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Isolation of a Cold-Responsive Gene (*Wcor14*) Encoding a Chloroplast-Targeted Protein from *Aegilops tauschii*

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Abstract: To determine the genetic nature of these mechanisms, several cold-responsive genes were identified. *Wcor14*, a member of the wheat cold-responsive (*Cor*) gene family, in this study has been isolated and characterized from ancient wheat ancestor *Aegilops tauschii*. The deduced polypeptide *Aegilops tauschii* WCOR14 is a hydrophobic polypeptide with 140 amino acids (MW = 13.5 kDa) showed high homology to the previously identified wheat and barley COR proteins. Analyses of the cDNA and genomic DNA sequences in this study suggested that, *Aegilops tauschii Wcor14* and its related sequences constitute a small multigene family with different intron sizes. The transcripts Analyses of *Wcor14* suggested that *Wcor14* transcripts were up-regulated within 3-6 h of cold acclimation at 4°C. We used SCRATCH server for protein structure prediction.

Key words: Cold-responsive (*Cor*) gene family, *Aegilops tauschii*, *Wcor14*

INTRODUCTION

Cold/freezing temperature is one of the most significant abiotic stresses restricting habitats of sessile plants and reducing crop productivity. Plants in temperate regions have evolved different degrees of ability to survive cold/freezing temperature stress. One prominent adaptive mechanism to this temperature stress is known as cold hardening or acclimation (Levitt, 1980). Cold acclimation is triggered by the exposure of plants to low but nonfreezing temperatures for certain periods of time. During this process, plants exhibit dramatic alterations in their gene expression profiles, which are characterized by the induction of a battery of cold-responsive (*Cor*) genes (Guy *et al.*, 1985; Guy, 1990). Importantly, this adaptive process is believed to be tightly associated with the development of cold/freezing tolerance (Thomashow, 1998, 1999).

Wheat and its relatives, which grow under widely different climatic conditions, exhibit a large genetic variation in cold/freezing tolerance (Fowler and Gusta, 1979; Veisz and Sutka, 1990). 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 (Houde *et al.*, 1992; Vazquez-Tello *et al.*, 1998; Ouellet *et al.*, 1998) and *Blt4.9* of barley (Dunn *et al.*, 1998). By contrast, significant study results are available for the dicotyledonous model plant Arabidopsis and it has now become a general view that the *Cor* genes are regulated through a specific signal transduction pathway. A functional cis-acting element of the Arabidopsis *Cor* genes, i.e., the CCGAC core motif known as a CRT(Crepeat)/DRE (dehydration responsive element) sequence, has been proved to play a pivotal role in the promoter function of *COR15A/RD29A* genes (Yamaguchi-Shinozaki and Shinozaki, 1994; Baker *et al.*, 1994). It has also been shown that the constitutive expression of the CRT/DRE binding protein genes (*CBF/DREB1*) in transgenic plants results in the expression of the CRT/DRE-controlled *Cor* genes without a low temperature treatment and increases their freezing tolerance (Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998). The levels of accumulation of *Cor*/*Lea* transcripts positively correlate with the levels of freezing tolerance in seedlings of common wheat cultivars (Kobayashi *et al.*, 2004). An ABA-independent wheat *Cor* gene *Wcor15* encodes a chloroplast-targeted COR protein analogous to the *Arabidopsis* protein COR15a (Takumi *et al.*, 2003). COR14b is, like *Arabidopsis thaliana* COR15a (Lin and Thomashow, 1992; Thomashow, 1994), a leaf-specific protein and transported into the stromal compartment of

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the chloroplasts during cold acclimation (Crosatti *et al*., 1995, 1999). In contrast to *Arabidopsis* COR15a, however, barley COR14b is considerably hydrophobic and its expression is light-stimulated (Crosatti *et al*., 1995, 1999). In wheat, one unique member of *Cor* genes designated as *Wcs19* encodes a leaf specific and basic (pI = 8.8) protein WCS19 which is also transported into the stromal compartment of the chloroplasts during cold acclimation (Chauvin *et al*., 1993; Gray *et al*., 1997). Since, WCS19 shows a low level of homology with the barley COR14b, we attempted to isolate the orthologue of *cor14b*. Bread wheat (*Triticum aestivum*), originated by hybridization of cultivated allotetraploid emmer wheat (*T. turgidum* ssp. *dicoccum*, 2n = 4x = 28, genomes *AABB*) with diploid *Aegilops tauschii* or *Ae. squarrosa* (2n = 2x =14, genome DD) (Caldwell *et al*., 2004). *Aegilops squarrosa* (n = 7) is the donor of the third or D genome to common wheat and also the donor of the pivotal genome to five polyploid species of section Vertebrata of the genus *Aegilops*. This diploid species grows as a predominantly autogamous wild grass or weed in the Middle East. *Ae. squarrosa* as well as *Triticum monococcum* and *Ae. speltoides* constitute a large unexplored gene pool for wheat breeding (Zohary *et al*., 1969). In this study we attempted to isolate the orthologue of *cor14* from *Aegilops tauschii*. We herein report the structural heterogeneity of cDNAs, distribution, low temperature-specificity and Protein structure of the identified *Aegilops tauschii Wcor14* gene.

MATERIALS AND METHODS

Plant materials: This study was conducted in spring of 2008 in Institute of Biotechnology, University of Shiraz. Three cultivars of Iranian common wheat (*Triticum aestivum* L.), winter-type Azare2, Sardari, Alamoot and wild diploid *Aegilops tauschii* were used. A winter hardy common wheat cv. Azare2 was bred in IRAN. It was reported as one of the hardiest cultivars among tetraploid and hexaploid common wheat tested for cold tolerance. seeds from each of the cultivars and *Aegilops tauschii* were planted as separate groups in the same pots (20×12 cm in width and 12 cm in depth) with soil and incubated in a growth chamber under the following standard temperature and light conditions; 24±0.5°C with a 14 h photoperiod at a light intensity of 100-110 μm photons/m^2/sec provided by cool white fluorescent lamps. 23-day-old Seedlings were cold-acclimated at 4±0.5°C for different periods under the standard light condition at intensity of 100-110 μm photons/m^2/sec provided by cool white fluorescent lamps with a 14 h photoperiod.

Genomic PCR analysis: Total DNA was extracted from leaves of 2-week-old seedlings were harvested and used for PCR amplification of the genomic *wcor14* sequence. A nested-PCR was conducted in the following way: the first amplification was performed using a forward primer (WcorF1:5'-CTCGTCCCACACCGTCAGC-3') and a reverse primer(WcorR1: 5'-TCATTTGCTCACATCCTCGACCGC-3') and the second amplification using a primer (WcorF2: 5'-CTGCCTGCAAACCCCTCCTA-3') and a primer (WcorR2: 5'-CCTCCTCCGTCGCCTGCTTCGCCT-3').

Total RNA extraction and cDNA synthesis: Total RNA was isolated from 100 mg of leaves with RNX-Plus™ kit according to the manufacturer's specifications. The concentration of total RNA was determined with NanoDrop by measuring absorbance at a wavelength of 260 nm (A260) and purity was assessed by the ratio of the absorbance values at 260 and 280 nm, wherein a ratio of about 2.0 was considered a good indication of purity. Before using RNA preparation in cDNA synthesis the accuracy of isolated RNA was established (by detection of RNA bands on an agarose gel). The best concentration of agarose gel for RNA electrophoresis is 1.5%. The voltage applied across the gel was 40 V. Staining was performed by ethidium bromide (0.5 mg mL^{-1}). Five milligram of total RNA was loaded in each well. The presence of two ribosomal RNA bands (28 and 18 S) and small smear between them, proved the precision of the RNA accuracy (Sambrook and Russell, 2001). For cDNA synthesis 1 μg total RNA and 0.5 μg oligo dT18 primer was added to a microtube and incubated for 5 min at 70°C, cooled on ice. dNTP (1 mM), Tris buffer (10 mM) and RNAse inhibitor (40 u/20 μL) was added and 5 min incubated at 37°C, finally 200 u M-MuLV enzyme was added to cocktail and incubated for 60 min at 37°C. Then incubated for 10 min at 70°C in order to inactivate the enzyme. Synthesized cDNA stored at -20°C (Sambrook and Russell, 2001).

Polymerase chain reaction: Each PCR reaction was performed in a total volume of 25 μL containing, Tris buffer (10 mM, pH = 8), $MgCl_2$ (2 mM), dNTP mix (0.8 mM, each dATP, dTTP, dCTP, dGTP 0.2 mM), Taq DNA polymerase (1 u), cDNA (2 μL), sense primer and antisense primer (each 0.5 μM), product size 423 bp (Fig. 1). An initial denaturation step of 5 min 94°C was followed by 30 cycles of: 45 sec 94°C, 45 sec 68°C, 2.3 min 72°C and end step 5 min 72°C. The following primer set was designed to isolate cDNA clones containing a complete Open-Reading-Frame (ORF), WcorF: 5'-ATGGCTTCTTCTTCCGTGCTGCT-3' and

WcorR: 5'-TCATTTGCTCACATCCTCGACCG C-3'. The amplified fragments were subcloned into pTZ57R/T vector (fermentas) and their sequences were determined. The genomic and cDNA sequences and the amino acid sequences were analyzed by Vector NTI 9.0. The sequence homology was searched with the BLAST algorithm (Karlin and Altschul, 1993) and a multiple alignment was calculated by blosum62mt2's matrix.

RESULTS AND DISCUSSION

Structural analysis of *Wcor14* cDNAs and their deduced WCOR14 polypeptides: A period of cold acclimation is an important factor for *Wcor14* transcript accumulation. An early study on the protein profile in response to low temperature in wheat demonstrated that two groups of translatable mRNAs were expressed during cold acclimation (Danyluk *et al*., 1991). The first group was transient and consisted of 18 mRNAs that reached their highest levels of induction after 1 day of low temperature exposure but thereafter decreased to undetectable levels. The second group consisted of 53 mRNAs that were also induced rapidly but maintained their high levels of expression during 4 weeks of the experiment. Among the second group, at least 34 were expressed at higher levels in freezing tolerant winter wheat cultivars than in less tolerant spring wheat cultivars and *Wcor14* is in the second group. The identical sequence (designated as *Aegilops tauschii Wcor14*, accession number FJ670451) had 423 bp containing an open reading frame which was predicted to encode a 13.5 kDa acidic (pI = 4.71) polypeptide of 140 amino acid residues (Fig. 1). Transcripts of *Wcor14* accumulated within 3-6 h of cold acclimation at 4°C and reached a maximum after 3 days. A search in the NCBI database found four sequences of *Triticum aestivum* encoding *Wcor14*: *Wcor14a* (accession number AF207545), *Wcor14b* (accession number AF207546), *WCOR14a* (Accession number AF491838) and *WCOR14c* (AF491837). The deduced amino acid sequence of *Aegilops tauschii Wcor14* protein (WCOR14) showed 70% identity with the barley COR14b (pI = 4.5) and 100% identity with WCOR14a (pI = 4.71) (Fig. 2a, b). Notably, a stretch of the N-terminal 51 amino acid residues of WCOR14 was nearly identical to that of the barley COR14b (98% identity with only one amino acid difference) and highly homologous (78% identity) to that of the wheat WCS19. The remaining downstream part of WCOR14 showed 54% identity with COR14b but only 34% identity with WCS19. In contract to *Arabidopsis* COR15a, WCOR14 was considerably hydrophobic (59% hydrophobic residues) similar to COR14b and WCS19 (56 and 55%, respectively).

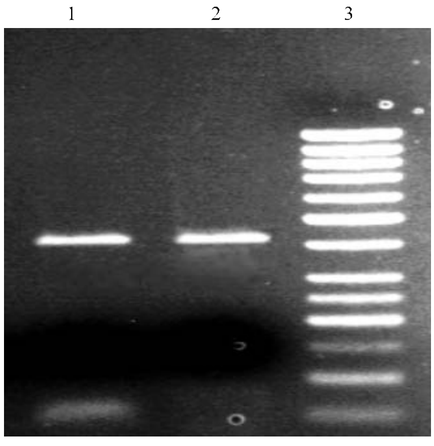

Fig. 1: Electrophoresis of *Wcor14* PCR products. Lane 1: *Triticum aestivum*, lane 2: *Aegilops tauschii* and lane 3 is 50 bp molecular weight marker

The barley COR14b immunologically cross-reacts with a related, chloroplast-imported protein COR14a (Crosatti *et al*., 1999). N-terminal microsequencing of COR14b was unsuccessful but that of the N-terminal 11 amino acids of COR14a purified from the chloroplast fraction suggested that it was encoded by a gene *cor14a* independent from *cor14b*. Because of the homology of this partial N-terminal sequence with the corresponding part of WCS19, the barley *cor14a* was suggested to be orthologous to the wheat *wcs19*. Similar to the barley COR14b, however, the N-terminal 51 amino acids of WCOR14a lacked the loosely defined consensus cleavage sequence of (Val/Lle)-X-(Ala/Cys)-Ala which is characteristic for transit peptides that target nuclear-encoded proteins to the stromal compartment of chloroplasts (Gavel and von Heijne, 1990). Except for this discrepancy, the sequence had several features in common with the reported chloroplast transit peptides. First, it had a relatively high serine plus threonine content (12%) but had no acidic residues. Second, it had an uncharged N-terminal domain (residues 1 to 23), a central domain (24 to 41) containing 3 positively charged residues and lacking acidic residues (Garnier *et al*., 1978).

Protein structure prediction: Knowledge of a protein's structure provides insight into how it can interact with other proteins, DNA/RNA and small molecules. It is these interactions which define the protein's function and biological role in an organism. Thus, protein structure and structural feature prediction is a fundamental area of computational biology. Its importance is exacerbated by large amounts of sequence data coming from genomics projects and the fact that experimentally determining

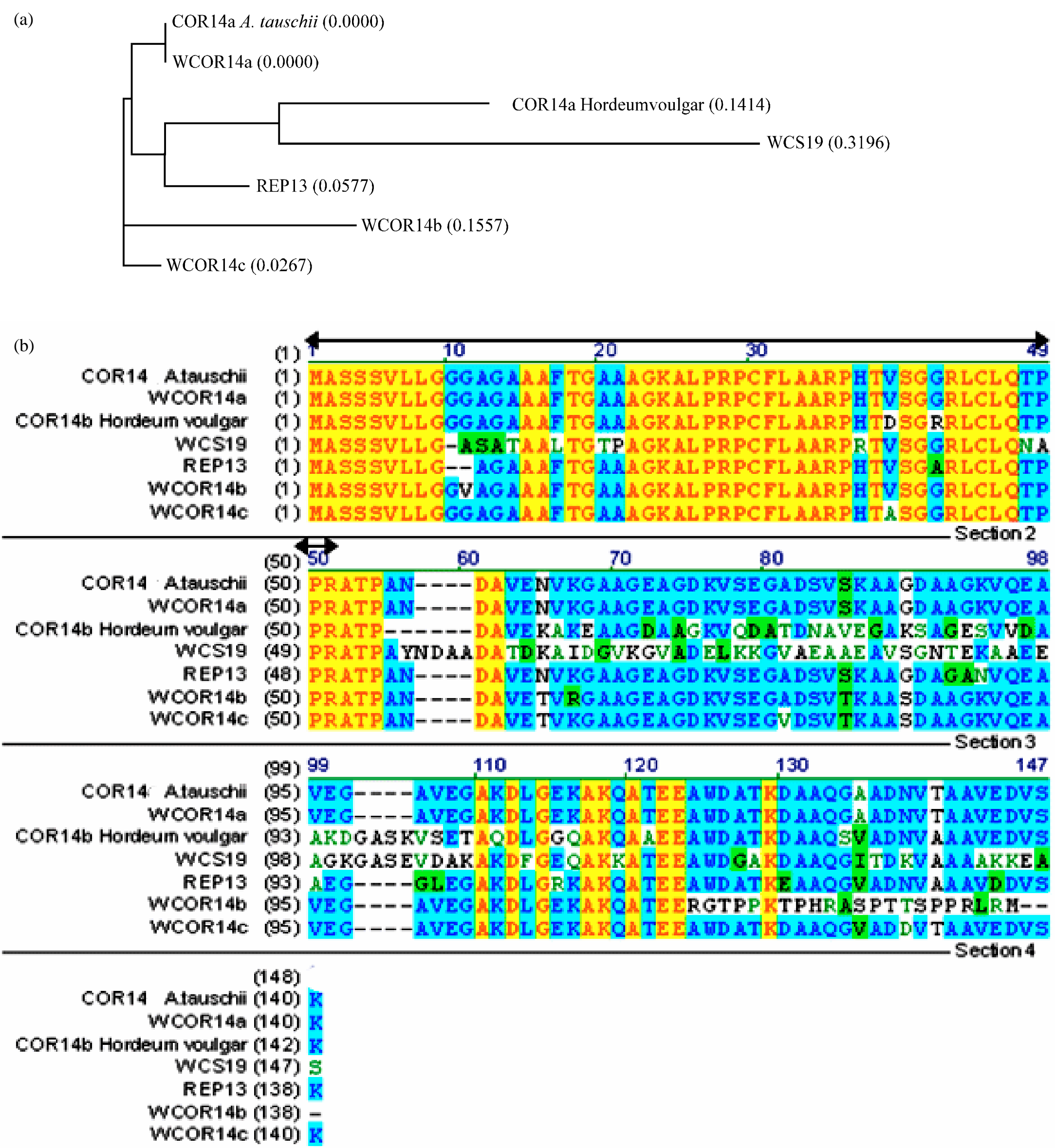

Fig. 2: (a) Phylogenetic tree based on amino acid sequences showing the relationship of *A. tauschii* COR14 with other homologous and (b) an alignment of the deduced WCOR14 polypeptide and its homologous proteins. The N-terminal amino acid residues shown with a bold line indicate putative chloroplast signal peptides

protein structures remains expensive and time consuming. We used SCRATCH server for protein structure prediction (Fig. 3a, b). The approach to tertiary structure prediction (3Dpro) combines the use of predicted structural features (Pollastri *et al*., 2002; Pollastri and Baldi, 2002), a fragment library (Simons *et al*., 1997) and energy terms derived from the PDB statistics. The structural features used are secondary structure, relative solvent accessibility and a residue level contact map at a distance cut-off of 12Å.

Genomic structure of *Wcor14*: To further analyze genomic structure of the *Wcor14* loci, nested genomic PCR amplification was carried out using primer sets of WcorF1-WcorR1 and WcorF2-WcorR2 In this study, total DNA extracted from *Aegilops tauschii* and three cultivars of Iranian common wheat seedlings were used as a template. The result showed that there was one sized intron (97 bp intron) delimited by GT-AG in *Aegilops tauschii Wcor14* coding region and there were two different sized introns (174 bp of intron and 97 bp of

(a)

CCCCCEECCCCHHHHHHHHHHHCCCCCCCCEEECCCCCCCCCEEEECCCC
CCCCCCHHHHHHHCCCCCCCCCHHHHHHHHHHHHHHHHHHHHHHHHHHH
HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHCCC

(b)

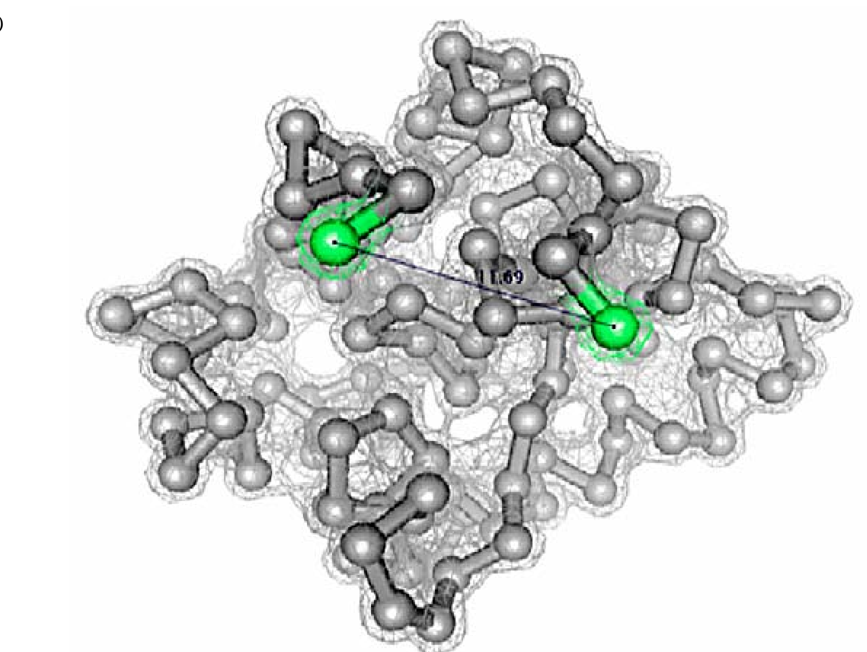

Fig. 3: (a) Predicted secondary structure (SSpro: Secondary structure prediction: H = Helix, E = Strand, C = The rest) and (b) predicted 3D structure of *Aegilops tauschii* WCOR14 protein

intron) delimited by GT-AG in the three cultivars of Iranian common wheat WCOR14 coding region.

Based on the present results, we conclude that *Aegilops tauschii* WCOR14 is a orthologue of the barley *cor14b*. The expression of WCOR14 is low temperature specific. Further, its turnover appeared to be rapid and the expression remains at a high steady-state level during the cold acclimation period. This finding suggests that a major regulatory switch governs the expression of Low temperature-responsive genes in wheat. The identification of this genetic system will certainly lead to a better understanding of how low temperature regulates this complex multigenic trait. Efforts can now be focused on the molecular characterization of this system and on the elucidation of the interaction occurring between this regulator and the promoter regions of Low temperature-responsive genes. These genes and their products are good candidate for functional analysis using bioinformatic, biochemical and genetic approaches. Future work should focus on studying the expression of additional cold acclimation genes and accumulation of proteins to get a better understanding of the complex gene interactions that regulate cold acclimation in the field. The level of protein accumulation should be studied to provide a better understanding of how protein accumulation relates to the level of cold tolerance.

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