RESEARCH ARTICLE

Allele mining across DREB1A and DREB1B in diverse rice genotypes suggest a highly conserved pathway inducible by low temperature

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Abstract

Low temperature stress is one of the major limiting factors affecting rice productivity in higher altitudes. *DREB1A* and *DREB1B*, are two transcription factors that have been reported to play key regulatory role in low temperature tolerance. In order to understand whether natural genetic variation in these two loci leads to cold tolerance or susceptibility, *OsDREB1A* and *OsDREB1B* were targeted across several rice genotypes showing differential response to low temperature. Expression data suggests induction of gene expression in shoots in response to low temperature in both tolerant and susceptible genotypes. Upon sequence analysis of 20 rice genotypes, eight nucleotide changes were identified including two in the coding region and six in the 5′UTR. None of the discovered novel variations lie in the conserved region of the genes under study, thereby causing little or no changes in putative function of the corresponding proteins. *In silico* analysis using a diverse set of 400 *O. sativa* revealed much lower nucleotide diversity estimates across two *DREB* loci and one other gene (*MYB2*) involved in DREB pathway than those observed for other rice genes. None of the changes showed association with seedling stage cold tolerance, suggesting that nucleotide changes in *DREB* loci are unlikely to contribute to low temperature tolerance. So far, data concerning the physiological role and regulation of DREB1 in different genetic background are very limited; it is to be expected that they will be studied extensively in the near future.

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Introduction

A large rice growing area in India (1.8 million ha) falls in the hilly regions, where a major production constraint is low temperature from seedling to grain filling stage. To enhance the productivity of rice in these regions, it is important to understand and harness the molecular mechanisms of cold tolerance for rice varietal development.

Numerous genes induced by low temperature in rice have been identified (Fowler and Thomashow 2002; Seki *et al.* 2002; Rabbani *et al.* 2003), including genes coding for regulatory proteins, such as transcription factors and protein kinases (reviewed by Nakashima *et al.* 2009). Among the transcription factors, dehydration responsive element binding (DREB) proteins, belonging to subfamily AP2/ERF transcription factors (Magnani *et al.* 2004) are well-studied.

Genes belonging to the DREB subfamily are thought to be important switches to regulate expression of many stress-inducible genes. The DREB group is divided into six subgroups (A-1 to A-6). The A-1 subgroup members like DREB1/CBF (C-repeat-binding factor) are induced by low temperature that activate the expression of many cold stress-responsive genes, whereas DREB2-like genes, belonging to the A-2 subgroup, are mainly involved in osmotic stress-responsive gene expression (Sakuma *et al.* 2002; Nakashima *et al.* 2009). The rice genome contains at least 10 DREB1 and four DREB2 type genes, among which *OsDREB1A* and *OsDREB1B* are induced by cold stress (Dubouzet *et al.* 2003). Having effective strategy to combat stress, it is essential to understand the role of allelic variations of known genes in pathways working in stress response leading to tolerance. In rice, which has a well-defined genetic structure and wide adaptability to various ecosystems, variations across different genic regions have to be looked into, to understand the role alleles play in stress response and tolerance.

With the availability of nucleotide information on 517 resequenced rice varieties (Huang *et al.* 2010), it is now clear that the single-nucleotide polymorphism (SNPs) are the most abundant type of polymorphism. This fact has been exploited for design of different types of SNP-based genotyping platforms. For example, with an Illumina GoldenGate

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SNP chip, 395 rice varieties have been genotyped using 1311 high-quality SNP markers (Zhao *et al.* 2010). Eventhough the SNPs are the most abundant type of polymorphism, only informative SNPs can be used either for biparental mapping studies or association mapping. In either case, it is essential that the phenotypic trait of interest be tagged to an SNP(s). Till date, studies using SNPs to associate with a trait of interest have used diverse germplasm (Tung *et al.* 2010; Chin *et al.* 2011; Famoso *et al.* 2011). But, given the number of rice accessions available in international (e.g. International Rice Genebank, maintained by IRRI, holds more than 117,000 types of rice) and national germplasm repositories of rice growing countries (e.g., India has more than 60,000 accessions in germplasm collection), it is clear that not all potential sources of polymorphism that may lead to subsequent crop improvement have been tapped. Further, SNPs used in genotyping assays are only a subset of the total SNPs and are not trait-specific. Therefore, to identify trait-specific allelic variation at a locus, genotyping a panel of germplasm that is diverse with respect to the trait of interest is a more targeted approach.

Till date, in the case of rice *DREB1* genes, natural allelic variations at nucleotide level and their relationship with abiotic stress tolerance have not been reported. Therefore, objectives of the current study were: (i) to study sequence polymorphism in a panel of diverse rice genotypes with respect to *DREB1A* and *DREB1B*, (ii) to identify cold tolerant and susceptible genotypes, and to study variation between them in terms of DREB1A and DREB1B sequence polymorphism and transcript levels and (iii) to use *in silico* analysis to find extent of nucleotide polymorphism in genes reported in the DREB1 pathway.

Materials and methods

Genomic DNA extraction, PCR and sequencing analysis

Genomic DNA was isolated from leaf tissue using the CTAB (N-acetyl-N, N,N-trimethylammonium bromide) method (Murray and Thompson 1980). PCR conditions were 94°C for 45 s, 55°C for 45 s and 72°C for 1 min for 30 cycles and a final extension at 72°C for 3 min. Amplified PCR products (2 μL) were resolved in 0.8% agarose gel stained with ethidium bromide (EtBr). Primers were designed for amplifying 5′UTR and CDS (coding sequences) of *OsDREB1A* and *OsDREB1B* using sequence available in GenBank (accession numbers between AF300970 and AF300972). The primer sequences were OsDREB1A_F: 5′-CTCGAGCAGAGCAAATACAG-3′, OsDREB1A_R: AGTAGTGTCCGTACAGTACC-3′, 5U_DB1A_Fb: 5′-CATAACCAAAACCGTGAGTCG-3′, 5U_DB1A_Rb: 5′-TGCTCCTGATTCCTGAACTGTA-3′, OsDREB1B_F: 5′-TCCAAGTCTCCAACCTCAGC-3′, OsDREB1B_R: 5′-CCCCCAATTTCTGGAGAATC-3′, 5U_DB1B_Fa: 5′-AAACGGAATAAACTATCTCCCAATC-3′ and 5U_DB1B_Ra: 5′- GGATGACTCTCTCTGGTTCACTTC-3′. PCR amplicons were obtained using the primer pairs with genomic DNA extracted from rice genotypes. All accessions produced single amplicon with four primer pairs when analysed by agarose gel electrophoresis. In this assay, the amplicons were directly sequenced from the PCR products using the CDS and UTR sets of forward and reverse primers. The PCR products were sent for sequencing to GCC Biotech Ltd (Kolkata, India). Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers from JQ885955 to JQ885966.

Table 1. Number of nucleotide variations across the rice DREB1A and DREB1B genes in 400 *O. sativa* accessions based on Gramene online tool (http://plants.ensemble.org/oryza_sativa/Info/Index?db=other). 5′UTR, 3′UTR, CDS are the variations reported upstream, downstream and in coding region of the genes.

Gene name	5′UTR	Gene CDS	3′UTR	Total
OsDREB1A	0	0	0	0
OsDREB1B	0	1	0	1

SNP discovery and validation

Sequences obtained from the genotypes were aligned using BioEdit ver. 5.0.6 (Hall 1999), and putative SNPs were located based on sequence homology. The SNPs were validated using SNP-based markers.

Estimation of nucleotide variation

Nucleotide variation (SNP; InDel) was searched for DREB 1A and DREB 1B using Gramene tool (http://archive.gramene.org/genome-browser/index.html).

Phenotyping

A set of 20 rice genotypes collected from different parts of India were evaluated for seedling stage low temperature response. To assay for cold tolerance, 14 day-old plant grown at 10°C for 12 days were compared with plant grown at 25°C under green house conditions. Parameters like plant height, root length, fresh weight, dry weight, leaf colour (using IRRI's Standard Evaluation System (SES) for rice) were recorded for both control and treated rice seedlings (table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/) and were used to phenotypically categorize the genotypes.

RNA isolation and cDNA synthesis

Leaf samples from 14 days old plant treated for 3 h at 10°C were used. Plants were grown in the greenhouse for a period of 14 days and then transferred to a growth chamber for 3 h. Total RNA isolated from shoots of rice seedling that were exposed to low temperature treatment and from control plants seedlings were isolated using Trizol (Sigma, St Louis,

USA) according to the manufacturer's protocol. Approximately 20 μg of total RNA was used to synthesize first-strand cDNA using the cDNA synthesis kit (Fermentas, Waltham, USA).

Semiquantitative PCR

First-strand cDNA(s) was used as template using gene-specific primers (DREB1A: F5′-CTCGAGCAGAGCAAA TACAG-3′ and R5′-AGTAGTGTCCGTACAGTACC-3′ and DREB1B: F5′-TCCAAGTCTCCAACCTCAGC-3′ and R5′-CCCCCAATTTCTGGAGAATC-3′). Rice ubiquitin gene was used as internal control. PCR reaction mixture consisted of 1× PCR buffer, 200 μM of each of dNTPs and 150 ng of each of the gene specific primers, in a 20 μL reaction. For analysing the housekeeping gene transcript, the PCR conditions were as follows: 94°C for 1 min; 55°C for 1 min; 72°C for 1 min for 30 cycles for ubiquitin gene, and for selected genes 94°C for 1 min; 55°C for 1 min; 72°C for 1 min for 33 cycles. At the end of PCR cycles, 3 μL of PCR products were resolved in 3% agarose gel, stained with ethidium bromide under UV light. For each sample, three biological repeats were performed. Band intensity of the PCR products was calculated using Gel-Quant software provided by biochemlabsolutions.com.

Results

Polymorphism across DREB1 loci in 20 rice genotypes differing in response to low temperature

A panel of rice genotypes, phenotyped for low temperature response along with international checks, *M 202* and *IR 24*, was sequenced across two DREB loci. The rice genotypes selected were representative of different rice ecosystems and included landraces and varieties (see table 1 in electronic supplementary material). Sequencing followed by the multiple alignment of 717-bp ORF of *OsDREB1A* across 20 genotypes revealed a total of two SNPs (figure 1). The nucleotide change (G to A) at position 201 from start codon was synonymous, while the SNP (C to G) at position 516 led to protein change at C-terminal (amino acid 172) where aspartate (D) changed to glutamate (E) in seven of the rice genotypes sequenced (figure 2, a&c). The change observed at protein level lies outside the reported conserved regions; EREBP-AP2-binding domain and VSEIRE region (lie between positions 14 to 19).

A singleton SNP was observed at position 427 (A to G) in the 657-bp *OsDREB1B* genic region in 11 genotypes (figure 1). This change led to N to D change in DREB protein (figure 2d). The C-terminal region of DREB1 genes is supposed to be transcription activator (Stockinger *et al.* 1997), but the changes observed in C-terminal regions of both OsDREB1A and OsDREB1B did not show any association with low temperature response of the rice genotypes (as suggested by nonsignificant *P* value; see figure 1).

Further, 553 bp of 5′UTR of DREB1A was sequenced to identify level of polymorphism in the immediate upstream regulatory region. The sequencing revealed three SNPs (C/–; A/– and T/C) and two InDels (2 bp and 10 bp) at positions –187, –176, –175, –128 and –15 from the start codon, respectively (figure 1). The variations at positions –15, –128 and –175 were rare, not occurring in more than two genotypes sequenced in the present study.

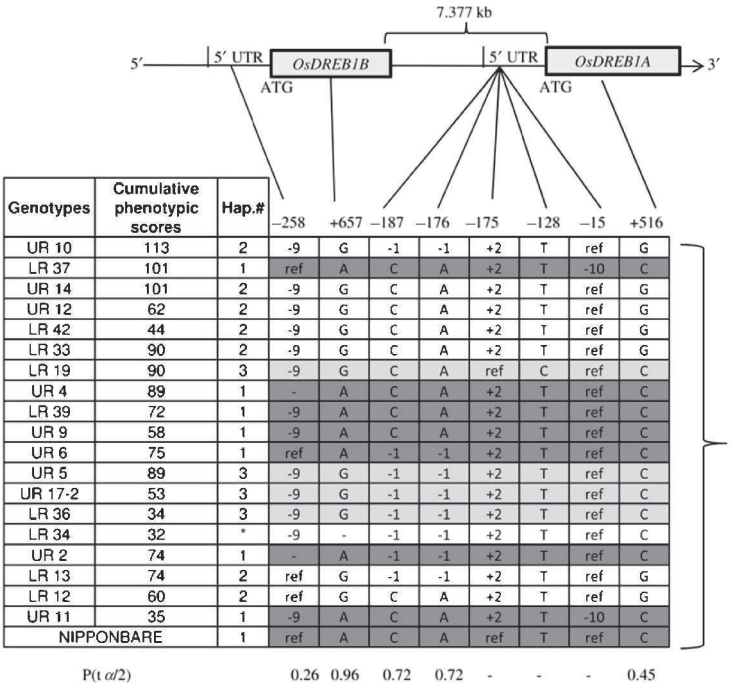
Upon sequencing of 549 bp region upstream of *DREB1B*, a 9 bp InDel at position –258 was found in 13 genotypes (figure 1). Analysis of the SNP and InDel for locating known *cis*-regulatory elements using plant database of *cis*-regulatory elements (PLACE) (Higo *et al.* 1999) revealed changes due to presence of two novel motifs when compared with reference sequence (figure 2, f&g). The 2-bp InDel (CA) found at position −175 in 5′UTR of DREB1A lies on a motif for anaerobic response (ANAERO1) with suggested role in abiotic stress as well (Mohanty *et al.* 2005). The 9 bp deletion found at position −258 in upland genotypes like UR5 and UR7 (figure 2g) lead to loss of a *cis*-acting element (myb). Myb motifs have been reported to play a role in drought stress (Abe *et al.* 1997). It is likely that Myb motif might play a role in response to low temperature as well. Further, the genotypes which carry insertion might respond differentially to stresses other than cold.

Two of the nucleotide changes observed in the 5′UTR of the DREB genes were validated using CAPS primer and allele-specific primer (figure 3). All the SNPs observed were novel. When compared with promoter regions of *Arabidopsis*, the number of SNPs/InDel found in rice DREB1B (one InDel) is too less (McKhann *et al.* 2008). On an average, eight SNPs were found in 1210 bp promoter region of *Arabidopsis* CBF1. However, for OsDREB1A, comparable number of variations were obtained (one SNP and four InDel in rice compared with four SNPs in *Arabidopsis*).

Nucleotide diversity (π) i.e., total number of SNPs / total length of aligned sequences for 5′UTR and CDS regions, was 0.037 and 0.027, respectively. The estimates of diversity were much lower than the average estimates reported for rice. The available SNP data from different rice genotyping platforms across two loci was checked for reported polymorphism using Gramene tool (table 1). The diversity estimates were found to be comparable with those obtained in the current study suggesting a highly conserved region in *Oryza sativa*.

Association of DNA polymorphism with low temperature tolerance at seedling stage

For screening of rice at seedling stage, traits like relative root length, relative shoot length, fresh weight, dry weight, fresh weight to dry weight ratio and leaf colour were recorded initially at 0 day and after 12 days of treatment. In low temperature condition, the rate of growth was low across all genotypes as compared with control condition. Using IRRI's SES for low-temperature stress, tolerance/susceptibility at



Genotypes	Cumulative phenotypic scores	Hap.#	−258	+657	−187	−176	−175	−128	−15	+516
UR 10	113	2	-9	G	-1	-1	+2	T	ref	G
LR 37	101	1	ref	A	C	A	+2	T	-10	C
UR 14	101	2	-9	G	C	A	+2	T	ref	G
UR 12	62	2	-9	G	C	A	+2	T	ref	G
LR 42	44	2	-9	G	C	A	+2	T	ref	G
LR 33	90	2	-9	G	C	A	+2	T	ref	G
LR 19	90	3	-9	G	C	A	ref	C	ref	C
UR 4	89	1	-	A	C	A	+2	T	ref	C
LR 39	72	1	-9	A	C	A	+2	T	ref	C
UR 9	58	1	-9	A	C	A	+2	T	ref	C
UR 6	75	1	ref	A	-1	-1	+2	T	ref	C
UR 5	89	3	-9	G	-1	-1	+2	T	ref	C
UR 17-2	53	3	-9	G	-1	-1	+2	T	ref	C
LR 36	34	3	-9	G	-1	-1	+2	T	ref	C
LR 34	32	*	-9	-	-1	-1	+2	T	ref	C
UR 2	74	1	-	A	-1	-1	+2	T	ref	C
LR 13	74	2	ref	G	-1	-1	+2	T	ref	G
LR 12	60	2	ref	G	C	A	+2	T	ref	G
UR 11	35	1	-9	A	C	A	+2	T	-10	C
NIPPONBARE		1	ref	A	C	A	ref	T	ref	C
P(t α/2)			0.26	0.96	0.72	0.72	-	-	-	0.45

Hap.#	DREB1B	DREB1A	Total
1	A	C	8
2	G	G	7
3	G	C	4

Figure 1. Summary of SNPs in DREB1A and DREB1B detected across the 20 genotypes sequenced along with diagrammatic distribution on chromosome 6 of rice. The numbers at the top and bottom of the figure indicate the position of the SNP and *t*-test *P* values of type 1 error for differences between two groups of SNPs, respectively. Haplotypes across the CDS is indicated on the right.

the seedling stage, the plant was scored from 1 (highly tolerant) to 9 (highly susceptible). The results obtained for different traits measured showed variation and no single genotype showed consistent performance across the seven parameters studied. Therefore, a score of '1' was assigned to the genotype with a minimum value for a trait and rest of the genotypes were assigned scores in ascending order with a maximum score of '30' (including SES scores). The scores obtained for a particular genotype across the seven parameters was added to arrive at a cumulative score (see table 1). The genotypes were then grouped into tolerant and susceptible based on cumulative scores. The tolerant genotypes like UR 10, LR 37, UR 14 etc. had higher score and susceptible genotypes like UR 11 and LR 36 had lower score (table 1 in electronic supplementary material). To study the association of SNPs/InDels in a panel of 20 genotypes, the genotypes were divided into two groups for each SNP/InDel, and the cumulative phenotypic score means of the two groups were compared using *t*-test. None of the SNP/InDel showed significant difference between groups, suggesting that the SNPs/InDels are not directly responsible for the phenotypic performance of genotypes under seedling stage cold stress.

Expressional analysis of transcripts across contrasting phenes using RT-PCR

A set of four rice genotypes differing in response to low temperature were selected for gene expression studies: *M 202* (tolerant variety), *ARR 09* (UR5, tolerant landrace), *IR24* (susceptible variety), Takyer (UR17-2, susceptible landrace) were used. UR5 and UR17-2 are landraces from northeast India, Arunachal Pradesh state, while the other two rice genotypes were selected on the basis of published report on their contrasting responses to low temperature obtained from IRRI, Philippines.

Analysis of DREB1A and DREB1B transcripts using RT-PCR for tolerant and susceptible genotypes showed around 10-fold transcript induction after 3 h of stress treatment (10°C) in leaves irrespective of tolerant and susceptible genotypes (figure 4), suggesting similar regulation of *OsDREB1A* and *OsDREB1B* in contrasting rice genotypes in response to low temperature. This is distinct from findings in *Arabidopsis*, where the tolerant genotype had higher CBF expression level and sensitive genotype had lower expression level (McKhann *et al.* 2008). The AP2 transcription factors containing GC-rich motifs like DREB are the most conserved set of genes upregulated in cold stress in both rice and *Arabidopsis* (Narsai *et al.* 2010), still the regulation of DREB1A and DREBlB appears to be different in rice and *Arabidopsis*.

Estimates of diversity across loci involved in cold regulation

At least two distinct cold stress signalling pathways involving MYBS3 and DREB are reported in rice (Su *et al.* 2010). We desired to understand the role played by natural sequence variation in determining cold stress regulation and the role played by sequence variation across genes involved in imparting tolerance to low temperature stress. The sequence information available for 400 *O. sativa* in Gramene

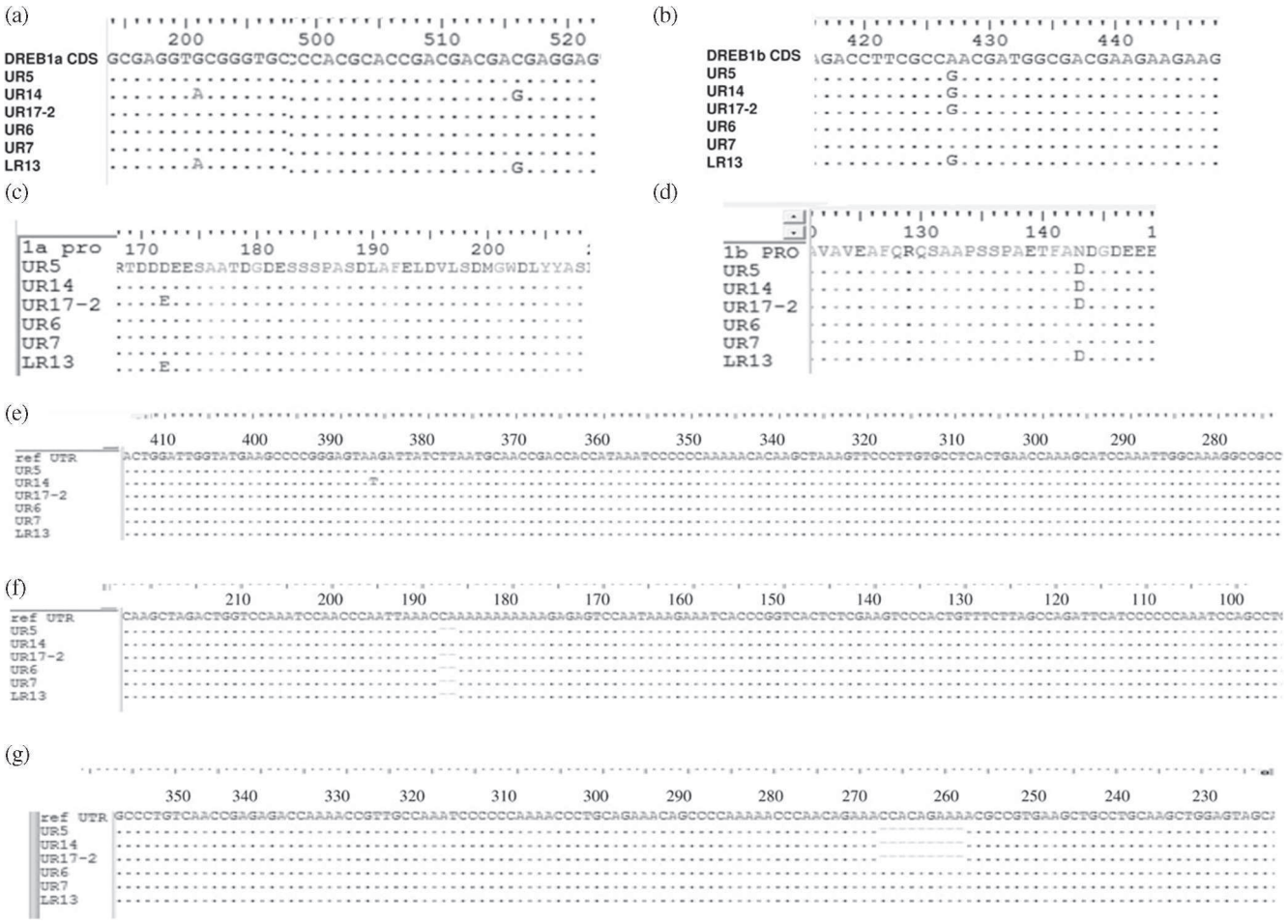


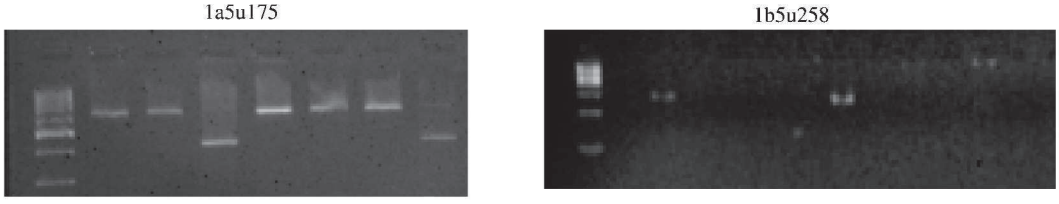
Figure 2. Representative multiple sequence alignment at DNA and protein levels for DREB1A and DREB1B showing SNPs and InDel identified in a set of representative six rice genotypes in comparison with reference *Nipponbare* sequence. Sequence at the top of each panel is the reference sequence and the numbers indicate the position of the sequence displayed.

(http://www.gramene.org) database was used for calculating sequence variation estimates for four genes (*SOC1*, *MYB2*, *TPP1* and *TPP2*) previously reported for cold regulatory pathway (table 2). The number of SNPs varied from a minimum of 2 to 640. Apart from *OsMYB2*, the other genes showed higher diversity estimates than rice DREB1A and DREB1B genes. This suggests that DREB genes are highly conserved in rice, whereas the upstream gene in DREB pathway (*SOC1*) and downstream genes (*TPP1* and *TPP2*) in MYBS3 pathway are not. The estimates obtained for DREB genes are less than the average nine SNPs per kb reported in rice (Huang *et al.* 2010).

Discussion

SNP analysis

Allele mining exploits the DNA sequence of one genotype to isolate useful alleles from related genotypes. A total of three SNPs and five InDels were observed across DREB1A and DREB1B regions. As DREBs are important transcription factors regulating stress-responsive gene expression, the highly conserved domains in DREB proteins are important for their specific biological functions and identifying SNPs in such critical domains would lead to better understand the role played by CBF regulon. In the coding region of *DREB1A* and *DREB1B* genes, a total of two SNPs were found. The SNPs discovered led to changes only in the C-terminal of the protein in both DREB1A and DREB1B suggesting that the N-terminal of the protein is highly conserved. The C-terminal region is transcription activator but the SNPs do not seem to provide any advantage for better performance under low temperature. Apart from conserved N terminal, which carries a conserved nuclear location signal, a conserved Ser/Thr-rich region containing the phosphorylation site for the regulation of DREB1 activity (Liu *et al.* 1998) adjacent to the EREBP/AP2 binding domain has been reported. Also residues in the β-sheet have been identified as key residues for DNA-binding activity of EREBP/AP2-type proteins. It has been observed that in these sites, Arg (R) and tryptophan (W) contact DNA, whereas alanine (A) and glycine (G)



(a) (b)

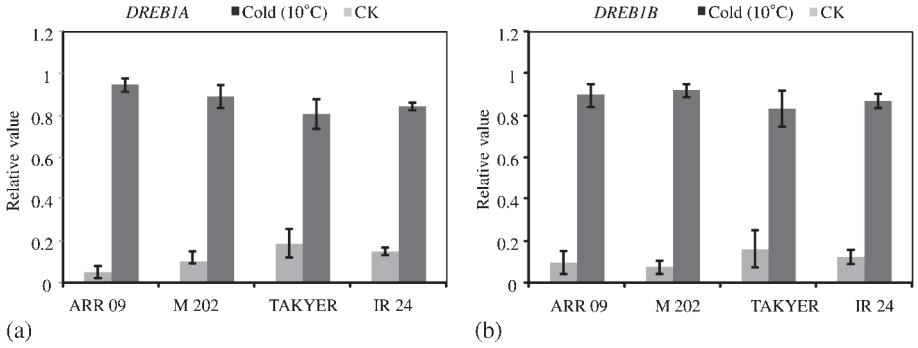
No	Forward sequence (5′–3′)	Reverse sequence (5′–3′)	Position targeted	Type of primer (allele)
1	TGGTCCAAATCCAACCCAATTAAACCA	CCTCGTATTTATAGGTGGTGGCGCG	5′UTR (–258)	CAPS
2	AACCCAACAGAAACCACAGAAAA	ATGACTCTCTCTGGTTCACTTCGCCGGC	5′UTR (–175)	ASM (CA/–)

(c)

Figure 3. SNP validation (a, b) using primers (c) for 5′UTR positions −175 and −258 in DREB1A and DREB1B, respectively.

form a hydrogen bond (Allen *et al.* 1998) and mutations in G caused malfunction in APETALA2 (Jofuku *et al.* 1994). The SNPs discovered in this study were not in the functional conserved domain and did not affect functionality of the protein / protein three dimensional structures (Dash *et al.* 2012). Similar results for DREB1 gene have been obtained in Durum wheat (Mondini *et al.* 2012). However, presence of SNP in EREBP/AP2 in highly tolerant wheat genotype could influence protein–DNA interactions.

The number of SNPs identified in this study was significantly less than previously discovered for coding regions of CBF1 and CBF2 in *Arabidopsis* (McKhann *et al.* 2008). It has been found that only 12% of cold-responsive genes in transgenic *Arabidopsis* overexpressing CBF are members of CBF regulon suggesting that other transcription factors play significant role in cold response (Fowler and Thomashow 2002). It has been suggested that high-level DREB1 expression is insufficient to sustain cold tolerance if the level of MYBS3 expression is too low (Su *et al.* 2010). Our present work also suggests that DREB induction and cold response are not directly related to tolerance mechanism. As yet, the natural variation across DREB factors in rice has not been studied. In the current study, the two genes were found to be highly conserved in a set of diverse rice genotypes. *In silico* analysis of three more genes of cold regulatory pathway revealed higher levels of sequence polymorphism than DREB genes hinting at involvement of other major genes in cold tolerance in rice. Previous work in *Arabidopsis* (McKhann *et al.* 2008), hints that a complex network of genes is involved in freezing tolerance and therefore, understanding the mechanism versus phenotype would require more comprehensive understanding of complex trait in rice. A similar work on *Hd1* gene, major QTL controlling heading date and the rice orthologue of the *Arabidopsis* CONSTANS (*CO*) gene (Yano *et al.* 2000) has also not revealed a clear evidence to prove that this orthologue is involved in



(a) (b)

Figure 4. Semiquantitative PCR analyses of *DREB1* genes on 14 day-old rice seedlings. x-axes are cold stress treatment across four different rice genotypes, namely, ARR 09 (UR5), M 202, Takyer (UR17-2) and IR 24. y-axes are scales of relative expression level. For cold stress, seedlings were sampled at 0 (CK) and 3 h (cold) after treatment. The bars are standard errors of technical repeats.

Table 2. Number of nucleotide variations across the four rice genes in 400 *O. sativa* accessions based on gramene online tool (http://plants.ensemble.org/oryza_sativa/Info/Index?db=other). Up, down, CDS are the variations reported upstream, downstream and in coding region of the genes.

		Gene					
Gene name	1 kb Up	5′UTR	Intron	CDS	3′UTR	1 kb Down	Total
OsSOC1	46	0	636	4 (splice variant)	0	4	640
OsMYB2	48	0	0	0	2	100	2
OsTPP1	40	1	3	2 (syn)	6	128	12
OsTPP2	34	0	1	6 (syn); 2 (mis)	1	23	10

natural variations in rice flowering time. This suggests that DNA level polymorphism in major transcription factors and QTLs may not be directly associated with a complex trait like cold tolerance across diverse genetic backgrounds in rice. Also genomic regions further upstream and downstream of DREB regions carrying higher diversity estimates (table 2 in electronic supplementary material) do not seem to have a regulatory role.

It might be possible that posttranscriptional regulatory mechanisms, such as mRNA export and small RNA-directed mRNA degradation are playing an important role in distinguishing responses of tolerant and susceptible genotypes to low temperature stress. Infact, studies have revealed that posttranscriptional regulation plays critical role during cold acclimation (Chinnusamy *et al.* 2007).

Presence of polymorphism within 5′UTR of the two genes suggests difference in regulation of the genotypes in response to other stresses and this variation seems to have very little to do with low temperature response as indicated by 3-h RT-PCR data and phenotypic scoring. In the present study, we used diverse landraces and cultivars and the *in silico* database used also uses sequence information generated on *O. sativa*. It may be possible that novel alleles exist in wild rice. This could be area of future studies.

Conclusion

The observed nucleotide diversity across *OsDREB1A*, *OsDREB1B* and MYB2 involved in cold response pathway were found to be much lower than those observed for other rice genes, suggesting a highly conserved pathway in rice. RT-PCR for *OsDREB1A* and *OsDREB1B* across diverse cold tolerant and susceptible genotypes revealed similar levels of induction under cold stress. Also, the sequence polymorphism observed in the two loci did not associate with tolerance or susceptibility. This suggests that functional allelic variation may not exist for *OsDREB1A* and *OsDREB1B* in rice, and cold tolerance may be due to further upstream or downstream regulators in the DNA or the pathway.

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