# Overexpression of *Arabidopsis* and Rice stress genes' inducible transcription factor confers drought and salinity tolerance to rice

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## Summary

Rice yield is greatly affected by environmental stresses such as drought and salinity. In response to the challenge of producing rice plants tolerant to these stresses, we introduced cDNA encoding the transcription factors *DREB1A* and *DREB1B* under the control of the stress inducible *rd29* promoter. Two different indica rice cultivars were used, BR29, an improved commercially cultivated variety from Bangladesh and IR68899B, an IRRI bred maintainer line for hybrid rice. *Agrobacterium* mediated transformation of BR29 was done independently with *DREB1A* isolated from rice and *Arabidopsis* and *DREB1B* isolated from rice, whereas biolistic transformation was done with rice- *DREB1B* in the case of IR68899B. Initial genetic integration was confirmed by PCR and Southern blot analysis. Salinity tolerance was assayed in very young seedlings. Drought stress tests were found to be more reliable when they were carried out at the pre-flowering booting stage. RNA gel blot analysis as well as quantitative PCR analysis was performed to estimate the transcription level under stressed and unstressed conditions. Agronomic performance studies were done with stressed and unstressed plants to compare the yield losses due to dehydration and salt loading stresses. Noticeably enhanced tolerance to dehydration was observed in the plants transformed with *DREB1A* isolated from *Arabidopsis* while *DREB1B* was found to be more effective for salt tolerance.

## Introduction

Environmental stresses have great negative effects on plant growth and productivity. Unfavorable growing environments routinely cause major reduction of the yield potential of crops. Abiotic stresses such as drought, low temperature and saline soils account for more losses of agricultural production than any other factors. Hence, it is a major challenge to sustain or to improve crop yields to feed the rapidly increasing world population.

Plants respond to different stresses by a number of biochemical and physiological adaptations that involve function of many genes. However, the number of genes that regulate the process and the way they are coordinated is not yet clearly understood. Although, different plant species have different thresholds for stress tolerance, most cultivated crop plants are highly sensitive when they are exposed to long periods of stress. Osmotic stress particularly water deficit in plants occur when water loss due to transpiration exceeds the supply of water from the soil. Prolonged water shortages affect metabolic activities and eventually result in severe reductions in plant productivity. Improvement of water stress tolerance in crop plants has a significant impact on agricultural productivity.

Breeding for osmotic stress tolerance in crop plants utilizes existing genetic stocks, which are limited. However, a transgenic approach may offer an effective alternative way of improving tolerance to dehydration by incorporating genes from any source with a better understanding of the mechanism involved. Various approaches have been used to produce transgenic plants with increased tolerance to osmotic stress. These include overproduction of enzymes responsible for biosynthesis of osmolytes, late-embryogenesis-abundant proteins and detoxification enzymes, which are involved in reducing the reactive oxygen species (Bohnert and Jensen, 1996). Each of these strategies involved transfer of a gene expressing a single specific stress protective protein. However, it is well established that plant tolerance to dehydration stress is mediated by a number of physiological and biochemical process which means a multigene trait. The activation of such genes must involve a distinctive set of transcription factors. By over-expressing transcription factor gene(s), it may be possible to change or to increase the level of expression of several downstream target genes responsible for dehydration tolerance at the same time (Varshney *et al.*, 2011).

Signal transduction during plant stress responses has been studied in various plants from a variety of angles to identify stress responsive genes and their regulatory mechanisms.

A large number of genes are regulated when plants are exposed to osmotic stress. The transcription factor *DREB1A* interacts with cis-acting DRE (dehydration responsive element) and regulates expression of many stress tolerance genes under drought, high salinity, and cold stress in *Arabidopsis* (Liu *et al.*, 1998). In plant, majority of the *DREB1* subfamily members are expressed under low temperature and increased level of drought or salt (Dubouzet *et al.*, 2003; Haake *et al.*, 2002; Huang *et al.*, 2007; Xiao *et al.*, 2006). It has been reported that homologous or heterologous expression of *DREB1*s can confer tolerance to multiple abiotic stresses as shown in rice (Wang *et al.*, 2008), peanut (Bhatnagar-Mathur *et al.*, 2007) and other plants (Varshney *et al.*, 2011).

Over-expression of *DREB1A* cDNA under the control of CaMV35S constitutive promoter in transgenic *Arabidopsis* plants activated the expression of many stress tolerance genes and resulted in increased tolerance to drought, salt loading and freezing (Gilmour *et al.*, 2000; Liu *et al.*, 1998). It has been reported that the over-expression of cDNA encoding *DREB1A* induced the expression of many stress tolerance genes such as *rd29A, kin1, Cor 6.6, Cor 15a, rd17, erd10, erd1* and *P5CS* in *Arabidopsis* (Kasuga *et al.*, 1999). Similarly, over-expression of *DREB1* genes can increase the expression of their direct downstream genes with DRE-cis element, such as RD29A, COR15A, ERD10, COR47 and GoLS2 (Maruyama *et al.*, 2004).

The strong constitutive *CaMV35S* promoter driving expression of *DREB1A* resulted in growth abnormalities under normal conditions. Use of the stress-inducible promoters such as rRab16A, 4XABRE, 2XABRC are effectively expressed in rice (Ganguly *et al.*, 2011). Similarly, *rd29A* promoter to drive the expression of *DREB1A* provided a better stress tolerance of the transgenic *Arabidopsis* under stress conditions with minimal effects on plant growth than when the expression of the same gene was driven by the 35S promoter (Kasuga *et al.*, 1999). Similar results were observed in rice plants over-expressing *OsDREB1A, OsDREB1B* or *OsDREB1F*, cloned from rice (Ito *et al.*, 2006; Wang *et al.*, 2008). Rice, OsDREB1-type proteins show high homology to *Arabidopsis* DREB1A proteins (Dubouzet *et al.*, 2003).

Abiotic stresses like drought and salinity are major environmental constraints of rice production in non-irrigated rice areas. Of the world's 130 million hectares of rice cultivated land, an estimated 20% are periodically subject to drought conditions, 30% contain enough salt to limit cultivation and reduce yield, and 10% occasionally experience low temperature (15 °C or below) (Lane, 2002). Conventional breeding approach has been used to exploit natural genetic variation in improving rice varieties. But until now, rice plants except for a few rice cultivars that are well adapted to water stress conditions, do not show osmotic adjustment, the most effective component of abiotic stress. Several stress-related genes have been cloned and introduced in rice to enhance tolerance to drought and salinity stress (Datta *et al.*, 2008).

In the research reported here, we introduced the transcription factor *DREB1A* and *DREB1B* cDNA driven by *rd29A* promoter into two different indica rice cultivars. *DREB1A* cDNA has been isolated from both *Arabidopsis* and rice plants. Over-expression of *DREB1A* cDNA isolated from *Arabidopsis* performed better in rice than the same isolated from rice when the plants are exposed to artificial drought stress in pots, whereas plants with inserted *DREB1B* cDNA from rice showed better performance in highly saline conditions.

## Results

### Transformation

Plants were transformed with a G29 AHS vector expressing the *DREB1A* cDNA isolated from either rice or *Arabidopsis* or *DREB1B* cDNA isolated from rice with the *rd29* promoter (Figure 1). Twenty six antibiotic resistant (hygromycin) BR29 plants carrying rice *DREB1A* (OsDREB1A), fourteen antibiotic resistant BR29 plants carrying rice *DREB1B* (OsDREB1B) and 23 hygromycin resistant BR29 plants with *Arabidopsis DREB1A* (AtDREB1A) were generated using the vacuum infiltration method for *Agrobacterium* mediated transformation. Twenty antibiotic resistant IR68899B plants carrying rice *DREB1B* (OsDREB1B) were obtained by biolistic transformation (Table 1). From the generated plants, 24 primary independent transgenics from variety BR29 and four primary independent transgenics from IR68899B were selected by PCR and DNA-blot analysis. Growth and fertility of the plants were compared with wild-type of their respective cultivars. Most of these independent primary transgenics showed a normal phenotype and were fertile. No significant difference was found in growth and fertility status of the primary transgenic plants with their respective wild type based on selected fast growing *in vitro* regenerants.

### Progeny

The $T_0$ plants after self pollination gave segregating $T_1$ progeny. PCR analysis was done for all the $T_1$ progeny to find out the segregation ratio and to identify the positive plants containing gene of interest. Most of the progeny lines showed the Mendelian segregation ratio of 3:1 (Figure 2). Southern blot analysis was done with the identified positive plants from different lines. The Southern-positive plants of BR29, transformed by *Agrobacterium* mediated transformation, showed a very simple pattern of integration with one to four copies of the transgene. Figure 3 represents one Southern blot showing integration of *AtDREB1A* in positive progeny of different lines of BR29 selected by PCR analysis (line BRSH29, BRSH24, and BRSH22) at the $T_1$ generation. All the plants showed the integrated 0.8 Kb fragment of *DREB1* gene.

### Drought tolerance at vegetative stage

Water stress tolerance experiments were conducted on homozygous plants at the $T_3$ generation. To study drought tolerance, three sets of experiments were performed for three phases of plant growth. For the vegetative stage drought tolerance experiment, 6-week-old non-transformed wild type and transgenic plants grown in soil were subjected to 21 days of continuous drought stress i.e. watering was stopped completely. After
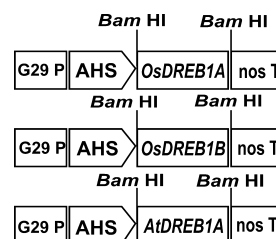


**Figure 1** Partial maps of the plasmid vectors of *DREB* genes driven by rd29 promoter used for Biolistic or *Agrobacterium*-mediated transformation in indica rice.

**Table 1** Transgenic indica rice developed with *DREB1* genes

| Cultivar | Gene of interest | Method | Promoter | No. of plants GGH | No. of Independent. Transgenic events |
|---|---|---|---|---|---|
| BR29 | OsDREB1A | *Agrobacterium* | *rd29A* | 26 | 12 |
| | OsDREB1B | *Agrobacterium* | *rd29A* | 14 | 5 |
| | AtDREB1A | *Agrobacterium* | rd29A | 23 | 5 |
| IR68899B | OsDREB1B | Biolistic | *rd29A* | 20 | 4 |

GGH, grown in greenhouse.

T$_1$ progenies of ML7 # 4
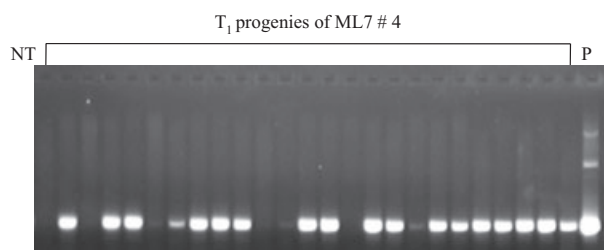


**Figure 2** PCR analysis showing the segregating *OsDREB1B* gene in T$_1$ transgenics of rice cv. IR68899B developed through biolistic transformation.

drought treatments, a variation of response was found in individual lines. Non transgenic wild type plants exhibited stress symptoms like wilting and drought induced leaf rolling within 7 days, whereas the transgenic lines did not show much difference within this short period of water stress. After drought stress and subsequent watering, recovery of the plants was observed depending on the damage caused by the stress. Sometimes new tillers came out and ultimately this stress at the vegetative stage did not cause much damage to the crop yield. Figure 4a–d represent differential phenotypic expression due to the water stress treatment and recovery of plants after watering in the case of BR29 (BRSH29 homozygous progeny). It has been observed that the lines with inserted *AtDREB1A* gene may be more tolerant to drought stress than the plants carrying *OsDREB1A* or *OsDREB1B*.

### Stomatal behavior

Experiments were carried out to understand the stomatal behavior of transgenic and wild type plants during a period of drought. Water was withdrawn from both transgenic and wild type plants for 6 days in greenhouse conditions and stomatal response was observed. The results showed that in wild type rice plants almost all the stomata were open; however in transgenic rice (BRSH29) most of the stomata were closed (Figure 5). The stomatal closure in transgenic plants during drought conditions may be due to the expression of the transgene regulating the functioning of guard cells in rice plants.

### Expression analysis of the transgenics

The expression of *rd29A DREB1* gene during drought stress at the vegetative stage was detected by RNA gel blot analysis. RNA was extracted from the leaves of transgenic plants and their respective wild type plants grown under normal condition and also after exposure to drought stress for 6 days. The induced expression of the inserted gene has been detected by the strong expression of mRNA extracted from leaves of dehydration stressed transgenic plants with At*DREB1A* gene controlled by *rd29* stress inducible promoter. Figure 6 represents mRNA expression of three different plants from different lines during each day after exposing to water stress.

Quantitative RT-PCR (qRT-PCR) analyses showed 10.8- to 14.4-fold induction of AtDREB1A mRNA levels in the drought stressed transgenic lines compared to that of well watered controlled transgenic lines (Figure 7). The fold-induction levels varied widely in the transgenics, the maximum induction being recorded in BRSH 22-22-25-7, which was 14.4-fold upon 6 days of drought treatment.

### Drought tolerance at flowering stage

To study drought tolerance at the pre-flowering stage, 10 week old plants in pots just before the emergence of flowers i.e. the booting stage, were subjected to drought stress for 7 days. After 7 days the soil became too hard. More damage was observed in the flowering of the wild type than in the different variable response in the transgenic plants at the pre-flowering stage water stress situation, which was also reflected in the crop yield. In Table 2 the effect of water stress on crop yield in some selected transgenic BR29 plants (homozygous progenies of BRSH22 and BRSH29) at the pre-flowering stage has been shown. Wild type BR29 severely affected by 7 days drought exposure at the pre-flowering stage whereas transgenics were less affected. The grain yield per plant was affected by water stress in all the plants which were compared with that of their normal watered plants of the same line but damage in yield
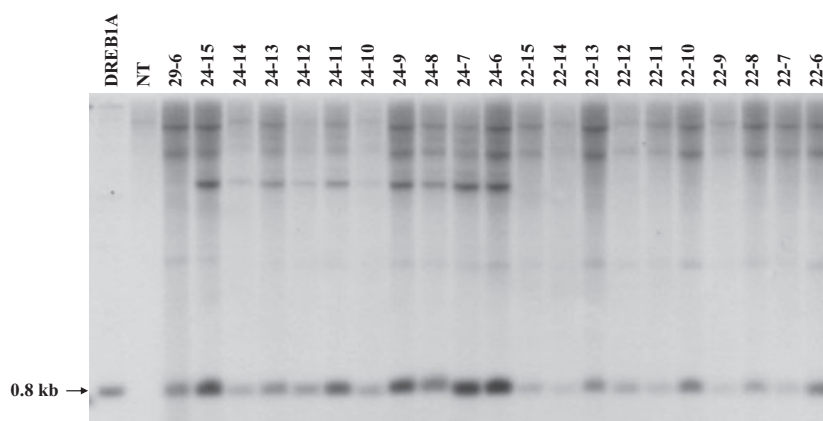


**Figure 3** Southern blot showing the stable integration of 0.8 kbp of *DREB1A* in progenies of different lines of BR29.
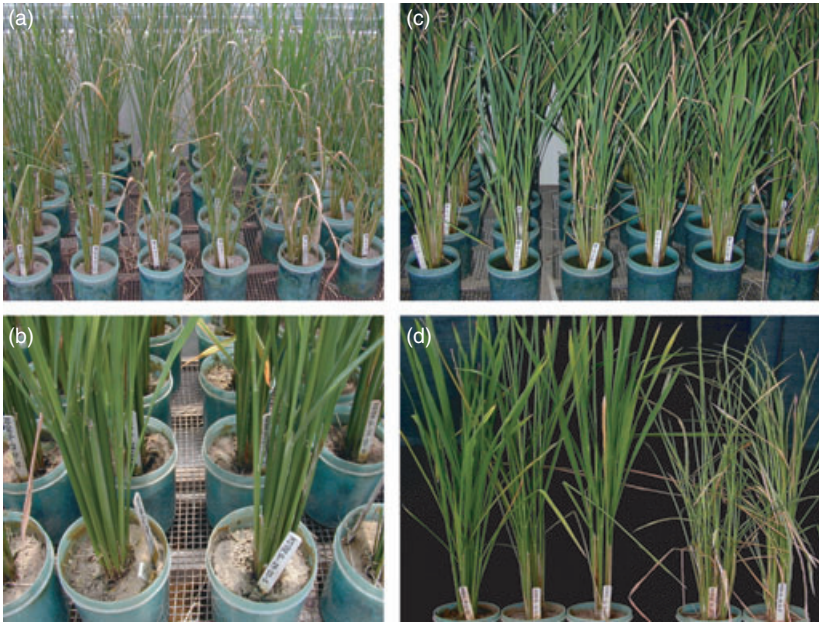
**Figure 4** Differential phenotypic expression due to water stress at vegetative stage (BRSH29 progeny). (a) Showing levels of tolerance after 14 days (b) selected transgenic plants surviving in hard soil after 21 days without water. (c) Quick recovery of water stressed transgenic plants after watering (d) recovery of water stressed plants after watering, two plants from right are non transgenic wild type.

was more in case of wild type (7.84 g/plant) as compared to that of transgenics (14.20–17.81 g/plant).

## Tolerance to salt

The technique for screening salt tolerance is based on the ability of seedlings to grow in high saline conditions. To examine the tolerance level of the transgenic plants, homozygous progeny of BRSH29 and BRSH22 containing *AtDREB1A* and progeny of IR68899B (designated as ML7) containing *OsDREB1B,* to salt stress, 8 day old seedlings were subjected to salt stress in hydroponic nutrient solution. Fourteen days of continuous salt stress with EC 12 salt level (with renewal of the nutrient solution every 7 days) the transgenic plants showed a moderately tolerant nature (Table 3). After 14-days in EC 12 the same plants were subjected to EC18 salt level for another 14 days. This stringent salt pressure killed almost all the plants excepting only a few salt exposed IR68899B (ML7) plants with *DREB1B* gene inserted (Figure 8). Five plants from the surviving line ML-4-17 were grown to soil after the experiment. Those plants showed much better seed setting than the non-transgenic wild type stressed plant but less seed setting than those plants which

were grown in normal condition without salt stress from seeding stage (Table 4).

## Discussion

Plant responses to water and salt stresses have much similarity in most metabolic processes. The high saline condition reduces the ability of plants to take up water. The water stress effect caused by drought on growth is similar to the osmotic effect of salt on the initial phase. Thus, any improvement in drought resistance may make a plant more adapted to saline soil.

Many plant genes have been reported to respond to abiotic stresses like drought, high salt condition and low temperature, and proteins encoded by these genes are expected to enhance the tolerance level of plants to these stresses. The analyses of these stress proteins and their corresponding genes provide us with the possibility of developing stress resistant plants.

In this present study, we were able to generate transgenic rice plants in which the *DREB1A* or *DREB1B* cDNAs were introduced to overexpress the DREB protein. The transgenic plants
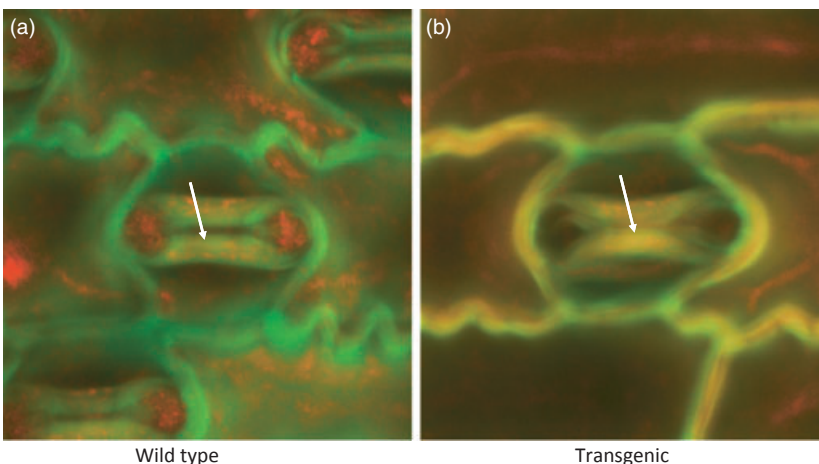


Wild type                    Transgenic

**Figure 5** Laser confocal micrographs of epidermal peels of transgenic and wild type rice plants under 6 days of drought condition stained with acridine orange. Wild type and transgenic (a) shows the stomata opening (wild type) and (b) closer of stomata (transgenic BRSH29 progeny).
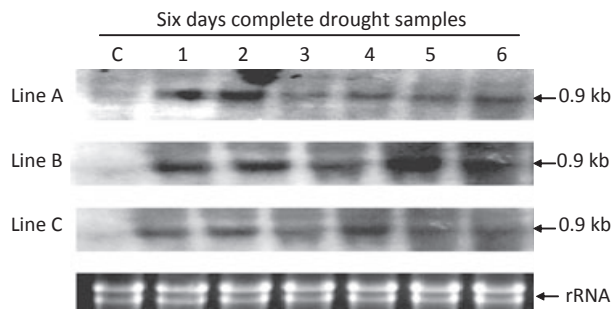
Six days complete drought samples



**Figure 6** RNA gel blot showing gene expression in different lines under drought stress condition. Line A: BRSH29-22-2-19-4; Line B: BRSH29-22-2-16-15; Line C: BRSH22-22-25-7-13; Days 1–6 represents each day after exposing to drought stress. Letter 'C' indicates well watered controlled transgenic plants.
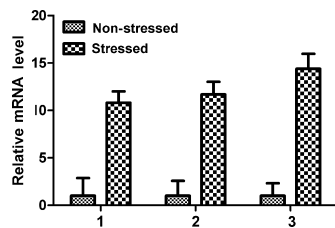


**Figure 7** qRT-PCR analyses showing the fold induction of AtDREB1A expression in response to drought stress for 6 days and well watered controlled transgenic plants of three different transgenic lines 1:BRSH29-22-2-19-4; 2:BRSH29-22-2-16-15; 3:BRSH22-22-25-7-13. The expression of gene determined using *tubulin* gene as an internal control. Results represent means ± SE, based on three replicates.

showed enhanced tolerance to drought and salinity because of overexpression of this single transcription factor governed by *rd29* stress inducible promoter. The stress response study of the

exposed plants at their different growth and developmental phases was based on the phenotypic changes due to stress and their ultimate effect on grain yield.

Homozygous lines with the *DREB1* gene have been developed and the phenotypic expression study was conducted with the over-expressed promising lines as has been selected by molecular analysis. It was observed that DREB1A transgenic rice plants remained green for longer time during drought stress condition when compared to DREB1B transgenic rice and the non-transgenic wild type. It has also been reported earlier that DREB1A *Arabidopsis* plants had significantly improved freezing and drought tolerance and downstream gene expression level in DREB1A were significantly higher than that in DREB1B *Arabidopsis* plants (Novillo *et al.*, 2007; Tong *et al.*, 2009).

Expression of *DREB1A cDNA* encoded by *rd29* inducible promoter in our study showed no significant phenotypic change in growth of transgenic rice plant. The stress inducible promoter appears to minimize the negative effects of the transgene on plant growth has been reported in *Arabidopsis* (Kasuga *et al.*, 1999).

Stomata closure to reduce water loss is an important feature to improve tolerance of plants under water limitation. Genetic determinants governing the stomatal function and consequent improvement of plant performance under water limitation have been identified (Pardo, 2010). Several transcription factors have been known which are involved in the regulation to the signaling network that controls stomatal movements in *Arabidopsis*. Transgenic plants expressing the transcription factor *MYB44* showed ABA-induced stomata closure response in transgenic plants (Jung *et al.*, 2008). Overexpression of *ARAG1*, a transcription factor gene, in rice plant may promote the synthesis of higher level of ABA in transgenic plants (Zhao *et al.*, 2010). The internal increase in concentration of phytohormone ABA may eventually lead to early stomata closer in transgenic plants which prevent major water loss (Apel and Hirt, 2004). Interestingly, in the present study, stomata closure in transgenic plants

**Table 2** Effect of water stress on agronomic trait in selected BR29 transgenic plants with At*DREB1A* gene at $T_3$ generation (pre flowering stage: 7 days of water stress)

| Plant line | Plant No. | No. reproductive tillers | No. filled grain | No. unfilled grain | Ratio (f/uf) | Grain yield/plant (g) |
|---|---|---|---|---|---|---|
| BRSh-22-24-6-11 | 6 | 10 | 670 | 488 | 1.37 | 16.20 |
| | 7 | 10 | 611 | 516 | 1.18 | 14.36 |
| | 9 | 7 | 601 | 283 | 2.15 | 14.20 |
| | Watered* | 9 | 937 | 126 | 7.43 | 22.59 |
| BRSh-29-22-2-16 | 10 | 8 | 614 | 501 | 1.22 | 14.52 |
| | 13 | 8 | 618 | 349 | 1.77 | 15.22 |
| | 15 | 9 | 739 | 362 | 2.04 | 17.81 |
| | Watered* | 8 | 767 | 142 | 5.40 | 20.17 |
| BRSh-29-22-2-19 | 4 | 6 | 687 | 279 | 2.46 | 16.16 |
| | 8 | 8 | 695 | 303 | 2.29 | 15.88 |
| | 12 | 6 | 541 | 294 | 1.8 | 13.20 |
| | Watered* | 7 | 681 | 119 | 5.7 | 16.35 |
| BRSh-22-22-25-6 | 9 | 8 | 685 | 522 | 1.31 | 16.63 |
| | Watered* | 6 | 797 | 137 | 5.81 | 18.84 |
| BRSh-22-22-25-7 | 13 | 9 | 583 | 533 | 1.09 | 17.14 |
| | Watered* | 7 | 809 | 127 | 6.37 | 19.40 |
| Non transgenic control | Treated | 7 | 339 | 525 | 0.64 | 7.84 |
| | Watered* | 6 | 754 | 98 | 7.69 | 18.56 |

*Data scored based on average five plants of the same transgenic line.

**Table 3** Response of transgenic lines transformed with *DREB1* genes to salinity

| Rice line | Gene used | EC-rating | |
| | | EC12* | EC18* |
| --- | --- | --- | --- |
| BR29-22-22-25-6 | At*DREB1A* | 5 | 9 |
| BR29-22-22-25-7 | At*DREB1A* | 5 | 9 |
| BR29-22-22-25-10 | At*DREB1A* | **3** | 9 |
| BR29-22-22-25-11 | At*DREB1A* | 5 | 9 |
| BR29-22-22-25-13 | At*DREB1A* | 5 | 9 |
| BR29-29-22-25-14 | At*DREB1A* | 5 | 9 |
| BR29-29-22-25-16 | At*DREB1A* | 5 | 9 |
| BR29-29-22-25-5 | At*DREB1A* | 5 | 9 |
| ML7-4-13 | Os*DREB1B* | 5 | 7 |
| ML7-4-16 | Os*DREB1B* | **3** | 9 |
| ML7-4-17 | Os*DREB1B* | **3** | **5** |
| ML7-4-18 | Os*DREB1B* | **3** | 7 |
| BR29 (control) | Check | 9 | 9 |
| IR29 (control) | Sensitive check | 9 | 9 |
| ML7 (control) | Check | 9 | 9 |

ML7 = IR6899B (Indica maintainer line).

*Data scored after 2 and 4 weeks (at EC12, 2 week & EC18 2 week);

9 rating represent maximum negative effect.

Bold EC 3 means tolerant and bold 5 indicates moderately tolerant.

during drought conditions may be due to the expression of the transcription factor transgene in rice plants as shown in Figure 5.

In rice plants, water stress at the vegetative stage does not have much effect on the grain yield as rice plants at the tillering stage can recover very quickly and new tillers can replace the damaged tillers. But in the pre-flowering stage and post flowering stage dehydration affects proper flowering and seed development. It was shown that the non-transgenic wild type plants under water stress produced more unfilled grains than filled grains and 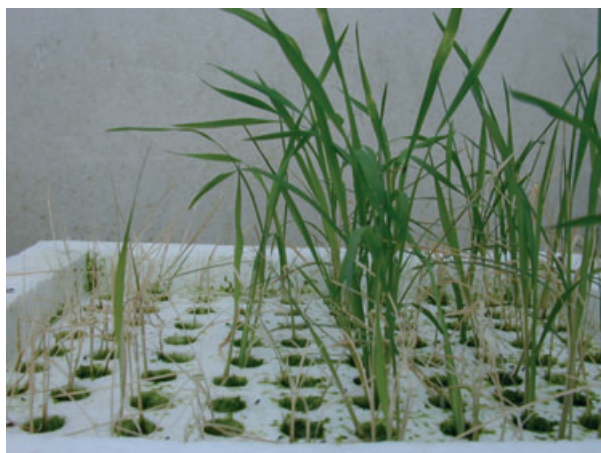consequently grain yield is very low, whereas yield loss in the case of the transgenic plants under water stress is much less. Water stress after flowering affects the grain filling of rice, thus causing severe reduction of the grain yield.

Transgenic plants with the *DREB1* gene showed more tolerance to salt stress when compared with their respective wild type plants. All the transgenic plants grown at the EC12 salt level for fourteen days were moderately tolerant [3–5 standard evaluation score (SES)] compared with their wild type (9 SES). The same plants after growing in nutrient solution with salinity level EC12 for 2 weeks were again grown in salinity level EC18 most of the transgenic plants died (i.e. SES 9), excepting only one line transformed with *DREB1B* gene which survived. The tolerance level of wild type variety BR29 and line IR68899B seems to be the same as is shown by the data scored after 2 weeks (SES 9), all died at salinity level EC12. It might be possible that for salinity tolerance, *DREB1B* gene may be more effective than the *DREB1A*.

Here we have demonstrated the enhancement of drought and salt tolerance with a single transcription factor gene encoded by a stress inducible promoter. It is known that different independent regulatory systems are involved in abiotic stress responsive gene expression. A better understanding of the physiological and biochemical basis of stress tolerance is still needed.

## Experimental protocol

### Plasmid DNA and bacterial strain

The plasmids pG29 AHS + Os*DREB1A*, pG29 AHS + Os*DREB1B* and pG29 AHS + At*DREB1A* were introduced into *Agrobacterium tumefaciens* strain EHA105. These plasmids contain marker genes for neomycin phosphotransferase (*nptII*), hygromycin phosphotransferase (*hph*) and the stress responsive gene Os*DREB1A*, Os*DREB1B* and At*DREB1A* controlled by *rd29* stress inducible promoter (Kasuga *et al.*, 1999). The bacteria were grown for 48 h in LB medium containing rifampicin (20 μg/mL) and kanamycin (50 μg/mL).

### Transformation

Two different rice cultivars BR29 and IR68899B were chosen for rice transformation. Embryogenic calli were obtained from 9 to 14 day-old immature embryos of BR29. Three-to-four-week-old actively growing embryogenic calli was taken as explants for *Agrobacterium* mediated transformation whereas for IR68899B, 10 day old immature embryos were used for biolistic transformation.

For *Agrobacterium*-mediated transformation we followed the method described by Datta *et al.*, 2000;. Vacuum infiltration was applied and calli were co-cultivated with *Agrobacterium* in 200 μM acetosyringone containing medium for 3 days. After 3 days of cocultivation, the *Agrobacterium* was removed by washing and the plant tissue was allowed to grow in medium containing 250 mg/L cefotaxime (to inhibit the growth of *Agrobacterium*) and 50 mg/L hygromycin (selective agent for transformation). After four cycles of selection the embryogenic calli were allowed to regenerate in regeneration medium. Regenerated plants were transferred to pots for natural growth. For biolistic transformation of IR68899B we followed the method described in earlier paper (Datta *et al.*, 1998). Selected regenerated putative transgenic plants were grown in pots in greenhouse.



**Figure 8** Effect of 14 days continuous salt stress with EC18; non-transgenic wild plants (left five rows), Transgenic IR68899B with Os*DREB1B* (b) (right five rows). EC has been measured in desi Siemens per meter (dS/m).

**Table 4** Effect of salinity stress on yield performance of salinity stress survived plants (IR68899B)

| Plant no. | Reproductive tillers | No. of filled grains | No. of unfilled spikelets | No. of total spikelets | Filled: unfilled | Grain yield per plant (g) |
|---|---|---|---|---|---|---|
| ML7-4-17-1 | 10 | 594 | 408 | 1002 | 1.45 | 12.14 |
| ML7-4-17-2 | 5 | 409 | 169 | 578 | 2.42 | 8.67 |
| ML7-4-17-3 | 9 | 355 | 135 | 490 | 2.63 | 7.10 |
| ML7-4-17-4 | 10 | 581 | 278 | 859 | 2.09 | 10.79 |
| ML7-4-17-5 | 11 | 602 | 338 | 940 | 1.78 | 11.96 |
| *IR68899B (without stress) | 10.4 | 1017 | 156 | 1173 | 6.51 | 19.95 |
| IR68899B (stressed control) | 5 | 365 | 257 | 622 | 1.42 | 7.3 |

*Average of five plants grown under normal condition up to maturity.

## Southern blot analysis

Ten microgram of DNA was digested with *BamHI* restriction endonuclease (Invitrogen, Carlsbad, CA). The digested DNA samples were separated by electrophoresis on 1% (W/V) TAE-agarose gel. Southern membrane transfer, hybridization and autoradiography were done as previously described (Datta *et al.*, 1998). The *BamHI* fragments containing the ~0.8 Kb *DREB* genes of *DREB1A* and *DREB1B* were radio labeled with '-α32P-dCTP and used correspondingly as hybridization probe.

## RNA gel blot analysis

Total RNA was isolated by the GITC (Guanidine isothiocyanate) method (Chomczynski and Sacchi, 1987). Equal amounts of total RNA (20 µg) were electrophoresed on 1.4% MOPS-Formaldehyde gel, transferred to nylon membrane (Hybond-N; Amersham place, Buckinghamshire, UK) and baked by 80 °C for 2 h. PCR amplified product of *DREB* gene were labeled by random prime method (Radiprime; Amersham) and used as a probe. Radio-labeled (50 µci α$^{32}$P-dCTP; Perkin-Elmer, Wellesley, MA) probes were denatured and hybridized to the membrane at 65 °C in a hybridization buffer (6× SSC, 5% Dextran sulphate, 0.05 M Sodium phosphate pH 7.2, 5× Denhardt's solution, 0.5% SDS, 0.0025 M EDTA and 100 µg/mL Salmon sperm DNA) for 12–16 h and washed for 15 min each with 1× SSC, 0.5% SDS and 0.5× SSC, 0.5% SDS. The hybrdization signal was observed on Kodak X ray film s after 2–3 days exposure (Chomczynski and Sacchi, 1987).

## Quantitative RT-PCR

The qRT-PCR was performed after 6 days of drought stress with gene specific primers and the cycle was as follows: 95° C for 30 s, 60 °C for 30 s and 72 °C for 30 s. The procedure was according to the manufacturer's instructions (CFX 96 Real time system; Biorad, Hercules, CA). The amplification of *tubulin* gene was used as internal control to normalize all data. To validate the qRT-PCR results, the experiments were repeated three times. The mean values for the expression levels of the genes were calculated from three independent experiments.

## Drought stress treatment

The screening for drought tolerance is based on the phenotypic changes and survival of plants due to the water stress condition and ultimately its effect on grain yield. It was performed at three phases of plant growth with three different sets of plants. For the vegetative phase watering of 6-week-old plants was stopped for 21-days or as long as the wild type plants have severe damage. In the case of pre-flowering stage screening, water supply was stopped for 7-days at the booting stage. For the flowering stage screening, plants were not supplied with water at the start of the flowering stage for 7-days. In all the three different sets of experiment, visual phenotype changes, recovery of the plants and its effect on grain yield were observed.

## Stomata studies

Water was withdrawn from transgenic and wild type rice plants of five different transgenic plants of BRSH29 for 6 days and leaf materials were collected and used for stomata studies. Epidermal peels were obtained from rice leaves 1-cm-long pieces of the leaves were scraped on the abaxial sides to remove most of the cells above the adaxial epidermis, then the isolated adaxial epidermis was stained with 0.1% acridine orange for 15 min (dye was prepared by dissolving 0.1 g of acridine orange in 0.05 M phosphate buffer), washed thoroughly in distilled water, mounted with dilute glycerin, observed and photographed using laser confocal microscope (LSM 510 with Carl Zeiss Axioplan-2, Göttingen, Germany).

## Screening for salinity tolerance

Salinity tolerance screening was done at the seedling stage. Four day old seedlings were placed on styrofoam seedling float with nylon net bottom on distilled water. The radicles were inserted through the nylon mesh. After 3 days when the seedlings were well established the water was replaced with salinized nutrient solution. Initial salinity was EC = 12 dS/m by adding NaCl to the nutrient solution. Renewal of the solution was done every 7-days and pH at 5.0. The first scoring was taken after 14-days at EC = 12 dS/m. After 14-days, the salinity level was increased to EC = 18 dS/m. The second scoring was taken after 14-days at EC = 18 dS/m. The modified SES in rating the visual symptom of salt toxicity was used (Gregorio *et al.*, 1997).

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