wrapped with saran-wrap and auto-radiographed overnight depending on the radioactive count by a Geiger-Mueller counter and visualized under a phosphor-imager (Typoon-5000 GE-Health care).

RT-PCR

Total RNA was extracted from the leaves of both stress imposed Southern positive transgenic lines as well as non- transformed control plants and RT-PCR was performed as per the standard protocol followed in our lab according to Rao et al. [15]. Primers used for *DREB1A* gene amplification were same as mentioned for PCR analysis.

Physiological Studies

To study the physiological parameters, transgenic rice lines of T3 generation were grown in plastic pots in triplicates, containing 5 kg of soil each. To maintain uniformity in the soil texture, soil was dried, finely ground and mixed thoroughly. Four to five seeds were germinated in each pot and after a week, a single healthy seedling was maintained per each pot. Drought stress experiments were conducted as per Liu et al. [16] and a ten day stress was imposed on the twenty-one day old seedlings by withdrawing irrigation and observations on different physiological and biochemical parameters were taken at two days interval from the day of imposition of stress.

Relative water content

Relative Water Content (RWC) was calculated as per formula given by Bonnet et al. [17]. Three kinds of weight viz., Fresh Weight (FW), Turgid Weight (TW) and Dry Weight (DW) were measured subjecting the leaves to three different conditions. Fresh weight was determined from the central portions of the fully expanded leaf samples collected during midday immediately after collection. These leaves were maintained in water for turgidity in closed petri-dish for 8 h under normal room temperature, blotted to remove excess surface moisture and weighed to determine turgid weight. Later, the samples were oven dried for 24 h at 80°C and weighed to determine dry weight.

Relative chlorophyll content

Chlorophyll concentration in rice leaves was determined using the chlorophyll meter as described by Markwell et al. [18] and such values have been shown to be positively correlated (r^2=0.93) with destructive chlorophyll measurements in rice [19].

Soil moisture content (SMC)

At the end of the stress period, soil moisture content was measured from a depth of 15 cm to 30 cm soil according to Brady [20].

Proline content

Proline content in the test sample from the standard curve was calculated using the following formula given by Bates et al. [21].

Gas exchange parameters

Gas exchange parameters were measured from the top second leaf in comparison to the standard parameters using a portable infrared gas analyzer according to Nataraja and Jacob [22]. Instantaneous Water Use Efficiency (WUE) is calculated as the ratio between net photosynthetic rate (A) and transpiration rate (T).

Chlorophyll fluorescence

Chlorophyll fluorescence w as measured a s given by Panda and Sarkar [23].

Results

A total of 72 putative transgenic plants were obtained and grown in the transgenic greenhouse till maturity. PCR assays, conducted could amplify the 560 bp fragment precise to the introduced target gene (*AtDREB1A*) and a 960 bp fragment specific to the selectable marker (*HPT* gene) in all the plants (Figures 2 and 3). Further studies were conducted with eight plants that were generated from different calli selected during the selection cycles. Southern hybridization studies, conducted using a 560 bp radioactive gene sequence as the probe, established the incorporation of the *AtDREB1A* gene in the rice genome. The presence of a strong signal in all the plants (D1-8) confirms the clear integration of the target gene (*AtDREB1A* gene) into the rice genome while hybridization signal was not detected in the non-transformed (wild type) plants (Figure 4). he signal pattern in the Southern blots was different in all the test plants, except for the plants 2 & 3 which appeared to be similar. The size of the hybridized fragment varied from plant to plant while the expected size of the fragment in the control (plasmid DNA) is 2.1 kb, suggesting the presence of at least seven independent events.

Figure 2: PCR amplification of *AtDreb1A* gene in the putative transgenics. L-100 bp ladder, C-(-) control (blank), P-(+) control (plasmid DNA), NT-Wild type (PB1). Nos. 1 to 8-putative transgenics.
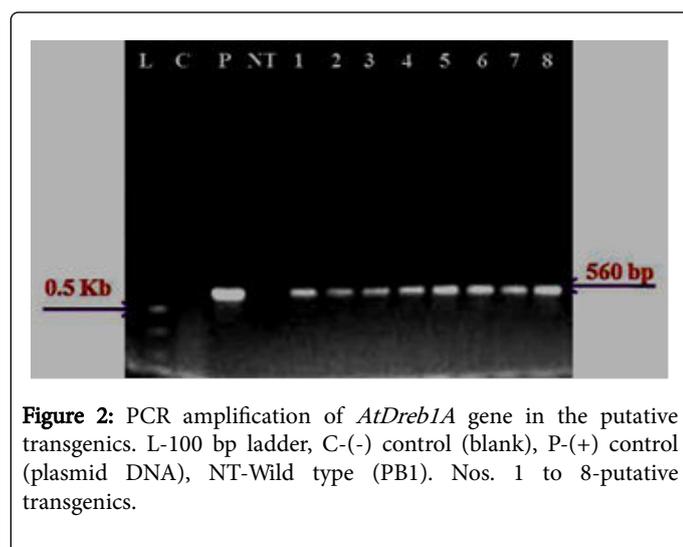
Expression of the introduced gene in the transformed plants was checked by reverse transcription polymerase chain reaction. The results suggest that in all the eight putative transgenics, the expression of introduced gene was observed (Figure 5). All these molecular analyses were performed on plants of T0 generation (putative transgenics) and studies continued to estimate the transmission rates through PCR assays and it was observed to be in Mendelian proportion.

Figure 3: PCR amplification of *hpt* gene in the putative transgenics. L-100 bp ladder, C-(-) control (blank), P-(+) control (plasmid DNA), NT-Wild type (PB1). Nos. 1 to 8-putative transgenics.

Figure 4: Southern hybridization results on putative transgenic plants for the presence of *AtDreb1A* gene. P-(+) control (plasmid DNA), E-(-) control (blank), NT-Wild type (PB1) Nos. 1 to 8-putative transgenics.
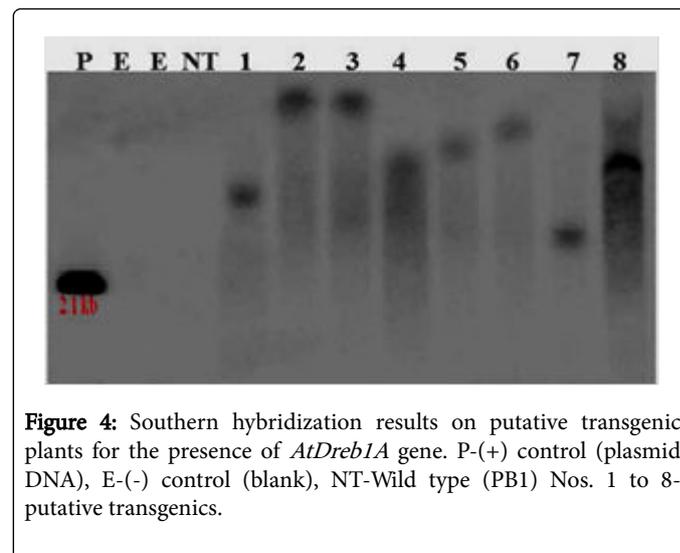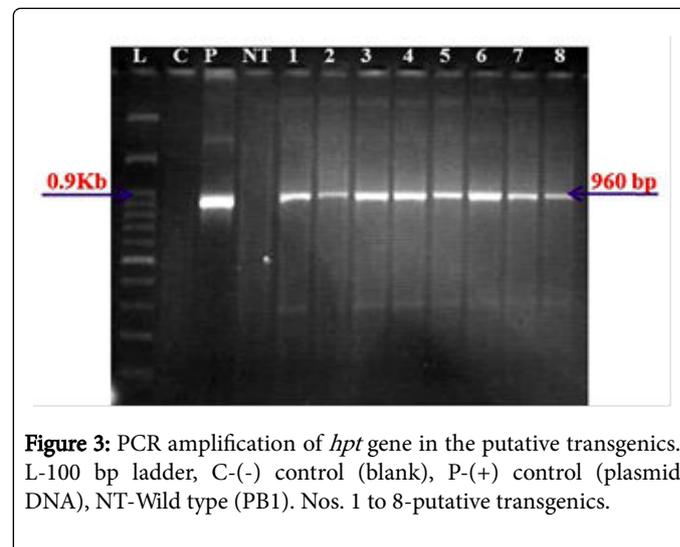
Physiological studies

Physiological studies were conducted on T4 generation lines and homozygous lines of each transgenic were grown for physiological studies to assess the lines for their level of drought tolerance with non-transformed PB1 plants as controls (Figure 6).

Relative water content (RWC)

During unstressed treatment there was not any practical difference in the leaf RWC between control and transgenic lines and was in the range of 84% to 93% while during stress (10 d), the RWC of both transformed and non-transformed leaves dropped quickly from 90.5% to 53.2%, in control, whereas the proportion of RWC in most of the transgenic plants were higher than in wild type which was of the following order: D-1(68.8%), D-2(62.1%), D-3(72.6%), D-4 (74.3%), D-5 (64.3%), D-6 (61.7%), D-7(65.2%) and D-8 (72.4%). After 10 days

of dehydration stress, the RWC of the transgenic lines had just decreased in the range of 11.6% to 34.7% in comparison to 37.3% as in case of control plants. However, the rate of decrease of RWC was less in lines such as D-6, D-2, D-5, D-7 and D-1 in comparison to lines, D-8, D-3 and D-4 (Table 1).
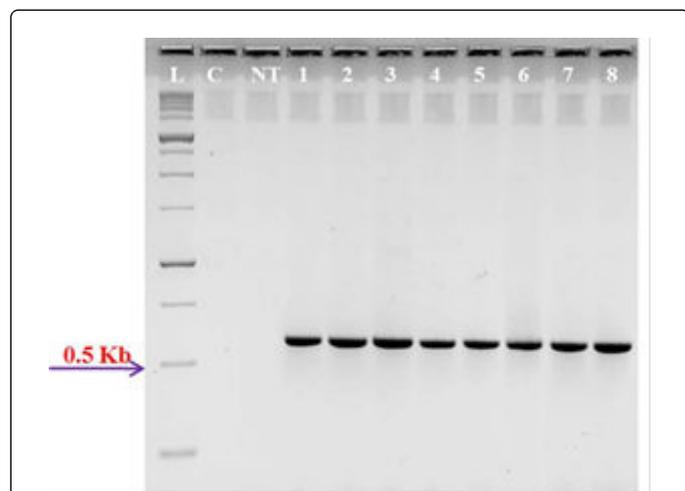
Figure 5: RT-PCR reaction for *Dreb1A* gene in the transgenic plants. L-1Kb ladder, C-PCR control (blank), NT-Wild type (PB1). Nos. 1 to 8-transgenic lines.
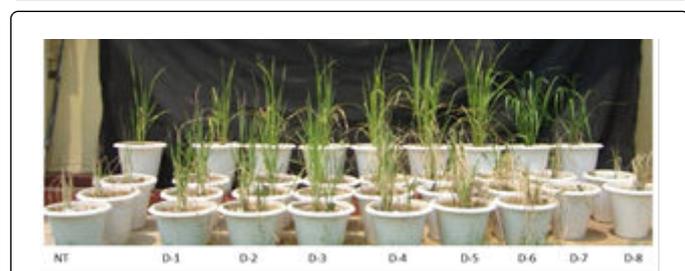
Figure 6: Evaluation of drought tolerance in the transgenic lines. NT- Wild type (PB1), Nos. D-1 to D-8 transgenic lines

Chlorophyll content

Soil Plant Analysis Development (SPAD) values of the transgenic and non-transgenic control plants were almost similar, and ranged from 32.3 to 42.4 before stress but after initiation of stress, the chlorophyll values of the stressed plants decreased ranging from 25.2 to 40.7 with most of the transgenic plants retaining 28.4% to 39.5% of the chlorophyll content on the 10th day of stress. However, after re-irrigation for 2 days, i.e., 12th day, increased levels of chlorophyll were observed in transgenic plants while such increase was not observed in control.

Proline content

During stress treatment, the levels of proline increased both in transgenic and control plants and it was found to be higher after 6 days in transgenic lines as follows: D-4 (216.2 μM/g fr wt.), D-5 (211.8 μM/g fr wt.), D-7 (186.1 μM/g fr wt.), D-6 (172.5μM/g fr wt.) and D-8 (145.3 μM/g fr wt.), but after 10 days of stress, proline content was highest as observed in D-4 (407.2 μM/g fr wt) followed by D-5 (405.9 μM/g fr wt), D-7 (396.2 μM/g fr wt), D-6 (391.9 μM/g fr wt) and D-1 (368.9 μM/g fr wt) whereas the unstressed plants showed the lower levels of proline ranging from 15.2 μM/g fr wt to 41.5 μM/g fr wt (Table 1), while there was not any difference in the proline levels of unstressed transgenic and control plants.

Photosynthetic gas exchange parameters

Rate of transpiration, stomatal conductance, and instantaneous water use efficiency of the transgenic lines (D1-D8) were measured in comparison to Non-Transgenic line (NT) under Well Watered (WW) and Water Stress (WS) conditions (Table 2). Transpiration rates were in the range of 9 to 13 (mM H_2O $m^{-2}s^{-1}$) under Well Watered (WW) condition but decreased in the range from 1.9 to 3.6 (mM H_2O m^{-2} s^{-1}) under Water Stress (WS) conditions. Transpiration rates in transgenic lines were more compared to control with D4 showing highest transpiration rate (3.6) followed by D1 (3.64), D2 (3.52), D7 (3.5), D8 (3.49), D3 (2.88) and D6 (2.87) in the decreasing order (Figure 7). Stomatal conductance ranged from 0.72 to 1.03 (M H_2O m^{-2} s^{-1}) under well watered condition but decreased within the range from 0.06 to 0.09 (M H_2O $m^{-2}s^{-1}$) under water stress conditions (Figure 8).

	Percentage of Relative Water Content				Relative chlorophyll content				Proline content (μ mole/g fresh wt.)				SMC	Drought score	Recovery score
	6-DAS		10-DAS		6-DAS		10-DAS		6-DAS		10-DAS				
	WW	WS	WW	WS	WW	WS	WW	WS	WW	WS	WW	WS			
PB1	92	59.8	90.5	53.2	32.3	28.2	32.2	27.1	17.7	46.5	17.2	140.6	11.36	9	9
D1	86.7	72.7	86.7	68.8	31.3	25.2	31.6	28.4	16.5	167.5	19.4	368.9	10.76	3	1
D2	91.7	75.9	91.4	62.1	30.6	29.4	30.4	29.8	19.3	119.8	19.2	277.1	9.12	1	1
D3	93	78.7	84.2	72.6	31.5	30.7	31.7	30.4	15.2	106.8	18.7	251.4	10.23	3	1
D4	91.3	67.2	91.2	74.3	34.8	27.6	33.2	30.5	15.8	216.2	21.1	407.2	11.05	3	1
D5	85.7	63.1	86.4	64.3	33.3	30.9	32	30.6	17.4	211.8	20.5	405.9	10.2	1	1
D6	90.5	68.3	96.4	61.7	34.2	31.5	33	30.8	22.4	172.5	18.4	391.9	10.05	3	1

| D7 | 84 | 67.4 | 83.3 | 65.2 | 42.4 | 40.7 | 41.2 | 39.5 | 35.1 | 186.1 | 17.8 | 396.2 | 10.29 | 3 | 1 |
| D8 | 86.7 | 78.1 | 84 | 72.4 | 41.4 | 39.7 | 40.7 | 37.7 | 41.5 | 145.3 | 21.8 | 347.8 | 10.92 | 1 | 1 |

Table 1: Mean data table of physiological and biochemical characterization. DAS-Days After Stress

S.N.	Transpiration rate		Stomatal Conductivity		Water Use Efficiency		F_v/F_m		Photosynthetic rate	
	WW	WS	WW	WS	WW	WS	WW	WS	WW	WS
NT	13.00 ± 0.59	1.93 ± 0.05	1.03 ± 0.07	0.08 ± 0.00	1.52 ± 0.02	1.74 ± 0.11	0.82 ± 0.05	0.70 ± 0.01	19.85 ± 0.71	03.37 ± 0.13
D1	09.87 ± 0.00	3.64 ± 0.23	0.88 ± 0.07	0.08 ± 0.02	2.21 ± 0.00	2.85 ± 0.15	0.81 ± 0.02	0.75 ± 0.05	21.87 ± 0.00	10.39 ± 0.12
D2	09.83 ± 0.11	3.52 ± 0.03	0.97 ± 0.00	0.09 ± 0.00	2.1 ± 0.06	2.91 ± 0.04	0.82 ± 0.03	0.75 ± 0.07	21.05 ± 0.86	10.27 ± 0.07
D3	09.23 ± 0.57	2.88 ± 0.86	0.72 ± 0.08	0.06 ± 0.01	2.45 ± 0.04	3.11 ± 0.57	0.81 ± 0.03	0.74 ± 0.01	22.62 ± 0.98	08.47 ± 1.01
D4	11.28 ± 1.40	3.68 ± 0.07	0.76 ± 0.15	0.06 ± 0.00	1.95 ± 0.35	2.62 ± 0.00	0.82 ± 0.06	0.74 ± 0.00	21.52 ± 1.27	09.67 ± 0.21
D5	09.18 ± 0.02	3.38 ± 0.05	0.76 ± 0.03	0.07 ± 0.00	2.49 ± 0.00	3.22 ± 0.03	0.81 ± 0.05	0.75 ± 0.01	22.88 ± 0.10	10.94 ± 0.24
D6	09.72 ± 1.56	2.87 ± 0.22	0.89 ± 0.06	0.06 ± 0.00	2.46 ± 0.54	3.90 ± 0.02	0.81 ± 0.02	0.76 ± 0.02	23.10 ± 1.36	11.21 ± 0.81
D7	09.90 ± 0.22	3.50 ± 0.04	0.84 ± 0.11	0.07 ± 0.00	2.32 ± 0.08	3.55 ± 0.18	0.82 ± 0.01	0.76 ± 0.01	23.06 ± 1.31	12.45 ± 0.48
D8	09.48 ± 0.21	3.49 ± 0.37	0.83 ± 0.10	0.06 ± 0.00	2.22 ± 0.02	3.39 ± 0.11	0.81 ± 0.05	0.74 ± 0.01	21.12 ± 0.63	11.83 ± 0.88

Table 2: Mean data table of photosynthetic studies in transgenic lines.
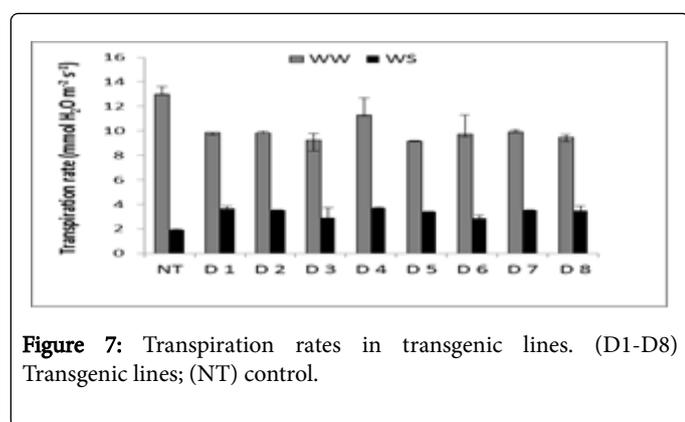
Figure 7: Transpiration rates in transgenic lines. (D1-D8) Transgenic lines; (NT) control.
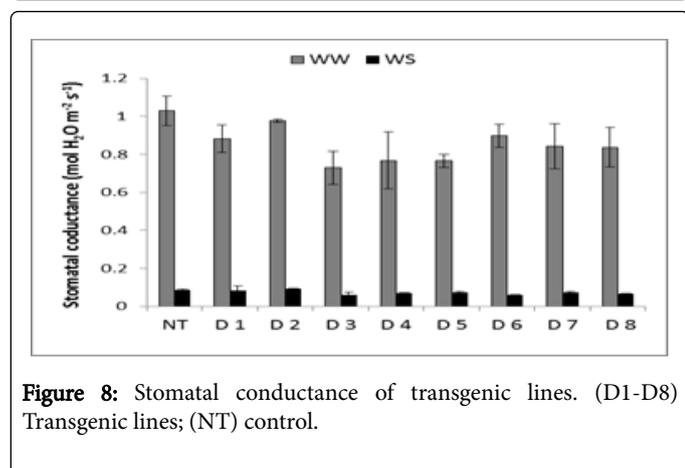
Figure 8: Stomatal conductance of transgenic lines. (D1-D8) Transgenic lines; (NT) control.

Water use efficiency ranged from 1.95 to 2.49 µM CO_2/mM H_2O under well watered condition but decreased within the range from 1.74 to 3.9 µM CO_2/mM H_2O under stress conditions. Water use efficiency rates in transgenic lines were higher in comparison to control (1.74). Line D6 showed highest water use efficiency (3.9 µM $CO_2 m^{-2} s^{-1}$) followed by D7 (3.55), D8 (3.39), D5 (3.22), D3 (3.11), D2 (2.91), D1 (2.85) and D4 (2.62) in the decreasing order (Figure 9).
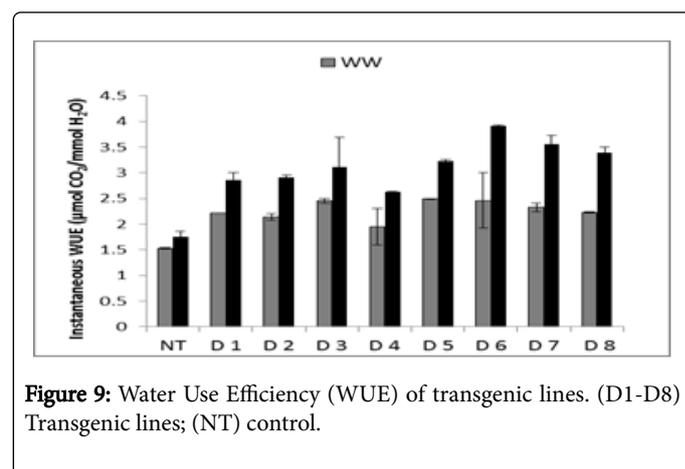
Figure 9: Water Use Efficiency (WUE) of transgenic lines. (D1-D8) Transgenic lines; (NT) control.

Photosynthetic rates ranged from 19.85 to 23.1 µM CO_2 $m^{-2} s^{-1}$ under well watered condition but decreased in the range from 3.37 to 12.45 µM $CO_2 m^{-2} s^{-1}$ under stress. Photosynthetic rates in transgenic lines were more compared to the control (3.37 µM $CO_2 m^{-2} s^{-1}$) under stress condition. Photosynthetic rates of the transgenic lines varied in the following decreasing order- D7 (12.45), D8 (11.83), D6 (11.21), D5 (10.94), D1 (10.39), D2 (10.27), D4 (9.67) and D3 (8.47) (Figure 10).
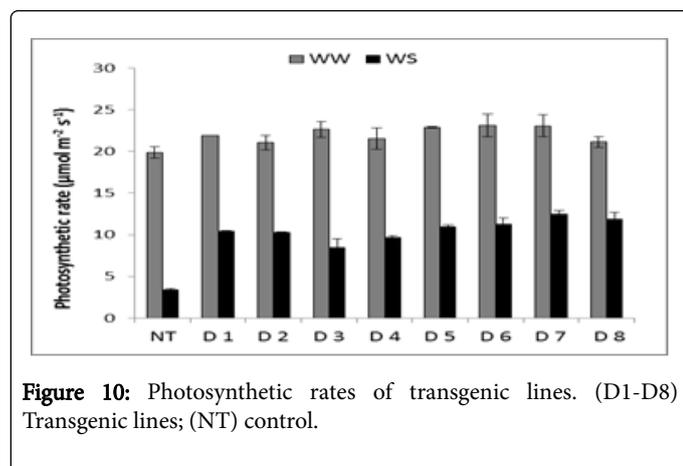
Figure 10: Photosynthetic rates of transgenic lines. (D1-D8) Transgenic lines; (NT) control.

The photosynthetic capacity and the organization of the Photo-System II (PSII) reaction centre in the dark adapted leaves are associated. Higher organization of the photosystem II as represented by F_v/F_m ratio was found to be almost similar in transgenic and non-transgenic lines with slight increase in case of transgenic lines compared to non-transgenic lines. It ranged from 0.7 (control) to 0.76 (D7) respectively under water stressed conditions compared to 0.82 (control) and 0.82 (D7) respectively under well-watered conditions (Figure 11).
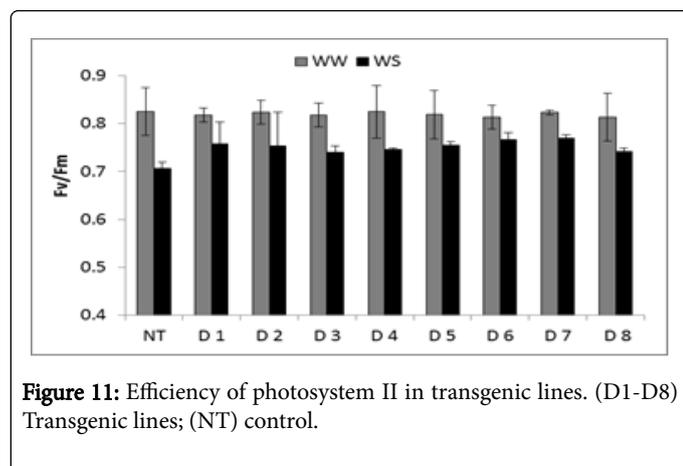
Figure 11: Efficiency of photosystem II in transgenic lines. (D1-D8) Transgenic lines; (NT) control.

This result suggests that the higher efficiency of PSII reaction centre of the transgenic lines contributed to the increased photosynthetic capacity.

Discussion

Drought is a frequent and periodic phenomenon occurring mainly in the drier months with varied intensities and is a major abiotic stress affecting about one fourth of the rice growing regions in Asia. About half of the total drought prone area in Asia belongs to Indian sub-continent. Surface water is evaporated due to increase in temperatures effecting the ground water levels and hydrologic cycle as a whole. Conventional breeding approaches were not able to address these challenges adequately as abiotic stress like drought is a polygenic trait. Transgenic approach provides a promising solution as the transfer of the gene of interest across the species is possible through this approach. In this study, an attempt was made to transfer the *DREB1A*

gene from *Arabidopsis thaliana* into Pusa Basmati, an elite *Indica* rice cultivar, through Agrobacterium mediated transformation.

Various genes involved in dehydration tolerance are controlled by transcription factors and the enhanced expression of these transcription factors had resulted in improved drought tolerance [24]. One of the important aspects to be taken care is that there are varied levels of drought tolerance with respect to their constitutive or stress inducible expression of promoter genes. Marginal growth defects were found by the applications of constitutive promoters with *DREB1A* genes. Hence to address this problem in the present study we had utilized stress inducible *RD29A* promoter with *AtDREB1A* gene and effects on growth were not observed thus confirming the utility of *RD29A* promoter in transformation studies [25].

The molecular analyses i.e. PCR and Southern analysis could validate the incorporation of the foreign gene in the transformed plants and the RT-PCR could successfully demonstrate the expression of the *DREB1A* gene in transformed plants in comparison to controls (Figure 5). Decrease in leaf chlorophyll content is considered to be an indicative symptom during water deficit condition which results from oxidation and degradation of photosynthetic pigments, sustained photo-inhibition and photo breeding [26]. High chlorophyll content ensures continued photosynthesis under moisture stress enabling plant to resume its growth and development [27]. In our experiment, all the transgenic lines showed higher SPAD value (Table 1), compared to NT after 10 days of stress which is similar with the findings of Ravikumar et al. [28], but maintenance of high chlorophyll content under severe stress is imparted by osmotic adjustment which leads to higher leaf water content. Higher leaf water content maintains turgidity of cells reflecting continued growth, and metabolic activity in tissues hence leaf chlorophyll content is considered as an important trait for water stress tolerance [29]. High RWC ensures continued transpiration leading to canopy cooling effect and continued water uptake by roots. In transgenic plants, stomata closure has been observed which is considered as a prominent feature of increased dehydration tolerance [30]. Nayyar and Gupta [31] had reported that there is a decrease in relative water content in response to dehydration stress in different plants.

Leaves exhibit large reductions in RWC and water potential when they are subjected to drought. In our study, the transgenic plants maintained 74.3% of RWC (Table 1) at 11.05% of SMC compared to NT which has 53.2% of RWC at 11.36% of SMC. These results are in congruence with earlier reports stating that plants tolerate drought stress by maintaining high RWC during dehydration stress periods [32].

Moreover, maintenance of high chlorophyll content or high RWC is under the control of osmotic adjustment contributed by accumulation of different osmolytes including proline. Accumulation of proline may reduce stress-induced cellular acidification or prime oxidative respiration to provide energy needed for recovery. We observed that highest levels of proline were accumulated on 8th day of the stress in transgenic rice lines whereas the control plants had very low levels of proline during the same period [33].

Stomatal conductance increases under heat stress conditions and thereby evapotranspiration increases to keep the canopy temperature cool. This is reflected in our studies where the transgenic lines exhibited more stomatal conductance and transpiration rates compared to control. High light radiation and heat stress affect the electron transport chain. Photosystem II is highly heat sensitive and is

affected during drought stress. Photo inhibition may occur during grain filling and this effect the rice yield. It was reported that drought stress reduces variable florescence (FV), and quantum yield [34]. Hence chlorophyll florescence measurement studies will help in understanding the equilibrium between metabolism and energy evolving processes which are affected during drought stress. Moreover chlorophyll florescence measurement is relatively simple technique which is non-destructive and less time consuming. Results in the present study show that there is a decrease in F_v/F_m ratio under stress conditions and this decrease is comparatively less in transgenic plants compared to control.

Transgenic lines and controls used in the study were given drought scores when subjected to stress as per IRRI, SES [35]. In this study, the controls (wild type) had a drought score 9 while transgenic lines D1, D3, D4, D6 and D8 had drought score 3 and the remaining transgenic lines D2, D5 and D7 showed a drought score 1. The recovery data suggests that all the transgenic lines showed recovery score of 1 (90% of all plants produced new leaves and tillers after irrigation applied for 1-2 days), whereas the control showed a recovery score of 9 (less than 50% of all plants produce new leaves and tillers after irrigation applied for 7days) (Table 1).

Conclusion

From the results it is evident that the transgenic lines developed did show proper integration, expression and inheritance of the introduced gene. The transgenic lines when screened using different physiological parameters that are associated with drought tolerance, did express high levels of tolerance to drought under controlled conditions. *AtDREB1A* gene was also known to provide salt tolerance and studies are underway to test the view. Assessment of drought tolerance under field conditions where various stresses come into play is really challenging and at present, limitations do exist as biosafety issues are involved.

However, the results obtained are highly promising and transgenic lines developed can address drought tolerance in present day climate change scenario and could serve as valuable source material for future breeding activities.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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