Research Article

Characterization and expression analysis of WOX2 homeodomain transcription factor in *Aegilops tauschii*

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Abstract

The WUSCHEL (WUS)-related homeobox (WOX) gene family coordinates transcription during the early phases of embryogenesis. In this study, a putative *WOX2* homolog was isolated and characterized from *Aegilops tauschii*, the donor of D genome of *Triticum aestivum.* The sequence consisted of 2045 bp, and contained an open reading frame (ORF), encoded 322 amino acids. The predicted protein sequence contained a highly conserved homeodomain and the WUS-box domain, which is present in some members of the WOX protein family. The full-length ORF was subcloned into prokaryotic expression vector pET-30a, and an approximately 34-kDa protein was expressed in *Escherichia coli* BL21 (DE3) cells with IPTG induction. The molecular mass of the expressed protein was identical to that predicted by the cDNA sequence. Phylogenetic analysis suggested that *Ae. tauschii* WOX2 is closely related to the rice and maize orthologs. Quantitative PCR analysis showed that *WOX2* from *Ae. tauschii* was primarily expressed in the seeds; transcription increased during seed development and declined after the embryos matured, suggesting that *WOX2* is associated with embryo development in *Ae. tauschii*.

Keywords: *WOX2*, *Aegilops tauschii*, phylogenetic analysis, real-time quantitative PCR.

Introduction

WOX genes comprise a large plant-specific gene family that belongs to the homeodomain (HD) class of transcription factors. WUS HDs are critical for early-phase embryogenesis and lateral organ development (Haecker *et al.*, 2004). HD proteins typically contain a highly conserved motif of 60 amino acids encoded by a characteristic DNA fragment called the homeobox and consisting of a helix-loop-helix-turn-helix structure (Kamiya *et al.*, 2003). Phylogenetic analyses have divided the *WOX* gene family into three major clades: the ancient clade with representatives from all lineages of land plants as well as green algae, and the intermediate and modern clades with representatives found only in ferns and seed plants (Nardmann *et al.*, 2009; van der Graaff *et al.*, 2009). In addition to the HD, the primary structures of WOX proteins in the modern clade also possess conserved structures called the WUS box and/or EAR-like motif (Kieffer *et al.*, 2006; Lin *et al.*, 2013).

Previous studies have identified 15 *WOX* gene family members in *Arabidopsis thaliana*: the *WUS* gene is specifically expressed in the shoot apical meristem (SAM) where it regulates stem cell fate (Mayer *et al.*, 1998; Haecker *et al.*, 2004; Lian *et al.*, 2014; Zhang *et al.*, 2010) and *WOX5* performs similar functions in the root apical meristem (RAM) (Sarkar *et al.*, 2007). *PRESSED FLOWER/AtWOX3* is required for the development of lateral stipules in the leaves and the lateral sepals and stamens of flowers (Matsumoto and Okada, 2001), while *AtWOX4* acts as a key regulator of cambium activity in the main stem of *Arabidopsis* (Suer *et al.*, 2011). *PRETTY FEW SEEDS2/AtWOX6* transcripts accumulate in developing primordia and differentiating organs and regulate ovule development (Park *et al.*, 2005). *STIMPY/AtWOX9* maintains cell division and prevents premature differentiation in the center zone of the SAM (Wu *et al.*, 2005). Moreover, *OsWOX11* mediates activation of crown root emergence and growth in rice (Zhao *et al.*, 2009). *AtWOX2* and *AtWOX8* are co-expressed in egg cells and zygotes and are involved in embryo formation and differentiation in *Arabidopsis* (Haecker *et al.*, 2004). In maize, *ZmWOX2A*, a homolog of *AtWOX2*, is expressed in the zygote, where it

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marks the apical cell fate and prepatterns the SAM position (Nardmann *et al.*, 2007). *PaWOX2*, which is the *Picea abies* homolog of *AtWOX2*, is highly expressed during early growth of the zygote and somatic embryo (Palovaara *et al.*, 2010).

Although there have been several studies of the *WOX* gene family in monocots, such as rice and maize (Kamiya *et al.*, 2003; Nardmann *et al.*, 2007), little is known about *WOX* genes in Triticeae plants. In this study, we characterized the *WOX2* gene structure and its expression from *Aegilops tauschii*, the D-genome progenitor species of hexaploid wheat. The objective of this study was to investigate the structural features of *WOX2*, determine its expression pattern at different tissues in *Ae. tauschii*, and examine the evolutionary history of the *WOX* genes.

Material and Methods

Plant material

Ae. tauschii AS2407 (2n = 2x = 14, DD) was cultivated in the field for sample collection. The seeds were provided by the Triticeae Research Institute of Sichuan Agriculture University, ChengDu, Sichuan, China. Genomic DNA was extracted from fresh leaves by using a CTAB procedure (Murray and Thompson, 1980). For gene expression analysis, root tissue was harvested from 1-week old seedlings. Leaf and stem tissues were from 3-weeks old seedlings. Stamina and ovaries were harvested before anthesis. Developing seeds were harvested from between 2 and 24 days after flowering at 2-day intervals. Immature embryos were cultured on MS callus induction medium (Özgen *et al.*, 1998) supplemented with 2 mg/L 2,4-dichlorophenoxyacetic acid. The cultures were maintained at 26 °C in the dark. Calli were collected after twelve days' culture. All samples were immediately frozen in liquid nitrogen and stored at -80 °C.

Database analysis and cloning of *WOX2* from *Ae. tauschii*

We searched Arabidopsis, rice, and maize *WOX2* DNA sequences from the National Centre for Biotechnology Information. After alignment using the ClustalW program (version 1.83) (Thompson *et al.*, 1994), the conserved sequences were used to design a pair of degenerate primers (HDF/R) (Table 1) for amplifying part of the *Ae. tauschii WOX2* gene. The amplified PCR product was purified and cloned into a pMD19-T vector (TaKaRa Biotechnology Co. Ltd. Dalian, China) for sequencing. BLAST searching of the GenBank database with the partial *Ae. tauschii WOX2* sequence identified one *WOX* ortholog. Based on this *WOX* ortholog, a pair of primers (WOX2_F/R) (Table 1) was designed to amplify the full-length gene from *Ae. tauschii* using cDNA and gDNA templates. PCR was performed with the LA PCR Kit Ver. 2.1 (TaKaRa Biotechnology Co. Ltd. Dalian, China). Sequence alignment and

Table 1 - Primer sequences.

Primer name	Primer sequence (5' →3')
HDF	TGGACVCCGACGVCGGAGCAGRT
HDR	GCCTTGTGGTTCTGGAACCAGTAGAA
WOX2_F	ACAGAGCAGATCCGTCCTCCTTAGCC
WOX2_R	CTCCTTGAGTCGGTGTTCAGCTTAGTGA
PET30a_F	TATCATATGAGAGCAGATCCGTCCTCCTTAGCC
PET30a_R	GTCGAATTCCTTGAGTCGGTGTTCAGCTTAGTGA
qWOX2F1	CCCCATCGAGGGCAAGAACG
qWOX2R1	CGGCGGAACTGCTTGGAGAA
actinF1	GTTCCAATCTATAAGGGATACACGC
actinR1	GAACCTCCACTGAGAACAACATTACC

protein prediction were performed in DNAMAN software (Ver. 5.2.10; Lynnon Biosoft).

Phylogenetic analysis

To examine the evolutionary history of the *WOX* genes and the evolutionary relationship of the *Ae. tauschii WOX2* with other plants, we screened sequences homologous to *AtWUS* and *AtWOX2* using TBLASTN in the NCBI database, with default parameters and the WUS/WOX2 homeodomain sequences as queries. We retrieved 76 *WOX* genes including 15 in *Arabidopsis*, 10 in rice, 15 in maize, 17 in *Populus tomentosa*, six in *Theobroma cacao*, and six in *Ginkgo biloba* (GenBank accession numbers are listed in the Supplemental Table S1). One *WOX2* gene was identified in each of the following species: *Glycine max*, *Vitis vinifera*, *Petunia x hybrida*, *Picea abies*, *Cicer arietinum*, *Fragaria vesca*, and *Solanum tuberosum* (Table S1). These sequences were used to construct a phylogenetic tree. Multiple sequence alignment was performed in ClustalW (version 1.83) (Thompson *et al.*, 1994). MEGA 5.0 was used to construct a Maximum Likelihood (ML) tree by using the Jones-Taylor-Thornton (JTT) model and with 1000 bootstrap replicates (Jones *et al.*, 1992; Tamura *et al.*, 2011).

Bacterial expression of cloned *Ae. tauschii WOX2* ORFs

To express the *Ae. tauschii WOX2* open reading frame (ORF) in bacteria, the PET30a_F/R primer (Table 1) sets were designed to amplify an ORF from the cDNA sequence. We modified the *WOX2* ORF using PCR mutagenesis, which is almost the same as general PCR except the primer added two restriction sites for *Nde*I and *Eco*RI. PCR conditions were identical to those described above except the template was plasmid DNA purified from the respective clones. The ORFs were cloned into the pET-30a expression vector (Novagen) and then expressed in BL21 *Escherichia coli* (DE3) cells. To induce bacterial expression, the cells were treated with 0.5 mM isopropyl β-d-thiogalactopyranoside (IPTG) for 3-5 h. The proteins were extracted with

extraction buffer (1% SDS, 5% 2-mercaptoethanol, and 0.25 M Tris-HC1, pH 6.8) (Santambrogio *et al.*, 1993), heated at 96 °C for 10 min, separated by 12% SDS-PAGE, and stained with Coomassie Blue R-250.

Expression analysis by real-time quantitative PCR (RT-qPCR)

Total RNA was extracted with Trizol reagent (Tiangen Biotech Co. Ltd, Beijing, China) according to the manufacturer's protocol. First-strand cDNA was synthesized with PrimeScript RT and gDNA Eraser (TaKaRa Biotechnology Co. Ltd. Dalian, China). RT-qPCR was performed with diluted cDNA and the SYBR *Premix* Ex Taq II Kit (TaKaRa Biotechnology Co. Ltd. Dalian, China). Reactions were performed in triplicate in a BioRad CFX96 thermocycler under the following cycling conditions: 5 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 60 °C, 40 s at 72 °C, and a final extension of 10 min at 72 °C. Negative (distilled water) and no-template (total RNA) controls were included in each run. The data were analyzed with the BioRad CFX Manager software. Relative expression was calculated based on the standard curve method described by Pfaffl (2001). Gene-specific primers for *WOX2* (qWOX2F1/R1) were used to detect *WOX2* RNA levels normalized to *actin* (actinF1/R1; GenBank accession number, AY145451) (Li *et al.*, 2010). Primer sequences are shown in Table 1.

Results

Isolation and sequence analysis of *WOX2* from *Ae. tauschii*

Based on the multiple alignments of homologous genes from *Arabidopsis*, rice, and maize, we amplified the HD region of *Ae. tauschii WOX2* with degenerate primers. After blasting against the GenBank database, we found a cDNA clone from wheat (AK335177) in which the HD is the same as the partial *WOX2* sequence we cloned in *Ae. tauschii* and highly homologous to *WOX2* in other species. This clone contained the complete ORF. To determine the coding sequence (CDS) of the putative *Ae. tauschii WOX2* gene, we isolated the gDNA and cDNA sequences from *Ae. tauschii*. Sequence analysis revealed that the *WOX2* gene is 2,045 bp in length, with a 969 bp ORF, a 29 bp 5' upstream sequence, and a 104 bp 3' downstream sequence (GenBank accession numbers KJ459391). Two exons and one intron were identified for *WOX2* after alignment of gDNA with the respective ORF. The putative *WOX2* gene in *Ae. tauschii* was predicted to encode a polypeptide of 322 amino acids. Our BlastP results against public databases indicated that the primary HD region of *Ae. tauschii* WOX2 (amino acids 40-105) is conserved in all WOX family members. The WOX2 HD in *Ae. tauschii* shared 89% and 85% similarity with OsWOX2 in rice and ZmWOX2A in maize, respectively. Its similarity to *Arabidopsis* AtWOX2 was, however, only 69%. Domain prediction analysis revealed that the putative WOX2 protein of *Ae. tauschii* also contains the WUS-box domain, which is present in the modern clade of the WOX protein family (Figure 1). We also compared the full protein sequences of *Ae. tauschii* WOX2 with *O. sativa*, *Z. mays*, *G. max*, *Arabidopsis*, *V. vinifera*, *P. hybrida*, *G. biloba*, *S. tuberosum*, *C. arietinum*, *F. vesca*, and *P. abies* (Figure 2). Multiple alignments showed that the WOX2 HD was highly conserved across all 12 species whereas the flanking sequences varied.

Expression of *Ae. tauschii WOX2* in *E. coli*

We used the clone of the *Ae. tauschii WOX2* ORF to analyze protein expression so as to verify whether the cloned gene expresses the corresponding protein product. Furthermore this also will allow the recombinant protein to be used as antigen to develop antibodies, which could be used to detect the expression of the transgenic progeny at the protein level in future studies. We ligated the *WOX2* ORF from *Ae. tauschii* into pET-30a by using modified restriction sites. The recombinant plasmid, pET30a-AetWOX2, was then expressed in *E. coli* BL21 (DE3) and protein expression was induced with IPTG. SDS-PAGE revealed a single protein band of approximately 34 kDa in the
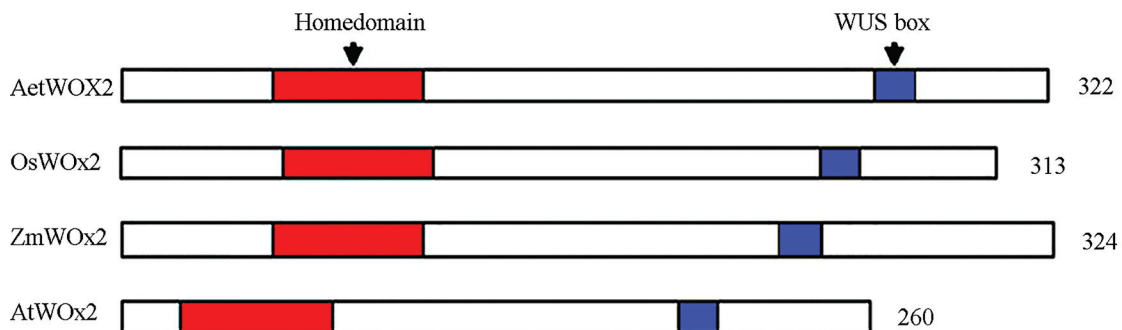
Figure 1 - Structure of WOX2 proteins in different species. Abbreviations are *Ae. tauschii*, Aet; *O. sativa*, Os; *Z. mays*, Zm; *A. thaliana*, At; The homeodomain (red) is the most prominent and defining feature of the family. The WUS-box motif (blue) was defined in a strict sense, as T-L-[DEQP]-L-F-P-[GITVL]-[GSKNTCV], consensus TLELFPLH.
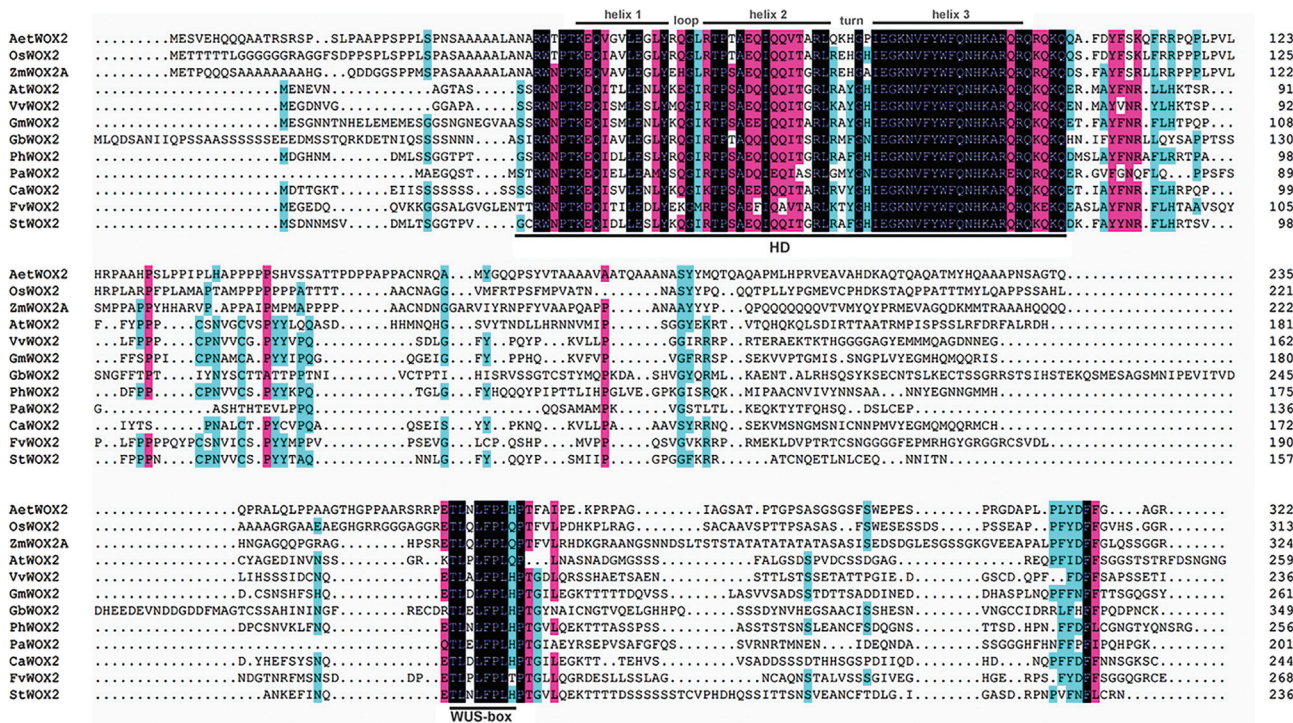
Figure 2 - Multiple sequence alignment of WOX2. Abbreviations are G. biloba, Gb; *G. max*, Gm; *V. vinifera*, Vv; *Petunia x hybrida*, Ph; *S. tuberosum*, St; *C. arietinum*, Ca; *F. vesca*, Fv; and *P. abies*, Pa.

induced extract (Figure 3). The molecular mass of the expressed protein was identical to that predicted by the *AetWOX2* cDNA sequence, so the single protein band is accurate representation of the *AetWOX2* encoding protein.

Phylogenetic analysis of *Ae. tauschii* WOX2

To confirm the classifications and analyze the phylogenetic relationships of the WOX proteins, multiple alignment analyses of the 77 WOX proteins were performed. The resulting ML tree divided the 77 WOX members into three clades: modern, intermediate, and ancient (Figure 4). There were 51 WOX proteins in the modern clade, which can be subdivided into five subgroups including WUS, WOX1/WOX4, WOX2, WOX3/NS, and WOX5; ten WOXs in the ancient clade of WOX10, WOX13, and WOX14 homologues; and sixteen WOXs in the intermediate clade consisting of WOX8, WOX9, WOX11, and WOX12. The WOX2 subgroup in the modern clade contained one member from each of twelve species. AetWOX2 in the WOX2 subgroup clustered with OsWOX2 and ZmWOX2A. WOX2 from *Ae. tauschii* is closely related to the rice and maize orthologs.

Expression pattern analysis

To understand the function of *WOX2* from *Ae. tauschii*, we investigated its expression in different tissues of *Ae. tauschii,* and in seeds at different stages of development. We set the expression level in roots as equal to 1, and calculated the expression in other tissues as relative

to roots. The qRT-PCR results revealed that *WOX2* is primarily expressed in seeds, with lower levels transcript levels in the other tissues (Figure 5). Expression of *Ae.*
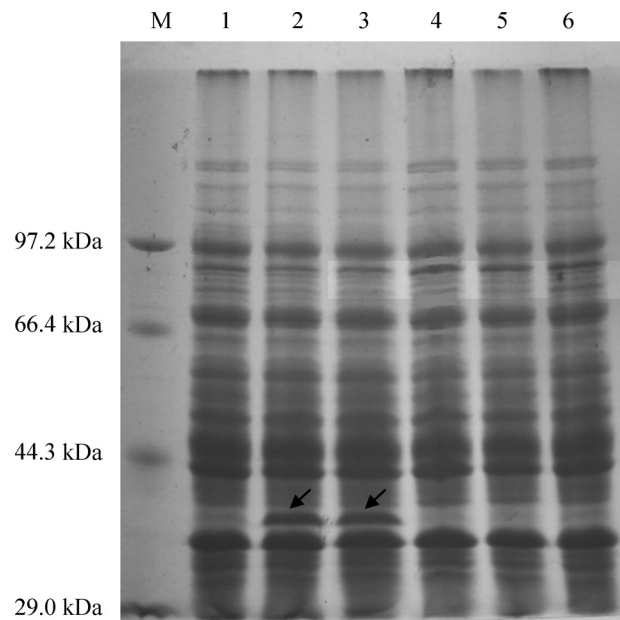
Figure 3 - Expression of a pET30a-WOX2 fusion protein. Lane 1, uninduced pET30a-WOX2; lanes 2-3, pET30a-WOX2 induced by 0.5 mM IPTG for 3 and 5 h, respectively; lane 4, uninduced pET30a; lane 5, induced pET30a for 3 h; lane 6, negative control *E. coli* BL21 (DE3); M, Protein Molecular Weight Marker (Low) (TaKaRa). The target protein is indicated with arrows.
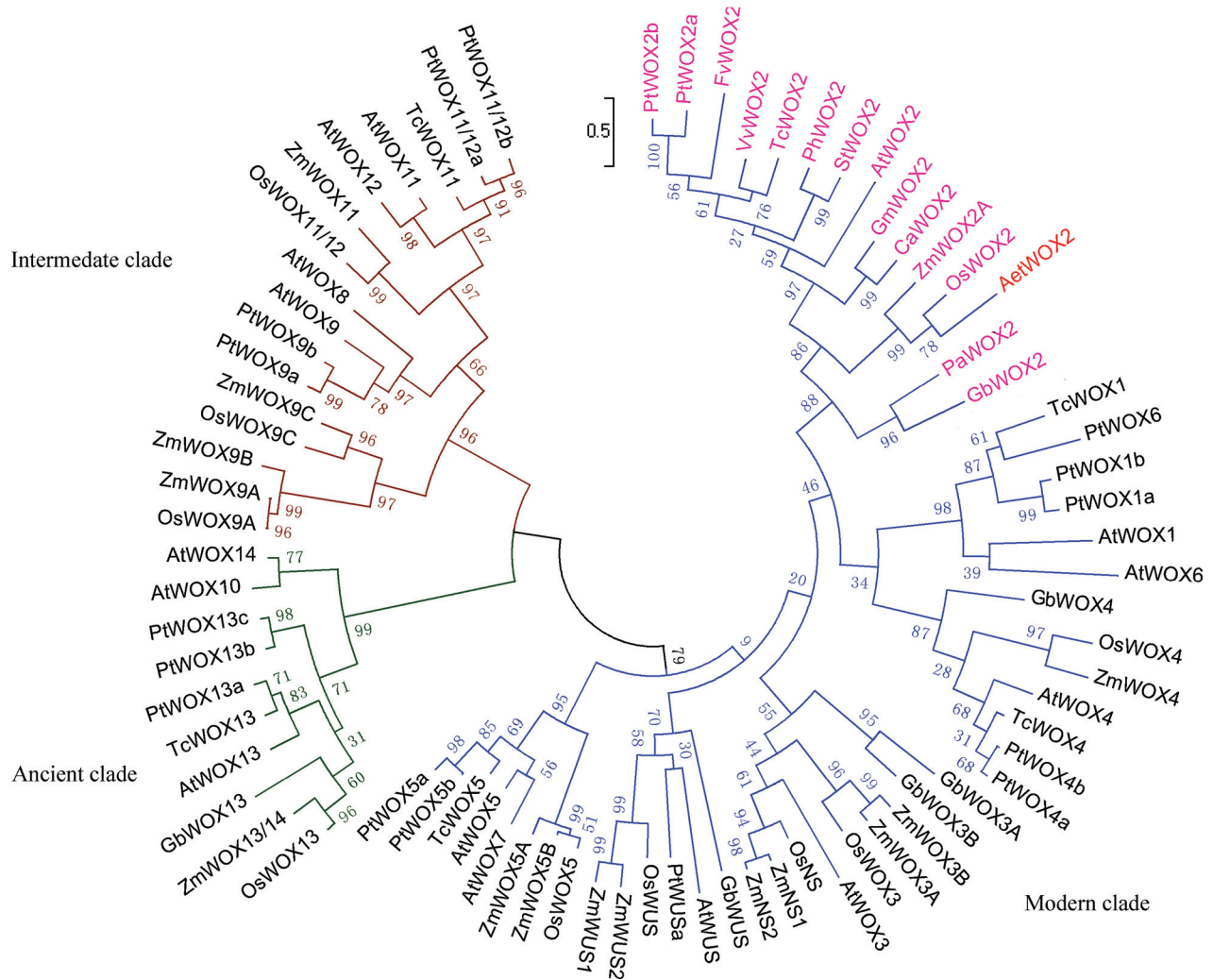
Figure 4 - Phylogenetic analysis of plant WUS/WOX proteins based on the Maximum Likelihood method. Abbreviations are P. tomentosa, Pt; and T. cacao, Tc. The three clades are color-coded, WUS/WOX1-7 (modern) in blue, WOX8, 9, 11, 12 (intermediate) in brown and WOX10, 13, 14 (ancient) in green. Protein names of the WOX2 subclade are in purple and AetWOX2 is red.

tauschii WOX2 continuously increased from 12 day after flowering (DAF), peaked at 18 DAF, and then declined until 24 DAF. Around 18 DAF is the stage at which embryos become mature.

Discussion

The coding region of *AetWOX2* shared 99% similarity with the clone AK335177 from wheat, a result agrees with the well-established fact that *Ae. taushii* is the donor of the D genome to the hexaploid wheat, *T. aestivum* (Mc and Sears, 1946; Gill *et al.*, 1996). As *Ae. taushii* is one of most important gene resources for improving common wheat, exploiting the *WOX* gene in *Ae. tauschii* may contribute to understanding the differentiation and evolution of the *WOX* gene in Triticeae species.

The *WOX2* gene from *Ae. tauschii* encodes a WUS/WOX-like HD protein that is highly conserved. The

WOX2 HD consists of about 65 amino acids with one extra amino acid in the loop between helices 1 and 2, and four extra amino acids in the turn between helices 2 and 3 (Figure 2). The WUS-box, another domain in *Ae. tauschii* WOX2, is located downstream of the HD and is found in most WOX proteins (Haecker *et al.*, 2004). We did not find an EAR-like motif in any the examined WOX2 sequences, but it is present in WUS/WOX5. The sequence similarity of AetWOX2 with OsWOX2 and ZmWOX2A is 89% and 85%, respectively. The orthology of AetWOX2 with OsWOX2 and ZmWOX2A in the modern clade (Figure 4) is well supported by the phylogenetic tree. Taken together, these results suggest that WOX2 is a *bona fide* member of the WOX family in *Ae. tauschii*.

The close phylogenetic relationship among WOXs in *Arabidopsis*, rice, maize, *P. tomentosa*, *T. cacao*, and *G. biloba* orthologs (Figure 4) suggested that WOXs are evolutionarily conserved. It is interesting that no rice and maize
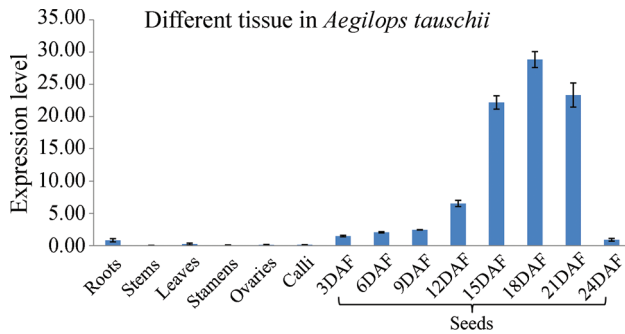
Figure 5 - Real-time PCR analysis of *WOX2* transcript levels in different tissues of *Ae. tauschii* and seed growth stages. Expression was normalized to an *actin* gene.

orthologs of *Arabidopsis* WOX1, 6, 7, 8, 10, and 14 were found in the phylogenetic tree. *Arabidopsis* has at least 15 *WOX* members, there are nine independent clones encoding WUS-type proteins from rice (Kamiya *et al.*, 2003), and research in maize indicated that orthologs of WOX1, 6, 7, 8, 10, and 14 are not present in grass genomes (Nardmann *et al.*, 2007). Therefore, the WOX family and functions of its member are distinct in different species.

WOX2 is associated with embryo patterning in *Arabidopsis*, maize, and spruce (Haecker *et al.*, 2004; Nardmann *et al.*, 2007; Palovaara *et al.*, 2010). In *Ae. tauschii*, the process of embryonic growth is consistent with seed development, so it is possible that the expression of *WOX2* in seeds corresponds to that in the embryo. Transcript levels of *Ae. tauschii WOX2* increased during the early process of seed development and declined after the embryo matured (Figure 5). Based on the function of members of the *WOX2* gene family already described in different plant species, and the expression pattern during seed development, the data suggest that *WOX2* in *Ae. tauschii* may indeed be associated with embryo development.

Acknowledgments

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Supplementary Material

The following online material is available for this article:
Table S1 - Accession numbers of WOX proteins.
This material is available as part of the online article from http://www.scielo.br/gmb.

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