4: Results
4.1 Isolation, cloning and in-silico characterization of DREB genes

4.1.1 Isolation, cloning and in-silico sequence analysis of cDNA encoding for OsDREB1A

The cDNA encoding for DRE-binding protein 1A (DREB1A), was isolated from Oryza sativa pokkali cDNA. Full length DREB1A gene was amplified by using gene specific primers such as forward 5’-CATATGTGCGGATCAAGCAGGA-3’ and reverse 5’-GTCGACCTAGTAGCTCCAGAG-3’) with the flanking restriction sites Nde I, and Sal I respectively. The size of amplified product was 0.7 kb, and this was confirmed by sequencing. The amplified PCR product was cloned in pGEM-T vector, and the clone was confirmed by restriction with Nde I, and Sal I (Fig. 4.2B). The cDNA clone was compared with genomic sequence and was not found to contain any introns. It encodes for 238 amino acid residues with a predicted molecular mass of 25.3 kDa.

BLAST search revealed that the deduced protein sequence of DREB1A contained a conserved DNA-binding domain, which showed high homology to the EREBP/AP2 domain. The multiple sequence alignment of rice DREB1A protein with other protein sequences such as barley, maize, arabidopsis, wheat, tobacco etc., are showing high homology at NLS, moderate homology in the AP2 domain region and very low conservation in the C-terminal acidic region. However, rice DREB1A proteins have a conserved valine in both 14th and 19th positions of AP2/EREBP (Fig. 4.1A). These amino acids are essential for the recognition and binding of the protein to the target DNA fragment (Sakuma et al., 2002). Phylogenetic analysis of DREB1A proteins with other plant DREB protein showed that the AP2 domain of this protein had high homology with Hordeum (HvCBF3, AAX23692), Arabidopsis (AtDREB1A, ABD14412), Nicotiana (NtDREB1A, ABD65969) (Fig. 4.2C). Multiple sequence and phylogenetic analysis of DREB1A proteins have been done by using Clustal W programme.
Results

A.

Fig. 4.1: Sequence and modular domain analysis of cDNA encoding for pokkali OsDREB1A: (A) Nuclear localization signal region at the N-terminus is indicated by dotted line. The AP2 conserved domain is underlined and two valine residues are at 14th and 19th position are in encircled. (B) DNA-binding domain found in transcription regulators in plants such as APETALA2 and EREBP. In EREBPs the domain specifically binds to the 11bp GCC box of the ethylene response element (ERE).
Fig. 4.2: Cloning and phylogenetic analysis of pokkali OsDREB1A:
(A) Graphical representation of pGEM-T vector. (B) Confirmation of OsDREB1A - pGEM-T clone by restriction digestion with by Nde I – Sal I. (C) Phylogenetic analysis by using Clustal W for OsDREB1A (AF300970) with other proteins named as HvCBF3 (AAY83360), AtDREB1A (ABD14412), NtDREB1A (ABD65969), TdDRF1 (AAY83360), TaDREB1 (ABA08424), ZmDBF1 (AF493800), GhDBP1 (AAO43165), BnCBF1 (AF370733), and AhDREB (AAF76898). (Scale indicates branch length)
4.1.2 Isolation, cloning and in-silico sequence analysis of the cDNA encoding for OsDREB1B

The cDNA encoding for DRE-binding protein 1B (DREB1B), was isolated from Oryza sativa cv pokkali cDNA. Full length DREB1B gene was amplified by using gene specific primers containing forward 5’- CATATGGAGGTGGAGGCG-3’ and reverse 5’-GGATCCTTATAGCTCCAGAG-3’ with the flanking restriction sites Nde I and BamH I respectively. The size of amplified product was 0.65 kb and this was confirmed by sequencing. The amplified PCR product was cloned in pCR-TOPO vector, and then confirmed by restriction Nde I and BamH I (Fig. 4.4B). The cDNA clone was compared with genomic sequence and was not found to contain any introns. It encodes for an open reading frame of 219 amino acid residues (Fig. 4.3) with a predicted molecular mass of 23.2 kDa.

The deduced protein sequence analysis showed it contains a highly conserved AP2/EREBP domain and two conserved functional valine residues at 14th and 19th sites which were responsible for binding between DREB transcription factors and DRE core sequences in the promoter. It also had an alkaline N-terminus amino acid region, which might act as a nuclear localization signal and acidic C-terminal region which might be functional in trans-activation activity (Fig. 4.3). Clustal W analysis of DREB1B protein with other plant DREB proteins showed that the AP2 domain of this protein had high homology with Hordeum (HvCBF3, AAX23692, Arabidopsis (AtDREB1A, ABD14412), Zea mays (ZmDBF1, AF493800) (Fig.4.4C).
Results

Fig. 4.3: Sequence analysis of pokkali OsDREB1B cDNA: DREB1B protein contains a basic region in the N-terminus that might function as a nuclear localization signal (dotted line), AP2/EREBP conserved domain was underlined, two valine residues that are encircled at 14th and 19th positions in the AP2 domain.
**Fig. 4.4:** Cloning and phylogenetic analysis of pokkali OsDREB1B: (A) Graphical representation of pCR-TOPO vector. (B) Confirmation of OsDREB1B-pCR-TOPO clone by digestion with Nde I and BamHI. (C) Phylogenetic analysis by using Clustal W for DREB1B (AF300972) with other protein named as HvBCBF1 (AF298230), AtDREB1B (FJ169278), TdDRF1 (AAY83360), TaDREB1 (ABA08424), ZmDBF1 (AF493800), GhDBP1 (AAO43165), BnCBF1 (AF370733), and AhDREB (AAF76898). (Scale indicates branch length)
4.1.3 Isolation, cloning, and in-silico sequence analysis of cDNA encoding for OsDREB2A

The full length cDNA encoding for DREB2A was amplified from Pokkali rice by using gene specific primers forward 5’-CATATGGAGCGGGGGAGGG-3’ and reverse 5’-GCGGCCGCTAATAGGAGAAAA-3’ with flanking restriction sites Nde I and Not I respectively. The size of amplified product was 0.82 kb, and it was confirmed by sequencing. The amplified product was cloned in pGEM-T vector, and the clone was confirmed by restriction with Nde I and Not I (Fig. 4.6B). The cDNA clone was compared with genomic sequence and was not found to be intronless. It encodes for 275 amino acid residues (Fig. 4.5) with a predicted molecular mass of 36 kDa and an estimated pI of 5.67.

The multiple sequence alignment of DREB2A protein with several DREB2-related proteins of other plant species such as sorghum, wheat, barley, soya beans, arabidopsis, pearl millet etc., are showed highly conservation at AP2/EREBP domain and also had two conserved functional valine (V) and glutamic acid (E) residues at 14th and 19th sites respectively (Fig. 4.5 and 4.8). The protein also possessed highly conserved alkaline N-terminal nuclear localization signal (NLS) and exhibited very low conservation at acidic C-terminal region (Fig. 4.8), which might be functional in trans-activation activity (Stockinger et al., 1997). Each DREB protein has a conserved DNA binding domain of 58 amino acids present in a large family of plant genes, including EREBPs of tobacco and AP2 of Arabidopsis. The alignment between all DREB2 proteins and the deduced phylogenetic tree revealed that this protein is related more closely with monocots (Hordeum DREB2, Sorghum DREB2) than to other dicot plants (Fig. 4.6C). Multiple alignment and phylogenetic analysis was performed by using Clustal W Software.

4.1.4 Phylogenetic relationship of DREB proteins

Sequence analysis showed that the predicted OsDREB proteins have all the characteristic structural domains of CBF proteins. BLAST search revealed that the deduced amino acid sequences contained a conserved DNA-binding domain, which showed high homology to the EREBP/AP2 domain (Fig. 4.7 & 4.8). Apart from the
Results

highly conserved AP2 domain, these predicted proteins also have the CBF signature motifs immediately upstream and downstream of the AP2 domain. Fourteen transcription factors from DREB family have been isolated from rice so far (DREB1 A, B, C, D, E, F, G, H, I; DREB2 A, B, C, D and DREB4-1, 4-2). The phylogenetic tree based on the deduced amino acid sequence of the various DREB proteins from rice showed high similarity between DREB1 A and 1B than between DREB1 C, E, G and H together, and very less similarity to DREB1D, F and I. DREB2 A, B, C, and D are very close to each other (Fig. 4.7). NLS consensus sequence i.e PKRPAGRTKRETRHP is well conserved in DREB1 type proteins which distinguish from rest of the AP2/ERF proteins. Most of these DREB1-type proteins display a well-conserved sequence DSAW and LWSY motifs at the end of their ERF/AP2 and C-terminal domains respectively (Fig. 4.7 and 4.8). All OsDREB1 proteins except OsDREB1D have short (4–5 aa residues) acidic sites in their C-terminus, but the distribution pattern/location is not well conserved. Based on homology it was found that the cDNA clone from pokkali has homology to DREB2A. The DREB2- type proteins also share extensive homologous regions beyond the ERF/AP2 domain especially in the N-terminus region (Fig. 4.8).

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Table 4.1: Completion of various DREB transcription factors known in the complete rice genome and their deduced protein characteristics
Results

A.

Fig. 4.5: Sequence and modular domain analysis of pokkali OsDREB2A: (A) The nuclear localization signal region in the N-terminus indicated by dotted line, AP2 conserved domain was underlined, valine and glutamic acid residues are conserved at 14th and 19th position are encircled, and acidic activation domain at C-terminal is indicated in box. (B) DNA-binding domain found in transcription regulators in plants such as APETALA2 and EREBP. In EREBPs the domain specifically binds to the 11bp GCC box of the ethylene response element (ERE).
Fig. 4.6: Cloning and phylogenetic analysis of OsDREB2A: (A) Graphical representation of pGEM-T vector. (B) Confirmation of OsDREB2A – pGEM-T clone by digestion with by Nde I and Not I. (C) Phylogenetic analysis by using Clustal W for DREB2A (AF300971) with other proteins named as SorDREB2 (ACA79922), TaDREB2 (AAX13274.1), HbDREB2 (AAU29412.1), AsDREB2 (ABS11171), PpDREB2A (AS59530), GmDREB2 (AAQ57226), NtDREB2 (ACE73694), HaDREB2 (AAS82861), AtDREB2A (BAA3379), AtDREB2B (BAA36706), SbDREB2 (ADE35085), AhDREB2A (ABC60025), PgDREB2A (ABB05044), OsDREB2B (AK09922), and FaDREB2 (CAG30548). Scale indicates branch length.
Fig. 4.7: Multiple sequence alignment of DREBs family proteins of rice (Continued)
Results

Fig. 4.7: Multiple sequence alignment of DREBs family proteins of rice: shaded letters is showing the conserved amino acid residues in each DREB protein. NLS indicated by straight line, AP2 domain showed as dotted line and triangle symbol indicated the 14th valine and 19th glutamic acid conserved amino acids in AP2/EREBP domain.
### Results

![Multiple alignment of amino acid sequences of OsDREB2A and related proteins](image)

**Fig. 4.8:** Multiple alignment of amino acid sequences of OsDREB2A and related proteins: (Continued..)
Results

Fig. 4.8: Multiple alignments of amino acid sequences of OsDREB2A and related proteins: shaded letters is showing the conserved amino acid residues in each DREB protein. NLS indicated by straight line, AP2 domain showed as dotted line and triangle symbol indicated the 14th valine and 19th glutamic acid conserved amino acids in AP2/EREBP domain.
4.2 Expression of OsDREB transcripts under different abiotic stresses

The expression of OsDREB1A and OsDREB2A transcripts were studied in response to osmotic, salt, and low temperature stress by northern blot analysis. RNA isolated from 15-d-old pokkali rice plants which were subjected to various abiotic stresses under different time intervals (0, 1, 5, 10, and 24 hrs). After 5 h of exposure to low temperature, DREB1A was activated and induced strongly, and transiently induced in response to salt and drought stresses (Fig. 4.9). The DREB2A transcript was induced within 5 h and gradually increased till 10 h of exposure to drought stress and constitutively expressed after 24 h of treatment. In response to salt, the DREB2A transcript was induced within 5 h and gradually decreased, whereas in response to cold stress it was not accumulated in all time points (Fig. 4.10). These results indicated that the transcription of the OsDREB1A gene is activated by cold stress and that of the OsDREB2A gene was by dehydration and salt stresses. Equal loadings of RNA in all samples were demonstrated by rehybridizing the blots with tubulin gene, in which single band with same intensity was detected (Fig. 4.9 and 4.10)
Fig. 4.9: Northern blot analysis of the OsDREB1A transcripts under various stress conditions: 14-d old rice seedlings were dehydrated (drought), salt (200 mM NaCl), and cold treated (4°C) at different time intervals (0, 1, 5, 10, and 24 hrs). Total RNA isolated from treated leaves, and then fractionated by electrophoresis in formaldehyde agarose gel. DIG labelled DREB1A and tubulin was used as a probe for hybridization.
Fig. 4.10: Northern blot analysis of the OsDREB2A transcripts under various stress conditions: Fourteen days old rice seedlings that had been dehydrated (drought), salt (200 mM NaCl), and cold treated (4ºC) at different time intervals (0, 1, 5, 10, and 24 hrs). Total RNA isolated from treated leaves, and then fractionated by electrophoresis in formaldehyde agarose gel. DIG labelled DREB2A and tubulin was used as a probe for hybridization.
4.3 Expression and purification of OsDREB2A protein from *E. coli*

In order to express and purify recombinant OsDREB2A protein, the *DREB2A* gene was cloned in the expression vector pET-28a (+) at the *BamH* I and *Sal* I restriction sites (Fig. 4.11B) and construct was transformed to *E. coli* rosetta strain. The histidine tag was at the N-terminal end of the protein. The cloning was confirmed by restriction digestion analysis (Fig. 4.11C). *E-coli* rosetta strain cells were grown in LB medium containing kanamycin at 37°C and induced for 3 hr at 1M isopropyl D-thiogalactoside (IPTG) (Fig. 4.12A). The recombinant protein could not be purified in its native form because of the probability of His-Tag being buried inside and thereby interfering with binding to the Ni-NTA affinity column. The recombinant protein was thereafter purified through affinity column (Ni-NTA column) in denatured form by using 8 M Urea and 150 mM Imidazole (Fig. 4.12B). Eluted fractions were dialyzed against decreasing gradient concentrations of Urea (8, 6, 4, 2, 1, 0.5 and 0.0 M). At each concentration dialysis was done at 4°C for at least two hours to allow refolding of the protein to its native form again (Fig. 4.12C). The purity of OsDREB2A recombinant protein was confirmed by using OsDREB2A specific antibodies and western analysis showed a band at the expected molecular weight ≈ 36 kDa (Fig. 4.12 D).
Results

Fig. 4.11: Cloning of OsDREB2A in pET 28a (+) expression vector: (A) Graphical representation of pET-28a(+) vector. (B) PCR amplification of DREB2A by using gene specific primers. (C) Confirmation of pET-28a(+)-OsDREB2A clone by restriction with SalI & BamHI.
Fig. 4.12: Expression and purification of recombinant OsDREB2A. (A) Protein induction in *E. coli* with 1mM IPTG at 37°C for 4 h. (B) Purified protein in denatured form. (C) OsDREB2A recombinant refolded protein purified within Ni-NTA column. (D) Western blot with OsDREB2A antibodies.
4.4 Isolation and *in-silico* analysis of *OsDREB2A* promoter

To identify the *cis*-acting elements responsible for the transcriptional regulation of *OsDREB2A* gene in Pokkali, we have isolated and analyzed promoter sequence. The promoter was amplified by using nested primers from the genomic DNA sequence database of Japonica rice for PCR reactions.

First PCR - Forward primer 5’ GCGTCGCGTCTGTTGCGTCA 3’
Reverse primer 5’ CAACTACTCCACAACCAACCA 3’

Nested PCR - Forward primer 5’ GTAGATCAGCGGCCATGGTAT 3’
Reverse primer 5’ CAGAGATTTCATCAGGAGA 3’

An upstream region of *OsDREB2A* gene was amplified from Pokkali rice (Fig. 4.13B). The amplified product (1.5 kb) was cloned in pCR-TOPO cloning vector and confirmed by sequencing (Fig. 4.13C). Promoter sequence search against the PLACE data base (http://www.dna.affrc.go.jp/PLACE) suggested that more than one copy of putative DRE (Dehydration Responsive Element) and ABRE (ABA Responsive Element) are existed. We found that this promoter containing TATA box and CAAT box elements. In addition, there are many consensus sequences matching the putative regulatory factor binding sites such as C-repeat Binding Factor (CBF), Dehydration Responsive Element (DRE), ABsiscic acid Responsive Element (ABRE), MYB, MYC, Heat Shock Element (HSE), WUN (wound responsive element), LTRE (Low Temperature Element), and WRKY (Fig. 4.14).

4.5 *rd29A*: a stress inducible promoter

An upstream promoter region of *rd29A* gene was isolated from *Arabidopsis* genomic DNA by using promoter specific primers (forward 5’- GGTACCGACTCAAAAACACCTTACTT-3’ and reverse 5’-CATATGAATCAACCCCTTTAT-3’) with the flanking restriction sites *Kpn* I and *Nde* I respectively (Fig. 4.15B). The amplified 0.82 Kb fragment was cloned in pCR-TOPO vector (Fig. 4.15C). The digested product of promoter was cloned in plant transformation vector. The upstream region of *rd29A* gene having the many number of TATA and CAAT box elements. In addition, there are many *cis*-acting elements such as DRE, CRT, ABRE, HSE etc., (Fig. 4.15A).
Fig. 4.13: Cloning and sequence analysis of OsDREB2A promoter from Pokkali: (A) Graphical representation of pCR-TOPO vector. (B) PCR amplification of 1.5kb OsDREB2A promoter from pokkali rice. (C) Confirmation of OsDREB2A promoter by colony PCR.
Fig. 4.14: Sequence of promoter region of OsDREB2A gene: The putative stress responsive cis-elements are marked in colours.
Fig. 4.15: Cloning and sequence analysis of Arabidopsis rd29A promoter: (A) Sequence of rd29A promoter showing putative cis-elements. (B) PCR amplification of rd29A promoter from Arabidopsis genomic DNA. (C) Confirmation of cloning of rd29A promoter in pCR-TOPO vector.
4.6 Constructions of plant transformation vector carrying OsDREB1A gene

The cDNA encoding for DREB1A gene product was cloned under the control of stress inducible rd29A promoter in N-terminus and poly A terminator at C-terminus region of binary vector pGreen0179. The promoter, gene and terminator were cloned in Kpn I-Nde I, Nde I-Xho I and Not I-Sac I respectively in MCS region of T-DNA (Fig. 4.17A). The colonies were selected on the kanamycin, were checked with by colony PCR. Promoter and gene cassette were digested with Kpn I and Xho I, which released the 1.7 kb fragment (Fig. 4.17B). Confirmed pGreen0179::OsDREB1A and pSoup plasmids were co-transformed in to Agrobacterium strain LBA 4404. It was confirmed by using promoter, gene and terminator specific primers to amplify rd29A (0.82 kb), DREB1A (0.71 kb) and poly A (0.2 kb) respectively (Fig 4. 17C).

4.7 Constructions of plant transformation vector carrying OsDREB1B gene

The cDNA encoding for DREB1B gene product was sub cloned under the control of stress inducible rd29A promoter in N-terminus and poly A terminator at C-terminus region of binary vector pGreen0179. The promoter, gene and terminator were cloned in Kpn I-Nde I, Nde I-Not I and Not I-Sac I respectively in MCS region of T-DNA (Fig. 4.18A). The colonies grown on the kanamycin selected plates were checked with by colony PCR using promoter specific primers to amplify the 0.82kb (Fig. 4.18C). Promoter and gene cassette were digested with Kpn I and Not I, which released the 1.4 kb fragment (Fig. 4.18B). Confirmed pGreen0179::DREB1B plasmid and pSoup plasmids were co-transformed in to Agrobacterium strain LBA 4404.
Fig. 4.16: Plant transformation binary vector: (A) pGreen 0179, the T-DNA containing cloning sites. (B) pSoup, a modified RK2 plasmid which carried the pSa replicase gene. (C) Rep A gene acts in trans upon the pSa Ori sequence.
A. Fig. 4.17: Construction of OsDREB1A plant expression vector pGreen0179: (A) Schematic representation of T-DNA of pGreen0179, in which LB and RB containing 35S-hygromycin phosphotransferase marker gene cassette and rd29A:OsDREB1A:terminator cassette respectively. (B) Digestion of construct with Kpn I and Sac I (1.7 kb). (C) PCR amplification of final construct with specific primers such as rd29A promoter (0.82 kb), OsDREB1A gene (0.71 kb), Poly A terminator (0.2 kb). M: molecular size 1kb marker.
Fig. 4.18: Construction of OsDREB1B plant vector pGreen0179: (A) Schematic representation of T-DNA of pGreen0179, in which LB and RB containing 35S-hygromycin phospho transferase (hpt) marker gene and rd29A:OsDREB1B:poly A respectively. (B) Digestion of construct with by Kpn I and Not I enzyme (1.4 kb). (C) PCR amplification of rd29A promoter (824bp) M: molecular size 1kb marker.
4.8 Rice transformation: Generation of drought tolerant rice

4.8.1 Construction of plant transformation binary vector pGreen0179 carrying OsDREB2A gene for rice transformation
The cDNA encoding for DREB2A was fused with rd29A stress inducible promoter in its N-terminus to drive the expression of the gene and poly A terminator at C-terminus region in T-DNA region of pGreen0179 vector. The promoter, gene, and terminator were cloned in Kpn I-Nde I, Nde I-Not I and Not I-Sac I respectively (Fig. 4.19A). The hpt gene (hygromycin phospho transferase) serves as the plant transformation selection marker which driven by the 35s cauliflower mosaic virus promoter. The colonies grown on the kanamycin selected plates were checked with by colony PCR. The gene (OsDREB2A; 0.82 kb) and whole construct (rd29A: OsDREB2A: polyA; 1.8 kb) were amplified by using gene specific and promoter forward-terminator reverse primers respectively (Fig. 4.19 B and C). Finally the construct was digested with Kpn I and Sac I for the confirmation of cloning. The digested product was 1.8 kb (Fig. 4.19D). The DREB2A expression construct and pSoup plasmids were mobilized in to Agrobacterium starin LBA4404 for rice transformation.

4.8.2 Development of rd29A::OsDREB2A transgenic rice plants
Embryogenic calli was induced from scutellar region of mature seeds of rice (Fig. 4.20A) swarna (MTU 7029) on MS medium with 2.5 mg/l 2,4-D (2,4-di-chloro phenoxy acetic acid). Twenty one day old globular, healthy, friable, and cream coloured embryogenic calli (Fig. 4. 20B & C) were used for transformation mediated by Agrobacterium. Approximately, 200 embryogenic calli were infected with Agrobacterium strain LBA4404 containing binary vector system, pGreen 0179 harboring the OsDREB2A expression cassette (rd29A:OsDREB2A:poly A-Fig. 4.19) for 10 min with gentle shaking. The infected calli was co-cultivated in MS medium (pH 5.2) supplemented with acetosyringone (100 μM ) for 72 hrs and incubated under dark condition at 27±28°C. After two days of co cultivation, calli washed with 250 mg/l cefotaxime to remove Agrobacterium and cultured on MS medium containing cefotoxime. Then the calli transferred on to MS selection medium containing 50 mg/l hygromycin as the selection agent. Hygromycin allowed clear distinction between transformed and non transformed calli (Fig. 4. 20E). The resistant calli were selected
after two rounds of selection pressures. Only few calli proliferated in the selection medium while others turned dark and eventually dried off. This was attributed to the expression of *hpt* gene in the transformed calli. Hygromycin resistant calli which survived two rounds of selection was transferred to shoot regeneration medium (Fig. 4. 20F). Number of regenerated plants per callus on regeneration medium was counted. Thirty seven plants were regenerated and plantlets were transferred onto ½ MS medium for rooting for 10 days (Fig. 4.20 G and H). The plants were subsequently transferred to vermiculite pots for hardening and finally transferred to pot cultures (Fig. 4.20I).
Fig. 4.19: Construction of OsDREB2A plant expression vector pGreen0179:
(A) Schematic representation of T-DNA of pGreen0179, in which LB and RB containing 35S-hygromycin phosho transferase marker gene and rd29A: OsDREB2A: poly A respectively. (B) Colony PCR: Amplification of OsDREB2A gene by using gene specific primers (0.82 kb). (C) Amplification of complete construct with by using rd29A F and poly A R primers. The colonies 3, 4, 10, and 12 are recombinant positive clones. (D) Confirmation of construct by restriction with by Kpn I and Sac I (1.8 kb).
Fig. 4.20: Generation of OsDREB2A transgenic plants: (A) structure of rice grain showing the callus inducing scutellar region. (B&C) callus induction from mature seeds. (D) callus excision (E) resistant callus on hygromycin containing selection media. (F) proliferation of resistant callus leading to regeneration. (G) shoot formation and rhizogenesis (H) plantlets growing in the bottles. (I) plants growing in the green house
4.9 Molecular analysis of transgenic rice plants

4.9.1 Molecular detection of transgene by polymerase chain reaction

The PCR amplification of transgenes was performed by using the genomic DNA isolated from the putative transgenic (T₀ and T₁ generation) and non transformed plants of swarna rice. The integration of the marker cassette and gene cassette into the genome of transgenic rice was initially detected by nested PCR using 35s-hpt and rd29A-OsDREB2A primers. We have analyzed 37 independent T₀ transformed lines, out of them, 11 plants (To-3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, and 35) were found to be positive for 35s-hpt selection marker gene (Fig. 4.21A) and rd29A-OsDREB2A gene cassette (Fig. 4.21B) of expected amplification products were 0.95 kb and 0.7 kb respectively. The PCR analysis of amplified product size in the positive plants is equal to the positive control and there was no amplification in the non transformed control plant. It provided evidence of the incorporation of T-DNA in to rice chromosome. There was no amplification in T₀-6 for hpt and T₀-7 for DREB2A, suggesting that transgene could be partly integrated in to the genome of these two plants (Fig. 4.22 A and B).

<table>
<thead>
<tr>
<th>Set no</th>
<th>Primer</th>
<th>Sequence</th>
<th>Purpose of primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>35s F1 HptR1</td>
<td>5'–AAGATGCCCTCTGCGACAGT-3' 5'–CCAGCCGAGCGAGCTTAGCG-3'</td>
<td>First PCR primers for Hpt</td>
</tr>
<tr>
<td>2</td>
<td>35sF2 HptR2</td>
<td>5’–TAAGGGATGACGACAATCCCAC-3' 5’–ATTGCCCGTCAACCAAGCTCTCA-3'</td>
<td>Nested PCR primers for Hpt</td>
</tr>
<tr>
<td>3</td>
<td>rd29aF1 DREB2AR1</td>
<td>5’–ACGAAATTTAAGGTAAGAATTTA-3' 5’–CACGTTTCAACCCATAGGCCTCTC-3'</td>
<td>First PCR primers for rd29a-DREB2A</td>
</tr>
<tr>
<td>4</td>
<td>rd29aF2 DREB2AR2</td>
<td>5’–GAAGGTGTGGGTCTGGTTAT-3' 5’–CCAGAGTTGTCATCTGGAA-3’</td>
<td>Nested PCR primers for rd29a-DREB2A</td>
</tr>
</tbody>
</table>

Table. 4.2: Primers used for analysis of transgenes in transgenic plants
4.9.2 Determination of gene copy number by Southern blotting

To confirm the integration of T-DNA and determination of the copy number southern hybridization was performed. To carry out this, Genomic DNA was extracted from putative PCR positive lines and from non transformed (control) line. Ten microgram aliquots of DNA were digested with 60 units of Xba I, which is non cutter of T-DNA portion. The T-DNA (35s-hpt and rd29A- OsDREB2A) coding sequence (4.4kb) was used as a probe for hybridization. The T-DNA of pGreen0179 has no Xba I site, so the number of hybridizing bands reflects the number of copies of integrated genes in the genome. Bioinformatics analysis revealed that there is only one endogenous copy of DREB2A gene in the genome of rice, so it could be was hybridized in all plants including non transgenic plant. The mobility of native DREB2A could be expected at 3.4kb. The detected bands represented the fragments more than 4.4 kb, which is the minimum size of hybridizing fragment expected. The mobility of the bands differed from plant to plant. The number of hybridized bands showed that the gene was integrated in to the genome as single or multiple copies. Plants regenerated from same colony of cells gave an identical pattern, indicating that these plants were clonal. As shown in Figure 4. 22 transgenic lines OsDREB2A- 3, 9, 10, 11, 12 and 13 gave an identical pattern containing single integration. Two copies were integrated in 14th; four and five copies were integrated in 5th and 8th lines respectively (Fig. 4.22). The hybridized bands representing more than 4.4 kb fragments, indicating that the whole T-DNA integrated in to genome.

<table>
<thead>
<tr>
<th>Calli cocultivated</th>
<th>Hyg$^r$ Plants regenerated</th>
<th>PCR positive plants</th>
<th>Southern positive plants (9)</th>
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<tr>
<td>200</td>
<td>53</td>
<td>37</td>
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<td></td>
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<td>1</td>
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</tbody>
</table>

Table. 4.3: Representation of transformation frequency and number of positive plants
Results

Fig. 4.21: PCR analysis of transgenic T₀ plants: A. Amplification of 0.95 kb region of hpt expression cassette, B. and 0.7 kb region of transgenic OsDREB2A expression cassette from genomic DNA derived from putative transgenic T₀ rice plants. P: Positive control (plasmid DNA template); N: Negative control (no DNA template); C: Untransformed control rice DNA template; Lanes represent putative transformed rice DNA templates. M: 1 kb DNA ladder.
Results

Fig. 4.22: Southern blot analysis of OsDREB2A transgenic rice (T₀) lines: DNA (10 µg) from each sample was digested with Xba I, a non-cutter restriction endonuclease in the T-DNA region and separated on a 0.8% agarose gel, transblotted onto nylon membrane and probed with rd29A-OsDREB2A expression cassette region. UD: undigested control rice genomic DNA; C: Control rice; Lane numbers: different transgenic lines.
4.10 Functional analysis of OsDREB2A transgenic plants under abiotic stress

To analyze the function of OsDREB2A in rice, we generated transgenic plants. OsDREB2A transgenic lines (T0-3, 5, 8, 9, 10, 11, 12, 13, and 14) were identified by PCR and Southern blot analysis (Fig 4.21 & 4.22). Among these, two independent homozygous lines named, T1-3 and T1-10 were chosen for conducting physiological experiments.

4.10.1 Effect of mannitol on germination, shoot and root length at the seedling stage

Overexpression of OsDREB2A under the stress-inducible promoter rd29A did not show any phenotypic abnormalities either in stress or non-stress growth conditions in these transgenic lines. During simulated osmotic stress conditions in the presence of mannitol (300, 400 mM) the seed germination percentage was 41-75%. The seedling survival of OsDREB2A transgenic lines was superior to control plants (25-41%) (Fig. 4.23A & B). All the transgenic lines showed a significant increase in rate of germination and survival of seedlings over the wild type during mannitol stress.

The transgenic plants in the presence of mannitol (300, 400 mM) the shoot length and root length was exhibited 10-14 cm and 8-14 cm, respectively. The shoots and roots of transgenic lines were more in length compare to wild type plants (5-7 cm; Shoot length and 4-6 cm; Root length) (Fig. 4.23C & D). During drought stress conditions employed by mannitol with water supply, the control plants showed wilting and loss of chlorophyll within 3-4 days, while transgenic lines wilted in 7-9 days with just few portions of their transgenic leaves affected by wilting. When we provided water to these samples, the transgenic seedlings exhibited recovery in growth, but most of the control seedlings died. It was interesting to observe that the DREB2A transgenic plants developed a more branched root system. The germination rates of transgenic plants on the liquid medium without mannitol were almost same as those of the wild type plants. These observations suggested that overexpression of the OsDREB2A conferred stress tolerance and protected rice seedling under osmotic stress.

4.10.2 Effect of salt stress on germination, shoot and root length at seedling stage

During simulated salt stress conditions in the presence of NaCl (100, 200 mM) the seed germination percentage was 41-66% and seedling survival of OsDREB2A
transgenic lines was superior than control plants (25-33\% NaCl stress) (Fig. 4.24 A & B). All the transgenic lines showed a significant increase in rate of germination and survival of seedlings over the wild type during salinity stress also. Approximately, 25 to 40\% of *OsDREB2A* transgenic rice seeds failed to germinate and seedlings could not survive either in salt stress. Comparatively, transgenic plants grew healthily whereas the surviving seedlings from control seed sample were dwarf and developed pale green leaves during salt stress (Fig. 4.24).

The transgenic plants in the presence of salt (100, 200 mM) the shoot length and root length was exhibited 8-11 and 10-14 cm, respectively. The shoots and roots of transgenic lines were more in length compare to wild type plants (4-6 cm; Shoot length and 5-7 cm; Root length) (Fig. 4.24C & D). These observations suggested that overexpression of the *OsDREB2A* conferred stress tolerance and protected rice seedling under salt stress. The germination rates of transgenic plants without salt were almost similar as those of the wild type plants. These transgenic (T1-3 and T1-10) lines were used to carry out for further segregation analysis and phenotypic characterization to determine the growth and productivity at matured stage (Fig. 4.26).

**4.10.3 Segregation analysis of T1 transgenic seeds by PCR**

The PCR amplification of transgene was performed with the genomic DNA isolated from T1 and control plants of Swarna rice. The integration of the marker cassette into genome was detected by nested PCR using 35s-*hpt* primers (Table 4.2). We have analyzed 40 plants of T1-3 and 45 plants of T1-10 line to check the segregation. 28 and 12 plants in T1-3 and T1-10 lines respectively were found to be positive for hygromycin gene. The amplified product size i.e. 0.95 kb in positive plants was same to that the positive control and there was no amplification in the control plant. The segregation percentages of T1-3 and T1-10 were 67\% and 37\% respectively (Fig. 4.25 A & B). Approximately, 25 to 40\% of *OsDREB2A* transgenic rice seeds failed to germinate and seedlings could not survive either in osmotic or in salt stress (Fig 4.23 & 4.24). Probably, these may pertain to non-transgenic segregating progeny in the T1 generation. Similar percentage of non-transgenic segregating progeny was observed for transgenic *OsDREB2A* rice plants in T1 generation as shown by PCR analysis.
Results

A.

Fig. 4.23: Drought tolerance analysis at seedling stage (Continued..)
Fig. 4. 23: **Drought tolerance analysis at seedling stage:** (A) Seeds of wild type and transgenic (T1-3 and T1-10) were germinated and grown on a Hoagland’s medium for 3 days, then transferred to Hoagland’s liquid medium plus 0, 300 mM, 400 mM mannitol for another 7 days (B) survival percentage (%) (C) shoot length in cm (D) root length in cm of transgenic wild type plants.
Results

A.

B.

Fig. 4. 24: Salt tolerance analysis at seedling stage (Continued..)
Results

C.  

**Fig. 4.24:** Salt tolerance analysis at seedling stage: Seeds of wild type and transgenic (T1-3 and T1-10) were germinated and grown on a Hoagland’s medium for plus 0, 100mM, 200mM NaCl for another 7 days, then recovered (A). Survival percentage (%) (B) shoot length in cm (C) and shoot length in cm (D) of wild type and transgenic plants.
Fig. 4.25: Segregation analysis of T₁ generation plants: Genomic DNA isolated from drought and salinity tolerant plants of T₁-3 and T₁-10. Agarose gel showing the PCR amplification of the 0.95kb hpt marker gene by using 35Scam F2-hpt R2 nested primers. PC: Positive control, NC: negative control, NT: non-transformed control line.
4.11 Functional evaluation of OsDREB2A transgenic (T$_1$) plants at maturity stage

The phenotypic characterization of progeny (T$_1$ generation) of rice plants which carried the OsDREB2A gene were compared to non transformed plants under greenhouse conditions. Even under simulated drought stress condition by limiting the water supply for the entire growth period the OsDREB2A transgenic rice plants performed relatively superior in comparison to their wild-type counterparts, in terms of several phenotypic growth parameters such as plant height, number of tillers per plant, panicle length, leaf length, root length, root weight, and seed weight (Fig. 4.26). The phenotypic characteristics of the wild and transgenic T$_1$ (OsDREB2A T$_1$-3 and OsDREB2A T$_1$-10) plants were scored at maturity/harvest. The details of phenotypic characteristics under simulated drought condition as followed.

4.11.1 Effect of drought stress on plant height

The plant height of wild type and transgenic T$_1$-3 and T$_1$-10 plants were scored at matured stage under three independent experiments. The height of the transgenic lines T$_1$-3 and T$_1$-5 were found to be 100±0.7 cm and 95±1 cm respectively, in contrast to only 78±0.9 cm of control plants under drought stress conditions (Fig. 4.26.1). The control and transgenic plants were grown to similar height under non stress conditions.

4.11.2 Effect of drought stress on tiller formation

The wild type plants were produced 17, whereas T$_1$-3 and T$_1$-10 produced 22 and 25 tillers respectively (Fig. 4.26.2). The number of tillers in T$_1$-3 was significantly more than T$_1$-10 plants. We have seen more tillers in transgenic plants than the wild type. The number of tillers per plants was same in control and transgenic plants which grown under non stress conditions. All these results indicate that the transgenic plants are showing better adaptation to the drought stress conditions than the wild type plants.
4.11.3 Effect of drought stress on panicle length
Transgenic plants had slightly longer panicles than wild type, and the numerical difference was small. The panicle length of wild type plants 17cm whereas, for both $T_1$-3 and $T_1$-10 plant panicle length was 19 respectively (Fig. 4.26.3). There was no significant difference of panicle length between control and transgenic plants.

4.11.4 Effect of drought stress on leaf length
The leaf length of wild type and transgenic $T_1$-3 and $T_1$-10 plants were scored at matured stage in triplicates. The average leaf length of $T_1$-3 and $T_1$-10 were seen 38 cm and 41 cm respectively, in contrast to only 30 cm of control plants (Fig. 4.26.4). The leaf length of transgenic plants was significantly higher than non transformed control plants. The leaf length of control and transgenic plants were shown similar length under non stress conditions.

4.11.5 Effect of drought stress on root length
The wild type plants have shown root growth of 30 cm where as $T_1$-3 and $T_1$-10 has shown 44 cm, respectively (Fig. 4.26.5). Transgenic plants have shown more lateral roots. Although the lateral root formation was observed even in the wild type, but the development was very slow. Lateral root development in transgenic plants was better than the wild type plants. All these results indicated that the transgenic plants were showed better adjustment to the drought stress than the wild type plants. The root length of control and transgenic plants were similar under non stress conditions.

4.11.6 Effect of drought stress on root weight
To study the effect of simulated drought stress on growth of roots at harvesting stage, we measured the root weight in triplicates. The wild type plants have shown root weight of 11.8 g/plant where as $T_1$-3 and $T_1$-10 has shown 12.5 and 14.5 g, respectively (Fig. 4.26.6). The root length of control and transgenic plants were similar under non stress conditions.
4.11.7 Effect of drought stress on seed yield

The seed yield of wild type and transgenic T1-3 and T1-10 plants were weighed after harvesting the seeds in three replications. The seed weights of T1-3 and T1-10 plants were 36 and 34 g/plant respectively, in contrast seed yield of control only 13 g. (Fig. 4.26.7). Transgenic plants produced more mature seeds per spikelet; consequently, the number of seeds per plant, grain weight per panicle was significantly higher compared with the wild type plants. Seeds derived from transgenic plants had high length/width ratios, whereas seeds of the wild type plants were shorter and narrower.
Results

Fig. 4.26: Comparative analysis of the growth competency for OsDREB2A transgenics and control rice plants during simulated drought stress: Transgenics (T₁-3 and T₁-10) and wild type plants were grown in greenhouse under simulated drought stress till mature the seeds. Phenotypic growth parameters such as plant height, number of tillers, panicle length, leaf length, root length, and seed weight were measured in triplicates. C: Control rice plants; C(DS): Control rice plants under stress T₁-3 and T₁-10; Transgenic rice plants.
**Results**

Fig. 4.26.1: Effect of simulated drought stress on plant height: Transgenic and wild type were grown in a greenhouse under simulated drought stress. X-axis represents the stress treatments and Y-axis represents the plant height in cm. C: Control rice plants; T: Transgenic rice plants. Data represents mean length ±SD derived from three separate experiments.
Fig. 4.26.2 Effect of simulated drought stress on tiller number: Transgenic and wild type was grown in greenhouse under simulated drought stress. X-axis represents the stress treatments and Y-axis represents the number of tillers per plant. C: Control rice plants; T: Transgenic rice plants. Data represents mean length ±SD derived from three separate experiments.
**Fig. 4.26.3 Effect of simulated drought stress on panicle length:** Transgenic and wild type was grown in green house under simulated drought stress. X-axis represents the stress treatments and Y-axis represents the panicle length in cm. C: Control rice plants; T: Transgenic rice plants. Data represents mean length ±SD derived from three separate experiments.
**Results**

Fig. 4.26.4 Effect of simulated drought stress on leaf length: Transgenic and wild type was grown in green house under simulated drought stress. X-axis represents the stress treatments and Y-axis represents the leaf length in cm. C: Control rice plants; T: Transgenic rice plants. Data represents mean length ±SD derived from three separate experiments.
Fig. 4.26.5: Effect of simulated drought stress on root length: Transgenic and wild type was grown in green house under simulated drought stress. X-axis represents the stress treatments and Y-axis represents the root length in cm. C: Control rice plants; T: Transgenic rice plants. Data represents mean length ±SD derived from three separate experiments.
Fig. 4.26.6: Effect of simulated drought stress on root weight: Transgenic and wild type was grown in greenhouse under simulated drought stress. X-axis represents the stress treatments and Y-axis represents the root weight in gms; C: Control rice plants; T: Transgenic rice plants. Data represents mean length ±SD derived from three separate experiments.
**Fig. 4.26.7: Effect of simulated drought stress on seed weight:** Transgenic and wild type was grown in greenhouse under simulated drought stress. X-axis represents the stress treatments and Y-axis represents the seed weight in gms; C: Control rice plants; T: Transgenic rice plants. Data represents mean length ±SD derived from three separate experiments.