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Rice *DREB1B* promoter shows distinct stress-specific responses, and the overexpression of cDNA in tobacco confers improved abiotic and biotic stress tolerance

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Abstract CBF/DREB (C-repeat binding factor/dehydration responsive element binding factor) family of transcription factors in plants is reported to be associated with regulation of gene expression under stress conditions. Here, we report the functional characterization of a DREB transcription factor, *DREB1B* gene from rice (*Oryza sativa* ssp. *indica*). The *OsDREB1B* gene was differentially regulated at the transcriptional level by osmotic stress, oxidative stress, salicylic acid, ABA, and cold. A 745 bp promoter region of *OsDREB1B* cDNA was fused to the *β*-glucuronidase (*GUS*) gene and introduced via *Agrobacterium tumifaciens* into the genome of *Arabidopsis*. Histochemical analysis of *GUS* expression in T_2 transgenic *Arabidopsis* plants indicated that *OsDREB1B* shows stress-specific induction pattern in response to a variety of stresses like mannitol, NaCl, PEG, methyl viologen, cold, ABA, and salicylic acid. Leaf-order-dependent induction pattern of the promoter was observed in response to both cold and ABA stresses. Further, *OsDREB1B* cDNA was introduced into tobacco plants under the control of *CaMV35S* promoter to investigate the role of *DREB1B* product in plant stress response. Transgenic tobacco plants have shown improved seed germination, root growth, membrane stability, and 2, 2-diphenyl-1-pycrilhydrazil hydrate (DPPH) free radical scavenging activity under inhibitory concentrations of mannitol. Importantly, transgenic plants accumulated higher fresh weight under long-term osmotic stress, and also have shown retention of more water than the wild type during drought stress. Overexpression of *OsDREB1B* in tobacco also improved the oxidative and freezing stress tolerance of transgenic plants. In addition, tobacco plants constitutively expressing *OsDREB1B* have shown decreased sensitivity to tobacco streak virus infection. Constitutive expression of *OsDREB1B* in tobacco also induced the expression of PR genes in transgenic plants. The data obtained provide strong in vivo evidence that *OsDREB1B* is involved in both abiotic and biotic stress responses, and confers broad-spectrum stress tolerance to transgenic plants.

Keywords Rice · *DREB1B* · *Arabidopsis* · Tobacco · Abiotic stress · Biotic stress

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Introduction

Terrestrial plants are usually subjected to many harsh environments all through their life cycle, and environment stresses such as drought, salinity, and low temperature severely limit the plant productivity worldwide. Plants respond and adapt to these sub optimal growth conditions by employing various biochemical and physiological processes, there by acquiring stress tolerance (Shinozaki et al. 2003). During stress condition, numerous genes are activated at transcriptional level in plants, and several of these genes have been described recently (Ingram and Bartels 1996; Shinozaki and Yamaguchi-Shinozaki 1996; Bray 1997; Reddy et al. 2002; Chaves and Oliveira 2004). Transcriptional regulation of such stress responsive genes is mediated by changes in the activity of DNA-binding transcription factors like Myb, Myc, basic-domain Leucine zipper (bZIP), Apetalous (AP2), Zn finger, Homeodomain (HD), and NAC (Carabelli et al. 1993; Shinozaki et al. 2003; Tran et al. 2004).

A subfamily of AP2 domain transcription factors, the Dehydration Responsive Element Binding (DREB)/C-repeat Binding Factor (CBF) proteins, were identified in many plant species in response to a wide spectrum of abiotic stresses. The DREB/CBF transcription factors have been shown to bind DRE (A/GCCGAC) and DRE-like *cis*-elements of several stress responsive genes and activate their expression (Stockinger et al. 1997; Liu et al. 1998). *DREB1B*, *DREB1C*, and *DREB1A* also referred as *CBF1*, *CBF2*, and *CBF3* respectively, were characterized in *Arabidopsis*, rice, and many other plant species (Stockinger et al. 1997; Gilmour et al. 1998; Liu et al. 1998; Shinwari et al. 1998; Dubouzet et al. 2003). Sequence analysis of all the *DREB1* genes showed that they are intronless genes, and duplication of them may have produced a small multigene family during species evolution (Haake et al. 2002). These transcriptional activators have been shown to regulate downstream genes in response to low temperature, water deficit, and also high temperature (Stockinger et al. 1997; Qin et al. 2007). Although DREB1 transcription activators were reported widely to function only in stress signal transduction pathways involving ABA independent gene expression, Haake et al. (2002) empirically proved that these also play a role in stress inducible ABA dependent gene expression. In *Brassica napus*, two groups of DREB transcription factors are present, group I and group II, functioning as *trans*-active and *trans*-inactive proteins respectively. Group I proteins act initially in the signal transduction pathways and activate down stream genes where as, group II proteins later replace the group I proteins and close the pathways in a competitive manner under cold stress (Zhao et al. 2006).

All the known DREB proteins contain a plants-specific ERF/AP2 domain, which is composed of 58 amino acid residues (Jofuku et al. 1994; Ohme-Takagi and Shinshi 1995). The binding specificity of DREB proteins is determined by the two amino acids of the domain, the 14th valine and the 19th glutamic acid (Liu et al. 1998; Sakuma et al. 2002). AP2 domain of DREBs present in dicots like *Arabidopsis* has valine and glutamic acid conserved at 14th and 19th positions respectively, but the domain of rice DREB1s has valine conserved at both positions like other monocots. OsDREB1A specifically binds DRE-related core binding motif, GCCGAC more preferentially than to ACCGAC unlike AtDREB1A, which shows efficient binding to both ACCGAC and GCCGAC (Dubouzet et al. 2003).

Constitutive expression of *DREB1* genes in plants resulted in the expression of numerous stress responsive genes even under non-stress conditions, and made the transgenic plants resistant to cold, drought, and high salt stresses (Liu et al. 1998; Kasuga et al. 1999; Gilmour et al. 2000; Oh et al. 2007). The transgenic *Arabidopsis* plants over expressing *AtCBF1/DREB1B* under *CaMV35S* promoter showed enhanced freezing tolerance and higher expression of *COR* genes (Jaglo-Ottosen et al. 1998). Constitutive overexpression of *CBF1* in tomato led to enhanced catalase activity and reduced H_2O_2 accumulation in transgenic plants signifying that *CBF1* has a role in oxidative stress tolerance in plants (Hsieh et al. 2002a). *CBF1* also imparted dehydration stress tolerance to tomato transgenic plants (Hsieh et al. 2002b). In rice, *DREB/CBF* genes have been induced in response to cold-, drought-, and high salt-stresses, but the expression of *DREB1A* and *DREB1B* genes have been observed only under cold stress (Dubouzet et al. 2003; Ito et al. 2006). Ectopic expression of *OsDREB1A* in *Arabidopsis* imparted high salt and freezing stress tolerance, and also activated the overexpression of several stress inducible genes in slightly growth retarded transgenic plants. *35S*::*OsDREB1A Arabidopsis* seedlings showed higher survival rate over the wild type under high salt and freezing stresses (Dubouzet et al. 2003). The *AtDREB1A/CBF3* transcription factor in tobacco improved the drought and low temperature stress tolerance, apart from increasing the accumulation of the group2 LEA proteins in *35S::AtDREB1A* transgenic tobacco plants (Kasuga et al. 2004). Unlike other *DREB* transgenic plants, rice transgenic plants constitutively expressing *AtDREB1A/CBF3* under the influence of *Ubiquitin* promoter did not show any growth retardation. Moreover, transgenic rice plants have shown elevated levels of tolerance to drought and high salt stresses, but very low level of tolerance to low temperature stress (Oh et al. 2005). Together these studies have demonstrated the potential use of *DREB*s as candidate genes in imparting stress tolerance capabilities to transgenic plants.

Among all the crop plants, rice is not only an important staple food crop providing sustenance to more than 50% of the world population, but also is a model monocot genetic system. As the DREB transcription factors are proving to be important in the stress tolerance in model plants like *Arabidopsis*, it is very important to analyze the *DREB*s and their regulation even in rice not only to understand the molecular mechanisms of stress tolerance in monocots, but also to use them in genetic engineering of plants for improved stress tolerance. Several *OsDREB* genes have been isolated and their functions analyzed (Chen et al. 2003; Dubouzet et al. 2003; Oh et al. 2005; Ito et al. 2006). Ito et al. (2006) reported that over expression of *OsDREB1B* imparts improved chilling tolerance to transgenic plants. In order to understand the in vivo functional role of *OsDREB1B* under stress, we chose to study the *OsDREB1B* promoter, and its cDNA in transgenic plants. Here, we report that the *OsDREB1B* has stress-dependent activity, and the overexpression of cDNA in tobacco (*Nicotiana plumbaginifolia* Viv.) imparts enhanced tolerance to osmotic, dehydration, oxidative stresses, and also to tobacco streak virus infection. Our results demonstrate that

OsDREB1B has a unique role in stress response, and protects the plants from a range of stresses.

Materials and methods

Plant materials and growth conditions

Rice (*Oryza sativa* ssp. *indica* cv. Nagina 22) seeds were imbedded in water at 37°C for 2 days with water changed every day, and then grown at 25°C on cheese cloth in a tray with the addition of water and mineral nutrient solution. *Arabidopsis* ecotype Columbia seeds were sown in pots filled with a compost mixture of 1:1:1 perlite:vermiculite:soilrite. The germination was synchronized by cold treatment for 48 h at 4°C, and the pots were placed in a controlled environment chamber (16 h day photoperiod, 20 ± 1°C constant day and night temperature). Plants were sub-irrigated with the standard nutrient solution [5 mM KNO_3, 2.5 mM KH_2PO_4, 2 mM $MgSO_4$, 2 mM Ca $(NO_3)_2$, 1× Fe-EDTA and 1× MS micronutrient mix (Murashige and Skoog 1962)]. Seeds of wild tobacco (*Nicotiana plumbaginifolia* Viv.) were germinated, transplanted in soil at two or three leaf stage, and maintained in greenhouse conditions at a day/night temperature of 25 ± 1°C.

Cloning of *OsDREB1B* promoter and agroinfiltration of *Arabidopsis*

5′ flanking region of *OsDREB1B* cDNA (−689 to +56) was PCR amplified from genomic DNA of *Oryza sativa* ssp. *indica* cv. Nagina 22 with *Pfu* polymerase. It was amplified by forward primer 5′-CGGGATCCGCGAGGTAAGCCATTAGCGCATG-3′ containing *BamH* I restriction site (under lined) and reverse primer 5′-CGGAATTCGGATGACTCTCTCTGGTTCAC-3′ containing *EcoR* I restriction site (under lined). Amplified promoter fragment was cloned into the *β*-glucuronidase (*GUS*) reporter gene cassette as *BamH* I and *EcoR* I fragment at corresponding restriction sites just upstream of the *GUS* gene in *pCAMBIA1391Z* binary vector to analyze the expression pattern of the promoter in vivo. Promoter fragment was sequenced by dye terminator automated DNA sequencing and the sequence has been submitted to GenBank (EF556551). *Cis*-element analysis of the promoter sequence was performed by searching the PLACE database, and TSS and TATA box of the promoter sequence were identified by the FGENESH analysis of www.softberry.com. The recombinant plasmid was mobilized into *Agrobacterium tumefaciens* strain LBA4404 by freeze-thaw method. *OsDREB1B* promoter::*GUS* chimeric construct was transferred to *Arabidopsis* plants by floral dip method (Clough and Bent 1998). Transformants were selected on MS agar plates containing 50 mg l^{-1} hygromycin, and further confirmed by PCR.

RT-PCR analysis

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was employed to investigate the expression profiles of *OsDREB1B* and its target genes. The RT-PCR was performed with 1 μg of total RNA using one step RT-PCR kit (Qiagen) according to the manufacturer's instructions. RT was carried out at 50°C for 30 min. A 15 min of denaturation at 95°C was included prior to initiation of the normal PCR cycles. Twenty-eight cycles (94°C for 1 min, 50–58°C for 1 min, 72°C for 1 min) of amplification, with a final extension at 72°C for 10 min was performed. *OsDREB1B* was amplified with the primers specific (forward: 5′-ATGGCGACGAAGAAGAAGAC-3′; reverse: 5′-AGTAGCTCCAGAGCGGCATA-3′) to the 225 bp 3′ terminal region of its ORF. To study the target genes of *OsDREB1B* that were activated in transgenic tobacco plants, RT-PCR was performed with eight ethylene-inducible and disease responsive PR genes such as *pathogenesis related-1 protein* gene (*PR1b*), *β-1,3-glucanse* (*PR2*), *chitinase* (*PR3*), *PR4, Osmotin* (*PR5*), *CHN50*, *ACC oxidase* (*ACO*), and *ACC synthase* (*ACS*) with their gene specific primers (Qin et al. 2006). RT-PCR experiments were repeated twice to validate the results. The RT-PCR for the house-keeping *Actin* gene was performed under the same conditions as described above to determine whether the equal amounts of total RNA were used in the RT-PCR reactions among samples.

Construction of plant expression vector and tobacco transformation

The full-length *OsDREB1B/CBF1* cDNA (AY166833) was isolated from a drought stressed rice cDNA library of *Oryza sativa* ssp. *indica* cv. Nagina 22 (Reddy et al. 2002). The fragment was inserted into the *Xho*I/*Xba*I site of the pRT100 vector placing the cDNA downstream of *CaMV35S* promoter. Expression cassette was introduced into the *Pst*I site of binary vector *pCAMBIA1301* containing a hygromycin resistance selectable marker. Tobacco transformation was done by co-cultivation of leaf discs, obtained from in vitro-grown tobacco (*Nicotiana plumbaginifolia* Viv.) plants, for 15 min with *Agrobacterium tumefaciens* strain LBA4404 harboring the recombinant binary vector *pCAMBIA1301* + *CaMV35S*::*OsDREB1B*. Transformed leaf discs were selected on MS agar supplemented with 6-benzylaminopurine (2.25 mg l^{-1}), napthalene acetic acid (0.1 mg l^{-1}),

cefotaxime (400 mg l^{-1}), and hygromycin (25 mg l^{-1}). Regenerated shoots were rooted on MS basal medium containing hygromycin (25 mg l^{-1}). The profusely rooted plantlets were transferred into pots and maintained in green house following acclimatization for 2 weeks.

DNA and RNA gel blot analysis

DNA and RNA gel blot analysis was performed according to Luo et al. (2005) with modifications. Thirty micrograms of the transgenic and wild type tobacco plant genomic DNAs were digested completely with *EcoR* I, separated by electrophoresis on a 0.8% agarose gel, and transferred by vacuum apparatus onto a Hybond-N^+ nylon membrane (Amersham Pharmacia) using 0.4 M NaOH/1.0 M NaCl. A 225 bp fragment of 3′ terminal region of *OsDREB1B* ORF was labelled with [α-^{32}P] dATP by the random priming method using a HexaLabel DNA Labeling Kit (MBI Fermentas). For RNA blot analysis, total RNA was extracted with TRIReagent (Sigma) and twenty micrograms of total RNA was resolved on a 1% formaldehyde denaturing gel and transferred to Hybond N^+ membrane using 20× SSC. Pre hybridization, hybridization for both DNA and RNA gel blot analysis were performed with ULTRAhyb hybridization buffer (Ambion) according to the manufacturer's instructions.

Histochemical localization of *GUS* activity

For histochemical *GUS* assays, plant tissues/seedlings were vacuum infiltrated in a solution containing 50 mM NaP buffer at pH 7.0, 5 mM K3Fe(CN)6, 5 mM K4 Fe(CN)6, 0.1% Triton X-100, and 1 mM X-Gluc (5-bromo-4-chloro-3-indole-β-D-glucuronide cyclohexyl ammonium salt) for several minutes and incubated overnight at 37°C, followed by 70% ethanol fixation and blue stain assessment and photomicroscopy (Jefferson 1987).

Seed germination under mannitol stress

For germination study, wild type and T_2 transgenic surface-sterilized tobacco seeds were sown on Petri dishes (9 cm diameter) containing 0.5× MS agar, 0.5× MS agar + 50 mM mannitol, 0.5× MS agar + 100 mM mannitol, 0.5× MS agar + 150 mM mannitol, and 0.5× MS agar + 200 mM mannitol. Germination assays were carried out in three replicates of 30–35 seeds and germination was scored 15 days after germination. Tolerance to mannitol stress was judged based on the ability of the seeds to germinate and grow on the amended media. The number of germinated seeds was expressed as a percentage of the total number of seeds.

Abiotic stress treatments

OsDREB1B promoter::*GUS Arabidopsis* plants were grown for 4 weeks under standard conditions on MS agar plates (9 cm diameter). Subsequently, plants were transferred to either normal MS agar plates or MS agar plates supplemented with 200 mM mannitol, 200 mM NaCl, 10%PEG, 10 μM methyl viologen, 100 μM ABA, 1 mM salicylic acid, separately for 2 days. For cold stress, *Arabidopsis* plants growing on MS agar plates were exposed to 4°C for 2 days. Seedlings/plants after each stress treatment were harvested from MS agar plates for qualitative *GUS* measurements. Transgenic tobacco (T_2 or T_3) plants constitutively expressing *OsDREB1B* were tested under various stress conditions. To observe the growth performance of the wild type and transgenic tobacco plants under stress, 5-day-old seedlings were transferred to Petri-plates (9 cm diameter) containing MS agar supplemented with 200 mM mannitol, 200 mM NaCl, 10%PEG, and 10 μM methyl viologen, separately. Further, root-growth experiments under laboratory conditions were performed by transferring the 5-day-old seedling to vertical Petri-plates (12 cm diameter) containing MS + 200 mM mannitol and MS + 200 mM NaCl, individually. Long term stress effect on transgenic plants was evaluated by transferring 5-day-old tobacco seedlings to glass bottles containing MS agar media supplemented with various concentrations of mannitol, PEG, and methyl viologen, and growth pattern was observed after 45 days. For salinity stress treatment under green-house conditions, 15-day-old seedlings were transferred to soil, and were irrigated with 200 mM NaCl until the maturity. For drought stress experiment, transgenic and wild type plants were grown in soil for 30 days, and watering was withheld for 21 days. During the water stress period, the amount of water retained by plants was estimated in terms of g water/g dry weight at regular intervals according to Hsieh et al. (2002b). For freezing stress, 15-day-old seedlings growing in soil were exposed to −5°C and maintained under continuous light for 48 h. After freezing stress, plants were allowed to recover for 8 days at 25 ± 1°C.

Estimation of the fresh weight, dry weight and chlorophyll

Plants growth in response to various stresses was determined by measuring fresh weight and dry weight of plants/seedlings challenged by a stress. The fresh weight of plants/seedlings or root portion was measured immediately after the harvest where as the dry weight was measured after drying the material for 48 h at 80°C. Amount of chlorophyll in leaves was measured spectrophotometrically after extraction in 80% acetone according to Arnon, (1949).

Measurement of malondialdehyde (MDA) content

The level of lipid peroxidation has been used as an indicator of free radical damage to cell membranes under stress conditions. Malondialdehyde (MDA) is a product of peroxidation of unsaturated fatty acids in phospholipids and is responsible for cell membrane damage. MDA contents were measured using thiobarbituric acid reactive substances (TBARS) assay (Heath and Packer 1968). About 0.5–1.0 g of tissue was homogenized in 5 ml of 5% (w/v) trichloroacetic acid and the homogenate was centrifuged at 12,000*g* for 15 min at room temperature. The supernatant was mixed with an equal volume of thiobarbituric acid [0.5% in 20% (w/v) trichloroacetic acid], and the mixture was boiled for 25 min at 100°C, followed by centrifugation for 5 min at 7,500*g* to clarify the solution. Absorbance of the supernatant was measured at 532 nm and corrected for non-specific turbidity by subtracting the A_{600}. MDA equivalents were calculated by the extinction coefficient of 155 M^{-1} cm^{-1}. Values of MDA contents were taken from measurements of three independent samples.

DPPH free radical scavenging activity assay

DPPH (2, 2-diphenyl-1-pycrilhydrazil hydrate) assay was performed according to Kang and Saltveit (2002). For this, 200 mg fresh leaf material of transgenic and wild type plants was excised. Each sample was homogenized at 4°C in 2.0 ml of absolute ethanol with mortar and pestle. A 0.5 ml aliquot was mixed with a 0.5 mM DPPH ethanol solution (0.25 ml) and 100 mM acetate buffer (pH 5.5; 0.5 ml). After standing for 30 min, the absorbance of the mixture was measured at 517 nm. %RSA (% radical scavenging activity) was calculated according to Yesil-Celiktas et al. (2007).

Virus inoculation

Inoculation of the tobacco plants with tobacco streak virus (TSV) was performed using a standard mechanical rubbing method. To prepare the inoculum, TSV-infected cowpea leaves were ground in 5 ml of potassium phosphate buffer (50 mM, pH 7.2), and 0.01% (w/v) celite was mixed immediately before inoculation. Fully expanded leaves of 15-day-old both transgenic (T_2) and wild type plants were inoculated by gently rubbing the upper leaf surface with 100 µl of the viral suspension inoculum, followed immediately by rinsing with deionized water. Like wise, 20–25 plants of each wild type and transgenic plants were inoculated. Following inoculation, plants were maintained at normal green house conditions.

Results

Expression pattern of *OsDREB1B* gene in response to various stresses

The expression pattern of the *OsDREB1B* gene was detected by semi quantitative RT-PCR with total RNA isolated from the rice leaves under normal and stressed conditions. The expression of the *OsDREB1B* gene transcripts were absent under normal conditions, where as transcript levels increased in response to several stresses. Stress treatments like 200 mM NaCl, 10% PEG6000, and cold (4°C) induced the expression of *OsDREB1B* very quickly, where as 200 mM mannitol, 10 µM methyl viologen, 1 mM salicylic acid, and 100 µM ABA stresses induced a delayed response (Fig. 1).While 100 µM ABA did not lead to any detectable increase in *OsDREB1B* transcripts up to 6 h, but low level of induction was observed after 12 h. Low temperature treatment among all the stresses induced a high level of gene expression. Rice seedlings treated by mannitol, NaCl, and PEG have shown the differential expression pattern initially, but similar transcript levels-accumulation was observed after 6 h of

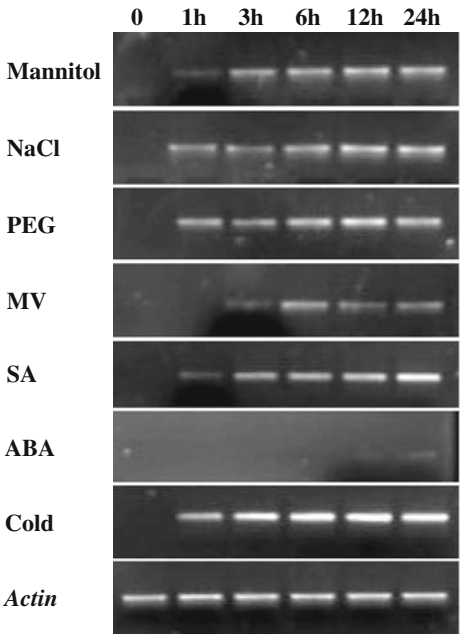


Fig. 1 Semi quantitative RT-PCR analyses of *OsDREB1B* gene in rice seedlings in response to various stress treatments. The rice seedlings grown for 20 days after germination were subjected to different stress treatments [200 mM mannitol, 200 mM NaCl, 10%PEG6000, 10 µM methyl viologen, 1 mM salicylic acid, 1 mM ABA, and cold (4°C)] and the total RNA was isolated from each treatment. Accumulation of *OsDREB1B* mRNA was determined by one step RT-PCR with 1 µg RNA, and with 28-cycle-amplification. The gene specific primers of *OsDREB1B* 3′ terminal region were used. The *Actin* gene was amplified as control. The PCR products were determined by electrophoresis on a 1% (w/v) agarose gel

treatment. Oxidative stress induced by methyl viologen accumulated transcripts up to 6 h at increasing levels, and then showed a decrease although constant levels were observed later. Expression pattern of *OsDREB1B* under 1 mM salicylic acid was observed to be similar to that of cold stress, though the transcript levels were relatively low.

OsDREB1B promoter and its activity in transgenic *Arabidopsis* plants

To analyze the induction pattern of OsDREB1B transcription factor in detail, 5′ flanking region of its cDNA was amplified from *Oryza sativa* ssp. *indica* cv. Nagina22. Sequence analysis of the 745 bp 5′ region showed that the transcription start site (TSS) of *OsDREB1B* is located probably 56 bp upstream of its translation start codon, and TATA box is located at −29. By searching the *OsDREB1B* promoter in the plant promoter database PLACE, a number of potential regulatory motifs corresponding to several known *cis*-acting elements related to tissue-specific gene expression, abiotic- and biotic-stress responses were predicted. In addition, several consensus *cis*-acting elements like ABRE, DRE, MYB, WBOX, and WRKY were also found (Table 1). DRE1COREZMRAB17 (ACCGAGA) *cis*-element was found at −270 in the nucleotide sequence of the promoter. The fact that many *cis*-elements related to various stresses are present in the promoter region hints that *OsDREB1B* gene must be controlled by a complicated mechanism, and responds to several stresses. This sequence has not shown any similarity with its respective promoter sequence of *Arabidopsis*.

Activity of the *OsDREB1B* promoter was tested in transgenic *Arabidopsis* plants transformed with *OsDREB1B* promoter::*GUS* fusion construct. To determine whether *OsDREB1B* promoter is developmentally regulated, we analyzed four independent T_2 transgenic lines by monitoring the *GUS* expression pattern in various organs through out the plant development by histochemical staining. In all the four lines, early stage seedlings showed intensely developed blue staining preferentially in the vascular region (Fig. 2a–c). But, the *GUS* staining was not detectable in any other stage of the developed seedlings/plants. Further, the 4-week-old transgenic *Arabidopsis* plants were exposed to different stresses for 2 days. In all the lines tested, each stress led to intense *GUS* staining in tissue/organ-specific manner (Table 2). However, the *GUS* induction was not observed in transgenic plants with out any stress (Fig. 2d). *GUS* expression was observed in leaves, stem and roots in response to both cold (Fig. 2e) and 100 μM ABA (Fig. 2f) stresses. But under these both stresses, intense *GUS* staining was observed in the lower and older leaves than in the younger leaves in the rosette of *Arabidopsis*. It indicates that the induction of *OsDREB1B* promoter by cold and ABA stresses was leaf-order dependent. Osmotic stress induced by 10% PEG (Fig. 2g), 200 mM NaCl (Fig. 2h), 200 mM mannitol (Fig. 2i), individually, and oxidative stress induced by 10 μM methyl viologen (Fig. 2j) induced the promoter strongly in both stem and roots but not in leaves of transgenic *Arabidopsis* plants. One mM salicylic acid (Fig. 2k) induced the promoter very uniquely in the histochemical experiments. It induced the *GUS* gene very strongly only in the stem portion but not in any other part of the seedling unlike

Table 1 Putative *cis*-acting elements in the *OsDREB1B* promoter

Cis-element	Position[a]	Sequence[b]
Myb binding elements		
MYBCORE	257	CCGTTG
MYB1AT	357	AAACCA
MYBCOREATCYCB1	531	AACGG
Light regulation elements		
GT1CONSENSUS	534	GGAAAA
SORLIP1AT	122	GCCAC
SORLIP2AT	115	GGGCC
−10PEHVPSBD	19,381	TATTCT
Tissue specific gene expression elements		
CACTFTPPCA1	35,333,411,467,475,647	TACT
DOFCORE	365,607	AAAG
DPBFCORE	409,566	ACACCGG
TAAAGSTKST1	365,607	TAAAG
POLLEN1LELAT52	206,229	AGAAA
AACACOREOSGLUB1	524	AACAAC
ROOTMOTIFTAPOX1	21	ATATT
BS1EGCCR	159	AGCGGG
Pathogen/elicitor response elements		
WBBOXPCWRKY1	451	TTTGACT
WBOXNTERF3	451	TGACT
WBOXATNPR1	452	TTGAC
WRKY71OS	452	TGAC
BIHD1OS	277	TGTCA
Abiotic stress response & other elements		
ABRERATCAL	118	CACGCGG
DRE1COREZMRAB17	270	ACCGAGA
ARR1AT	615,667	TGATT
CAATBOX	513	CAAT
CPBCSPOR	654	TATTAG
CCAATBOX1	513	CCAAT
SURECOREATSULTR11	150,267	GAGAC
ANAERO2CONSENSUS	162,165,168,171	AGCAGC
RAV1AAT	210	CAACA
PREARPRODH	464	ACTCAT

[a] Position of the *cis*-elements are with respect to upstream position of the transcription start site

[b] Sequences are indicated from 5′ to 3′

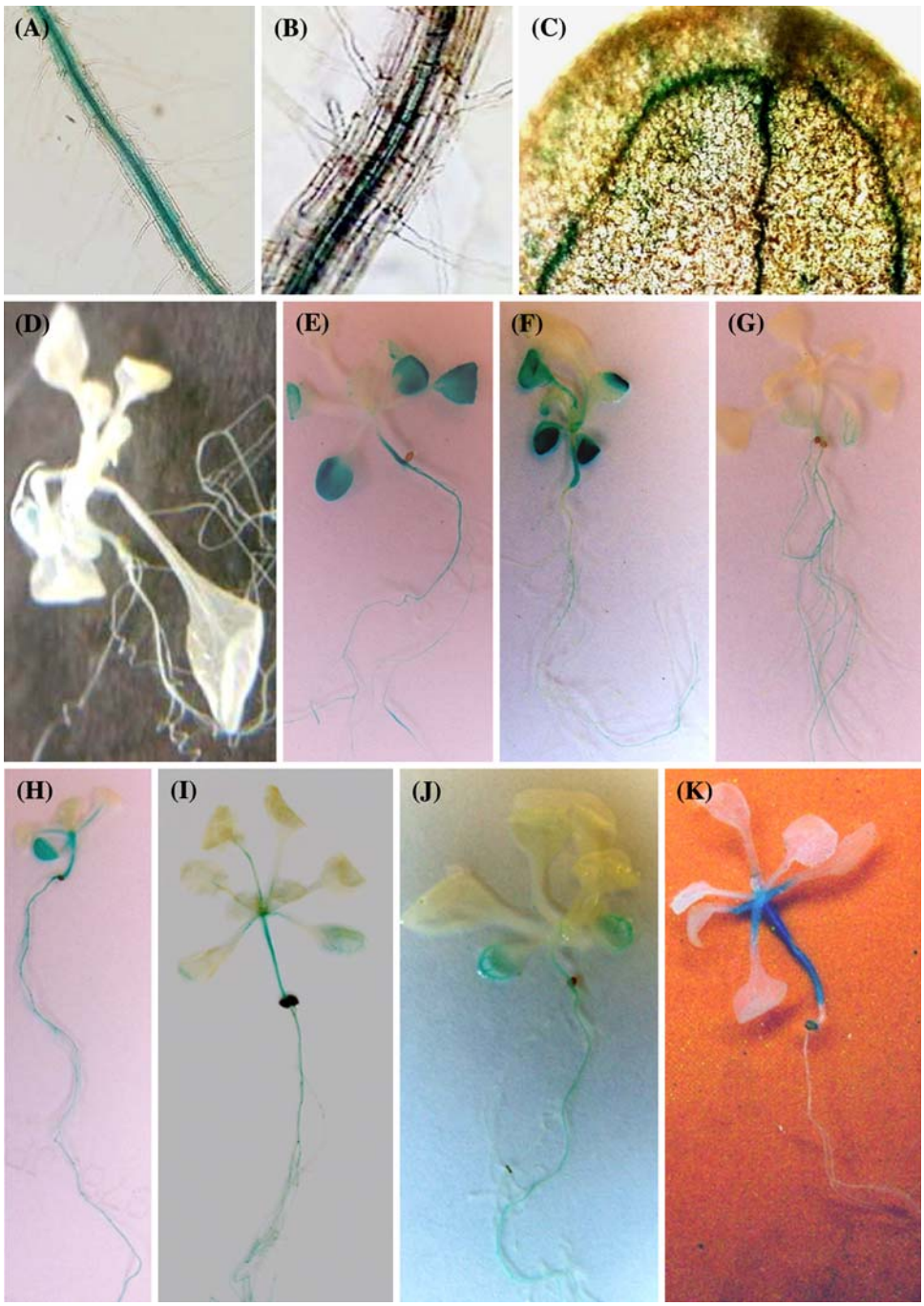


Fig. 2 Pattern of expression of the *OsDREB1B* promoter::*GUS* gene fusion in transgenic *Arabidopsis* plants. (**a**–**c**) Histochemical localization of *GUS* activity in early stage seedlings. Three-day-old seedlings were used for these experiments. (**a**–**b**) Staining in primary root. (**c**) Staining in cotyledons. (**d**–**k**) Stress-specific induction pattern of *OsDREB1B* promoter in response to various stresses. Four-week-old T_2 *Arabidopsis* plants were challenged with various stresses for 2 days and the harvested seedlings were incubated with X-gluc overnight; (**d**) non-stress, (**e**) cold, (**f**) ABA, (**g**) PEG, (**h**) NaCl, (**i**) Mannitol, (**j**) Methyl viologen, (**k**) Salicylic acid

any other stresses imposed on *OsDREB1B* promoter::*GUS* *Arabidopsis* plants.

Constitutive overexpression of *OsDREB1B* in transgenic tobacco shows no negative effect on plant growth

To evaluate the effect of overexpression of *OsDREB1B* in tobacco, *OsDREB1B* cDNA was fused to cauliflower mosaic virus *35S* promoter and transferred to tobacco genome using *Agrobacterium tumifaciens*-mediated transformation. After selection on hygromycin-containing medium, the putative transgenic tobacco plants were further identified by leaf senescence assay, *GUS* histochemical staining assay, and PCR. Twenty independent transgenic lines were obtained, and DNA gel blot analysis demonstrated that the transgene is stably integrated into the genome of putative transgenic tobacco lines, (Fig. 3a) with each carrying either one or two copies of the transgene. RNA gel blot analysis of transgenic plants grown under normal conditions confirmed that the transgene is expressing constitutively in transgenic plants where as, no expression was observed in wild type

Fig. 3 Molecular analysis of *35S*::*OsDREB1B* transgenic tobacco plants. (**a**) DNA gel-blot analysis of wild type and *OsDREB1B* expressing tobacco plants. (**b**) RNA gel-blot analysis of wild type and *OsDREB1B* expressing tobacco plants. The ethidium bromide stained gel demonstrates equivalent RNA quantities loaded in each lane (2, 7, 9, 10, 11, 12, 18, and 20 denotes DB2, BB7, DB9, DB10, DB11, DB12, DB18, and DB20, respectively are the *35S*::*OsDREB1B* independent transgenic tobacco lines; WT, wild type tobacco). (**c**) Growth phenotypes of wild type and *OsDREB1B* transgenic tobacco seedlings. Seeds of wild type and transgenic lines (DB2, DB11, DB12, and DB18) are sown in the soil and the growth phenotype of 15-day-old seedlings was observed. (**d**) Fresh weights of 15-day-old seedlings growing in (**c**). Fifteen-day-old seedlings were uprooted ▶ from the soil and fresh weights were measured. Both wild type and transgenic seedlings have not shown any significant difference between them in terms of fresh weight. Each bar value represents the mean ± sd ($n = 12$) of triplicate experiments (student's t test; $P < 0.05$ versus wild type). (**e**) Growth phenotypes of wild type and *OsDREB1B* transgenic tobacco plants at maturity stage. Plant heights of both wild type and transgenic lines (DB2, DB11, DB12, and DB18) were recorded at maturity stage. Both wild type and transgenic seedlings have not shown any significant difference between them in terms of plant height. Each bar value represents the mean ± sd ($n = 20$) (student's t test; $P < 0.05$ versus wild type)

Table 2 Induction pattern of *OsDREB1B* promoter in transgenic *Arabidopsis* plants transformed with *pCAMBIA1391Z* + *OsDREB1B* promoter::*GUS* in response to various stresses

Stress treatment	Leaves	Stem	Roots
Cold (4°C)	Y	Y	Y
1 mM ABA	Y	Y	Y
10% PEG	N	Y	Y
200 mM Mannitol	N	Y	Y
200 mM NaCl	N	Y	Y
10 μM Methyl viologen	N	Y	Y
1 mM Salicylic acid	N	Y	N

Four-week-old transgenic *Arabidopsis* plants were challenged with various stresses for 2 days and *GUS* expression pattern was observed. Y, *GUS* Induction; N, No *GUS* Induction

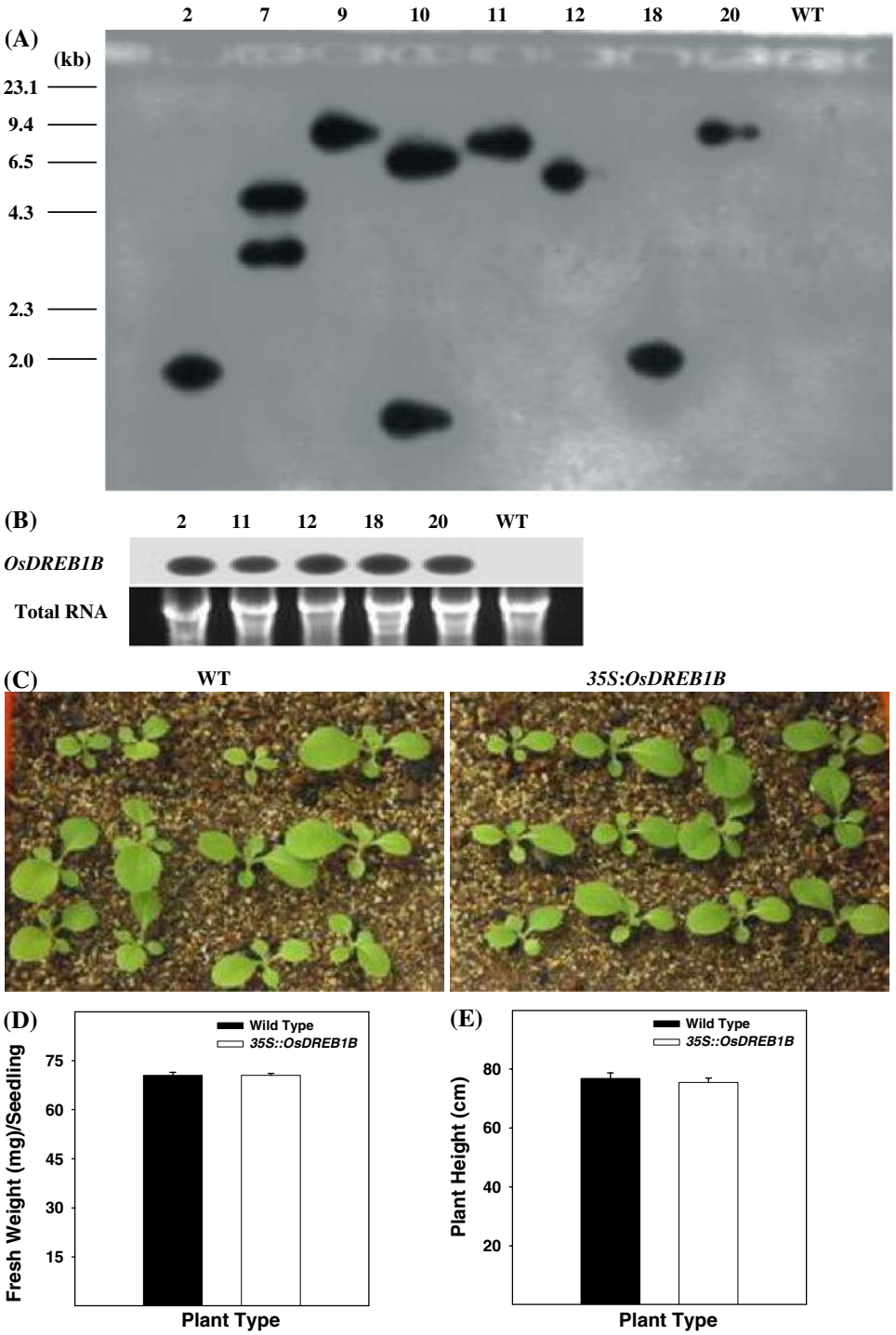
plants, as expected (Fig. 3b). The transgenic plants have not shown any growth inhibition or phenotypic alterations with the wild type. Irrespective of the growth stage, transgenic tobacco plants developed normally, and have not shown any type of growth retardation. Seed setting was also normal. Fresh weight (Fig. 3c, d), and plant height (Fig. 3e) of both wild type and transgenic tobacco plants recorded at seedling and maturity stages respectively showed that there is no significant difference between them.

Transgenic tobacco plants show improved tolerance to mannitol stress

To test the ability of seeds to germinate under osmotic stress conditions, seeds from wild type and homozygous transgenic lines (DB2, DB11, DB12, and DB18) were surface sterilized and placed on 0.5× MS agar medium supplemented with 100 mM mannitol. Observations were recorded 15 days after placing the seeds on the amended MS agar medium. On the 0.5× MS medium (0 mM mannitol), germination of all the lines was about 99%. However, wild type and transgenic tobacco lines have shown different rates of germination in 0.5× MS + 100 mM mannitol (Fig. 4a, b). It was observed that the germination rate of transgenic lines was 40–85%. But, the wild type has shown only 3–5% germination indicating that the seed germination was almost inhibited in the wild type under stress conditions. All the transgenic lines have shown significant increase in rate of germination over the wild type.

Further, 5-day-old seedlings of both wild type and transgenic lines were grown on MS agar supplemented with 200 mM mannitol, and observed their performance under stress. All the transgenic seedlings grew well, and accumulated more fresh weight over the wild type (Fig. 4c, d). In another set of experiments, wild type and transgenic plants (DB18) were continuously exposed to 100 mM, 150 mM and 200 mM mannitol supplied with MS agar medium for 45 days. It was observed that the transgenic plants were growing healthy, whereas the wild type plants were dwarf and developed pale green leaves. After 30 days, stress-induced necrotic spots were observed on the wild type leaves (Fig. 4e). Wild type and transgenic plants have shown about 80% and 41% reduction in chlorophyll content, respectively over the plants growing in unstressed conditions (Table 3). Under normal conditions, both wild type and transgenic tobacco plants grew very well. It was also observed that fresh weight accumulation was similar in both plants. But under stress, transgenic plants have shown significant decrease in growth inhibition over the wild type (Fig. 4f, g). In addition, a densely developed root system was observed in transgenic plants grown on MS agar + 200 mM mannitol. Both transgenic and wild type plants have also shown development of narrow leaves, and shorter internodal length under stress relatively with their respective growth under control conditions.

To get a clear picture of root growth under mannitol stress, 5-day-old transgenic and wild type seedlings were transferred to vertical agar plates containing MS agar supplemented with 200 mM mannitol. The roots of transgenic plants have shown rapid root elongation on amended media than that of wild type plants (Fig. 4h, i). Thus, these results demonstrate that *35S::OsDREB1B* transgenic tobacco plants exhibit a significant resistance to mannitol stress.



Effect of constitutive expression of *OsDREB1B* on membrane stability and free radical scavenging activity under mannitol stress

Five-day-old both transgenic and wild type seedlings were transferred to MS + 200 mM mannitol, and allowed to grow for 30 days. The level of lipid peroxidation in both transgenic and wild type plants was estimated in terms of amount of malondialdehyde (MDA) equivalents by thiobarbituric acid reactive substances (TBARS) assay (Table 4). DB12 and DB18 transgenic lines have shown significantly lower MDA content than the wild type under mannitol stress. Free radical scavenging activity of transgenic plants growing under mannitol stress was estimated by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) as a model free radical as explained in materials and methods. Transgenic

Fig. 4 Mannitol stress tolerance of *35S::OsDREB1B* transgenic tobacco plants. (**a**) Seed germination under 200 mM mannitol stress. Representative images of the germination of respective lines are shown here. (**b**) Germination (%) of wild type and all the transgenic lines shown in (**a**). Each bar value represents the mean ± sd of triplicate experiments (student's *t* test; * $P < 0.05$ versus wild type). (**c**) Phenotype of plants under 200 mM mannitol stress. Five-day-old seedlings were transferred to Petri dishes containing MS agar + 200 mM mannitol, and the phenotypes were observed after 10 days. (**d**) Fresh weights of wild type (WT), and transgenic plants (DB2, DB11, DB12, and DB18) in (**c**) were measured after 10 days of growth under stress, and represented in bar graph. Each bar value represents the mean ± sd ($n = 14$) of triplicate experiments (student's *t* test; * $P < 0.05$ versus wild type). (**e**) Wild type and transgenic tobacco plant leaves under mannitol stress. Wild type showed development of stress induced necrotic spots, where as the transgenic plant leaves were normal after 20 days of growth under mannitol stress. (**f**) Growth performance of tobacco transgenic plants under long term mannitol stress. Five-day-old seedlings were transferred to bottles having various concentrations of mannitol on MS agar medium as mentioned above the images and growth phenotypes were observed after 45 days. (**g**) Growth inhibition of both wild type and transgenic plants growing in (**f**) after 45 days of growth under mannitol stress. Decrease in fresh weight relatively with the plants growing under control conditions (MS + 0 mM mannitol) is calculated as % growth inhibition. Each bar value represents the mean ± sd ($n = 5$) (student's *t* test; * $P < 0.05$ versus wild type). (**h**) Root elongation of transgenic tobacco plants under mannitol stress. Five-day-old seedlings were transferred to MS medium containing 200 mM mannitol. (**i**) Root elongation of wild type (WT), and transgenic lines (DB2, DB11, DB12, and DB18) in (**h**) growing on MS + 200 mM mannitol. Each bar value represents the mean ± sd ($n = 5$) (student's *t* test; * $P < 0.05$ versus wild type) ▶

Table 3 Chlorophyll amount of *CaMV35S::OsDREB1B* tobacco transgenic plants under mannitol stress

Genotype	Chlorophyll (mg/g FW)
Control	3.75 ± 0.05
WT	0.73 ± 0.04
DB18	2.21 ± 0.14

Five-day-old seedlings were transferred to MS medium containing 200 mM mannitol, and observations were recorded after 45 days in a triplicate experiment ($n = 5$). Control plants are same old, unstressed tobacco plants growing on MS agar with out mannitol. Values are mean ± SE

plants have shown significantly higher free radical scavenging activity over the wild type under mannitol stress (Table 5). They have shown 2–2.5 folds higher %RSA over the wild type. These results show that the *35S::OsDREB1B* tobacco plants suffer lower membrane damage due to lipid peroxidation, and also show higher free radical scavenging activity under the osmotic stress induced by mannitol.

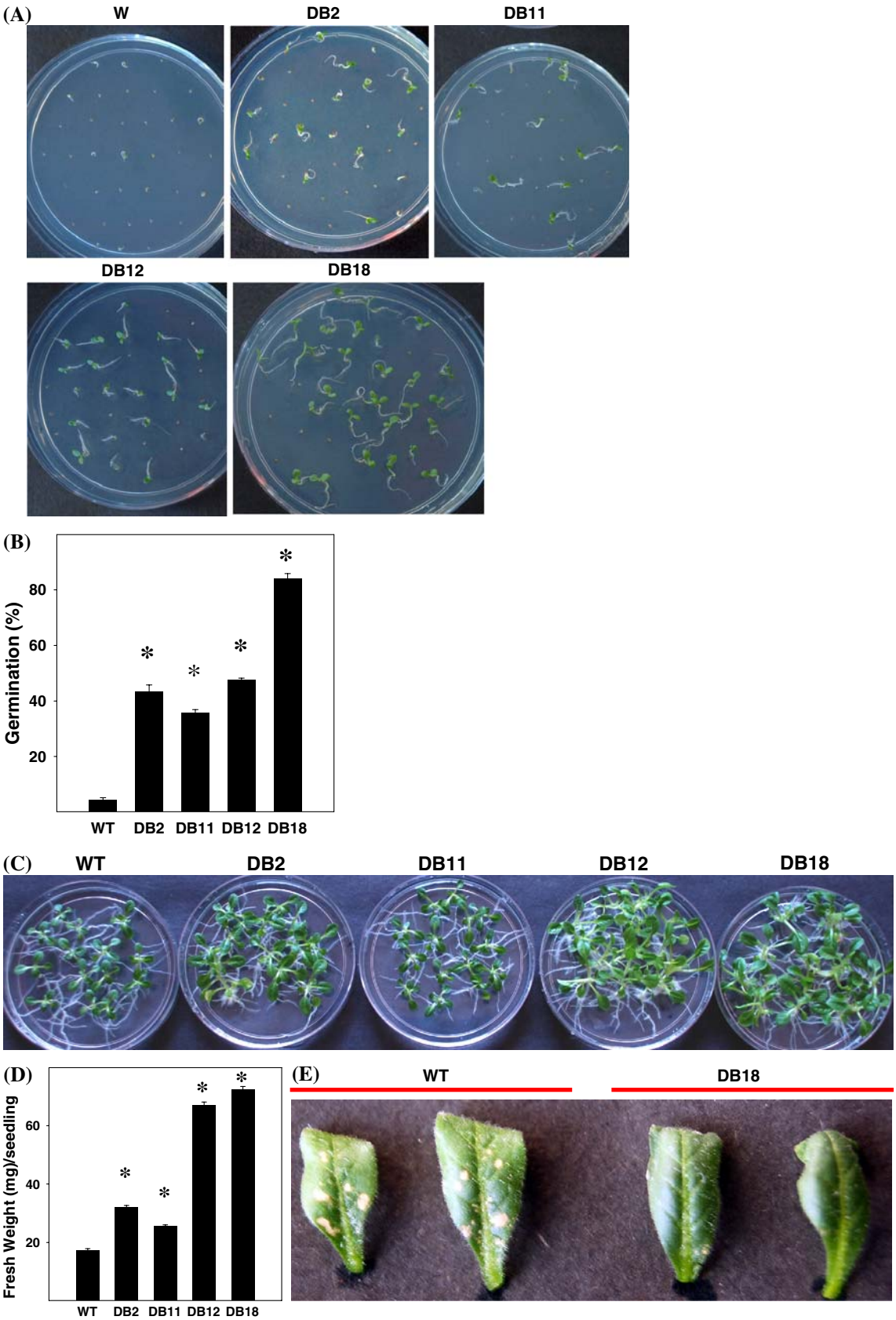
Transgenic tobacco plants show higher biomass accumulation and rapid root elongation under high salt stress

To test for high salt stress tolerance, 5-day-old seedlings of both wild type and transgenic lines were grown on MS agar supplemented with 200 mM NaCl, and observed their performance under stress. All the transgenic seedlings grew well, and accumulated significantly higher fresh weight over the wild type (Fig. 5a, b). Further, to understand the root elongation under high salt stress, 5-day-old seedlings were transferred to vertical agar plates containing MS agar supplemented with 200 mM NaCl. Root elongation was observed to be significantly faster in all the transgenic seedlings compared to that of wild type under high salt stress after 30 days (Fig. 5c, d).

To assess the effect of long-term high salt irrigation, 15-day-old T_3 transgenic (DB18) and the wild type seedlings were watered on alternate days with 200 mM of NaCl until the maturity. Growth of the wild type plants was severely inhibited by the salt-water treatment, whereas the growth of the *OsDREB1B*-expressing transgenic plants was good. It was observed that the canopy of transgenic tobacco plants was significantly bigger than the wild type even 20–30 days after salt treatment. Both the transgenic and wild type plants under stress have shown a significant growth inhibition compared with unstressed plants, but the growth inhibition was relatively less in transgenic plants (data not shown). Transgenic plants have shown delayed flowering, where as the wild type plants did not reach the reproductive stage at all (Fig. 5e). Root volume of the wild plants was relatively lower than that of transgenic plants (Fig. 5f), although both the plants have shown a reduced root system compared to the unstressed plants. Several critical growth parameters such as plant height, root length, and biomass accumulation of both the transgenic and wild type plants were scored as an indicator of tolerance to high salt stress (Fig. 5g–l). The height of transgenic plants was about 62.78% more than that of wild type where as the fresh- and dry-shoot weight of transgenic plants were 40–60% larger than the wild type. The root length of transgenic plants was 64.15% higher than the wild type. It was also observed that the fresh- and dry-root weight of transgenic plants were about 2.5-folds greater than those of wild type tobacco. These data suggest that the overexpression of *OsDREB1B* confers a high degree of tolerance to high salt stress in transgenic tobacco plants.

Transgenic tobacco plants show improved tolerance to drought stress

To evaluate the water stress tolerance of *35S::OsDREB1B* transgenic tobacco plants, plants were challenged by water stress conditions mimicked by poly ethylene glycol (PEG)


(A)
W
DB2
DB11
DB12
DB18
(B)
Germination (%)
80
60
40
20
WT
DB2
DB11
DB12
DB18
(C)
WT
DB2
DB11
DB12
DB18
(D)
Fresh Weight (mg)/seedling
60
40
20
WT
DB2
DB11
DB12
DB18
(E)
WT
DB18

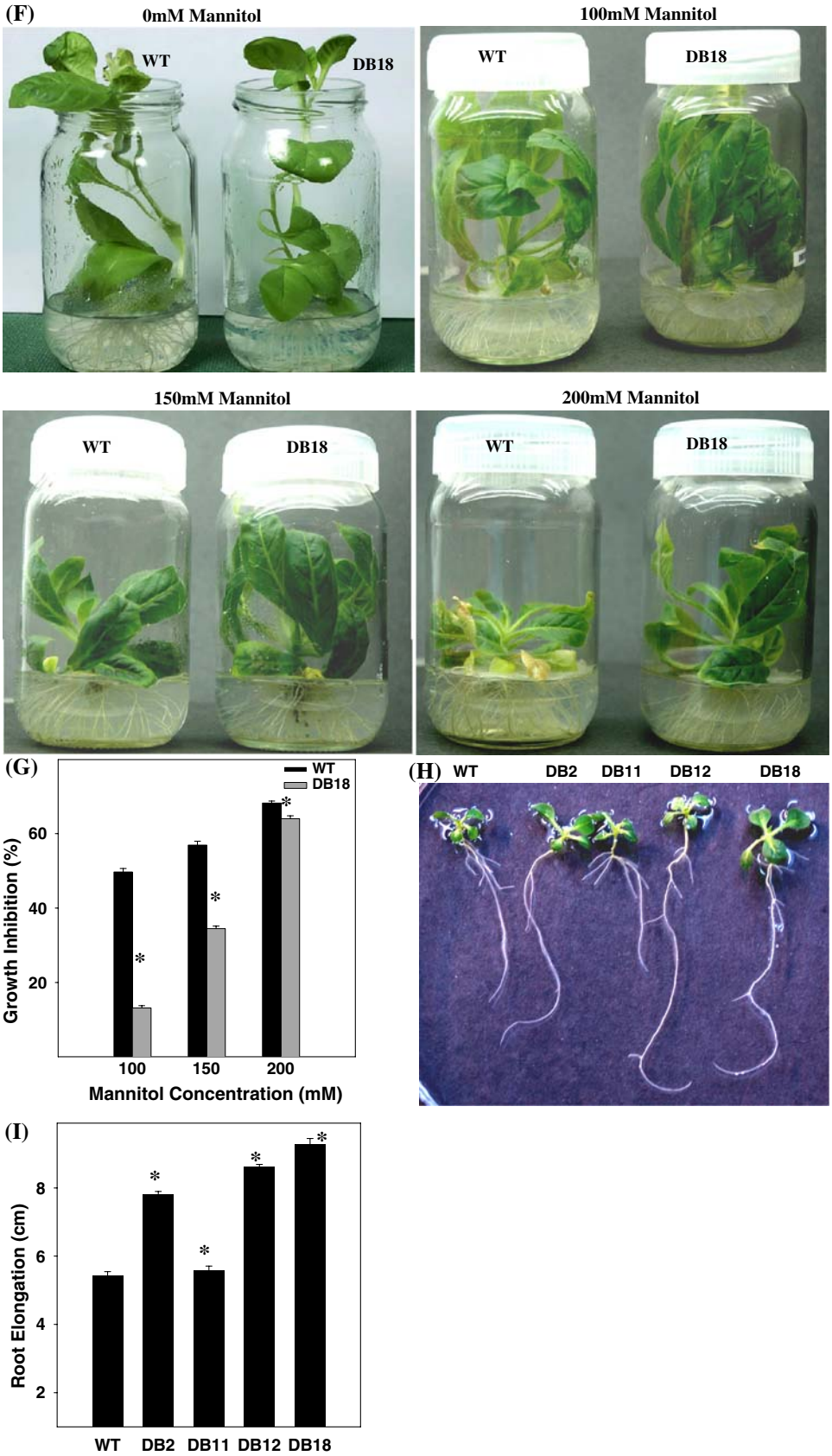


Fig. 4 continued

Table 4 Lipid peroxidation under mannitol stress

Genotype	MDA (μM/g FW)	Decrease in lipid peroxidation over WT (%)
WT	18.54 ± 0.29	–
DB12	16.91 ± 0.24*	8.79
DB18	15.46 ± 0.10*	16.61

Five-day-old seedlings were transferred to MS medium containing 200 mM mannitol, and observations were recorded after 30 days in a triplicate experiment. Malondialdehyde (MDA) released due to osmotic stress was measured in terms of μM/g FW using thiobarbituric acid reactive substance (TBARS) assay. MDA values are mean ± SE ($n = 8$). Student's t test; * $P < 0.05$ versus wild type

Table 5 DPPH free radical scavenging activity under mannitol stress

Genotype	% RSA
WT	9.94 ± 0.78
DB12	28.97 ± 0.64*
DB18	34.54 ± 0.46*

Five-day-old seedlings were transferred to MS medium containing 200 mM mannitol, and observations were recorded after 30 days in a triplicate experiment. Free radical scavenging activity of plants growing under mannitol stress was tested with DPPH assay. Values are mean ± SE ($n = 8$). Student's t test; * $P < 0.05$ versus wild type

6000 stress and also natural water limiting conditions. Five-day-old seedlings of both wild type and transgenic lines were grown on MS agar supplemented with 10% PEG, and observed their performance under stress. All the transgenic seedlings grew well, and accumulated significantly more fresh weight over the wild type (Fig. 6a, b). Similarly, transgenic (DB18) and wild type seedlings were transferred to glass bottles containing MS agar infused with various concentrations of PEG 6000. Both transgenic and wild type plants have shown a significant growth inhibition at all the concentrations of PEG. But, the transgenic plants have shown higher fresh weight accumulation over the wild type (Fig. 6c, d). However, both the transgenic and wild type plants have shown a severe growth inhibition at 20% PEG. Transgenic plants have shown significant decrease in growth inhibition over the wild type only at the 15% PEG6000 concentration.

Further, transgenic (DB18) and wild type plants were grown in soil for 30 days under unstressed conditions. Then, watering was stopped for 21 days. Three to five days after the drought treatment, plants started showing curling and wilting of leaves. However, the wilting of transgenic plants was delayed compared to the wild type. Water content of plants was recorded as g water/g dry weight of the plants at regular intervals (Fig. 6e). After 21 days of water withdrawal, even the transgenic plants showed severe wilting, but retained relatively more water than the wild type. Transgenic plants contained 17.16%, 89.20%, and 135.85% more amount of water at 7, 14, and 21 days, respectively over the wild type. So, it appeared that the water content of transgenic plants remained high during drought treatment than the wild type, which lost its water content very rapidly. These results indicate that the overexpression of *OsDREB1B* can significantly improve drought stress tolerance in tobacco plants.

Enhanced tolerance of *35S::OsDREB1B* tobacco plants to methyl viologen and freezing stresses

Tolerance to a well known oxidative stress inducer, methyl viologen (MV), was assessed in terms of seedling/plant growing capability under methyl viologen induced toxicity. Five-day-old seedlings of both wild type and transgenic lines were grown on MS agar supplemented with 5 μM methyl viologen, and observed their performance under stress. A few of the wild type seedlings survived upto 10 days but even the surviving wild type seedlings succumbed to the MV induced oxidative stress in 15–20 days. All the transgenic seedlings survived and accumulated fresh weight (Fig. 7a, b).Then transgenic (DB18) and wild type seedlings were transferred to bottles containing MS agar medium supplemented with 5 μM, 10 μM, and 25 μM methyl viologen, separately (Fig. 6a) to test the tolerance of transgenic plants at higher concentrations of methyl viologen. At 5 μM methyl viologen concentration, transgenic tobacco seedlings (DB18) survived well, whereas the wild type seedlings were not able to grow and died with in 5–10 days after transfer of the seedlings to the amended medium. However, both the transgenic and wild type plants could not survive at 10 μM and 25 μM methyl viologen concentrations (Fig. 7c).

Freezing stress tolerance was tested in 15-day-old seedlings of transgenic and wild type tobacco plants growing on MS agar 120 mm-Petri plates. Seedlings were exposed to freezing at −5°C for 2 days and returned to normal conditions (25 ± 1°C) for recovery. After 8 days, survival rate and fresh weight were examined (Fig. 8a–c). Survival rate of transgenic plants was observed to be not significantly higher over the wild type. Transgenic seedlings accumulated significantly more fresh weight over the wild type. These observations indicated that the *OsDREB1B* gene conferred freezing stress tolerance in the transgenic tobacco plants.

Constitutive overexpression of *OsDREB1B* resulted in enhanced disease resistance against tobacco streak virus

Transgenic tobacco plants were screened for a newly emerging and rapidly spreading virus threat, tobacco streak virus (TSV), for many crops in India. Wild type and transgenic tobacco plants were inoculated with TSV by

Fig. 5 High-salt stress tolerance of transgenic tobacco plants over expressing *OsDREB1B*. (**a**) Phenotype of plants under 200 mM NaCl stress. Five-day-old seedlings were transferred to Petri dishes containing MS agar + 200 mM NaCl, and the phenotypes were observed after 10 days. (**b**) Fresh weights of wild type (WT), and transgenic plants (DB2, DB11, DB12, and DB18) in (**a**) was measured after 10 days of growth under stress, and represented in bar graph. Each bar value represents the mean ± sd ($n = 14$) of triplicate experiments (student's t test; * $P < 0.05$ versus wild type). (**c**) Root elongation of transgenic tobacco plants under high salt stress. Five-day-old seedlings were transferred to MS medium containing 200 mM NaCl. (**d**) Root elongation of wild type (WT), and transgenic lines (DB2, DB11, DB12, and DB18) in (**c**) growing on MS + 200 mM NaCl. Each bar value represents the mean ± sd ($n = 5$) (student's t test; * $P < 0.05$ versus wild type). (**e–f**) Both transgenic and wild type plants were watered with 200 mM NaCl solution alternate days from seedling stage until maturity. (**e**) Transgenic and wild type tobacco plants growing under long-term high salt stress. (**f**) Root portion of transgenic and wild type plants growing under long-term high salt stress. (**g–l**) Various growth parameters of plants (maturity stage) stressed with continuous watering of 200 mM NaCl were recorded in a triplicate experiment ($n = 10$) and plotted in bar graphs. Black and gray bars represent wild type and transgenic tobacco (DB18) plants, respectively. (**g**) Plant height. (**h**) Shoot fresh weight. (**i**) Shoot dry weight. (**j**) Root length. (**k**) Root fresh weight. (**l**) Root dry weight ▶

mechanical sap inoculation. Mosaic and necrotic lesions were seen on the leaves of the wild type and transgenic tobacco plants 2–3 days after inoculation however, the transgenic plants developed a few number of necrotic lesions. Virus infection spread to young leaves immediately in wild type plants where as, the mosaic and necrotic lesions were observed after 25–30 days on younger leaves of the transgenic plants. While the wild type plants have shown completely stunted growth, transgenic plants have shown a reduction in growth only 25–30 days after inoculation (Fig. 9a–c). The stem portion of the wild type showed extensively developed, coalesced necrotic lesions (Fig. 9d), while the transgenic plants have shown sparsely developed smaller lesions (Fig. 9e). Transgenic plants have shown significant decrease in growth inhibition over the wild type in terms of number of seed capsules and plant height at maturity stage (Fig. 9f). These results indicated that the overexpression of the *OsDREB1B* in tobacco led to an enhanced disease resistance against TSV.

Constitutive overexpression of *OsDREB1B* activates PR gene expression in transgenic tobacco plants

The *35S::OsDREB1B* transgenic tobacco plants have shown moderate tolerance to virus infection, apart from tolerance to various abiotic stresses. We therefore tested whether the *OsDREB1B* transgene had any influence on the expression of disease-responsive genes in transgenic tobacco with out stress treatment. Expression of the eight ethylene-inducible PR genes was analyzed by semi quantitative RT-PCR (Fig. 10). In this study, expression of PR genes, such as *pathogenesis related-1 protein* gene (*PR1b*), *β-1,3-glucanse* (*PR2*), *chitinase* (*PR-3*), *PR4, Osmotin* (*PR5*), *CHN50*, *ACC oxidase* (*ACO*), and *ACC synthase* (*ACS*) was analyzed in both the transgenic and wild type plants with out any stress. The transcripts of the *PR1b* were observed in both transgenic and wild type tobacco plants, but the expression levels were more in all the transgenic lines over the wild type. The expression of the GCC-box containing *PR2*, *PR3*, *Osmotin* (*PR5*) and *CHN50* genes was observed in all the transgenic plants in different levels, but not in wild type tobacco plants. However, the expression of the *PR4*, *ACO*, and *ACS* was not detected in both the wild type and transgenic tobacco plants. Based on these results, we suggest that *OsDREB1B* specifically induces some of the PR genes in transgenic tobacco plants, which were showing moderate stress tolerance to biotic stress induced by tobacco streak virus (TSV) infection.

Discussion

We have isolated the *OsDREB1B* gene from a cDNA library constructed from rice seedlings in response to drought stress. The expression pattern of *OsDREB1B* in response to several stresses, such as mannitol, NaCl, PEG, drought, methyl viologen, salicylic acid, ABA, and cold was studied. A differential expression pattern of the gene was observed in all the stress treatments (Fig. 1). Earlier reports show that *OsDREB1B* is induced in response to only low temperature stress (Dubouzet et al. 2003 and Ito et al. 2006). On the contrary, the *BjDREB1B* lead to enhanced expression in response to NaCl, drought, low temperature, ABA, heavy metal, and salicylic acid stresses (Cong et al. 2008). The difference observed in the expression pattern of *OsDREB1B* from the earlier reports could be due to genetic background differences and/or due to different experimental conditions. Sensitivity of the *OsDREB1B* in response to methyl viologen and salicylic acid indicates that *OsDREB1B* has a role in oxidative stress and disease response in plants. In order to understand its induction pattern, the 5′ region of the *OsDREB1B* was amplified from the rice genome, and cloned into a promoter-less *GUS* gene containing binary vector, *pCAMBIA1391Z,*. The *OsDREB1B* promoter has abiotic- and biotic-stress responsive *cis*-elements apart from several tissue-specific expression and light-responsive elements (Table 1). The analysis of *OsDREB1B* promoter::*GUS* fusion construct in transgenic *Arabidopsis* plants clearly demonstrated that the −745 bp promoter region of *OsDREB1B* directs the expression of the gene in a temporal and stress-specific manner (Fig. 2). Vascular specific activity of *OsDREB1B* promoter specifically in

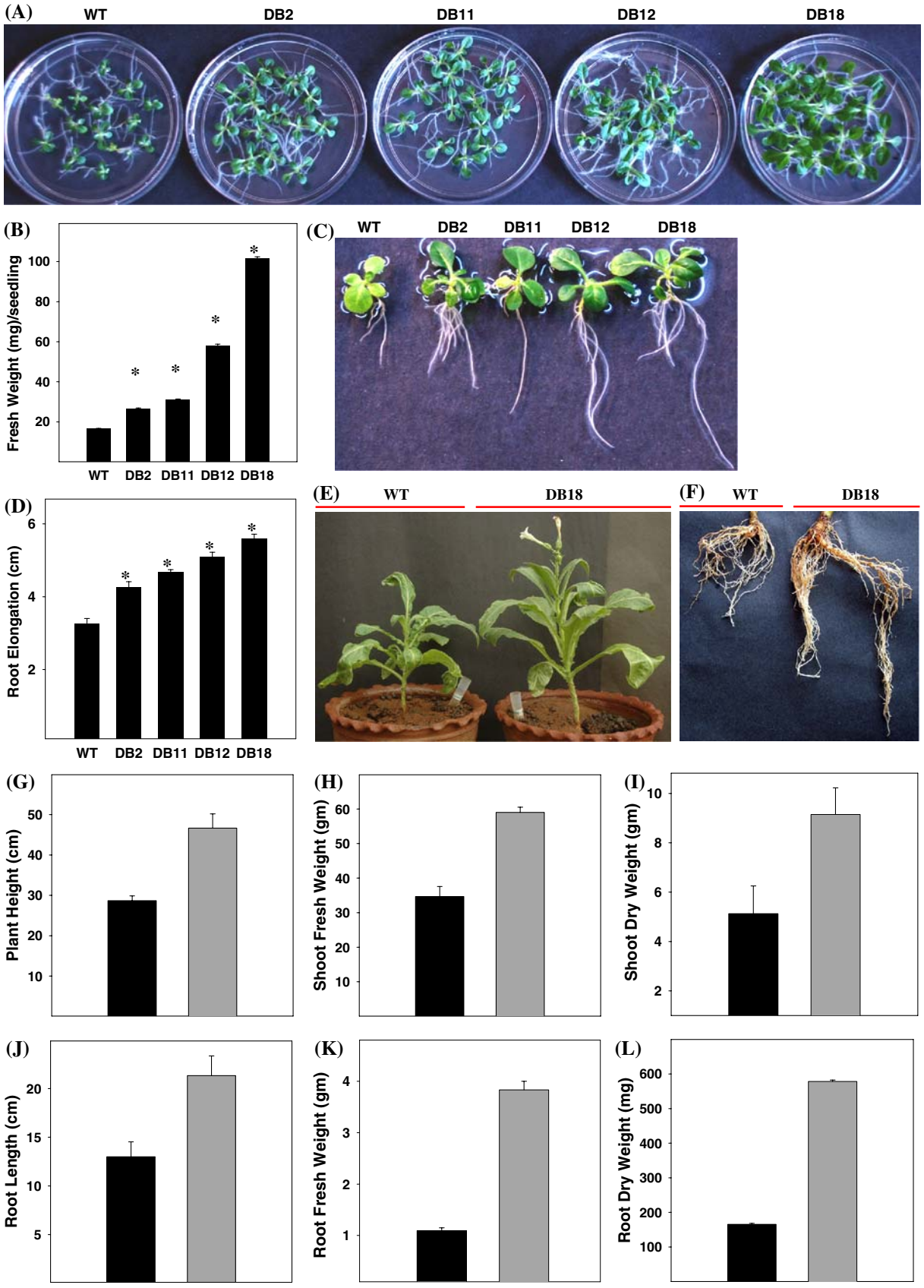
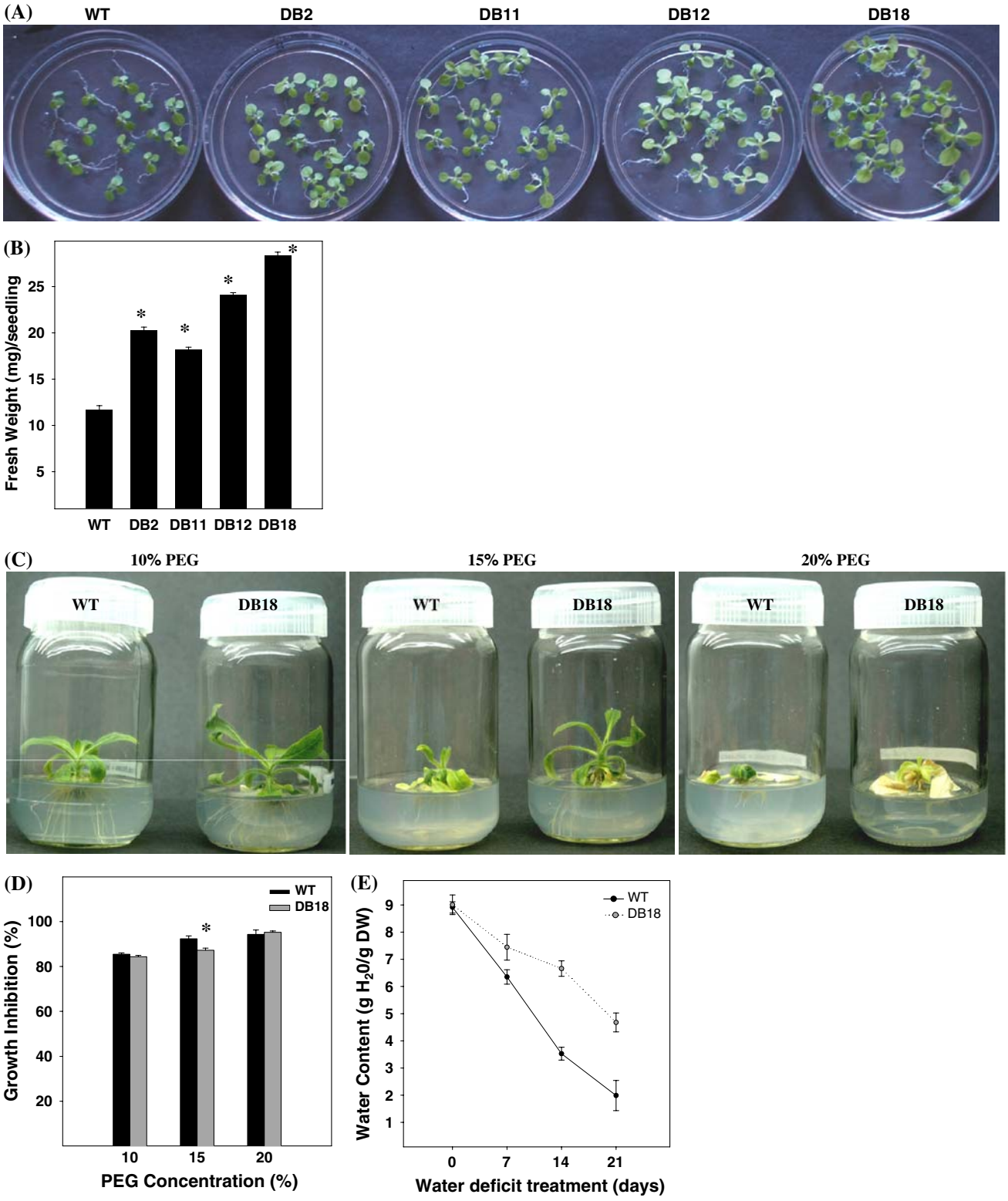

(A)
WT
DB2
DB11
DB12
DB18
(B)
Fresh Weight (mg)/seedling
100
80
60
40
20
WT DB2 DB11 DB12 DB18
(C)
WT DB2 DB11 DB12 DB18
(D)
Root Elongation (cm)
6
4
2
WT DB2 DB11 DB12 DB18
(E)
WT
DB18
(F)
WT
DB18
(G)
Plant Height (cm)
50
40
30
20
10
(H)
Shoot Fresh Weight (gm)
60
50
40
30
20
10
(I)
Shoot Dry Weight (gm)
10
8
6
4
2
(J)
Root Length (cm)
20
15
10
5
(K)
Root Fresh Weight (gm)
4
3
2
1
(L)
Root Dry Weight (mg)
600
500
400
300
200
100

Fig. 6 Drought stress tolerance of *35S::OsDREB1B* transgenic tobacco plants. (**a**) Phenotype of plants under 10% PEG6000 stress. Five-day-old seedlings were transferred to Petri dishes containing MS agar + 10% PEG6000, and the phenotypes were observed after 10 days. (**b**) Fresh weights of wild type (WT), and transgenic plants (DB2, DB11, DB12, and DB18) in (**a**) was measured after 10 days of growth under stress, and represented in bar graph. Each bar value represents the mean ± sd ($n = 14$) of triplicate experiments (student's t test; * $P < 0.05$ versus wild type). (**c**) Growth performance of transgenic plants under long term PEG 6000 stress. Five-day-old seedlings were transferred to bottles having various concentrations of PEG on MS medium as mentioned above the images and growth phenotypes were observed after 45 days. (**d**) Growth inhibition in both wild type and transgenic plants under PEG stress. Growth ▶ inhibition of both wild type and transgenic plants growing in (**c**) after 45 days of growth under mannitol stress. Decrease in fresh weight relatively with the plants growing under control conditions (MS) is calculated as % growth inhibition. Each bar value represents the mean ± sd ($n = 5$) (student's t test; * $P < 0.05$ versus wild type). (**e**) Water content of transgenic tobacco and wild type plants during drought stress. Watering was withheld for one-month-old wild type and T_3 transgenic tobacco line (DB18) in greenhouse conditions for 21 days. Water content was measured in terms of g water/g dry weight at regular intervals and plotted in line graph. Data shown are average and SE values of five independent experiments ($n = 5$)

Arabidopsis early stage seedlings may be due to the presence of several vascular specific elements present in the nucleotide sequence of the promoter. This expression pattern suggests a possible role for *OsDREB1B* in cell differentiation at radicle and plumule development. The rice *DREB4* genes show a differential tissue specific expression pattern in rice seedlings, and are controlled by specific aspects of differentiation and development (Tian et al. 2005). Further, it was observed that various external stimuli could induce the *OsDREB1B* promoter::*GUS* expression in transgenic *Arabidopsis* in a stress-specific and organ-specific manner. Clearly, the promoter was induced in response to osmotic-, cold-, ABA- and salicylic acid-stresses.

The *OsDREB1B* promoter is active in various tissues in response to different stresses albeit, the significance of the unique expression pattern is not clear at present. Under high salt stress, *DREB1* of *Atriplex hortensis* shows higher expression only in roots and the root-specific response of *AhDREB1* might be due to the requirement of more gene product to re-establish the disturbed endogenous environment caused by salt stress in roots (Shen et al. 2003). Thus, under given stress conditions, the *DREB1B* transcription factor is activated in the respective tissues in transgenic *Arabidopsis* plants to activate downstream genes in turn to protect the plants from stress induced damage. Our results indicate that the older leaves of rosette have shown more *GUS* staining than the top-order younger leaves under cold and ABA stresses. In a previous report, a leaf-order-dependent expression of *CBF/DREB1* transcripts was observed by northern analysis in *Arabidopsis* rosette leaves in response to cold stress (Takagi et al. 2003). Interestingly, *OsDREB1B* promoter was induced even in response to 100 μM ABA stress in transgenic plants. *CBF1-3* genes were considered to be showing ABA independent expression in plants (Liu et al. 1998; Medina et al. 1999; Dubouzet et al. 2003). But, *CBF*-dependent *GUS* transcript levels for all the three genes (*CBF1*, *CBF2*, and *CBF3*) increased after 1 h in response to ABA stress, indicating that the addition of ABA could induce the *CBF*s transcription (Knight et al. 2004). The *OsDREB1B* promoter has DRE1 element in the proximal region of promoter unlike the *DREB1B* promoter of *Arabidopsis*. The DRE1 element (ACCGAG) (Busk et al. 1997) found in this promoter is not exactly similar to the classical DRE/CRT (A/GCCGAC) element found in several target genes of *DREB1B* either in rice or *Arabidopsis*. So, it can be assumed that the *OsDREB1B* may not be involved in self-regulation, although only further experimental evidence would make this clear. Apart from several ABA responsive *cis*-elements, ABRERATCAL was observed at −118 in the promoter. ABA dependent expression pattern may be attributable to the presence of these elements. In *rd29A*, it was reported that ABRE cannot work independently, and it needs the cooperative action of DRE/CRT as a coupling element (Narusaka et al. 2003). In *Arabidopsis*, DREB1B was reported as an ABA independent protein and DRE/CRT element was not found in its promoter region (Shinwari et al. 1998). But, in this case DRE/CRT element was observed in the *OsDREB1B* promoter. We have also observed DRE1 and ABRE *cis*-elements in rice *DREB1D* promoter at −111 and −271, respectively, as part of our studies on other rice *DREB1* genes (data not shown). These data suggest that the DRE1 element of *OsDREB1B* promoter might also be working as a coupling element of the ABRE. Induction of *GUS* by *OsDREB1B* promoter in response to 1 mM salicylic acid stress in transgenic *Arabidopsis* indicates that the *OsDREB1B* could be playing a role even in disease, wounding and senescence responses. An EREBP/AP2 gene, *Tsi1*, was also induced by salicylic acid (SA) apart from salt, ethylene, methyl jasmonate, and wound treatments (Park et al. 2001).

Interestingly, constitutive expression of *OsDREB1B* in tobacco plants has resulted in neither growth retardation nor visible phenotypic alterations in transgenic plants (Fig. 3c). But, transgenic rice plants constitutively expressing *OsDREB1B* have shown growth retardation (Ito et al. 2006). Several other transgenic plants constitutively expressing *DREB* genes have shown growth retardation (Hsieh et al. 2002a; Dubouzet et al. 2003; Haake et al. 2003; Kasuga et al. 2004; Cong et al. 2008). However, Dubouzet et al. (2003) observed that *Arabidopsis* transgenic plants over expressing *OsDREB1A* under *CaMV35S*



promoter have shown growth retardation but at the time of bolting only. However, Oh et al. (2005) reported that the transgenic plants (*Ubi1::CBF3*) exhibited neither growth inhibition nor visible phenotypic alterations despite the constitutive expression of *AtDREB1A/CBF3* in rice. From these various reports and our present results, we understand that the growth abnormalities observed in many *DREB* transgenic plants is not a universal phenomenon, and it may be specific to the source of the gene, promoter, host plant, growth stage of the transgenic plant, and the set of

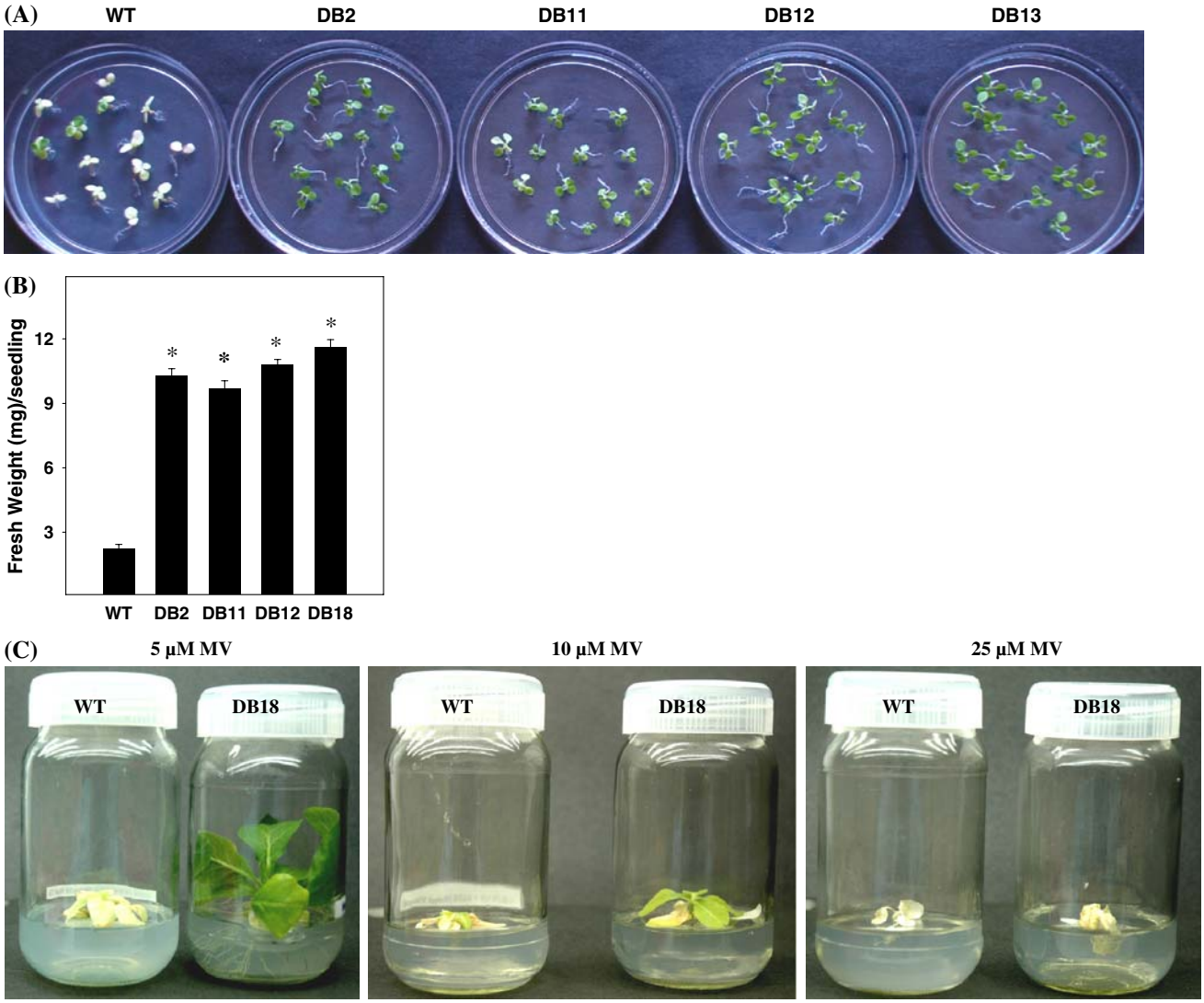


Fig. 7 Methyl Viologen (MV)-induced oxidative stress tolerance of *35S*::*OsDREB1B* transgenic tobacco plants. (**a**) Phenotype of plants under 5 µM methyl viologen stress. Five-day-old seedlings were transferred to Petri dishes containing MS agar + 5 µM methyl viologen, and the phenotypes were observed after 10 days. (**b**) Fresh weights of wild type (WT), and transgenic plants (DB2, DB11, DB12, and DB18) in (**a**) was measured after 10 days of growth under stress, and represented in bar graph. Each bar value represents the mean ± sd ($n = 14$) of triplicate experiments (student's t test; * $P < 0.05$ versus wild type). (**c**) Growth performance of transgenic plants under long term methyl viologen stress. Five-day-old seedlings were transferred to bottles having various concentrations of methyl viologen on MS medium as mentioned above the images and growth phenotypes were observed after 45 days

target genes. Likewise, a fewer number of target genes could be activated by *OsDREB1B* in tobacco than in rice, and thus the negative effect on plant growth might be minimized in tobacco.

Transgenic tobacco lines have shown improved stress tolerance to osmotic stress as evidenced by the improved seed germination, fresh/dry weights of plants, and root growth under mannitol and high salt stresses (Figs. 4, 5). Constitutive overexpression of *OsDREB1B* might be leading to the induction of several genes responsible for maintaining osmotic adjustment in transgenic plants and enabling them to survive well. Under high salt stress, lateral root growth of transgenic tobacco plants was more than the wild type. Many environmental and endogenous factors were reported to affect the process of lateral root formation. Alteration in the levels of phytohormones like ethylene, auxin and ABA or even the mutations in the corresponding signaling pathways would influence the lateral root formation (Brady et al. 2003; Tian et al. 2004). In the present study, *OsDREB1B* promoted lateral root formation under high salt stress, and *OsDREB1B* promoter was induced by ABA. A previous report (Hsieh et al. 2002a) also indicates that *AtDREB1B* could be interfering with the gibberellic acid pathway. Modulation of expression of phytohormones could be the reason for the rapid lateral root formation and rapid growth of *35S::OsDREB1B*

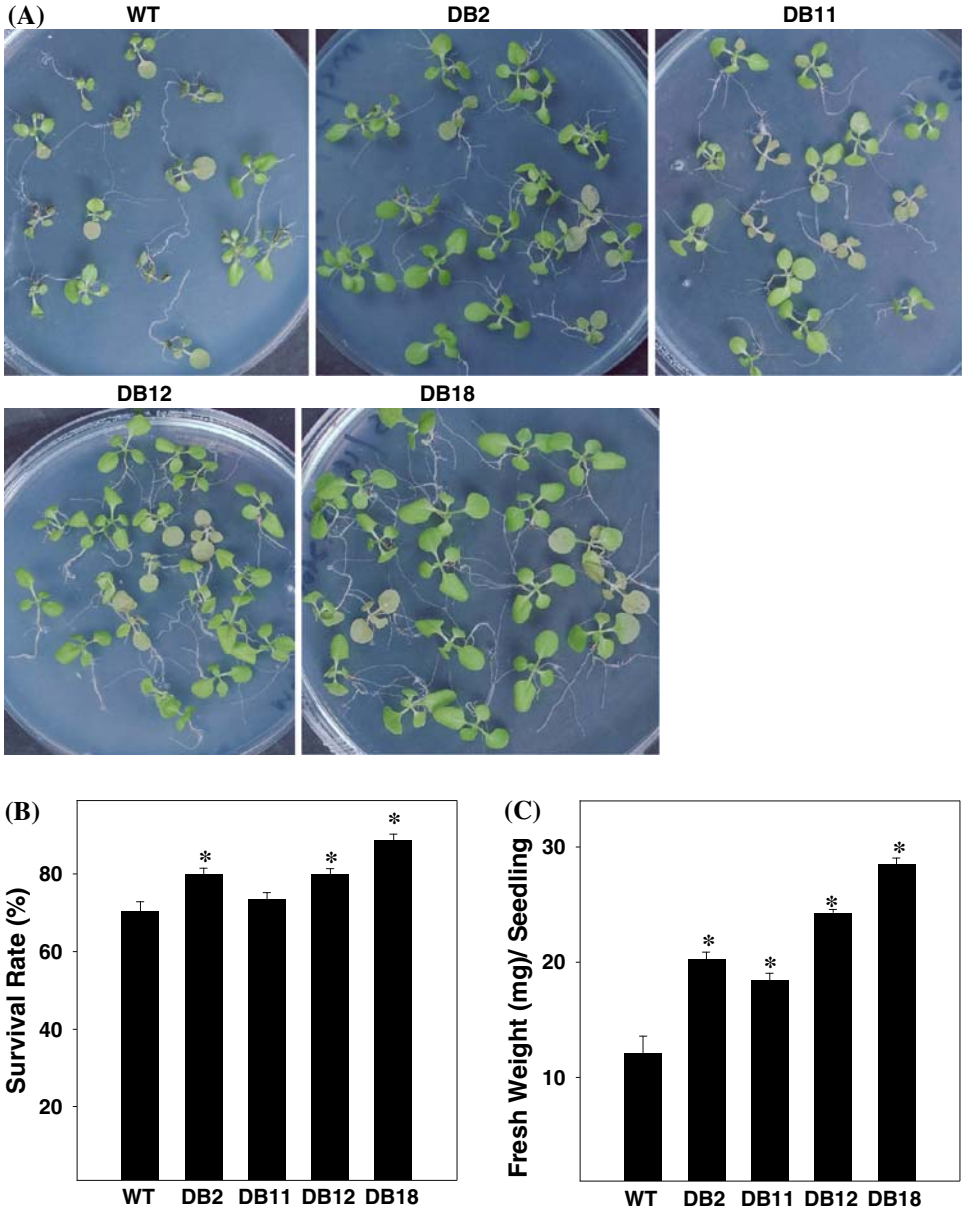


Fig. 8 Freezing stress tolerance of *35S*::*OsDREB1B* transgenic tobacco plants. Fifteen-day-old seedlings growing on MS agar plates (15–20 seedlings/plate) were placed at −5°C growth chamber for 48 h and survival rate was observed 8 days of recovery at normal conditions. (**a**) Phenotype of the wild type (WT), and transgenic lines (DB2, DB11, DB12, and DB18) after recovery at normal temperature. (**b**) Survival rate of wild type (WT), and transgenic lines (DB2, DB11, DB12, and DB18) growing in (**a**) after recovery. Each bar value represents the mean ± sd of triplicate experiments (student's *t* test; * $P < 0.05$ versus wild type). (**c**) Fresh weight of wild type (WT), and transgenic lines (DB2, DB11, DB12, and DB18) growing in (**a**) after recovery. Each bar value represents the mean ± sd of triplicate experiments (student's *t* test; * $P < 0.05$ versus wild type)

tobacco plants compared to the wild type plants under high salt stress.

Biotic and abiotic stresses cause the accumulation of reactive oxygen species (ROS), such as superoxide radical and H_2O_2 (Sunkar et al. 2003). Transgenic tobacco plants, constitutively expressing *OsDREB1B*, have shown moderately lower lipid peroxidation due to mannitol stress (Table 4). Free radical scavenging activity of the transgenic tobacco plants was also significantly higher over the wild type as evidenced by the capability of transgenic plants to scavenge DPPH free radical (Table 5). The damage induced by methyl viologen was less severe in transgenic plants (Fig. 7). It was reported that the reduced susceptibility to paraquat toxicity proves the increased ability of transgenic *Arabidopsis* plants, over expressing spermidine synthase, to scavenge ROS efficiently (Kasukabe et al. 2004). Thus, it is conceivable that the ROS-scavenging and membrane-protecting properties of *OsDREB1B* accounts, at least partially, for the enhanced tolerance to oxidative stress in our transgenic tobacco plants. *AtDREB1B* also imparted improved oxidative stress tolerance in tomato along with the increased levels of catalase (Hsieh et al. 2002a).

Transgenic tobacco plants showing constitutive expression of *OsDREB1B* were more tolerant to dehydration (Fig. 6). Transgenic tobacco plants have put up more fresh weight over a period of time, in comparison to the wild type plants under water stress conditions induced by PEG 6000. Transgenic plants retained more amount of water over the wild type tobacco plants in drought stress

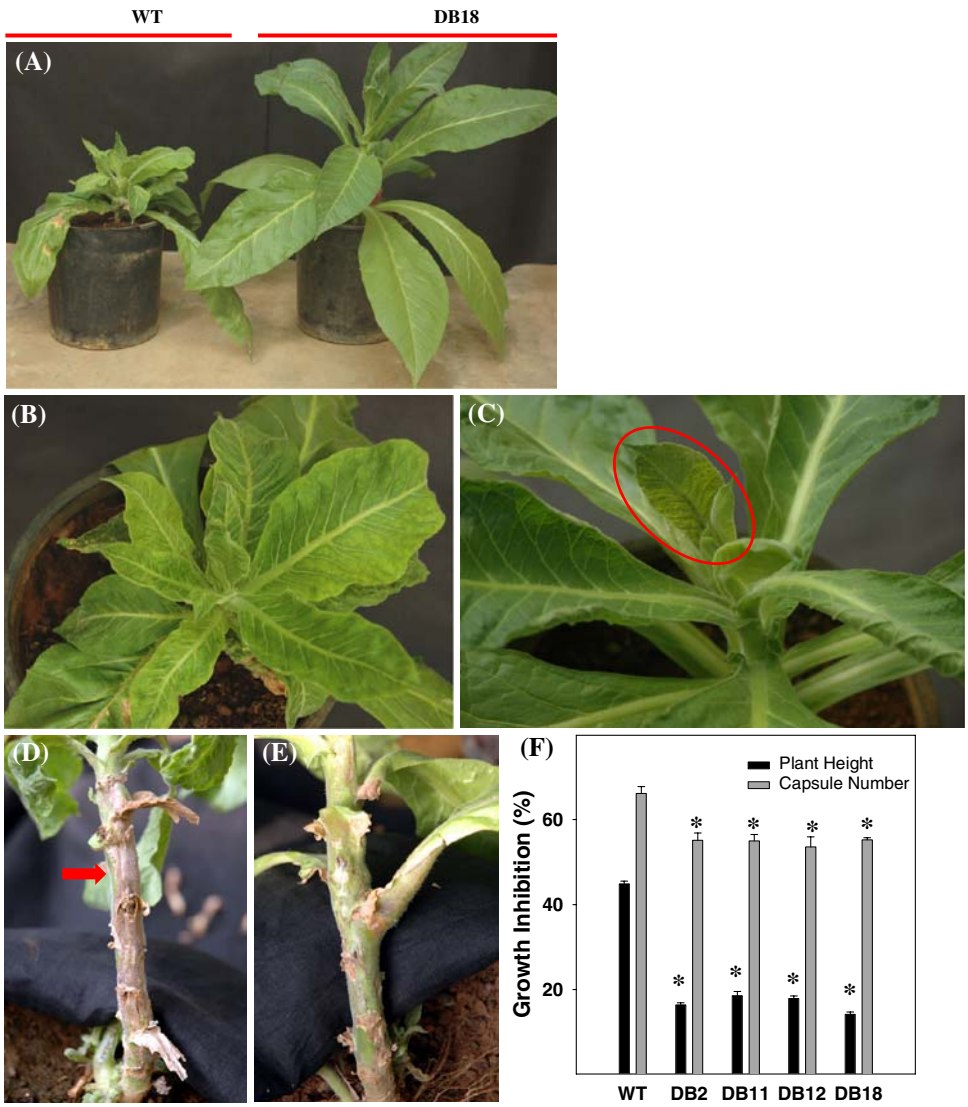


Fig. 9 Enhanced disease resistance in *OsDREB1B*-overexpressing transgenic tobacco plants against tobacco streak virus. *35S::OsDREB1B* and wild type tobacco plants were inoculated with tobacco streak virus (TSV) by mechanical sap inoculation. Fifteen-day-old plants were infected with TSV inoculum. (**a**) Wild type, and transgenic tobacco plants infected with TSV after 45 days of inoculation. (**b**) Wild type plants with mosaic and necrotic lesions after 45 days of inoculation. (**c**) Transgenic plants have shown mosaic and necrotic symptoms on early leaves 25–35 days after inoculation. (**d**) Wild type plants showing extensive stem necrosis at maturity stage. (**e**) Transgenic plants showing low level of stem necrosis at maturity stage. (**f**) Growth inhibition in wild type and transgenic plants at maturity stage due to TSV disease. Decrease in capsule number and plant height relatively with their respective uninfected tobacco plants is calculated as % growth inhibition. Each bar value represents the mean ± sd ($n = 20$) (student's t test; * $P < 0.05$ versus wild type)

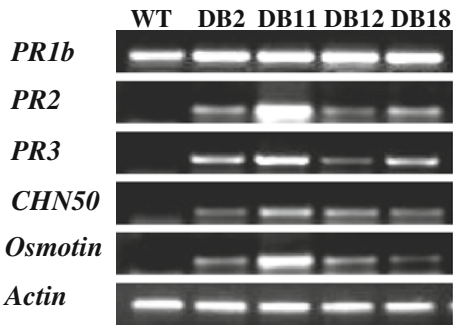


Fig. 10 Expression of the *OsDREB1B* target genes in *35S::OsDREB1B* transgenic tobacco and wild type plants. WT-wild type tobacco plants; DB2, DB11, DB12, and DB18 are T_2 independent transgenic tobacco lines. Transcript levels of various target genes of *OsDREB1B* were determined by reverse transcriptase-polymerase chain reaction (RT-PCR) using gene specific primer-pairs of the corresponding genes. The *Actin* gene was used as equal loading control

experiments. Similarly, water content of transgenic tomato plants constitutively expressing *AtDREB1B* remained high during water stress period. On the contrary, a striking decrease in water content was observed in the wild type tomato plants (Hsieh et al. 2002b).

DREB proteins in various plants were reported to be activating the overexpression of many osmoprotectants like LEA proteins, which have been reported for contributing improved drought stress tolerance. Overexpression of *OsDREB1B* in rice leads to the improved tolerance to drought stress in transgenic plants (Ito et al. 2006). One stress inducible *DREB1A*-responsive gene, *rd29A,* encodes a protein similar to the LEA proteins (Yamaguchi-Shinozaki and Shinozaki 1993). *DREB1*-target genes like *Cor47/rd17* and *erd10* encode group 2 LEA proteins (Dure et al. 1989; Kasuga et al. 2004). Transgenic rice plants over expressing *OsDREB1B* gene have also shown improved

tolerance to drought stress than control plants (Ito et al. 2006). Thus, it is possible that the drought stress tolerance obtained in tobacco plants constitutively expressing *OsDREB1B* might be due to the activation of a large number of its target genes involved in various stress response pathways.

Under freezing stress, transgenic tobacco plants have shown a moderately higher survival rate over the wild type plants (Fig. 8). Constitutive expression of *AtCBF1/DREB1B* in *Arabidopsis* improved the freezing tolerance and causes an enhanced expression of *COR* genes (Jaglo-Ottosen et al. 1998). Target genes of both the *DREB1A* and *CBF1* encode proteins that function in protecting cells from freezing stress (Kasuga et al. 1999). Cold acclimation is associated with the synthesis of novel proteins (Guy et al. 1985) and the expression of *COR* and several other stress-inducible genes (Thomashow 1999). *AtDREB1B* imparts significantly improved tolerance to freezing stress than any other stress where as, *OsDREB1B* have limited effect on tolerance to freezing stress than osmotic and dehydration stresses. Rice is a monocotyledonous plant and it does not cold acclimate unlike *Arabidopsis*, which acclimates to cold effectively (Oh et al. 2005).

Our results showed that *OsDREB1B* is responsive to salicylic acid stress, and its promoter contains several disease responsive *cis*-acting elements. These findings provide new evidence that *OsDREB1B* gene product may also have a role in plant disease resistance. Upon infection of both the wild type and transgenic tobacco plants with tobacco streak virus (TSV), transgenic tobacco plants have shown a few necrotic spots on infected leaf, and delayed development of systemic infection over the wild type (Fig. 9). It has been shown that the overexpression of AP2/EREBP-type transcription factor, *Tsi1*, in tobacco results in enhanced tolerance of transgenic plants to salt and pathogens. Size and density of the lesions, induced by *Pseudomonas syringae* pv. *tabaci*, were very low in *35S*::*Tsi1* tobacco plants over the wild type (Park et al. 2001). These results imply that *OsDREB1B* could also contribute for biotic stress tolerance, apart from osmotic-, dehydration-, and oxidative-stress tolerance.

Constitutive expression of *OsDREB1B* cDNA in transgenic tobacco plants induced the expression of different PR genes such as *pathogenesis related-1 protein* gene (*PR1b*), *β-1,3-glucanse* (*PR2*), *Chitinase* (*PR-3*), *Osmotin* (*PR5*), *CHN50*. But the expression of *PR4, ACC oxidase* (*ACO*), and *ACC synthase* (*ACS*) was not observed in both wild type and transgenic plants (Fig. 10). Interestingly, *OsDREB1B* induced the GCC-box containing *PR1b*, *PR2*, *PR3*, *PR5*, and *CHN50* genes, which were reported as the target genes of various EREBP transcription factors by Ohme-Takagi and Shinshi, (1995). Constitutive expression of *Tsi1* gene in tobacco induced the expression of disease responsive genes such as *PR1*, *PR2*, *PR3*, *PR4*, *Osmotin*, *SAR8.2* in transgenic plants with out any stress. Further, it was also observed that *Tsi1*, EREBP transcription factor also binds DRE/CRT element, apart from the GCC box. Therefore, it is suggested that the biotic and abiotic signal pathways may interact to activate or repress biotic and abiotic response genes in plants and the *Tsi1* protein may have a function. Further, the GCC-box contains GCCGCC core sequence and resembles the DRE/CRT (C/GCCGNC) common core sequence (Park et al. 2001). All these observations suggest that *OsDREB1B* activates several target genes containing GCC-box in addition to the genes having DRE/CRT element in their promoters.

In conclusion, we demonstrate that *OsDREB1B* participates in early stage seedling development, and responds to multiple stresses including, dehydration, ABA, methyl viologen and salicylic acid in a stress-specific manner. Constitutive expression of the cDNA in tobacco leads to a marked increase in tolerance to several stresses in transgenic plants with out any growth inhibition. Under osmotic stress, transgenic plants show higher biomass accumulation, rapid root growth, higher free radical scavenging activity and membrane stability. In addition, transgenic plants overexpressing *OsDREB1B* show improved tolerance to tobacco streak virus, and *OsDREB1B* activates the expression of several ethylene responsive PR genes in transgenic plants. Our findings provide new insights to understand the functional role of OsDREB1B transcription factor in both abiotic and biotic stress response in plants.

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