

# Expression Profiling and Bioinformatic Analyses of a Novel Cold Stress-Regulated and Chloroplast-Targeted Protein from *Triticum aestivum* and *Aegilops tauschii*

E. Valiellahi (Corresponding author)

Institute of Biotechnology, University of Shiraz, Shiraz, Iran

E-mail: e\_valiellahi@yahoo.com

A. Niazi

Institute of Biotechnology, University of Shiraz, Shiraz, Iran

E-mail: Niazi47@yahoo.com

M. Farsi

Department of Biotechnology and Plant Breeding, College of Agriculture

Ferdowsi University of Mashhad, Mashhad, Iran

E-mail: Mohfarsi@yahoo.com

## Abstract

Cold acclimation is a multigenic trait that allows hardy plants to develop efficient tolerance mechanisms needed for winter survival. To determine the genetic nature of these mechanisms, several cold-responsive genes of unknown functions were identified from cold-acclimated wheat (*Triticum aestivum*). To identify the putative functions and structural features of these new genes, integrated genomic approaches of data mining, expression profiling and bioinformatic predictions were used. We herein report the structural heterogeneity of cDNAs, distribution, low temperature-specificity and protein structure of the identified *Wcor14* gene. Analyses of the cDNA and genomic DNA sequences by Vector NTI 9.0 software, suggested that, *Wcor14* and its related sequences constitute a small multigene family with different intron sizes. The deduced WCOR14 polypeptide, a hydrophobic polypeptide with 140 amino acids (MW=13.5 kDa), showed a high homology to the previously identified wheat and barley COR proteins. No homologous sequences were found in other organisms suggesting that this family is unique to the plant kingdom. The highly homologous signal peptides of WCOR14, BCOR14b and WCS19 contained one putative 14-3-3 protein recognition motif. In this motif, S-residue was predicted as a phosphorylation site and besides this, four other putative phosphorylation sites in WCOR14 were predicted by the NetPhos version 2.0 software. Comparative analyses of gene expression profiling shows that the expression of this gene is correlated with freezing tolerance in cereals.

**Keywords:** *Triticum aestivum*, Abiotic stress, Cold-acclimation, Expression profile, *Wcor14*

## 1. Introduction

To achieve the complete life cycle and reproduction in temperate regions, hardy plants like winter wheat (*Triticum aestivum*) have developed two major evolutionary adaptive mechanisms: vernalization and cold acclimation (CA). Cold acclimation is triggered by the exposure of plants to low temperature, for certain period 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). Overwintering plants sense the upcoming winter and delay flowering by postponing the transition from the vegetative to the cold-sensitive reproductive phase (Simpson *et al.* 1999). In addition, they develop a high degree of freezing tolerance (FT) needed for winter survival (Fowler *et al.* 1999). Following low temperature (LT) acclimation, some winter cereals can tolerate temperatures as low as -33°C. The regulatory mechanisms underlying these two processes and how they are interconnected are far from being fully understood. To gain further knowledge on the strategies that plants use for winter survival, the identification of

cold-regulated (*Cor*) genes are needed. A survey of the literature reveals that the expressions of a large number of genes are altered during the process of CA (Thomashow 1999; Breton *et al.* 2000; Seki *et al.* 2002).

The genes responsible for CA could be classified into four groups, based on the presumed function of the encoded proteins. The first group comprises genes encoding structural proteins that may be involved in protecting the cells during LT stress. The second group represents those genes that regulate gene expression and signal transduction pathways, such as transcription factors, protein kinases, phosphatases and the enzymes involved in phosphoinoside metabolism. The third group represents genes encoding enzymes involved in the biosynthesis of different osmoprotectants, membrane lipids and those of the antioxidative response. The fourth group contains cold-induced genes encoding proteins of unknown function. To gain insight into the function of these novel proteins, a combination of expression profiling and bioinformatic tools, can be used to predict properties and features that may be important for their function.

Previous studies have shown that, compared with winter varieties, the less hardy spring wheat varieties cannot maintain the expression of *Cor* genes (e.g. the *Wcs19* family) at a high level and this differential expression is closely associated with their low degree of FT (Sarhan *et al.* 1997). As a subsequent step, each novel LT-regulated protein sequence can be analyzed using available bioinformatic tools. These tools help in the identification of sorting signals, conserved posttranslational modifications, transmembrane helices, secondary and tertiary structures. The most recent prediction softwares incorporate machine-learning algorithms in the form of a neural network and a hidden Markov model (Blom *et al.* 1999; Krogh *et al.* 2001).

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). As WCS19 shows a low level of homology with the barley COR14b, we attempted to isolate the orthologue of *cor14b*. We herein report the structural heterogeneity of cDNAs, distribution, low temperature-specificity and protein structure of the identified *Wcor14* gene. To identify the putative functions and structural features of this new gene, integrated genomic approaches of data mining, expression profiling, and bioinformatic predictions were used. 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 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).

## 2. Materials and Methods

### 2.1 Plant materials

Three cultivars of Iranian common wheat (*T. aestivum* L.), winter-type Azare2, Sardari, Alamoot and wild diploid *Ae. tauschii* were used. Azare2 has been reported as one of the hardiest cultivars among tetraploid and hexaploid common wheat tested for cold tolerance. Seeds from each of wheat cultivars and *Ae. tauschii* were planted as separate groups in the same pots (20 cm × 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;  $24 \pm 0.5^{\circ}\text{C}$  with a 14h photoperiod at a light intensity of  $100\text{-}110 \mu\text{m photons m}^{-2}\text{s}^{-1}$  provided by cool white fluorescent lamps. Twenty three days-old seedlings were cold-acclimated at  $4 \pm 0.5^{\circ}\text{C}$  for different periods under the standard light condition at intensity of  $100\text{-}110 \mu\text{m photons m}^{-2}\text{s}^{-1}$  provided by cool white fluorescent lamps with a 14 hour photoperiod.

### 2.2 Genomic PCR analysis

Total genomic DNA, extracted from leaves of two weeks-old seedlings, were harvested and used for PCR amplification of the genomic *Wcor14* sequence. A nested-PCR carried out, where 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 (*WcorR2*: 5'-CCTCCTCCGTCGCCTGCTTCGCCT-3').

### 2.3 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. For cDNA synthesis one  $\mu\text{g}$  total RNA and 0.5  $\mu\text{g}$  oligo-dT primer was added and incubated for 5 min at  $70^{\circ}\text{C}$  and then cooled on ice. dNTP (1 mM), Tris buffer (10 mM) and RNase inhibitor (40 u/20  $\mu\text{l}$ ) was added and for

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 was stored at -20°C (Sambrook and Russell 2001).

#### 2.4 Semi-quantitative RT-PCR

Relative semi-quantitative PCR was performed to study the gene expression of *Wcor14*. Each PCR reaction was performed in a total volume of 25 µl containing, Tris buffer (10 mM, pH = 8), MgCl<sub>2</sub> (2 mM), dNTP mix (0.8 mM, each dATP, dTTP, dCTP, dGTP 0.2 mM), Taq DNA polymerase (1u), cDNA (2 µl), sense primer; 5'-CTGCCTGCAAACCCCTCCTA -3' and antisense primer 5'-CCTCCTCCGTCGCCTGCTTCGCCT-3' (each 0.5 µM). The product size of 215 bp was expected. To avoid false positives from pseudogenes of contaminating genomic DNA, primer sequences were designed to span intron regions. The PCR condition were as follows, initial denaturation step of 5 min at 94°C was followed by 30 cycles, 45 sec at 94°C, 45sec at 68°C, 2.3 min at 72°C, and end step 5 min at 72°C.  $\beta$ -tubulin, house keeping gene was used as the reference gene for *Wcor14* (Fig.1). The following primer set was designed to isolate a complete open-reading-frame (ORF) from cDNA, product size 423 bp (Fig.2): WcorF: 5'-ATGGCTTCTTCTCCGTGCTGCT-3' and WcorR: 5'-TCATTTGCTCACATCCTCGACCGC-3'.

#### 2.5 Gel Electrophoresis

The semi-quantitative PCR products were loaded onto ethidium bromide stained 1 % agarose gels in TBE buffer. Documentation of agarose gel was done and quantification of amplified products was performed by Total lab software. The intensities of the *Wcor14* mRNA bands were normalized relative to that of  $\beta$ -tubulin bands by dividing the former by the  $\beta$ -tubulin specific PCR product densities.  $\beta$ -tubulin acted as a referenc for sample to sample variation in reverse transcription and PCR conditions, and the extent of degradation and recovery of RNA.

#### 2.6 Protein isolation and analysis

Protein extract were prepared from four days cold-acclimated plant by homogenizing 500 mg of leaves in 1 ml of extract buffer (Tris-HCl 0.05 M, pH 8, 0.02% SDS, 30.3% urea, and 1% 2-mercaptoethanol) was added to each micro tube, and centrifuged at 13000 rpm for 10 min at 4°C. The supernatant contained dissolved extracted protein ready for experiment purposes. The protein concentration was determined by NanoDrop and 100ng protein was used for experiment. SDS-PAGE gel preparation, running and staining were carried out as standard procedures.

#### 2.7 Structural Analyses

For detection of specific targeting sequences, we used PSORT, iPSORT (Nakai and Kanehisa 1992; Bannai *et al.* 2002; <http://psort.nibb.ac.jp>), and TargetP v1.01 (Emanuelsson *et al.* 2000; <http://www.cbs.dtu.dk>). For TMD prediction, TMHMM (<http://www.cbs.dtu.dk>) was used (Krogh *et al.* 2001). Phosphorylation site predicted by NetPhos version 2.0 software (<http://genome.cbs.dtu.dk/services/NetPhos>). Predictions of secondary structure were carried out by SOPMA available in [www.expasy.org](http://www.expasy.org) (Jaakola *et al.* 2001). We used I-TASSER server for protein structure prediction (Zhang 2008).

### 3. Results and Discussion

#### 3.1 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 *Ae. 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 *Ae. tauschii Wcor14* coding region and there were two different sized introns (174 bp of intron and 97 bp of intron) delimited by GT-AG in the three cultivars of Iranian common wheat *Wcor14* coding region (Fig.3).

#### 3.2 Structural analysis of *Wcor14* cDNAs and their deduced WCOR14 polypeptides

A period of cold acclimation is an important factor for *Wcor14* transcript accumulation. A previous 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 consisted of 18 mRNAs that reached their highest levels of induction after one 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 all along four weeks of the experiment period. 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 sequences have been submitted to the GenBank databases (designated as *Ae. tauschii Wcor14*, accession number FJ670451, *T. aestivum* cultivar Adle cross cold-responsive protein WCOR14, accession number FJ605270 and *T. aestivum* cultivar Adle cross cold-responsive protein WCOR14, accession number FJ655857) 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. A search in the NCBI database resulted four sequences of *T. aestivum* encoding *Wcor14*: *Wcor14a* (accession number AF207545), *Wcor14b* (accession number AF207546), *WCOR14a* (Accession number AF491838) and *WCOR14c* (AF491837). The deduced amino acid sequence of *Ae. tauschii Wcor14* protein (WCOR14) showed 70% identity with the barley COR14b ( $pI = 4.5$ ) and 100% identity with WCOR14a ( $pI = 4.71$ ) (Fig. 4).

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 downstream part of WCOR14 showed 54% identity with COR14b, but only 34% identity with WCS19. In contrast to *Arabidopsis* COR15a, WCOR14 was considerably hydrophobic (59% hydrophobic residues) similar to COR14b and WCS19 (56% and 55%, respectively). 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*. The homology of this partial N-terminal sequence is high 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).

### 3.3 COR14 Proteins Contain Conserved Putative Phosphorylation Sites

Motif searches using the PROSITE, Pfam and Smart databases, did not detect any known motifs. However, the neural network-based NetPhos phosphorylation site prediction software generated several interesting findings (Blom *et al.* 1999). NDong *et al.* (2002), reported that the highly homologous signal peptides of WCOR14 and WCS19 contained one putative 14-3-3 protein recognition motif. This amino acid motif was conserved in WCOR15. In this motif, S-residue was predicted as a phosphorylation site by the NetPhos version 2.0 software (<http://genome.cbs.dtu.dk/services/NetPhos>). Besides this, there were four other putative phosphorylation sites in WCOR14 (Fig. 5). Generally, the 14-3-3 recognition motifs are phosphorylated and continuously interact with the 14-3-3 proteins (May and Soll 2000). The binding of the 14-3-3 proteins to the signal peptides is necessary for the chloroplast precursor proteins to be efficiently transported into chloroplasts (May and Soll 2000). Since both of the WCOR14 and WCOR15 proteins contain a putative 14-3-3 recognition motif in the chloroplast-targeting signal and WCOR15 at least is targeted into chloroplasts in transgenic tobacco plants (Takumi *et al.* 2003), these phosphorylated proteins might interact with the 14-3-3 proteins to be efficiently transported into chloroplasts of both monocotyledonous and dicotyledonous plants. Analysis using the TMHMM2.0 program (server for prediction of transmembrane helices in proteins) revealed that there is no transmembrane helices present in WCOR14 sequences.

### 3.4 Gene Expression Studies

A reverse transcriptase-PCR experiment was carried out to compare the mRNA profile of plants grown at 24°C with those of plants exposed at 4°C. The previous studies showed higher freezing tolerance of winter-hardy common wheat (*T. aestivum* L.) cv. Mironovska 808 compared with that of a spring-type common wheat cv. Chinese Spring by the simple one-point assay (Kume *et al.* 2005). It has been demonstrated that such cultivar difference in freezing tolerance could be partly caused by the differential accumulation levels of COR/LEA transcripts during cold acclimation (Vágújfalvi *et al.* 2000; Kobayashi *et al.* 2004). We also studied *Wcor14* transcript accumulation under the low temperature condition and compared the expression profiles of three Iranian cultivars *Wcor14* genes, and found that they were also induced early and at high levels in Azar2 and Sardari. Transcripts of *Wcor14* rapidly accumulated within 3–6 hours after cold acclimation at 4°C. The expression patterns clearly showed rapid response to LT in Azar2 and Sardari than Alamoot. There was a sharp increase in *Wcor14* transcripts in the leaves of all three genotypes after four days of LT acclimation (Fig. 6). In the non-acclimated control plants, no *Wcor14* transcripts were detected.

To examine the effect of long-term acclimation on *Wcor14* transcript accumulation, 23-day-old seedlings were placed under the LT condition and kept for 35 days. The amount of *Wcor14* transcript showed a gradual decrease under the long-term acclimation condition. The amount of the major transcript reached a maximum at day 4 and thereafter leveled down under the long-term acclimation condition (Fig. 6). The electrophoretic profiles of soluble proteins in three genotypes (Azar2, Alamoot and Sardari) showed the contrasting cold tolerance nature (Fig. 7). Comparison of profiles of proteins extracted from four day acclimated plants indicated that polypeptides of about 14 kDa were the major proteins that accumulated during LT condition. This approximately 14-kDa band was present at a very higher intensity in the acclimated plants than that of control plants (Fig. 7). Immunoblot analysis of the *Wcor14* proteins, using polyclonal antibodies showed that accumulation of *Wcor14* protein increased during cold acclimation condition (Kume *et al.* 2005).

### 3.5 Protein structure prediction

Knowledge of a protein 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. Protein prediction is exacerbated by large amounts of sequence data from whole genome projects and experimentally determining protein structures remains expensive and time consuming. The predicted secondary structure of *T.aestivum* cultivar Adle cross WCOR14 protein consisted of 68.57%  $\alpha$  helix, 25% random coil, 5% extended strand and 1.43%  $\beta$  turns and *Ae. tauschii* WCOR14 protein consisted of 65%  $\alpha$  helix, 25.71% random coil, 7.86% extended strand and 1.43%  $\beta$  turns. The random coil and alpha helix constituted domain comprised the main part of the secondary structure in these proteins. We used I-TASSER server for protein structure prediction (Fig. 8). I-TASSER is a hierarchical protein structure modeling approach based on the secondary-structure enhanced Profile-Profile threading Alignment (PPA) and the iterative implementation of the Threading ASSEMBLY Refinement (TASSER) program (Zhang 2008).

Our results strongly suggest that cold acclimation induced protein accumulation plays a crucial role in freezing tolerance of these genotypes. However, subsequent studies have suggested that LT tolerance is affected by other interacting regulatory circuitries (Chinnusamy *et al.* 2007), besides low temperature, genetic potential, and the factors determining developmental stage, such as vernalization and photoperiod (Mahfoozi *et al.* 2001). Regulatory loci at the *Vrn-A1/Fr1* loci on chromosome 5A have been shown to control expression of *Wcor14* (Vágújfalvi *et al.* 2000; Kobayashi *et al.* 2005), more specifically regions associated with *cbf*-lik sequences (Crosatti *et al.* 2003; Kobayashi *et al.* 2005). A *cis* acting element, named C-repeat (CRT) or LT responsive element (LTRE), containing A/GCCGAC motif that forms the core of the DRE sequence, have been shown to regulate LT inducible promoters in *Arabidopsis* (Baker *et al.* 1994; Stockinger *et al.* 1997), *Barascica* (Jiang *et al.* 1996), rice (Rabbani *et al.* 2003) and wheat (Takumi *et al.* 2003). The transcription factors that interact with the CRT/DRE element are the C-repeat Binding Factor/DRE Binding protein 1 (CBF/DREB1), which first was found in a yeast one-hybrid screen by Stockinger *et al.* (1997). Furthermore, Kume *et al.* (2005) demonstrated that a *cbf* homologue, *Wcbf2*, when activated by LT led to induction and increase in accumulation of *Cor/Lea* genes such as *Wcor14* and *Wcor15*. Thus the discrepancy in expression between Azar2, Sardari and Alamoot could be due to the influence of the *Vrn-A1* locus.

Based on the present results, we conclude that *Ae. tauschii Wcor14* is an 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 bioinformatics, 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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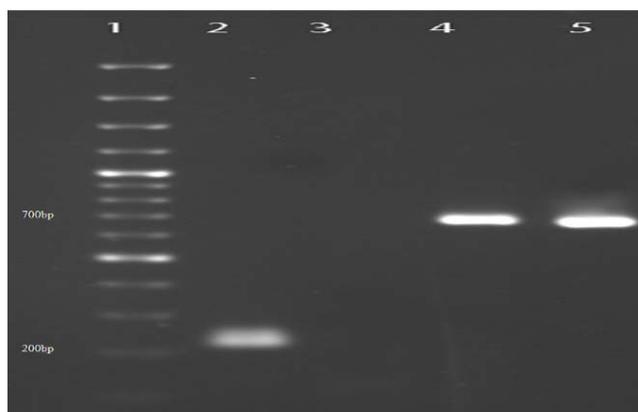


Figure 1. Semiquantitative RT-PCR of *Wcor14* mRNA from acclimated plant (lane 2) and control plant (lane 3) and  $\beta$ -tubulin as the reference gene (lanes 4, 5). Lane 1 is 100bp molecular weight marker

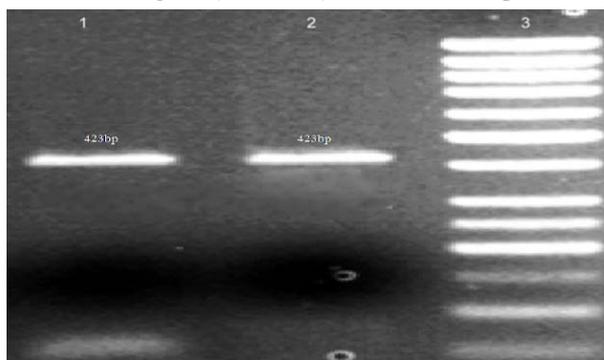


Figure 2. Electrophoresis of *Wcor14* PCR products. Lane 1: *Triticum aestivum*, Lane 2: *Ae. tauschii* and Lane 3 is 50bp molecular weight marker

intron Adle 174	(1)	GTACGTGCACTTGATGTTATTACTCTCTCTGTAAAGAAATATAAGAG
intron Adle 97bp	(1)	GTACGTGCACTTGATGTTACTAC-----
intron Aegilops	(1)	GTACGTGCACTTGATGTTACTAC-----
	51	100
intron Adle 174	(51)	TGTTTAGATCACTACTAACAATGATCTAACACTCTTATAATTTTTTACGG
intron Adle 97bp	(27)	-----
intron Aegilops	(27)	-----
	101	150
intron Adle 174	(101)	AGAAAGTACTTGCTGGTGTTCGTTCTTTGCGATCTTACGTATGCTGACT
intron Adle 97bp	(27)	---CACACTTGCTGGTGTTCGTTCTTTGCGATTTACGTATGCTGACT
intron Aegilops	(27)	---CACACTTGCTGGTGTTCGTTCTTTGCGATTTACGTATGCTGACT
	151	175
intron Adle 174	(150)	GTTTGTGATTCATTGTCGTCAG
intron Adle 97bp	(73)	GTTTGTGATTCATTGTCGTCAG
intron Aegilops	(73)	GTTTGTGATTCATTGTCGTCAG

Figure 3. Alignment of intron sequences of *Wcor14* loci. Introns were amplified by the nested PCR using total DNA from *Triticum aestivum* L. and *Ae. tauschii* as templates. Sequences of two different sized introns (174 bp and 97 bp) were detected



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MASSSVLLGGGAGAAAF TGAAAGKALPRPCFLAARPHTVSGGRLCLQTPPRATPANDAVENVKGAAGEAGDKVSEGADSV      80
SKAAGDAAGKVQEAVEGAVEGAKDLGEKAKQATEEAWDATKDAAQGAADNVTTAAVEDVSK                      160
.....S.....T.....S.....S.
S.....
    
```

Phosphorylation sites predicted: Ser: 4 Thr: 1 Tyr: 0

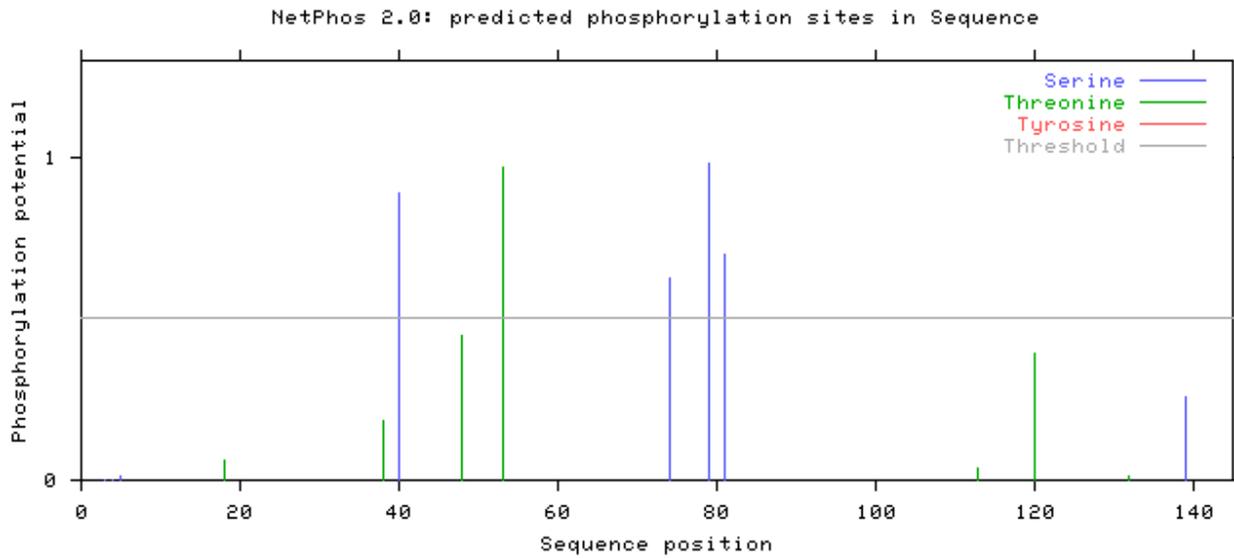


Figure 5. Predicted phosphorylation site in WCOR14 sequence.  
 Box show a putative 14-3-3 protein recognition motif.

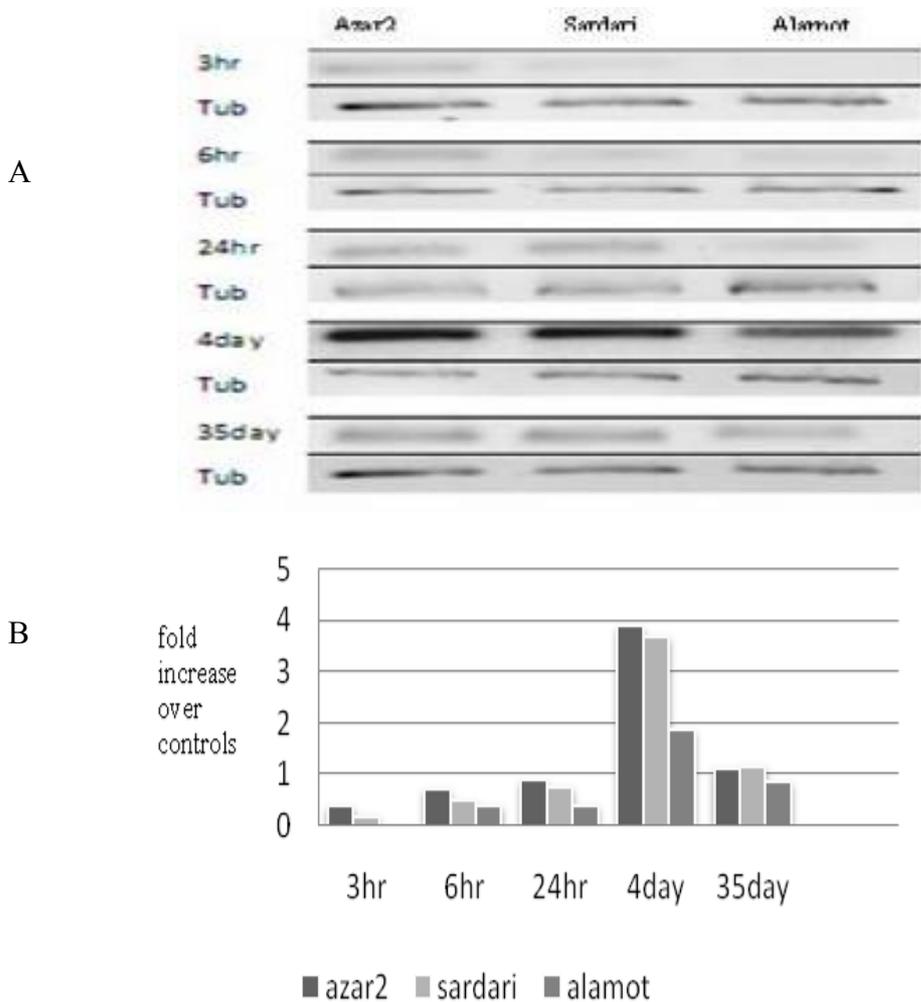


Figure 6. (A) Transient enhancement of the *Wcor14* gene expression by cold stress. Total RNA was isolated from seedling leaves of Azar2, Alamoot and Sardari at the indicated time points. Semi-quantitative RT-PCR analysis was conducted to detect the *Wcor14* gene transcript. The  $\beta$ -*tubulin* gene was used as a control. (B) Quantification of the *Wcor14* transcripts in different time. Using the internal standard  $\beta$ -*tubulin* RT-PCR signal as the denominator as described in the materials and methods section.

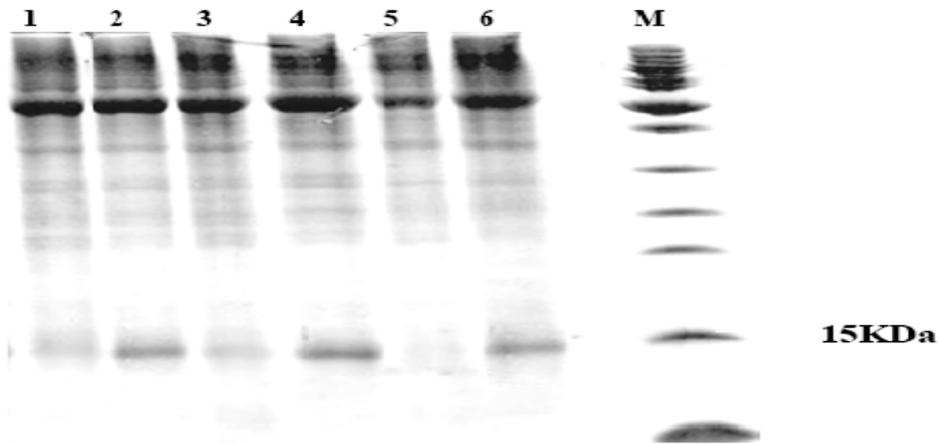
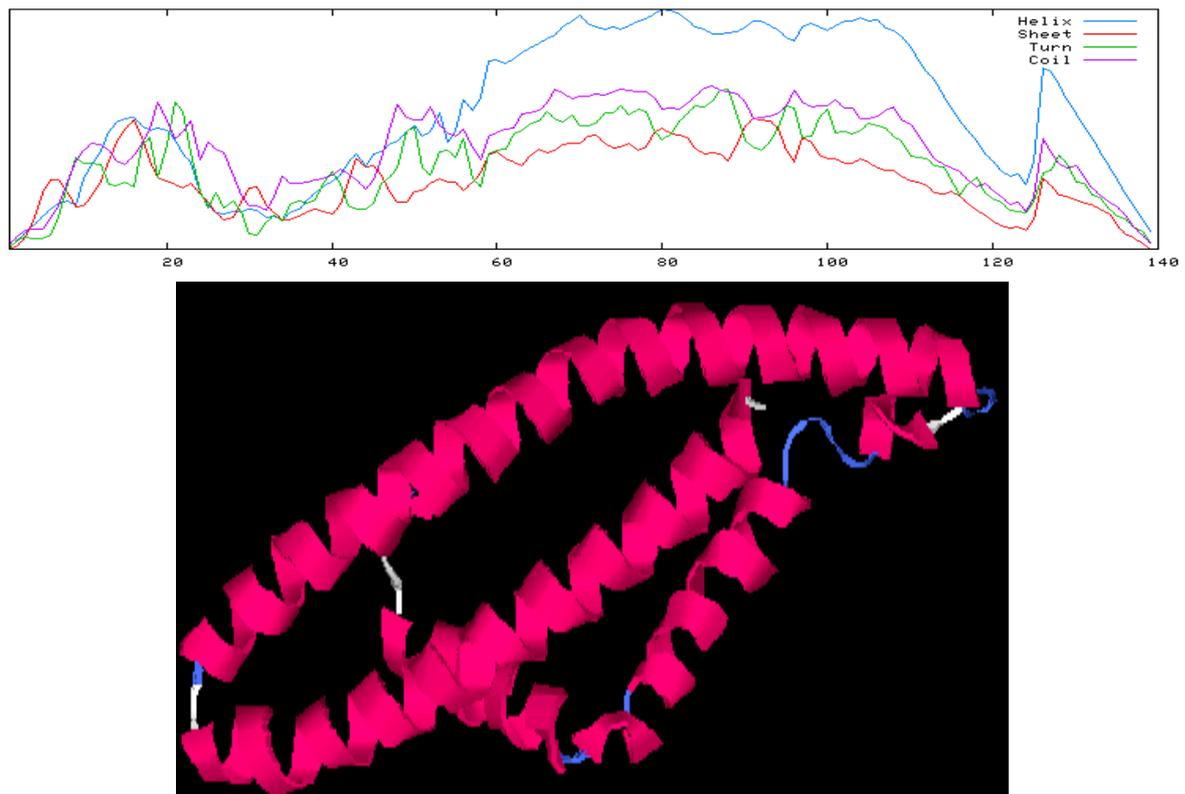


Figure 7. Electrophoresis profiles of soluble proteins in three genotypes (Azar2, Alamoot and Sardari). Lanes 1, 3, 5 profiles of Azare2, Alamoot and Sardari non-acclimated control plant, respectively. Lanes 2, 4, 6 profiles of Azare2, Alamoot and Sardari 4days cold-acclimated plant, respectively

A



B

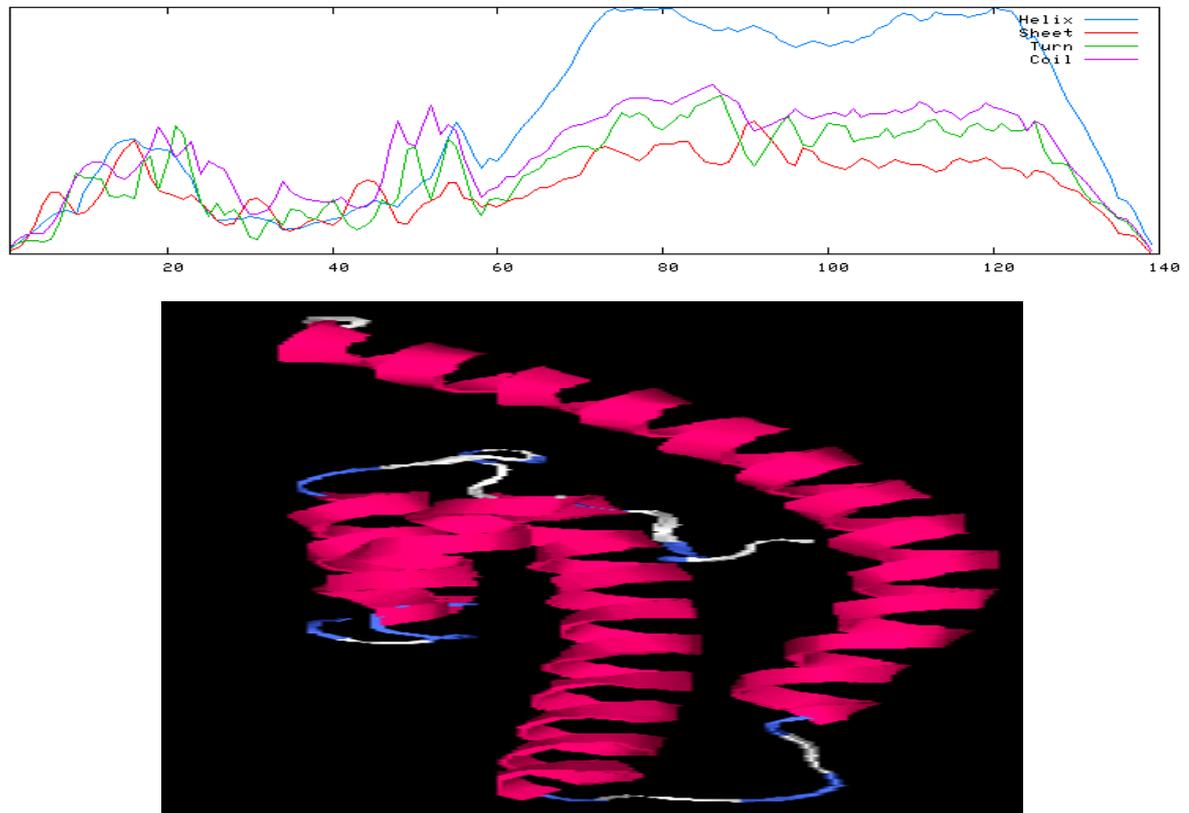


Figure 8. Predicted secondary and tertiary structure of *Triticum aestivum* cultivar Adle cross WCOR14 protein (A) and *Aegilops tauschii* WCOR14 protein (B)