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Identification of DREB Homologous Genes in Bread Wheat via CODEHOP PCR Primer Design

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Abstract: In this study, we exploit the useful described CODEHOP primer design and RT-PCR strategy for targeted isolation of homologues in large gene families. The method was tested with two different objectives. The first was to apply CODEHOP strategy for design degenerate oligonucleotide primers in a broad range of plant species. The second was to isolate an orthologus of the transcription factor of dehydration-responsive element binding protein (DREB) and to determine the complexity of gene family in bread wheat. We used a new primer design strategy for PCR amplification of unknown targets that are related to multiply-aligned protein sequences. Each primer consists of a short 3' degenerate core region and a longer 5' consensus clamp region. Only 3-4 highly conserved amino acid residues are necessary for design of the core, which is stabilized by the clamp annealing to templates molecules. This provides the possibility of isolating numerous additional DREB genes by Polymerase Chain Reaction (PCR) with degenerate oligonucleotide primers. The relationship of the amplified products to DREB genes was evaluated by several sequence and genetic criteria. Present data show that expression of DREB and its homologues, is induced by low temperature stress. Towards this step, it found that the expression of DRE-regulated genes increased freezing tolerance in plants.

Key words: *Triticum aestivum* L., DREB, cold stress, expression pattern, CODEHOP strategy

INTRODUCTION

Plants have evolved extremely diversified gene families as tools to cope with a harsh environment. (Shinozaki and Yamaguchi-Shinozaki, 1996; Thomashow, 1994). Some of these families such as DRE-binding protein (DREBs) reflect the extra ordinary biochemical versatility of plants and across plant species and represent a very valuable source of gene for biotechnologies (Zou *et al*., 2006). The DREB genes that have been molecularly characterized so far can be grouped into several classes based on similarities in the function or amino acid sequence of the proteins they encode (Bohnert *et al*., 1995).

The products of these genes can be classified into two groups: those that directly protect against environmental stresses and those that regulate gene expression and signal transduction in the stress response (Shinozaki and Yamaguchi-Shinozaki, 1997). Gene expression profiling using cDNA microarrays or gene chips has identified many more genes that are regulated by cold, drought, or salt stress (Bohnert *et al*., 2001; Kawasaki *et al*., 2001; Seki *et al*., 2001). Although the signaling pathways responsible for the activation of these genes are largely unknown, transcriptional activation of some of the stress-responsive genes is understood to a great extent, owing to studies on a group of such genes represented by RD29A (also known as COR78/LT178). The promoters of this group of genes contain both the ABRE and the DRE/CRT (Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger *et al*., 1997). Transcription factors belonging to the EREBP/AP2 family that bind to DRE/CRT were isolated and termed CBF1/DREB1B, CBF2/DREB1C and CBF3/DREB1A (Stockinger *et al*., 1997; Gilmour *et al*., 1998; Liu *et al*., 1998; Medina *et al*., 1999). These Transcription factor genes are induced early and transiently by cold stress and they, in turn, activate the expression of target genes. Similar transcription factors DREB2A and DREB2B are activated by osmotic stress and may confer osmotic stress induction of target stress-responsive genes (Liu *et al*., 1998). The ability of the CBF/DREB1 transcription factors to active the DRE/CRT class of stress-responsive genes was further demonstrated by observation that over expression or enhanced inducible of CBF/DREB1 could activate the target genes. Over expression also increased tolerance of the transgenic plants to freezing, salt, or drought stress

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(Jaglo-Ottosen *et al*., 1998; Kasuga *et al*., 1999; Thomashow, 2001), suggesting that regulation of the CBF/DREB1 class of genes in plants is important for the development of stress tolerance.

Three of the corresponding genes; DREB1A, DREB1B, DREB1C, seem to be specifically cold inducible (Gilmour *et al*., 1998; Liu *et al*., 1998; Stockinger *et al*., 1997). The cDNAs encoding the DRE-binding proteins, DREB1A and DREB1B, have been isolated by yeast one-hybrid screening (Liu *et al*., 1998). Both proteins specifically bind and activate transcription of genes containing the DRE sequence in *Arabidopsis* (Liu *et al*., 1998). With the growing availability of gene sequences plus information regarding their diversity and phylogeny, increasingly sophisticated PCR techniques have been developed to target gene families (Zhang, 2001).

In this study, we report on the high potential of the recently described COnsensus-DEgenerate Hybrid Oligonucleotide Primers (CODEHOP) strategy (Rose *et al*., 1998) of primer design, ensuring optimal match and PCR amplification focused on very short conserved sequences, for the isolation of orthologues in evolutionarily distant species and for the focused or systematic exploration of gene families in plants with a very large genome. The method was tested to analyze of DREB gene family in bread wheat (*Triticum aestivum* L.). This family was suggested to play important and essential roles in plant stresses responses (Thomashow, 2001).

MATERIALS AND METHODS

Plant material, growth conditions and experimental treatments: Seeds of wheat (*Triticum aestivum* L.) were sterilized for 5 min in 1% (w/v) sodium hypochloride and were finally washed in distilled water. Then seeds (obtained from Karaj Agriculture Herbarium) were grown on Murashige and Skoog medium (GIBCO) containing 1.5% sucrose. When the primary leaves were about 3 cm in length, were exposed to temperature of +2°C for 5-8 weeks (for hardening), then was put low temperature from -2 to -20°C (-2, -4, -8, -12, -16 and -16°C, respectively). Leaves were harvested for RT-PCR after 1, 2, 4, 8 and 16 h. All of samples were put into liquid nitrogen just after harvested and stored at -70°C.

Primer design: The CODEHOP program utilized all of information available in the input alignment and takes into account the codon usage of the target genome to aid in primer design. The program first converts protein multiple sequence alignments into scoring matrices that consider sequence redundancy and amino acid conservation. Then the primers designed.

Primers completely degenerate at their 3'-end and ensuring high probability of annealing to DREB (dehydration-responsive element binding protein) specific sequences were designed using the CODEHOP strategy based on the multiply-aligned sequences: AY064403, AF448789, AY646223, AY196209, EF672101, NM-00111, NM-117777, NM-001036760, NM-111939, NM-120628, NM-118680, for DREB.

The multiple alignment was first generated using CLUSTALW (Thompson *et al*., 1994), then cut into blocks using the Block Maker server (blocks www server). Primers were designed using the default parameters of the CODEHOP server (http://blocks.fhcrc.org/codehop.html). It was assumed that barley codon usage proposed by the server was close enough to that of wheat to obtain effective primer design. From the primer solutions proposed by the server, 2 primers were selected, both of which provided the large PCR fragments and avoided the most conserved consensus regions common to a large number of DREB.

RT-PCR analysis: RNA was isolated using a RNeasy plant mini kit (Qiagen Inc.) according to the manufacturer's instructions. The first strand cDNA was obtained by RT using M-MLV Reverse Transcriptase (Fermentase) in a 20 μL reaction volume on RNA prepared from leaves. Reverse transcription-polymerase chain reaction (RT-PCR) was used to semi-quantitatively determine the expression profile of the DREB gene. Five microgram of RNA was reverse-transcribed into cDNA as described earlier. The sense primer was 5'-CCGTGGACCGGAAGGAYGCNGARGC-3' and the antisense primer was 5'- CTCTTGGCGCCTTGTT-GGRTCRTGYTC -3'.The expected length of the amplified fragment was 300 and 600 bp. The total volume of PCR reaction was 25 μL, containing 1 μL of the first-strand cDNA, 0.4 μM of each primer, 1xPCR Buffer, 0.2 mM of dNTP and 1U of *Taq* DNA polymerase. The reaction was denatured at 94°C for 3 min and then subjected to 35 cycle of 94°C for 1 min, 60°C for 30 sec and 72°C for 30 sec, plus a final extension at 72°C for 7 min. The PCR product were separated on 1.5% agarose gel and quantified using the higher performance ultraviolet transilluminator (GDS-8000, Gel Documentation system, USA). The experiments were repeated three times with the similar results and one of them is presented. This PCP product was purified from the gel and then was sequenced directly. This sequence was determined by automated DNA sequencing.

RESULTS

The degenerate primer strategy was tested on problems in which the target sequence for amplification

was unknown but could be predicted from multiply aligned protein sequences. In the first test, plants are induced during cold acclimation and gene expression is identified under cold stress. The second test utilized the automated CODEHOP prediction program to design optimal primers from Block Maker generated alignments of several DREB's plants. Predicted CODEHOPs were used to identify members of a new subfamily of DREB from different plant genes.

Chasing the DREB genes in wheat: Our first aim was thus to test if it possible to detect several genes belonging to the DREB family expressed in the seedlings from wheat, a major crop plant with a very large genome. Primers were designed using the CODEHOP strategy. Visual examination of the alignment revealed two blocks that contained invariant regions suitable for primer prediction. One block was chosen for primer design after evaluation of codon degeneracy within the block and distance between blocks (Fig. 1). Primer were designed from this region using all codon possibilities for the 3'degenerate core and the most frequent nucleotide in each position for the 5'consensus clamp. The design strategy is shown for the most conserved sequence block (Fig. 2). RT-PCR product from samples using the primers as described in material and methods was provided. The length of this amplified fragment was 300 and 600 bp. A PCR product of the correct size was detected on an electrophoretic gel (Fig. 3).

Expression of DREB: As shown in Fig. 3A-C, DREB showed different expression level in different temperature. Freezing tolerance of the plants correlated with the level of expression of the stress-inducible DREB-responsive gene under low temperature. In contrast, the freezing tolerance in samples 4, 8 and 16 h, respectively, was much stronger than the others, even though the expression of the DREB gene was weak under -2°C (All of data not shown). These results indicate that strong expression of the DREB-responsive gene under stress conditions correlated with tolerance to freezing. Therefore, the

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ID      TDREB BLOCK A
DE      family
DREB1         37    QPNFRGVRMRQWGKWVSEIREPRKKSRIWLGTFSTPEMAARAHDVAALAIKG
DREB1         76    RCSFRGVRQRIWGKWVAEIREPNRGSRLWLGTFPTAQEAASAYDEAAKAMYG
DREB like     75    HCSFRGVRQRIWGKWVAEIREPKIGTRLWLGTFPTAEKAASAYDEAATAMYG
DREB         553    HPTFRGVRMRAWGKWVSEIREPRKKSRIWLGTFPTAEMAARAHDVAALAIKG
DREB2         71    HPSYRGVRRRSWGKWVSEIREPRKKSRIWLGTFPTAEMAARAHDVAALAIKG
DREB2B        92    HPTFRGVRMRAWGKWVSEIREPRKKSRIWLGTFPTAEMAARAHDVAALAIKG
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Fig. 1: Block representation of highly conserved region within the family of DREB's plant. The ungapped multiple sequence alignment of Block A of the DREB was obtained from a comparison of six sequences: AY064403 (DREB1), AF448789 (dreb1), AY646223 (DREB LIKE), Y196209 (DREB), EF672101 (DREB2), NM-111939 (DREB2B) using Block Maker. Information regarding the size of the block and its position within the original sequences is indicated. The block output of Block Maker is hypertext linked to the CODEHOP designer

```
          M  R  I  W  G  K  W
oligo:5'-GGATGCGGATCtggggnaartg-3' degen = 8 temp = 61.0
          M  R  I  W  G  K  W
oligo:5'-GGATGCGGATCtggggnaartgg-3' degen = 8 temp = 61.0
          M  R  I  W  G  K  W  V
oligo:5'-GGATGCGGATCTGgggnaartggg-3' degen = 8 temp = 61.0
          M  R  I  W  G  K  W  V
oligo:5'-GGATGCGGATCTGGggnaartgggt-3' degen = 8 temp = 61.0
          K  K  S  R  I  W  L  G  T
oligo:5'-GGAAGAAGTCCCGGATCtggytnggnac-3' degen = 32 temp = 63.2
          P  R  K  K  S  R  I  W  L  G
oligo:5'-CCAAGGAAGAAGTCCCGGhtntggytngg-3' degen = 96 temp = 60.6
          P  R  K  K  S  R  I  W  L  G  T
oligo:5'-CCAAGGAAGAAGTCCCGGATntggytnggna-3' degen = 128 temp = 62.6
```

Fig. 2: CODEHOP designer output. The biased Block A of DREB's plant (Fig. 1) was used to design CODEHOP PCR primers using the default 60°C annealing temperature parameter. The consensus amino acid residues and predicted CODEHOP PCR primers (5'-3') are shown. The preferred CODEHOP has 32-fold degeneracy. The primers shown here are very similar but not identical to those utilized in our previous study to identify new DREB. This codon usage table was used for plant. The degeneracy and length for each core and the length and annealing temperature of each clamp are indicated

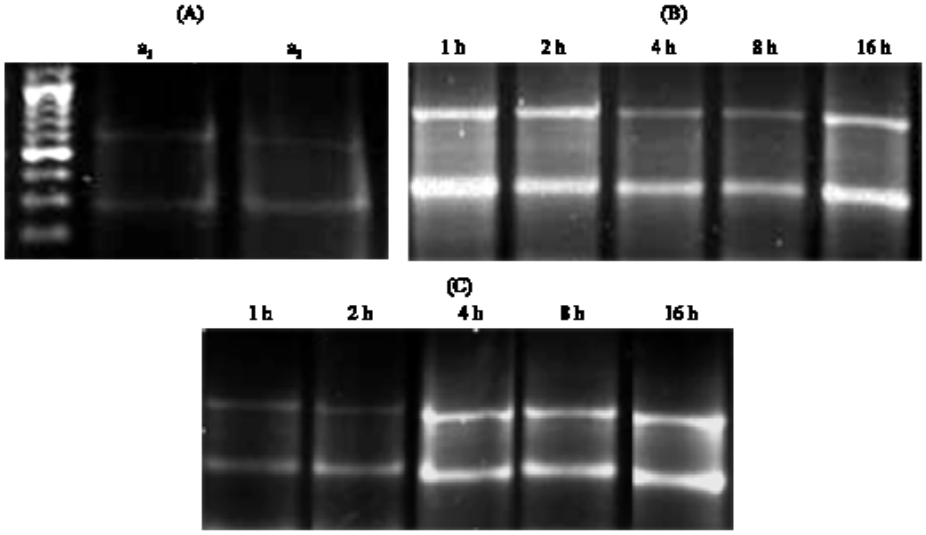

Fig. 3: Expression patterns of the DREB gene in leaves tissues and in response to cold stress, (A) expression patterns of gene at +2°C (a_2) and on the normal room temperature (a_1), (B and C) expression patterns of gene in -4°C and -8°C, respectively, after 1, 2, 4, 8 and 16 h. Two bands appearance on the gels are 300 and 600 bp. (M = 100 bp ladder)

expression pattern of this gene was different between the various temperatures of freezing. Additionally, we did not amplify the corresponding band from -20°C (data not shown). RT-PCR analysis was also performed to investigate the DREB expression in response to cold stress treatments. The results shown in Fig. 2A-C indicated that the DREB could be induced by cold stress.

Isolation and sequence analysis of DREB: RT-PCR products (especially band 300 bp), after detection on electrophoretic gel, were purified by the glass milk method (silica method) and then sequenced directly. Several sequences were obtained from several experiments that had 245-250 nucleotides. This sequence was blasted and submitted to the GenBank data base under the accession number 1112701. Multiple alignment of the sequence with DREBs was performed with CLUSTALW program (Fig. 4). By analyzing this sequence in http://au.expasy.org.tools/dna.html, three 5'3'frame were predicated. The analysis results also showed that there was in-frame 2, two stop codon TAA and TAG upstream from the first initiation codon ATG at the 5'-end. And there was also an in-frame 3 stop codon at the 5'-end. These indicated that it was a full-length gene probably. Based on the sequencing result, further analysis via Plants program (Gribskov *et al*., 2001) indicated that the deuced amino acids, DREB, was a protein kinase catalytic domain similar to *Arabidopsis thaliana* and also was a protein kinase catalytic domain similar to *Oryza sativa*. Blasting protein homologies in NCBI by the deduced amino acid sequence of DREB showed the greatest identities to DREB group from *Arabidopsis* (Fig. 4), suggesting that DREB is a member of this group.

DISCUSSION

Isolation of an unknown sequence related to known sequences is a powerful method for investigation biological function. The sequence of an unknown protein in one organism may be homologous to those of known proteins from different organisms, or may be related to a known protein sequence belonging to a multi gene family within an organism. In many cases, low-stringency hybridization or PCR methods have succeeded in obtaining such desired genes. However, as the degree of protein similarity decreases, so does success in gene isolation. When only a single sequence is known, low-stringency hybridization is used, although a fairly long region of similarity may be needed. Moreover, considerable effort is required to determine whether a candidate clone is a correct one. If a family of proteins is available, then consensus or degenerate PCR methods may be used, because region of high sequence similarity can be identified and utilized in the design of PCR primer. PCR methods are not only faster and easier than low-stringency hybridization, but product size and homogeneity can also be used to judge probable success. However, consensus primers may be too dissimilar to an

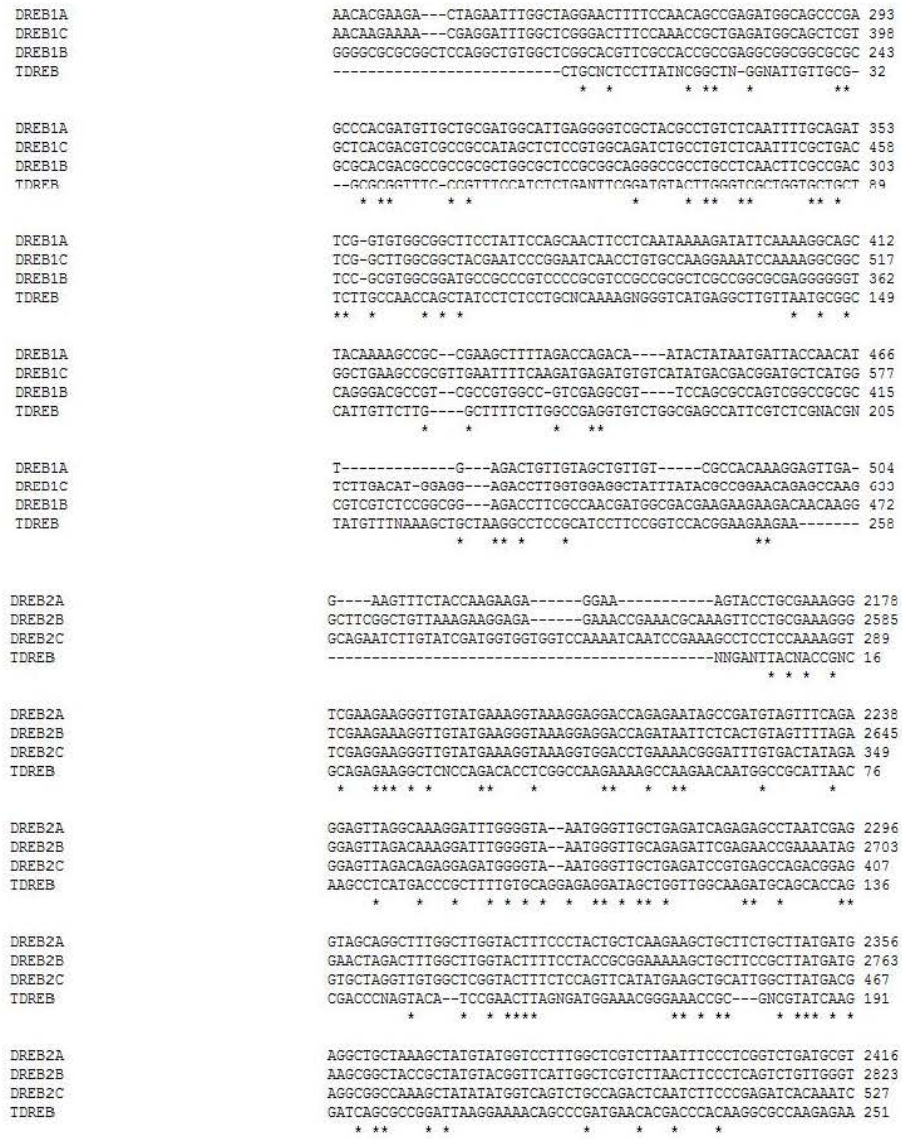

Fig. 4: Alignment of nucleotide sequence of TDREB with that of other closely related sequences from plant: *Arabidopsis* DREB1A and DREB2A (GeneBank accession numbers NM-120623 and NM-111939), *Oryza sativa* DREB1B and DREB2B (GeneBank accession numbers AYo644o3 and AY646223), *Zea mays* DREB1C and DREB2C (GeneBank accession numbers AF448789 and NM-00111611). In the consensus sequence, asterisk (*) indicate positions at which the sequence are identical or similar. Sequences were aligned using Clustal w and Genedoc

unknown target to efficiently anneal to the original template and degenerate primers may be too dissimilar to each other to efficiently amplify the synthesized product.

Using the CODEHOP strategy on subgroups of phylogenetically related genes, families or clades within large super families such as DREB is a powerful approach for exploring their complexity in various genomes. It is a very effective tool for the construction of expression libraries for agrochemical and other industrial applications. It can also be used for identifying genes from a given subgroup expressed at specific stage of development.

Many genes have been demonstrated to respond to drought, high salt levels and cold stress (Nordin *et al*., 1991; Wang *et al*., 1995) and the proteins encoded by

these genes are thought to function in protecting cell from these stresses (Jiang *et al*., 1996; Ouellet *et al*., 1998). One stress inducible DREB-responsive gene, encodes a protein similar to the LEA proteins (Yamaguchi-Shinozaki and Shinozaki, 1993). These proteins are quite hydrophilic and are believed to function by directly protecting plant cells from these stresses (Ingram and Bartels, 1996; Bray, 1997). This similarity in structure and expression suggest that the products of this gene may have similar function in plants. In *Arabidopsis*, these genes are called *rd* (responsive to dehydration), *erd* (early responsive to dehydration), *lti* (low-temperature induced), *kin* (cold-induced) and *cor* (cold-regulated). These genes are also induced by dehydration (due to water deficit or high salt) and abscisic acid and can be collectively called cold-responsive genes. In wheat and its relatives, which grow under widely different climatic conditions, exhibit a large genetic variability in cold/freezing tolerance. A number of genes classified in the families of *Lea* (late embryogenesis abundant), *Dhn* (dehydrin), *Rab* (responsive to abscisic acid) have been isolated and their mode of expression has been characterized in wheat, barley and rye. However, the molecular structure and the function of their promoter sequences are largely unknown and, in fact, such important information is only available for the two genes, *Wcs120* of wheat and *Blt4.9* of barley. Also, *Wcor15*, a member of the wheat cold-responsive (cor) gene family, has been isolated and characterized (Takumi *et al*., 2003).

An important step towards understanding of the cold-responsive gene regulation was isolation of a gene encoding a CRT/DRE-binding protein, called CBF1, from *Arabidopsis* by Stockinger *et al*. (1997). Later, five independent genes encoding DREBs were isolated from *Arabidopsis* using a yeast one-hybrid screening. Both DREB1 and DREB2 can specifically bind to the CRT/DRE elements and trans activate cold-responsive genes in yeast and *Arabidopsis* protoplasts. Expression of DREB1A and its homologues, DREB1B and DREB1C, is induced by low temperature stress (Liu *et al*., 1998). Thus two independent families of DREB proteins, DREB1 and DREB2 function as transcriptional factors in low temperature and dehydration signal transduction pathways, respectively, to activate CRT/DRE *cis*-element. Liu *et al*. (1998) showed that DREB1 is induced by cold (4°C) within 40 min, reaches maximum expression by 2 h and then slowly decreases to a minimum level by 24 h at 4°C. Analysis of promoter regions of DREB1-family genes of *Arabidopsis* revealed that the 5' upstream regions contain motifs similar to G-box and ABRE (abscisic acid responsive element) sequences.

In this study, at first gene expression pattern were studied at -2, -4 and -8°C, then the DREB sequence analyzed and characterized. The deduced amino acid sequence showed more than 50% identity whit DREB from *Arabidopsis* and *Oryza sativa*. However, an alignment of amino acid sequence revealed that the DREB were closely related to the transcription factors which are known to be the typical DREB1 group. All these above indicate that DREB is a member of plant's DREB. Using the PlantsP program, a protein kinase catalytic domain was found in this group. This domain plays a vital role in signal transduction in plant responses to environmental stress (Gilmaur *et al*., 1998). Significantly, a large number of DREB-related sequences are found among wheat ESTs isolated from wheat challenge with stress. Recombinant expression of such genes should provide a valuable library for environmental stress investigations and for studying evolutionary ecology of plant stress interactions (Rieseberg, 2001).

CONCLUSIONS

The CODEHOP strategy appears as a powerful method for exploring the complexity of gene families in plants with a large genome and conservation of genes across evolution. DREB are genes evolved early and are highly conserved during evolution, as expected for genes with an essential role in transcription and protecting plant cells from a biotic stresses. Conversely, The DRE are more variable and it found in many plants species, are also present in wheat. The great variability of this subfamily in wheat strongly suggests a role in environmental adaptation and plant response. It is important to continue to explore in agronomically important crops, such as rice, wheat, maize, soybean, tomato etc., which suffer from low/freezing temperature, whether similar cold signaling modules are employed. If different mechanisms are found, then future work will identify the novel components.

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REFERENCES

Bohnert, H.J., D.E. Nelson and R.G. Jensen, 1995. Adaptations to environmental stresses. Plant Cell, 7: 1099-1111.

Bohnert, H.J., A. Patricia, B. Chris, R.A. Bressan and R.L. Burnap *et al*., 2001. A genomics approach towards salt stress tolerance. Plant Physiol. Biochem., 39: 295-311.

Bray, E.A., 1997. Plant responses to water deficit. Trends Plant Sci., 2: 48-54.

Gilmour, S.J., D.G. Zarka and E.J. Stockinger, 1998. Low temperature regulation of the Arabidopsis CBF family of AP2 transcriptional activators as on early step in cold-induced cor gene expression. Plant J., 16: 433-442.

Gribskov, M., F. Fana, J. Harper, D.A. Hope and A.C. Harmon *et al*., 2001. Plants P: A functional genomics database for plant phosphorylation. Nucleic Acids Res., 29: 111-113.

Ingram, J. and D. Bartels, 1996. The molecular basis of dehydration tolerance in plants. Ann. Rev. Plant Physiol. Plant Mol. Biol., 47: 377-403.

Jaglo-Ottosen, K.R., S.J. Gilmour, D.G. Zarka, O. Schabenberger and M.F. Thomashow, 1998. *Arabidopsis* CBF1 overexpression induced cor genes and enhances freezing tolerance. Science, 280: 104-106.

Jiang, C., B. Iu and J. Singh, 1996. Requirement of a CCGAC cis-acting element for cold induction of the *BN115* gene from winter *Brassica napus*. Plant Mol. Biol., 30: 679-684.

Kasuga, M., Q. Liu, S. Miura, K. Yamaguchi-Shinozaki and K. Shinozaki, 1999. Improving plant drought, salt and freezing tolerance by gene transfer of signal stress-inducible transcription factor. Natl. Biotechnol., 17: 287-291.

Kawasaki, S., C. Borchert, M. Deyholos, H. Wang and S. Brazille *et al*., 2001. Gene expression profiles during the initial phase of salt stress in rice. Plant Cell., 13: 889-905.

Liu, Q., Y. Sakuma, H. Abe, M. Kasuga and S. Miura, 1998. Two transcription factor, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction phatways in drought and low temperature-responsive gene expression, respectively, in Arabidopsis. Plant Cell, 10: 1391-1406.

Medina, J., M. Bargues, J. Terol, M. Perez-Alonso and J. Salinas, 1999. The *Arabidopsis CBF* gene family is composed of three genes encoding AP2 domain-containing proteins whose expression is regulated by low temperature but not by abscisic acid or dehydration. Plant Physiol., 119: 463-470.

Nordin, K., P. Heino and E.T. Palva, 1991. Separate signal pathways regulate the expression of a law-temperature-induced gene in *Arabidopsis thaliana*. Plant Mol. Biol., 16: 1061-1071.

Ouellet, F., A. Vazquez-Tello and F. Sarhan, 1998. The wheat *wcs120* promoter is cold-inducible in both monocotyledonous and dicotyledonous species. FEBS Lett., 423: 324-328.

Rieseberg, L.H., 2001. Polyploidy evolution: Keeping peace at genomic reunions. Curr. Biol., 11: R925-R928.

Rose, T.M., E.R. Schults, J.G. Henikoff, S. Pietrovski, C. Mc Callum and S. Henikoff, 1998. Consensus-degenerate hybrid oligonucleotide primer for amplification of distantly related sequences. Nucleic Acids Res., 26: 1628-1635.

Seki, M., M. Narusaka, H. Abe, M. Kasuga and K. Yamaguchi-Shinozaki *et al*., 2001. Monitoring the expression pattern of 1300 Arabidopsis genes under drought and cold stresses by using a full-length cDNA microarray. Plant Cell, 13: 61-72.

Shinozaki, K. and H. Yamaguchi-Shinozaki, 1996. Molecular responses to drought and cold stress. Curr. Opin. Biotechnol., 7: 161-167.

Shinozaki, K. and K. Yamaguchi-Shinozaki, 1997. Gene expression and signal transduction in water-stress response. Plant Physiol., 115: 327-334.

Stockinger, E.J., S.J. Glimour and M.F. Thomashow, 1997. *Arabidopsis thaliana* CBF1 encods an AP2 domain-containing transcription activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proc. Natl. Acad. Sci. USA., 94: 1035-1040.

Takumi, S., A. Koike, M. Nakata, S. Kume, R. Ohno and C. Nakamura, 2003. Cold-specific and light-stimulated expression of a wheat (*Triticum aestivum* L.) Cor gene *Wcor15* encoding a chloroplast-targeted protein. J. Exp. Bot., 54: 2265-2274.

Thomashow, M.F., 1994. Arabidopsis thaliana as a model for studying mechanisms of plant cold tolerance. Cold Spring Harbor Monograph. Series, 27: 807-834.

Thomashow, M.F., 2001. So what's new in the field of plant cold acclimation? Lots! Plant Physiol., 125: 89-93.

Thompson, J.D., D.G. Higgins and T.J. Gibson, 1994. Clustal w: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res., 22: 4673-4680.

Wang, H., R. Datla, F. Georges, M. Leowen and A.J. Cutler, 1995. Promoters from kin1 and *cor6.6* two homologous *Arabidopsis thaliana* genes: Transcriptional regulation and gene expression induced by low temperature, ABA osmoticum and dehydration. Plant Mol. Biol., 28: 605-617.

Yamaguchi-Shinozaki, K. and K. Shinozaki, 1993. Characterization of the expression of a desiccation-responsive *rd29* gene of *Arabidopsis thaliana* and analysis of its promoter in transgenic plants. Mol. Gen. Genet., 236: 331-340.

Yamaguchi-Shinozaki, K. and K. Shinozaki, 1994. A novel cis-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell, 6: 251-264.

Zhang, G.F., 2001. PlantsP: A functional genomic database for plant phosphorylation. Nucleic Acids. Res., 29: 111-113.

Zou, H., X. Zhang, J. Zhao, Q. Yang, Z. Wu, F. Wang and C. Huang, 2006. Cloning and characterization of maize *ZmSPK1*, a homologue to non fermenting 1-related protein Kinase2. Afr. J. Biotechnol., 5: 490-496.