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Controlled Over-Expression of *AtDREB1A* Enhances Tolerance against Drought and Salinity in Rice

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Abstract: Engineering transcription factors (TF) hold promise in enhancing abiotic stress tolerance in plants. In this study, one of the popular rice varieties of South India, namely ADT 43, was engineered with a TF *AtDREB1A* driven by a stress-inducible *rd29A* promoter. PCR and Southern hybridization were employed to confirm the integration and copy number of the transgene. Transgenic lines (T_1) of ADT 43 showed enhanced tolerance to drought and salinity compared to the non-transgenic ADT 43. Transgenic lines were found to maintain higher RWC %, lower leaf temperature, and partially closed stomata, enabling better survival under stress conditions. qRT-PCR analysis revealed the strong induction of *AtDREB1A* transcripts during drought. Transgenic lines of ADT 43 exhibited increased germination and retention of chlorophyll in their leaves under salinity. Evaluation of transgenic lines under transgenic screen house conditions revealed that line # A16 exhibited *on par* agronomic performance against its non-transgenic counterpart under normal conditions. Under drought, non-transgenic ADT 43 showed >20% reduction in the total number of spikelets per panicle, whereas transgenic line # A16 registered only a 2% reduction. Non-transgenic ADT 43 recorded 80% yield reduction under drought, whereas line # A16 recorded only 54% yield loss. The above results demonstrated the effectiveness of controlled expression of DREB1A in regulating dehydration responses in rice.

Keywords: DREB1A; drought; salinity tolerance

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1. Introduction

Drought, salinity, and temperature extremes are the most common environmental factors limiting crop productivity. Sustained increase in food grain production depends mainly on developing climate-resilient crop varieties [1]. Accelerated genetic improvement of complex traits like drought/salinity tolerance has been achieved through marker-assisted selection and genetic engineering. A thorough understanding of molecular responses is a prerequisite for discovering candidate genes underlying stress tolerance. Plants respond to abiotic stresses through a complex network of physiological, biochemical, and molecular mechanisms. Among the various molecular processes, the mechanism of stress perception and cell signaling plays an essential role in altering the plant's ability to survive under abiotic stress conditions [2–7]. In the tolerant genotypes, perception of stress signals initiated early during the onset of stress reprograms the activation of a large set of stress-responsive genes through different transcription factors (TFs) [8,9]. Recently, numerous studies have

shown that Transcription Factors (TFs) play an important role in regulating the responses against various stresses in plants [10]. A large number of TF families' *viz*., AP2/ERF, NAC, bZIP, ABRF, MYB, WRKY, homeodomain, and bHLH have been reported to play crucial roles in abiotic stress tolerance in plants [11–13].

Among various stress-responsive TFs, the APETALA2/ETHYLENE RESPONSIVE FACTOR (AP2/ERF) family has drawn the attention of several researchers due to its key role in modulating gene expression in response to multiple stresses and hormones [14–16]. Dehydration Responsive Element Binding (DREB) TFs are one of the key members of ERF family of transcription factors involved in the ABA-independent signal transduction pathway and are responsible for controlling osmoprotection and metabolism [17] and modulating gene expression against cold, drought, salinity, etc. [18]. The dehydration responsive element (DRE) with a 9 bp conserved core sequence (5′-TACCGACAT-3′) was first identified in the promoter of the drought-responsive gene *rd29A* [19] to which *DREB* transcription factor binds and regulates the expression of many stress-responsive genes. DREB1 /CBFs were reported to be induced by cold stress, and ectopic expression of CBF1, CBF3, and CBF4 significantly improved plant's tolerance to low-temperature and activates the expression of COR genes [20]. DREB2A and DREB2B are induced by drought and salinity but not by cold [21–23]. Over-expression of *DREB1A* conferred increased tolerance against drought, salinity, and cold stresses in Arabidopsis [21,24]. *DREB1A* was reported to activate several stress tolerant genes *viz*., *rd29A*, *kin1*, *Cor 6.6*, *Cor 15a*, *rd17*, *erd10*, *erd1,* and *P5CS*, as well as the expression of their direct downstream genes with DRE-cis element, such as *RD29A*, *COR15A*, *ERD10*, *COR47* and *GoLS2* [25,26].

Over-expression of DREB1A driven by a constitutive CaMV35S promoter resulted in growth abnormalities [27]. Such adverse effects were mitigated through the engineered expression of DREB1A driven by a stress-inducible promoter *rd29A* [28–30]. In this study, *AtDREB1A* cloned under the control of stress-inducible promoter *rd29A* was used for engineering drought tolerance in a popular rice variety ADT43. Transgenic rice lines (T_1) were subjected to thorough molecular characterization and superior lines were evaluated for measuring their responses against drought and salinity.

2. Results

2.1. Development of Transgenic ADT 43 Lines and Molecular Analysis

Embryogenic calli from immature embryos of ADT 43 were co-cultivated with *A. tumefacians*-harboring pCAMBIA 1300 engineered with *AtDREB1A* (Figure 1a,b). A total of 47 putative transgenic plants were regenerated and used for further characterization. Putative transgenic plants (T_0 generation) were confirmed by PCR analysis using primers specific to hygromycin (*hpt*) and the transgene *AtDREB1A*. Out of 47 progenies, 45 showed the presence of expected amplicon of 548 bp (*AtDREB1A*) and 630 bp (*hpt* gene) (Figure 1c). Southern hybridization analysis using radio-labeled (P^{32}) *hpt* gene (selectable marker) identified both single copy and multiple copy insertions (Figure 1d). Transgenic lines (T_0) *viz*., A16, A17, A19, B5, B9, and B16 and were found to have single-copy integration of the transgene construct while few other lines *viz*., C3 and B13 were found to have multiple copy insertions.

2.2. Evaluation of Transgenic ADT 43 (T_1 Generation) against Drought and Salinity

2.2.1. Drought Responses

Transgenic ADT 43 Lines Exhibited Enhanced Tolerance against Drought

Non-transgenic ADT43 plants showed leaf rolling and wilting symptoms on 18 DAIS (days after imposing stress) at which the transgenic lines (B9, A16, and C3) did not show any rolling or wilting symptoms. Leaves of transgenic lines were found to be turgid and green even on 18 DAIS at which non-transgenic plants developed severe leaf drying symptoms (Figure 2). Transgenic lines # B9 and # C3 did not show any delay in their flowering behavior (Figure 2). Upon re-watering, all the transgenic plants exhibited better recovery, whereas the non-transgenic plants of ADT 43 failed to recover (Figure 2).

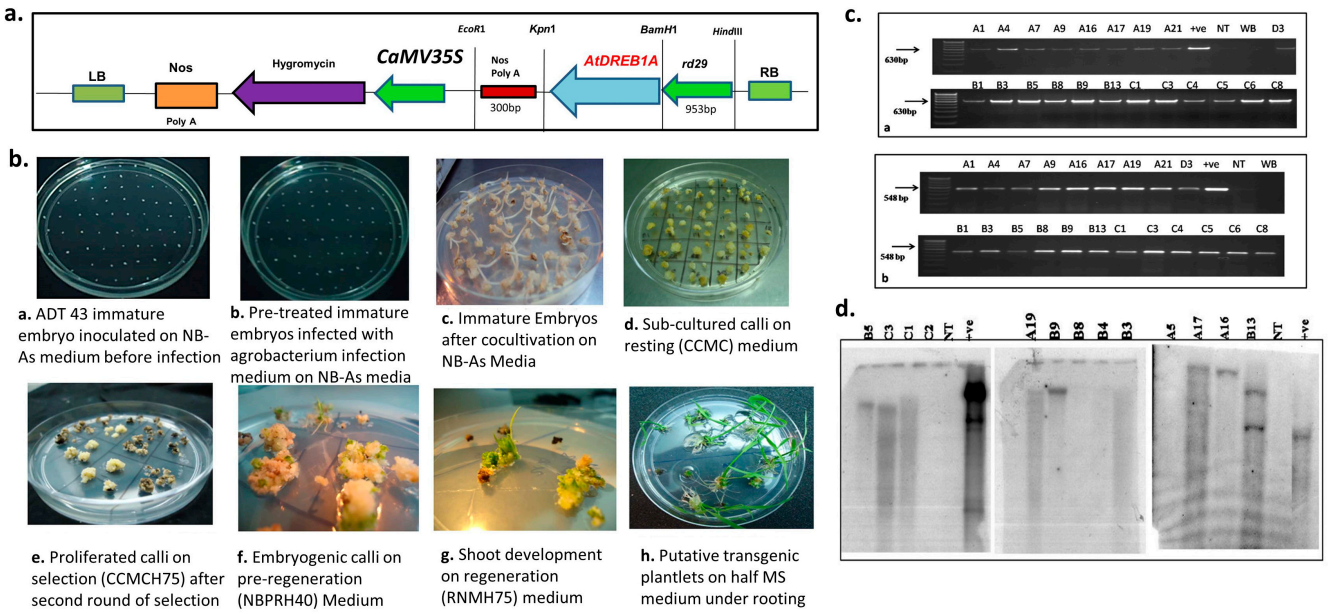

Figure 1. Development of transgenic rice (ADT 43) lines and molecular analysis (**a**) map of pCAMBIA 1300 harboring *AtDREB1A* under the control of stress-inducible promoter *rd29A* (**b**) Agrobacterium-mediated transformation of rice (ADT 43) (**c**) PCR analysis of transgenic and non-transgenic (NT) ADT 43 lines showing the amplification of hygromycin (*hpt)* gene (630 bp) and *AtDREB1A* gene (548 bp), WB—Water Blank (**d**) Southern hybridization analysis of transgenic and non-transgenic ADT 43 lines; 25 µg genomic DNA digested with *BamHI* and transferred to nylon membrane and hybridized with radiolabeled hygromycin probe. Blots show different transgenic, non-transgenic (NT), and positive plasmid-harboring *AtDREB1A* (+ve).

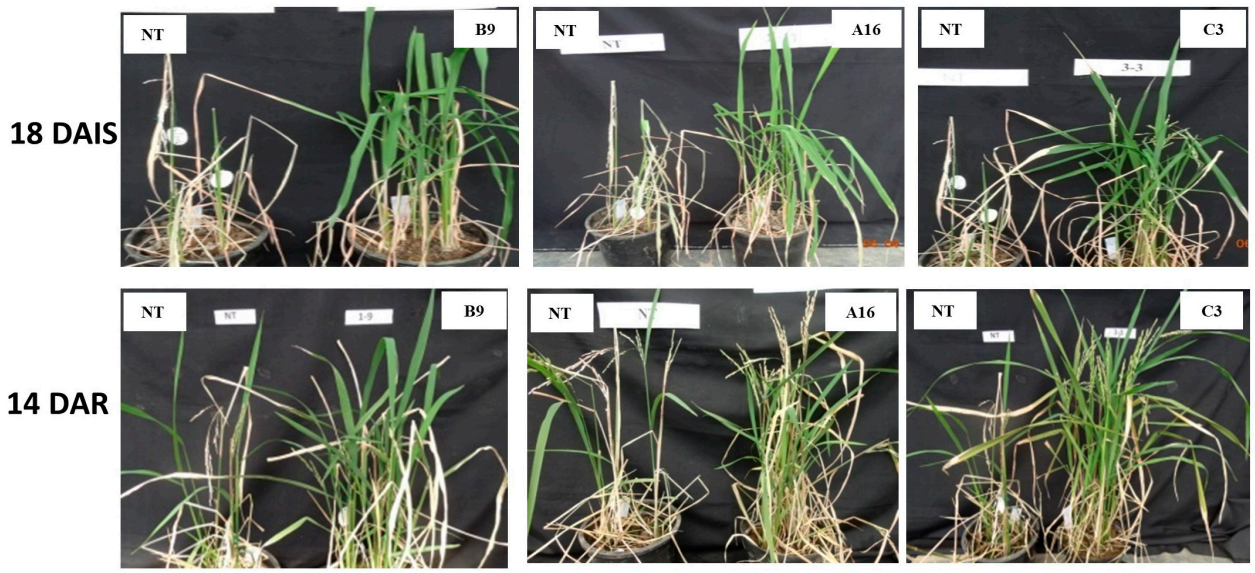

Figure 2. Responses of transgenic and non-transgenic ADT43 (T_1) lines to drought (18 DAIS) and re-watering (14 days after re-watering).

Transgenic Lines Maintained Higher RWC and Lower Leaf Temperature

Relative water content in the leaves of both transgenic and non-transgenic ADT43 was found to decrease during the progression of drought. On 13 DAIS, leaves of non-transgenic ADT 43 were found to retain 77.7% of RWC, whereas it ranged between 79.6–90% among the transgenic lines (Figure 3a). On 18 DAIS, RWC in the leaves of non-transgenic ADT

43 reached 65.29%, whereas it ranged between 75–84% among the transgenic lines. The transgenic lines *viz.*, B9 and A16 were found to contain >80% RWC (Figure 3a).

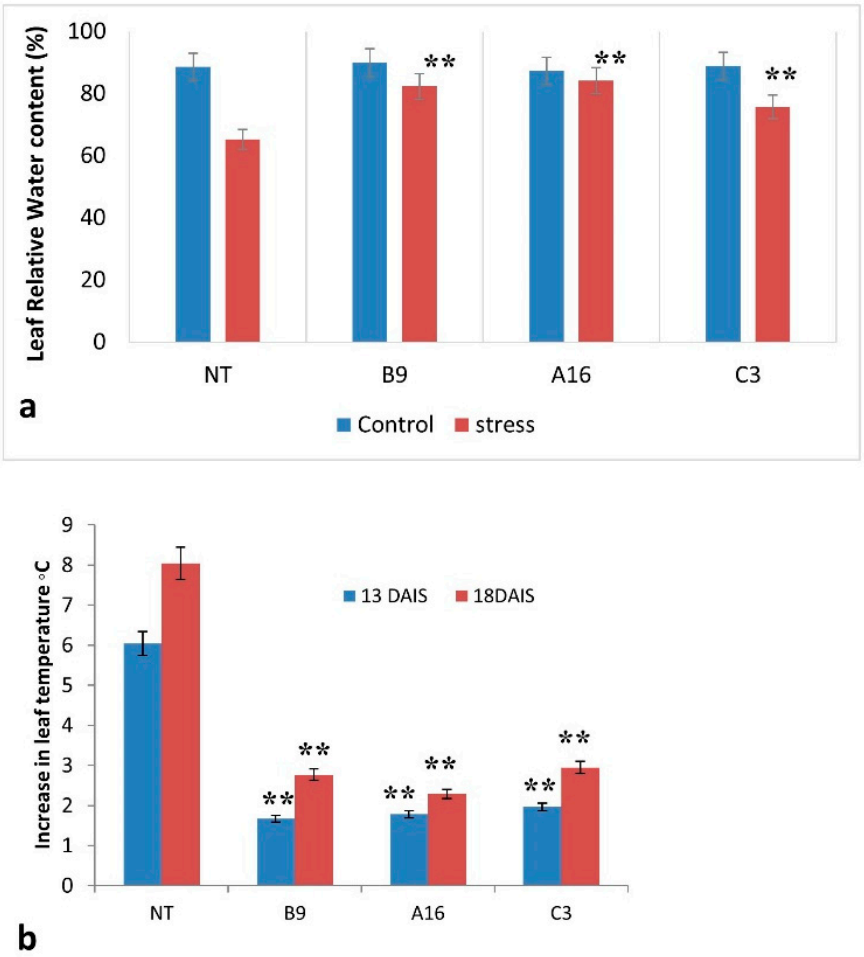

Figure 3. Relative water content (RWC) and temperature (increase over control) in the leaves of transgenic and non- transgenic ADT 43; (**a**) Relative water content measured on 18 DAIS; (**b**) increase in the leaf temperature of stressed plants over controls on 13 DAIS and 18 DAIS; NT, Non-transgenic ADT 43. Values are mean of three replications and ** indicates statistical significance at $p < 0.01$ (ANOVA compared to non-transgenic data).

Internal tissue temperature of the leaves was measured in the control and drought subjected plants of both transgenic and non-transgenic ADT 43 (once on 13 DAIS and secondly on 18 DAIS). Leaf temperature was found to increase during the progression of drought, and the increase was more in the non-transgenic plants (Figure 3b). Drought-stressed (13 DAIS) plants of non-transgenic ADT 43 recorded an increase of 6 °C when compared to its well-watered control, whereas the transgenic plants recorded an increase of up to 2 °C (Figure 3b). On 18 DAIS, the NT plants with clear leaf drying symptoms showed an increased leaf temperature of 8 °C than their control plants. The transgenic lines (B9, A16 and C3) showed only 2–4 °C increase in their leaf temperature against their respective controls (Figure 3b).

Transgenic Plants Exhibited Better Stomatal Control to Maintain Cellular Activities

Regulation of stomatal opening/closure is one of the vital mechanism(s) in maintaining CO_2 assimilation and canopy cooling. Intermittent drought stress caused the complete closure of stomata in the leaves of non-transgenic ADT 43, whereas stomata remained partially opened in the leaves of transgenic ADT 43 lines. Scanning Electron Microscopic

analysis revealed that transgenic plants were found to contain intact papillae and leaf hairs during drought, whereas they were found to be deformed during the drought in the non-transgenic ADT 43 (Figure 4a,b).

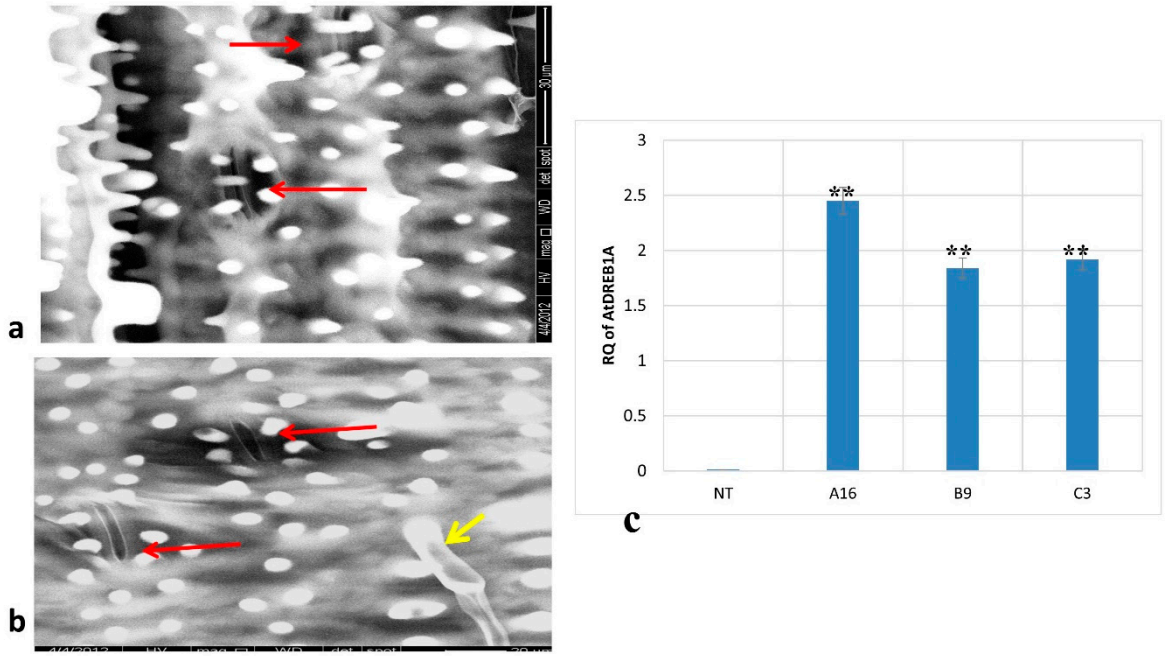

Figure 4. Scanning electron microscopic view of leaves from non- transgenic and transgenic ADT 43 (Event # A16) at 18 DAIS; (**a**) Non-transgenic leaf showing fully closed stomata on 18 DAIS (30 µm); (**b**) Transgenic (# A16) leaf showing partially opened stomata at the same time. Red arrow indicates the stomata, and yellow arrow indicates the leaf hairs; (**c**) qRT-PCR analysis of *AtDREB1A* transcripts in non-transgenic (NT) and transgenic ADT43 lines. Values are mean of three replications, and ** indicates statistical significance at $p < 0.01$ (ANOVA compared to non-transgenic).

Transgenic Plants Exhibited a Strong Induction of AtDREB1A during Drought Stress

Total RNA isolated from control and drought-stressed (18 DAIS) leaf samples of transgenic lines A16, B9 and C3 were subjected to qRT-PCR analysis of the transgene *AtDREB1A* along with non-transgenic ADT 43. The qRT-PCR analysis revealed the strong induction of *AtDREB1A* transcripts only in the transgenic lines, whereas there was no expression in non-transgenic ADT 43 and control plants (Figure 4c).

2.2.2. Salinity Responses

Transgenic Plants Exhibited Higher Germination Percentage under Salinity Stress

Transgenic lines were evaluated for their ability to germinate under salinity by germinating them under different concentrations of NaCl along with their non-transgenic counterpart. The germination percentage was calculated based on the number of seeds germinated on 8 DAS (days after sowing), which reduced with increasing NaCl stress in both non-transgenic and transgenic ADT 43 (Figure 5a). At 50 mM and 100 mM NaCl stress, the transgenic lines were found to exhibit significantly greater percentage of germination than the non-transgenic ADT 43 (Figure 5b). Transgenic lines showed 70–80% germination at 50 mM and 60–70% germination in 100 mM NaCl (Figure 5b). At 200 mM NaCl stress, only the two transgenic lines B9 and A16 showed 10% germination. Shoot length and root length were recorded on 8 DAS. All the lines exhibited significant growth retardation under NaCl stress, but the effect was more pronounced in the non-transgenic ADT 43. At 50 mM NaCl stress, the mean shoot length of non-transgenic ADT 43 was 2.45 cm, whereas the transgenic lines recorded 2.87–4.2 cm (Figure 6a). Non-transgenic ADT 43 plants did not develop any roots under salinity (50 mM and 100 mM), whereas the transgenic lines

recorded 0.23–1.36 cm of root length at 50 mM and 0.17–0.4 cm at 100 mM NaCl (Figure 6b). At 50 mM and 100 mM NaCl stress, all the transgenic lines were found to have a greater vigor index than the non-transgenic ADT 43 (Figure 6c).

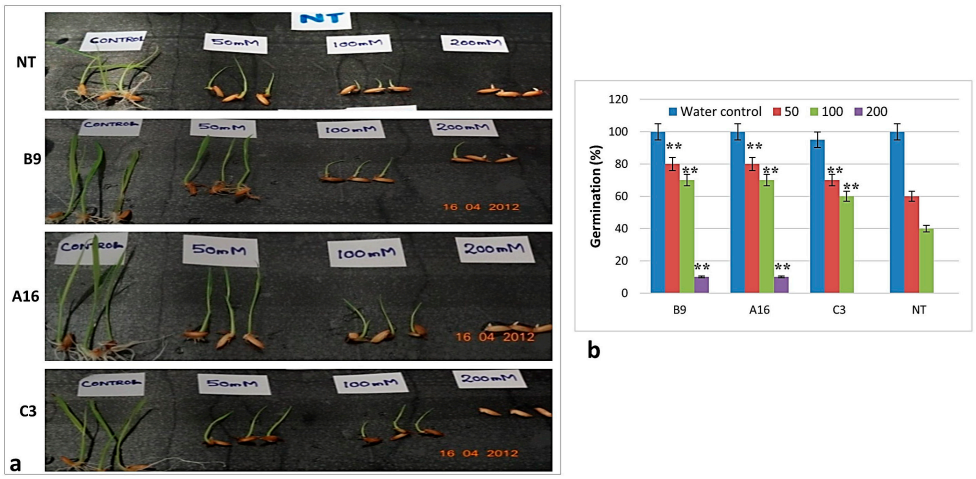

Figure 5. Evaluation of transgenic and non-transgenic ADT 43 lines (T_1) for their ability to germinate under 50, 100, and 200 mM NaCl stress; (**a**) Phenotype of transgenic and non-transgenic ADT 43 seedlings germinated at different concentration of NaCl; (**b**) Germination percentage of transgenic and non-transgenic ADT 43 (NT). Values are mean of fifteen replications and ** indicates statistical significance at $p < 0.01$ (ANOVA compared to non-transgenic ADT 43).

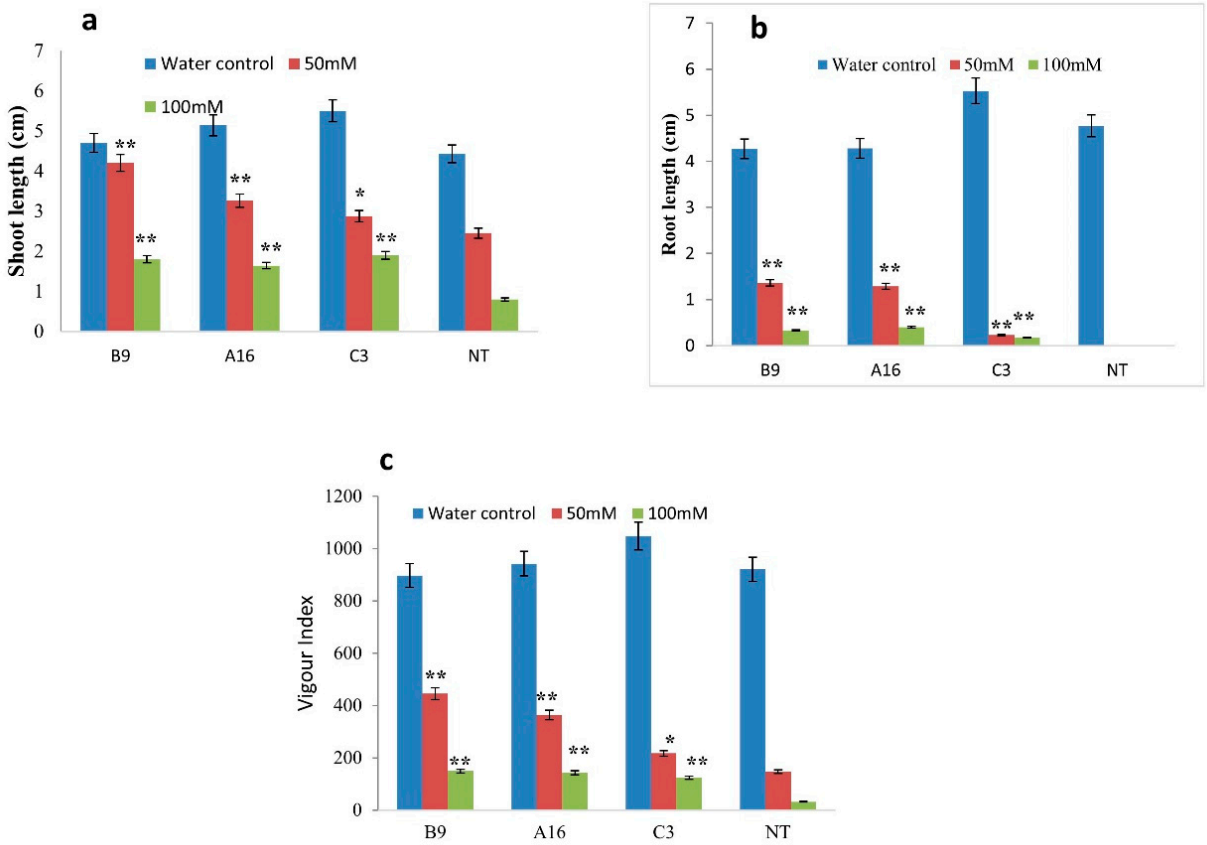

Figure 6. Shoot length; (**a**) root length; (**b**) and Vigor Index; (**c**) measured in non-transgenic (NT) and transgenic ADT 43 (T_1) seedlings germinated at different levels of NaCl concentration. Each value is a mean of fifteen replications. ** indicates statistical significance at $p < 0.01$ and * indicates significance at $p < 0.05$ (ANOVA compared to non-transgenic salinity stressed).

Leaf Discs of Transgenic Plants Exhibited Slower Degradation of Chlorophyll

Leaf discs collected from the non-transgenic and transgenic plants were floated on 50 mM, 100 mM, and 200 mM NaCl solution. Water was used as a control. Periodical observations were made to measure the development of yellowing symptoms on the discs. Leaf discs of non-transgenic ADT43 started showing visible yellowing symptoms at about 72 h after putting into the NaCl solution at which all the leaf discs of transgenic ADT43 remained green. Leaf discs of transgenic ADT43 started showing yellowing at 96 h after salinization (100 mM and 200 mM NaCl) at which all the non-transgenic ADT43 discs turned completely yellow (Figure 7).

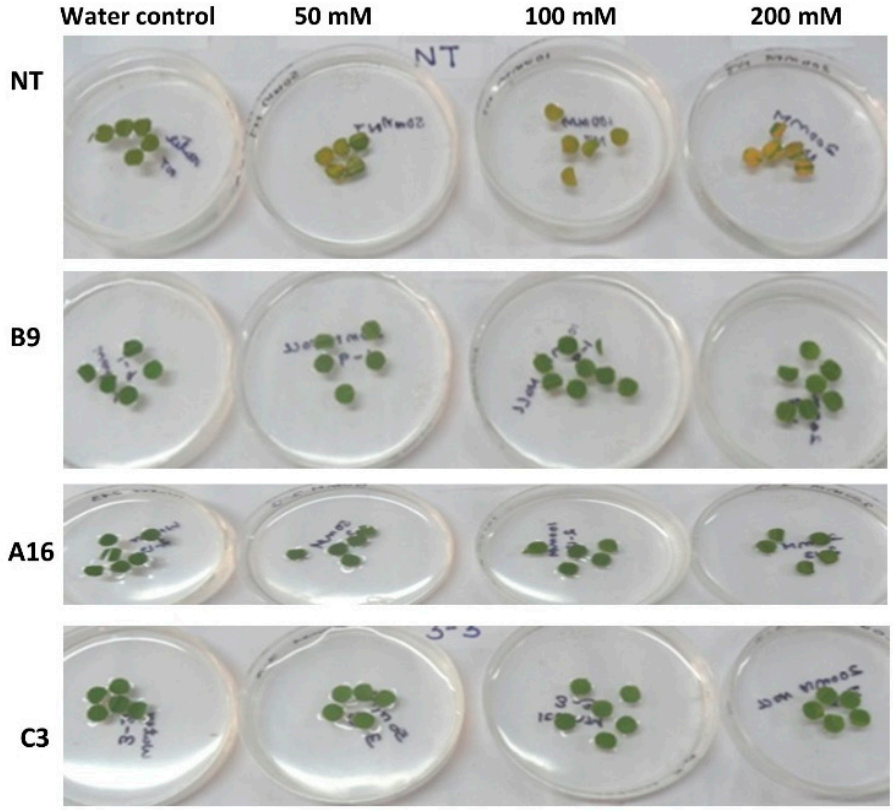

Figure 7. Chlorophyll stability assay in the leaf discs of non-transgenic (NT) and transgenic ADT 43 (T_1) lines at various levels of NaCl stress.

2.3. Evaluation of T_2 and T_3 Progenies of Line # A16 against Drought

The transgenic line #A16 possessing single copy integration and exhibiting a superior level of drought tolerance in T_1 generation was forwarded to T_2 and T_3 generation and evaluated for its responses against drought. On 12 DAIS, leaves of the transgenic line remained intact and green without any symptoms of leaf rolling and leaf drying (Figure 8a). Leaves of the transgenic line # A16 maintained 72.69% RWC, whereas the non-transgenic ADT 43 retained only 56.28% of RWC (Figure 8c). The leaf temperature of the transgenic line was 24.82 °C, whereas the non-transgenic plants registered 28.51 °C. Non-transgenic plants showed an increase of 4–5 °C during drought over its well-watered control, whereas the transgenic A16 showed an increase of only 1.63 °C over its control (Figure 8d). A similar drought response was exhibited by the transgenic line # A16 in the T_3 generation (Figure 8b,d).

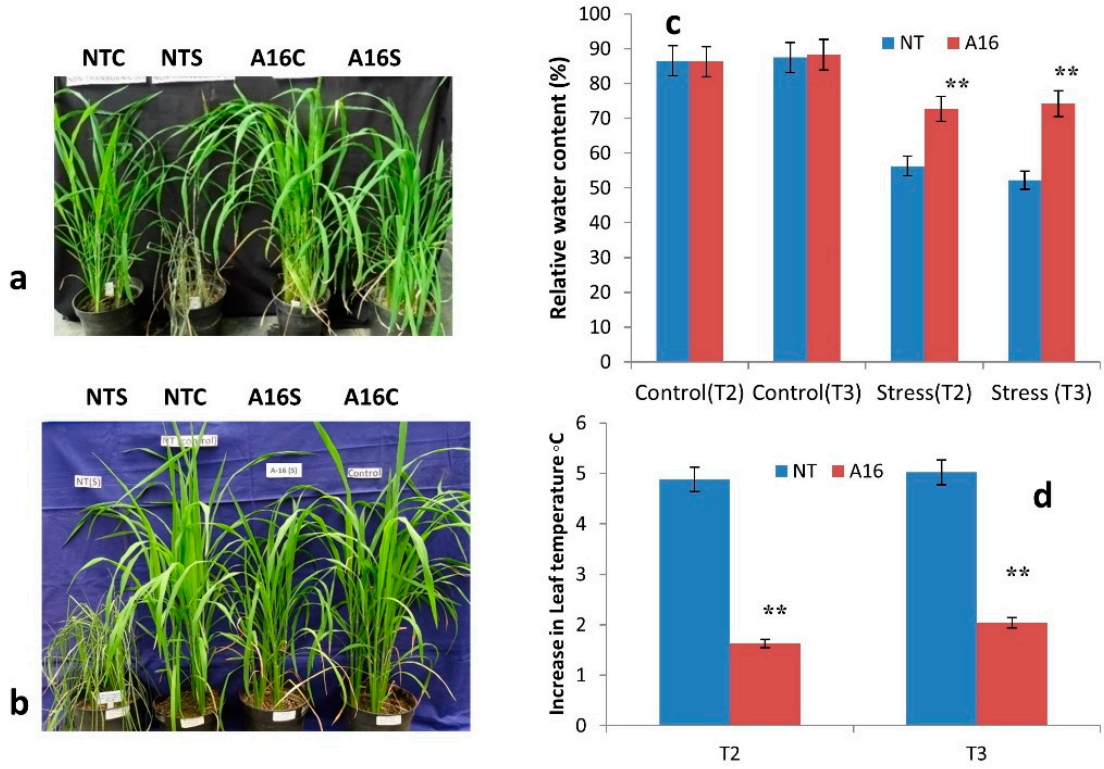

Figure 8. Responses of ADT 43 non-transgenic (NT) and transgenic (# A16) line (T_2 and T_3 progenies) to drought stress; (**a**) Performance of # A16 (T_2) at 12 DAIS; (**b**) Performance of # A16 (T_3) at 14 DAIS; NTC, Non-transgenic control; NTS, Non-transgenic stress; A16C, Control plants of # A16; A16S, Stressed plants of # A16; (**c**), Relative water content in the leaves of Non-transgenic and transgenic ADT 43; (**d**) increase in the leaf temperature of stressed plants over the respective controls; The values in the graph are the mean of three replications. ** indicates statistical significance at $p < 0.01$ (ANOVA compared to non-transgenic drought-stressed).

2.4. Enhanced Salinity Tolerance of A16 (T_2 and T_3) Progenies during Vegetative Stage Stress

In 100 mM NaCl stress, the seedlings of non-transgenic ADT43 plants showed wilting of terminal leaves at 15 days after stress compared to the A16 progenies (T_2), which were found to be healthy. After 45 days of salinity stress non-transgenic plants exhibited growth retardation and wilting of terminal leaves compared to the healthy A16 transgenic plants (Figure 9a). All the transgenic lines retained greenness in leaves and had higher root length than non-transgenic plants (Figure 9a). The non-transgenic ADT43 plants showed leaf chlorosis and leaf rolling symptoms after 30 days of salinity exposure at 100 mM NaCl stress, while the superior transgenic line A16 progenies (T_3) did not show any symptoms of salinity stress at this stage (Figure 9b).

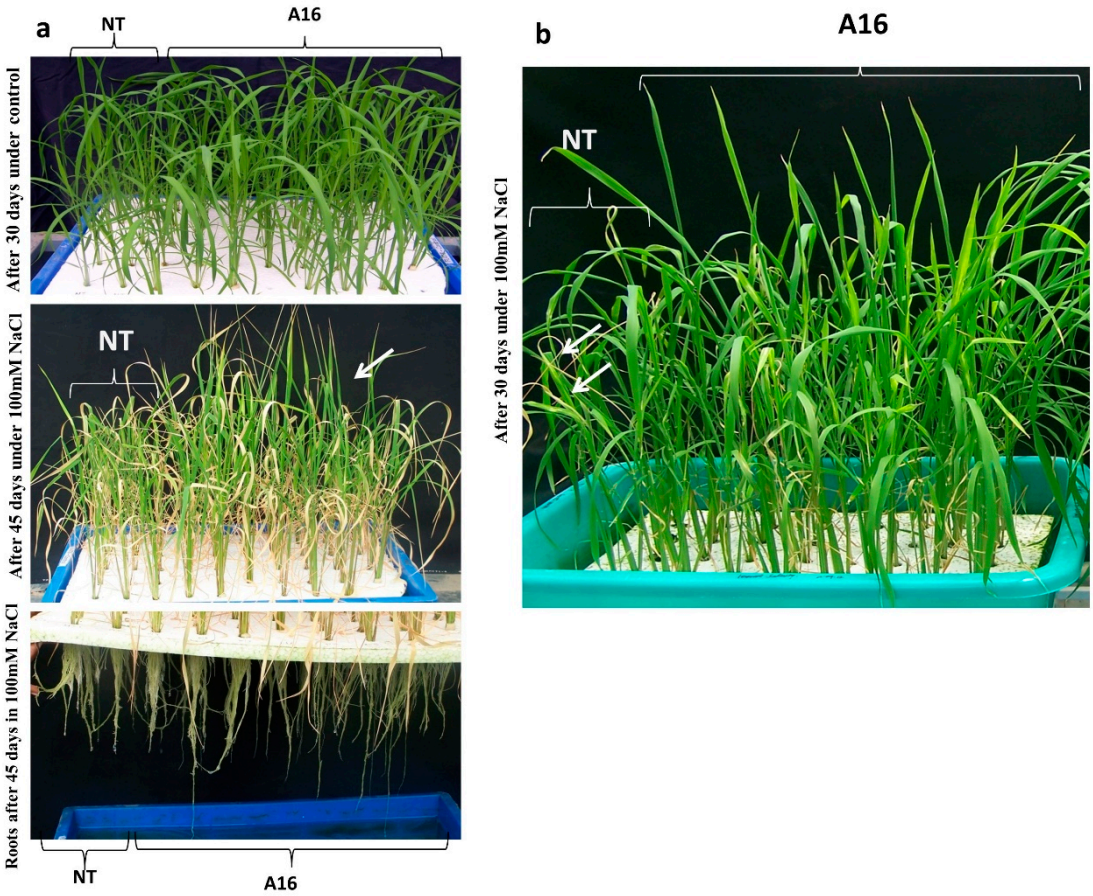

Figure 9. Responses of non-transgenic and transgenic ADT 43 (# A16) seedlings to 100 mM NaCl stress; (**a**) Morphology of leaves and roots of transgenic (T_2) and non-transgenic ADT 43. White arrow indicates the erect leaves of A16 transgenic line; (**b**) Responses of transgenic (T_3 progenies of # A16) at 30 DAIS. The white arrow indicates the leaf rolling and chlorosis symptoms in non-transgenic plants.

2.5. Agronomic Evaluation of Transgenic ADT 43 under Drought

The transgenic line A16 (T_4) was evaluated for its agronomic performance under well-watered and drought stress conditions in a transgenic screen house facility simulating field conditions (Figure 10a). Transgenic line # A16 recorded a lesser reduction in the growth and yield traits during drought when compared to the non-transgenic ADT43. Under well-watered conditions, transgenic line # A16 exhibited on par performance against its non-transgenic counterpart in its agronomic traits viz., plant height (cm), number of tillers, panicle length (cm), number of grains per panicle, grain yield per plant (g), spikelet fertility (%) and biomass per plant (g) (Table 1). Non-transgenic ADT 43 plants showed 22% reduction in the total number of spikelets per panicle during drought, whereas the transgenic plants showed only 2% reduction (Figure 10b). Non-transgenic plants showed 28% increase in their spikelet sterility, whereas A16 showed only 16% increase in its spikelet sterility (Table 1, Figure 10b). Transgenic line # A16 did not show any significant yield reduction over its non-transgenic counterpart ADT 43 under normal conditions. Drought-induced yield reduction was significantly higher in the non-transgenic ADT 43 (74%) than the transgenic line # A16 (57%). (Table 1, Figure 10b).

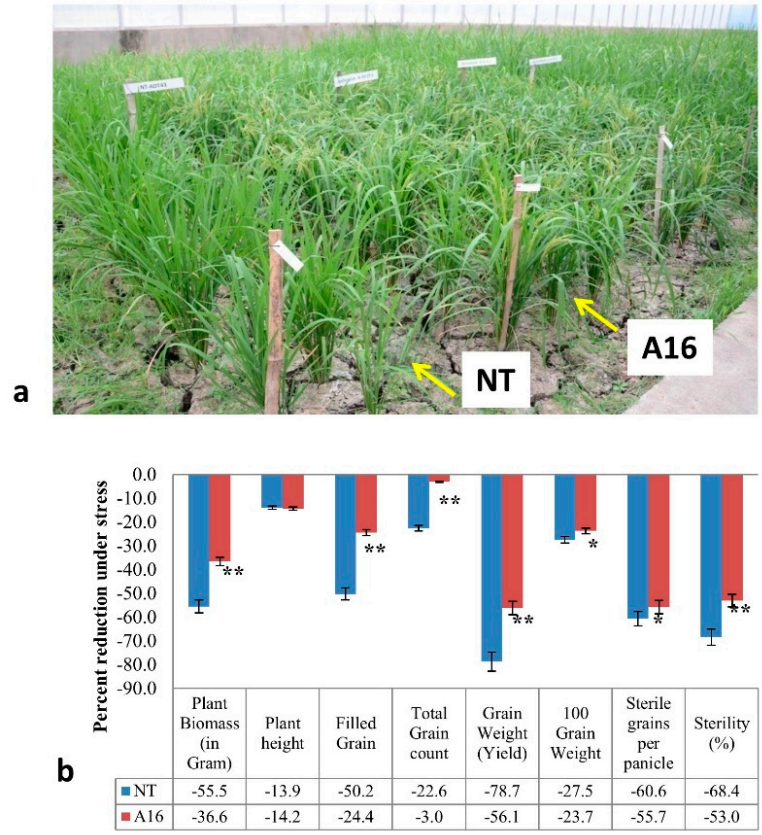

Figure 10. Performance of # A16 under drought in the transgenic screen house facility. (**a**) Overall view of the performance of # A16 over the non-transgenic ADT 43; (**b**) Percent reduction of various yield traits under drought stress. Values are mean of fifteen replications, ** indicates statistical significance at $p < 0.01$ and * indicates significance at $p < 0.05$ (ANOVA compared to non-transgenic drought-stressed lines).

Table 1. Agronomic performance of non-transgenic and transgenic ADT 43 (T_4 progenies of line # A16) under normal and drought conditions. Values are mean ± standard error of fifteen replications. Means with different alphabet letters are statistically significant using Fisher's Least Significant Difference.

Traits	NT		A16		*p*-Value	LSD at 5%
	Control	Stress	Control	Stress		
Plant height (cm)	96.1 ± 2.0 ^{A}	82.7 ± 2.13 ^{B}	97.9 ± 1.40 ^{A}	83.95 ± 1.12 ^{B}	0.001	6.428
No. of tillers per plant	20.7 ± 1.10 ^{A}	20.4 ± 2.03 ^{A}	19.6 ± 1.35 ^{A}	17.2 ± 1.09 ^{C}	0.01	2.456
No. of panicles per plant	20.5 ± 1.04 ^{A}	19 ± 1.85 ^{A}	19.6 ± 1.38 ^{A}	15.5 ± 0.75 ^{B}	0.001	3.125
Plant Biomass (g)	72.89 ± 7.5 ^{A}	32.45 ± 3.1 ^{C}	70.16 ± 2.9 $^{A\,B}$	44.51 ± 2.66 ^{BC}	<0.0001	20.998
Panicle Length (cm)	20.87 ± 0.40 ^{B}	16.97 ± 0.35 ^{C}	21.99 ± 0.22 ^{A}	22.155 ± 0.34 ^{A}	<0.0001	1.575
Number of spikelets per panicle	199.5 ± 10.8 ^{A}	154.5 ± 11.30 ^{B}	201.9 ± 16.90 ^{A}	195.78 ± 7.0 ^{A}	0.0032	46.313
No. of filled spikelets	176.5 ± 11.3 ^{A}	87.89 ± 8.1 ^{B}	177.66 ± 15.60 ^{A}	134.35 ± 6.70 ^{A}	<0.0001	39.731
No. of sterile spikelets	25.28 ± 1.2 ^{B}	64.10 ± 2.10 ^{A}	24.55 ± 1.30 ^{B}	59.50 ± 3.50 ^{A}	0.0001	22.204
Spikelet sterility (%)	13.02 ± 1.10 ^{C}	41.25 ± 4.1 ^{A}	14.50 ± 1.20 ^{C}	30.88 ± 2.4 ^{B}	<0.0001	12.383
Grain yield per plant (g)	44.24 ± 4.5 ^{A}	9.42 ± 3.1 ^{B}	43.73 ± 1.7 ^{A}	19.18 ± 1.2 ^{B}	<0.0001	12.583
100 Grain Weight (g)	1.6 ± 0.03 ^{B}	1.16 ± 0.02 ^{D}	1.78 ± 0.02 ^{A}	1.36 ± 0.02 ^{C}	<0.0001	0.1428

3. Discussion

A sustained increase in rice production is facing serious threats from the increased occurrence of abiotic stresses like drought, salinity, flooding, and temperature extremes. Out of several abiotic stresses, drought and salinity remain at the top in affecting rice productivity. This is expected to be aggravated by the predicted effects of climate change. Several management practices viz., alternate wetting and drying (AWD) method of irri-

gation and direct seeded rice (DSR) cultivation have shown better water use efficiency in rice cultivation [31]. However, non-availability of high yielding cultivars adapted to water-saving rice cultivation methods like AWD and DSR limits their widespread adoption. Developing drought and salinity tolerant rice cultivars seems to be a viable approach in achieving sustained increase in rice production under marginal environments and thereby to meet global demand. Conventional breeding has met with limited success in developing drought/salinity tolerant rice varieties as tolerance is controlled by complex mechanisms and lack of reliable phentoyping procedures. Marker-assisted breeding and genetic engineering enabled us to make substantial progress in developing drought/salinity tolerant rice varieties [32,33]. Under genetic engineering, one of the promising strategies is to modulate the expression levels of stress tolerance related TFs that might regulate a wide array of downstream genes/pathways and thus bringing the desired levels of tolerance to plants [21,34]. TFs play vital roles in modulating expression levels of genes involved in many biological processes such as development, growth, cell division, and responses to environmental stimulus [35].

Among the various transcription factors, MYB, NAC, bZIP, WRKY, and AP2/EREBP members play a major role in the ABA-dependent and ABA independent signaling pathways controlling drought responses in plants [36]. The DREBs (dehydration responsive element binding) are members of the ERF family of transcription factors and regulate stress (cold, drought, temperature stress) responsive gene expression through the ABA-independent signal transduction pathway [29]. In Arabidopsis, DREBs/CBFs specifically interact with the dehydration responsive element/C repeat (DRE/CRT) *cis*-active elements, controlling the transcription of several stress-responsive genes [37]. The over-expression of DREB1A was found to enhance tolerance against drought in *A. thaliana* [29], tobacco [38], wheat [39], and potato [40]. Even though DREB1A was found to enhance tolerance against dehydration, its constitutive over-expression was associated with growth abnormalities and thereby leading to reduced yield. This was overcome by over-expressing the DREB1A under the control of a stress-inducible promoter rd29A, which reduced its undesirable effects on growth and development [29,30,41,42].

With the confidence gained from the above reports, attempts were made in this study to engineer drought tolerance in a popular rice variety ADT 43 through controlled over-expression of *AtDREB1A*driven by a stress-inducible promoter *rd29A.* Transgenic ADT 43 lines exhibiting engineered expression of *AtDREB1A* driven by *rd29A* promoter did not show any growth abnormalities when compared to non-transgenic ADT 43 (Table 1). The stress-inducible promoter *rd29A* has been reported to minimize the adverse effects of growth retardation in *Arabidopsis* [30,43]. This study also recorded and reconfirmed the earlier observations.

Putative transgenic lines of ADT43 exhibiting controlled over-expression of AtDREB1A showed enhanced tolerance against drought. Transgenic lines of ADT 43 showed delayed rolling and wilting symptoms as against their wild type, which is in accordance with the earlier reports [44]. This may be due to the fact that the transgenic lines maintained higher internal water status at relatively equal level of moisture stress. Reduction in the relative water content (RWC) of leaves and stomatal closure are the two indicators of stress progression [45]. RWC is an index to measure the metabolic activity of tissues during dehydration and the level of tolerance against dehydration is positively correlated with RWC. In the present study, transgenic lines of ADT 43 engineered with AtDREB1A maintained relatively higher RWC as compared to its non-transgenic counterpart reflecting its greater degree of dehydration tolerance. Similar observations were recorded when AtDREB1A was over-expressed in groundnut and rice [41,42,46]. Loss of internal water and stomatal closure is associated with an increase in leaf temperature affecting cellular activities. Non-transgenic plants recorded an increase of about 8 °C at 18 DAIS as compared to their well-watered controls, whereas transgenic lines showed an increase of only 2–3 °C against their controls (Figure 3b). This increase in leaf temperature may be associated with stomatal closure and reduced transpiration (Figure 3a,b). Transgenic lines of ADT 43 were able to

maintain their stomata under partially closed conditions and thus maintain minimum level of transpiration to cool the canopy and thereby reduce the canopy/leaf temperature. Such an increase in leaf temperature during dehydration due to increased respiration and decreased transpiration has been reported earlier [43]. DREB1A was reported to alter the metabolism of osmoprotectants like proline and sugar accumulation during dehydration, thereby leading to enhanced tolerance [47]. Enhanced dehydration tolerance of transgenic ADT 43 through maintenance of higher RWC% and partially opened stomata may be due to the better turgor maintenance involving accumulation osmoprotectants.

DREB1A is known to be induced by salinity, drought, and low temperature [21,24,43]. Transgenic line of ADT 43 engineered with *AtDREB1A* showed increased tolerance to salinity (NaCl) in terms of germination (Figure 5a) and retention of chlorophyll (Figure 7). Reduced rate of degradation of chlorophyll exhibited by transgenic plants over-expressing *DREB1A* through preventing the photo-oxidation and degradation of chlorophyll has already been reported [42,48,49].

Effect of any QTL/gene(s) involved in drought tolerance can be precisely estimated based on the agronomic performance and yield of NILs introgressed with target QTLs or transgenic plants over-expressing putative candidate gene(s). Out of several experiments demonstrating the influence of putative candidate genes on drought tolerance in rice [5,8,44,50–53], only very few transgenic rice exhibiting enhanced tolerance has been taken to the field evaluation. This may be due to the fact that the majority of the reports involving the development of drought-tolerant rice through transgenic method have been carried out under controlled laboratory conditions at the seedling stage or in pot culture experiments containing a limited volume of soil. Only a very few attempts have been made to demonstrate the effects of transgenes on rice yield under field drought conditions [54–58]. A major breakthrough in the development of drought-tolerant rice cultivars through conventional/molecular breeding was achieved during recent years when the yield was considered as a direct selection criteria under well-watered and drought conditions [59]. This warrants the development or adoption of a similar strategy of evaluating transgenic plants under field or near field conditions for yield under stress. In this study, transgenic lines of ADT 43 were evaluated under a controlled transgenic screen house facility simulating field conditions along with non-transgenic ADT 43 (Figure 10a). Transgenic lines subjected to drought by withholding irrigation from 65th day after sowing onwards did not show any growth abnormalities or yield penalty when compared to its wild type under well-watered conditions as demonstrated earlier [29]. Non-transgenic ADT 43 showed 74% yield reduction under drought, whereas the transgenic ADT 43 line (# A16) showed 15–20% yield advantage over ADT 43. This increased yield reduction in the non-transgenic ADT 43 may be attributed to increased spikelet sterility (28%). This may be again due to rapid loss of water in the non-transgenic ADT 43, as evident from its lower RWC% and reduction in the associated cellular metabolic activities. The strong up-regulation of *AtDREB1A* in transgenic lines during dehydration showed the effectiveness of *rd29A* promoter in controlling the expression of transgene during dehydration, as reported earlier [60]. Through the overall co-ordinated regulation of stomatal control, reduced water loss, and cellular turgor maintenance, transgenic lines of ADT 43 performed better than their non-transgenic counterparts.

4. Materials and Methods

4.1. Construction of Plant Transformation Vector-Harboring AtDREB1A

A cDNA encoding for DREB1A was isolated from *Arabidopsis thaliana* based on the nucleotide sequence information available at NCBI (EF523124) and cloned in a plant transformation vector pCAMBIA1300 under the control of a stress-inducible promoter *rd29A* and a selectable marker encoding for hygromycin (*hpt*) resistance (obtained as a kind gift from Dr. M. Parani, Head, Dept. of Genetic Engineering, SRM University, Chennai). This plasmid was then introduced into *Agrobacterium tumefaciens* strain LBA4404 by triparental mating and used for genetic transformation of a popular but drought susceptible rice

variety ADT43 through *Agrobacterium*-mediated transformation [61,62]. Embryogenic calli were obtained from 14 day-old immature embryos of ADT43 and co-cultivated with the *Agrobacterium* strain *LBA4404* harboring pCAMBIA1300 engineered with *AtDREB1A*. Putatively transformed calli were selected in a medium containing 50 g/mL hygromycin. After two cycles (10 days of the first selection and 7 days of second selection) of selection, resistant calli were transferred to the regeneration medium. Regenerated plantlets (shoots) with well-established roots were hardened and maintained under transgenic greenhouse conditions.

4.2. Molecular Characterization of Putative Transgenic ADT43 Lines Engineered with AtDREB1A

PCR Analysis and Southern Hybridization Analysis

Genomic DNA was extracted from the leaves of putative transgenic plants by using modified CTAB method [63,64]. Primers specific to hygromycin phosphotransferase (*hpt*) (Forward: 5′AGAAGAAGATGTTGGCGACCT3′_ and Reverse: 5′GTCCTGCGGGTAAATAGCTG3′) and *AtDREB1A* (Forward: 5′CGAGTCTTCGGTTTCCTCA3′; Reverse: 5′ACTGTACGGACGGAAGCGGC3′) were used for screening the putative transgenic plants along with non-transgenic ADT 43 using PCR. PCR amplification using *hpt* primers was performed by following conditions *viz*., 94 °C for 5 min; 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min; finally, at 72 °C for 5 min. PCR amplification using gene-specific (*AtDREB1A*) primers was carried out at 94 °C for 5 min leading to 35 cycles of amplification (denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min and extension at 72 °C for 1 min) followed by a final extension at 72 °C for 10 min.

Copy number of the transgene (*AtDREB1A*) was determined by southern hybridization analysis using the *hpt* gene as a probe. About 25 µg of genomic DNA from each sample was digested with *BamH1*, fractionated on 0.8% agarose gel transferred to a nylon membrane (Hybond-N+; Amersham, Sigma Aldrich, Chennai, India) by alkaline transfer [65] and baked at 80 °C for 1 h. The probe was labeled by using Random Primer labeling kit (M/s. Bangalore Genei Pvt. Ltd., Bangalore, India) and the radiolabeled P^{32} probe was denatured and hybridized to the membrane at 42 °C. After 16 h of hybridization, the blots were washed with 1xX SSC, 0.5% SDS (*w/v*) at 42 °C for 15 min and exposed to X-ray film (KODAK) for 24 h and auto-radiographed.

4.3. Evaluation of Transgenic ADT43 Lines Engineered with AtDREB1A against Drought

Transgenic ADT43 lines (T_1) were evaluated for their performance against drought along with its non-transgenic counterpart ADT43. A superior single copy transgenic event (# A16) was forwarded up to T_4 generation through selfing. Line # A16 was evaluated for the inheritance of transgene and drought tolerance in T_2 and T_3 progenies. Non-transgenic and transgenic ADT43 plants were grown up to ten weeks, and one set of plants were subjected to water stress by withholding irrigation. Before withholding irrigation, all the pots were equilibrated by irrigating up to field capacity.

4.3.1. Measurement Soil and Leaf Water Status

Progression of drought stress was monitored by measuring relative water content (RWC) in the leaves of control and drought-stressed plants of non-transgenic and transgenic ADT43 [66]. Soil moisture content was measured using the gravimetric method [67]. For measuring RWC, fresh weight of three leaf segments of third leaf (10–15 cm length) collected from control and drought-stressed plants of transgenic and non-transgenic ADT43 were recorded and leaves were put into screw cap tubes containing water by keeping the lower end of leaves in water. Turgid weight of leaf segments was recorded after 5–6 h and leaves were dried at 65 °C for 48 h before recording the dry weight. RWC was calculated as follows:

$$\mathrm{RWC}(\%) = \frac{(\text{Fresh weight} - \text{Dry weight})}{(\text{Turgid weight} - \text{Dry weight}))} \times 100$$

4.3.2. Measurement of Leaf Temperature

Tissue temperature in the leaves of control and drought-stressed plants of transgenic and non-transgenic ADT 43 was measured using an infra-red gun (Model APOGE MI200). IR thermometer was held at a right angle so that the sensor viewed only the leaf surface and thus preventing the thermometer from sensing the soil surface. Three temperature readings were recorded for each plant.

4.3.3. Monitoring Drought-Responsive Stomatal Behavior in the Transgenic and Non-Transgenic ADT 43

Drought responsive stomatal behavior was studied in the drought-stressed leaves of a transgenic (Event # A16) and non-transgenic ADT 43 (T_1) using Scanning Electron Microscope (FEI Quanta 250, Icon analytical, Mumbai, India). Leaves were collected from the drought-stressed (18 days after imposing stress (DAIS) plants of transgenic line # A16 and non-transgenic ADT 43 line and used for SEM analysis. Leaf samples were mounted on round aluminum stubs with the aid of double side adhesive tape, and the adaxial surface of leaf samples was scanned and photographed.

4.4. Measuring Transcript Abundance of AtDREB1A through RT-PCR

Leaf tissues collected from the control and drought-stressed plants of transgenic lines and non-transgenic ADT 43 (T_1) was used for profiling the expression of *AtDREB1A* through qRT-PCR. Total RNA was isolated from leaf tissues using Trizol reagent (Biobasic, Alberta, Canada). An equal amount of DNAse treated total RNA (about 1 µg) was converted into sscDNA using Transcriptor High Fidelity cDNA Synthesis Kit (M/s. Roche, Germany) and used for qRT-PCR analysis using StepOne Plus qPCR (M/s. Applied Biosystems, ABI, Chennai, India) by following default cycling conditions (10 min 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 60 °C). The reaction mixture contained SYBR Green Master mix (Roche Diagnostics, Roche, Chennai, India) 300 nM of *AtDREB1A* gene-specific primers and 2 µL of diluted cDNA (1:10) in each 15 µL reaction. Blank controls containing all ingredients of the RT-PCR reaction except the cDNA template were also maintained. The abundance (relative quantity) of mRNAs, was calculated using the comparative Ct (ΔΔCt method) [68]. An adequate number of biological replications (three replications) was used and each biological replication was repeated three times. Ubiquitin was used as an endogenous reference gene for the normalization of Ct values.

4.5. Evaluation of Transgenic ADT43 Lines against Salinity

4.5.1. Germination Ability

Transgenic lines (T_1) were evaluated for their ability to germinate under salinity. Seeds of transgenic and non-transgenic ADT 43 were germinated in different concentrations of NaCl solutions (50, 100 and 200 mM NaCl). Fifteen seeds in each line were germinated in Petri dishes containing different concentrations of NaCl and kept at 24 ± 1 °C along with suitable controls (water). The number of germinated seeds was recorded at every 24 h interval for 9 days. Germination percentage was scored on 8 DAS. Observations on the number of germinated seeds, shoot length (cm) and root length (cm) were recorded and used for calculating the vigor index of seedlings grown under control and salinity conditions. Germination percentage was calculated by using the formula as described by [69].

$$\text{Germination \%} = \frac{\text{No.of germinated seeds}}{\text{No.of seeds sown}} \times 100$$

4.5.2. Chlorophyll Retention Capacity under Salinity

Leaf discs from three months old transgene positive plants were excised from the leaves of both transgenic and non-transgenic plants (T_1) of ADT 43. Excised leaf discs were floated on solutions containing various concentrations of NaCl (50 mM, 100 mM, 200 mM

NaCl) along with a control (water). Depletion of chlorophyll (yellowing of leaf discs) was assessed regularly and photographed.

4.5.3. Salinity Tolerance during the Vegetative Phase

The T_2 and T_3 progenies of superior single copy transgenic event (#A16) were evaluated for their salinity tolerance during vegetative stage along with the non-transgenic lines. Seeds of both non-transgenic and transgenic lines were germinated in petri plates (up to 7 days) and then PCR positive transgenic plants were transferred to a hydroponics system in trays filled with Yoshida solution. The plants were grown under Yoshida solution for 35 days and salinity stress was imposed by adding 100 mM NaCl to the Yoshida solution. Effect of salinity stress on both transgenic and non-transgenic plants was assessed based on the development of wilting and drying of leaves.

4.6. Agronomic Evaluation of Transgenic Lines under Transgenic Screen House Conditions

T_4 progenies were evaluated for their agronomic performance under irrigated and drought conditions against its non-transgenic counterpart ADT43. Twenty-five old seedlings of transgenic and non-transgenic ADT43 were planted (20 × 20 cm) in transgenic screen house facility. Standard practices of fertilizer application (100; 50; 50 Kg of N, P and K per ha), irrigation and weeding were followed till 40 days after transplanting. Plants were grown under well-watered conditions up to 40 days and a set of plants were subjected to drought by withholding irrigation by maintaining appropriate well-watered control plots. Stressed plots were left without irrigation till maturity and harvest (110 days after sowing). Observations on soil moisture, RWC %, plant height, number of tillers, days to flowering, panicle length, number of grains per panicle, spikelet fertility, hundred grain weight, grain yield per plant and biomass per plant were recorded in both well-watered and drought-stressed plants.

4.7. Statistical Analyses

Experimental data were statistically analyzed using analysis of variance (ANOVA) and the multiple comparisons were carried out using Fisher's Least Significant Difference (LSD) test. All the statistical analyses were carried out using Statistical Analysis System package (SAS 9.2).

5. Conclusions

Over-expression of the transcription factors regulating activation of a cascade of stress-responsive downstream genes seems to be a promising strategy in developing stress-tolerant crop varieties. In this study, transgenic lines of a popular rice variety ADT 43 engineered for controlled expression of *AtDREB1A* were developed and demonstrated for their enhanced tolerance to drought and salinity. Transgenic rice lines were found to exhibit stronger induction of *AtDREB1A* transcripts during the drought when compared to non-transgenic ADT43. The transgenic line # A16 (T_4 generation) was demonstrated for the stable inheritance of the transgene *AtDREB1A* and enhanced drought tolerance under simulated field conditions. Transgenic ADT43 engineered with *AtDREB1A* exhibiting enhanced tolerance to drought could be a valuable genetic material to grow under water-limiting and saline conditions.

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