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# Characterization and expression analysis of *WOX5* genes from wheat and its relatives

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ABSTRACT

The WUSCHEL (WUS)-related homeobox (WOX) gene family plays an important role in coordinating gene transcription in the early phases of embryogenesis. In this study, we isolated and characterized *WOX5* from common wheat and its relatives *Triticum monococcum*, *Triticum urartu*, *Aegilops speltoides*, *Aegilops searsii*, *Aegilops sharonensis*, *Aegilops longissima*, *Aegilops bicornis*, *Aegilops tauschii*, and *Triticum turgidum*. The size of the characterized *WOX5* alleles ranged from 1029 to 1038 bp and encompassed the complete open reading frame (ORF) as well as 5′ upstream and 3′ downstream sequences. Domain prediction analysis showed that the putative primary structures of wheat WOX5 protein include the highly conserved homeodomain besides the WUS-box domain and the EAR-like domain, which is/are present in some members of the WOX protein family. The full-length ORF was subcloned into a prokaryotic expression vector pET30a, and an approximate 26-kDa protein was successfully expressed in *Escherichia coli* BL21 (DE3) cells with IPTG induction. The *WOX5* genes from wheat-related species exhibit a similar structure to and high sequence similarity with *WOX5* genes from common wheat. The degree of divergence and phylogenetic tree analysis among *WOX5* alleles suggested the existence of three homoeologous copies in the A, B, or D genome of common wheat. Quantitative PCR results showed that *TaWOX5* was primarily expressed in the root and calli induced by auxin and cytokinin, indicating that *TaWOX5* may play a role related to root formation or development and is associated with hormone regulation in somatic embryogenesis.



## 1. Introduction

Somatic embryogenesis is a process that initiates the formation of the somatic plant tissue in plant embryos. Somatic embryogenesis represents an attractive model system for studying early embryo development in plants since somatic embryos (SEs) follow a set developmental pathway that, morphologically and temporally, resembles that of their zygotic counterparts (Zimmerman, 1993). Callus and wheat cell suspension cultures were first developed in 1968 (Trione et al., 1968), and regenerated plants were obtained from immature wheat embryos in 1969 (Shimada et al., 1969). Importantly, somatic embryogenesis can be utilized to introduce novel genes into wheat cells *in vitro*. Somatic embryogenesis of wheat is influenced by many factors such as plant genotype, explant type, and culture medium. Hormones play a key role in somatic embryogenesis; specifically, they are required to induce embryo formation and differentiation *in vitro* (Feher et al., 2003). Some hormonal components involved in somatic embryogenesis include auxins, such as 2,4-dichloro-phenoxyacetic acid (2,4-D), and cytokinins, such as 6-benzyl-adenine (BA).

Advancements in the characterization of the plant transcriptome and proteome have led to the identification of genes involved in somatic embryogenesis, such as *WUS*, *SERK*, *LTP*, *BBM*, *LEC*, and *LEC1-LIKE* (Boutilier et al., 2002; Che et al., 2006; Kwong et al., 2003; Mayer et al., 1998; Schmidt et al., 1997; Stone et al., 2001). *WOX* genes comprise a large gene family specifically expressed in plants and belong to the homeodomain (HD) class of transcription factors. Typically, HD proteins contain a highly conserved motif of 60 amino acids, which is encoded by a characteristic DNA fragment, called the homeobox.

*Abbreviations:* AP, adaptor primer; BLAST, basic local alignment search tool; CDA, callus in differentiation medium after days; CIA, callus in induced medium after days; CTAB, cetyltrimethyl ammonium bromide; EAR, ERF-associated amphiphilic repression; EC, embryogenic callus; HD, homeodomain; IAA, indole-3-acetic acid; IPTG, isopropyl β-D-thiogalactopyranoside; MS, murashige and skoog medium; NAA, 1-naphthoxyacetic acid; NEC, non-embryogenic callus; NJ, neighbor-joining; ORF, open reading frame; QHB, quiescent center-specific homeobox; RAM, root apical meristem; RT-qPCR, real-time quantitative PCR; SAM, shoot apical meristem; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; SNPs, single nucleotide polymorphism; TAIL-PCR, thermal asymmetric interlaced PCR; UTR, untranslated transcribed region; USDA-ARS, United States Department of Agriculture—Agricultural Research Service; WUS, Wuschel; WOX, WUS-related homeobox; 2,4-D, 2,4-dichlorophenoxyacetic acid; 6-BA, 6-benzyl-adenine.

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Notably, HD transcription factors are critical for early-phase embryogenesis and lateral organ development (Haecker et al., 2004). Phylogenetic analyses have helped divided the WOX family into three major clades: the ancient clade containing the WOX10, WOX13, and WOX14 homologues; the intermediate clade consisting of WOX8, WOX9, WOX11, and WOX12; and the modern clades including WUS/WOX1–7 (Van der Graaff et al., 2009). The WOX proteins in the modern clade also contain conserved structures, the WUS box, and/or the EAR-like motif (Kieffer et al., 2006; Lin et al., 2013). Previous studies have identified 15 *WOX* gene family members in *Arabidopsis thaliana* (Haecker et al., 2004), of which the *WUS* gene was found to be specifically expressed in the shoot apical meristem (SAM) where it promotes stem cell fate (Haecker et al., 2004), whereas *WOX5* performs similar functions in the root apical meristem (RAM) (Sarkar et al., 2007). *Arabidopsis WOX5* orthologs have been identified in rice (*QHB*/*OsWOX5*) and maize (*ZmWOX5*), where they act through similar molecular mechanisms to initiate formation of the root meristem (Kamiya et al., 2003; Nardmann et al., 2007). In legumes, the *WOX5* gene has been shown to be involved in nodule meristem development (Osipova et al., 2011). Moreover, *WOX2* and *WOX8/9* are involved in embryo formation and differentiation of both *Arabidopsis* (Haecker et al., 2004; Lie et al., 2012) and the conifer (Palovaara et al., 2010). Another gene, *WOX3*/*PRS* is required for the development of lateral stipules in the leaves and lateral sepals and stamens of flowers (Matsumoto and Okada, 2001), while *WOX4* acts as a key regulator of cambium activity in the main stem of *Arabidopsis* (Suer et al., 2011). *WOX11* has been shown to be involved in the activation of crown root emergence and growth in rice (Zhao et al., 2009).

Until now, in monocotyledons, a few members of the *WOX* gene family have been characterized in rice and maize (Kamiya et al., 2003; Nardmann et al., 2007), whereas the *WOX* gene has not yet been explored in wheat and related species. In this study, we characterized the *WOX5s* gene structure and its expression in wheat and wheat-related species. The objective of this study is to investigate the structural features of the *WOX5* gene, determine its expression pattern in different wheat tissues, and identify any evolutionary relationship between *WOX5* genes within *Triticeae*.

## 2. Materials and methods

### 2.1. Plant materials

Forty-two accessions of *Triticum monococcum* (2n = 2x = 14, $A^mA^m$), *Triticum urartu* (2n = 2x = 14, $A^uA^u$), *Aegilops speltoides* (2n = 2x = 14, SS), *Aegilops searsii* (2n = 2x = 14, $S^sS^s$), *Aegilops sharonensis* (2n = 2x = 14, $S^{sh}S^{sh}$), *Aegilops longissima* (2n = 2x = 14,$S^1S^1$), *Aegilops bicornis* (2n = 2x = 14, $S^bS^b$), *Aegilops tauschii* (2n = 2x = 14, DD), *Triticum turgidum* (2n = 2x = 28, AABB), and *Triticum aestivum* (2n = 6x = 42, AABBDD) were used in this study (Table 1). Seeds for *Ae. tauschii* and *T. aestivum* and two of the *T. turgidum* seeds were provided by the Triticeae Research Institute of Sichuan Agriculture University, ChengDu, Sichuan, China. The remaining seeds were kindly provided by USDA-ARS (http://www.ars-grin.gov).

The plants listed above were cultivated in the field from October 2011 to May 2012. The immature embryos were cultured on MS callus induced medium (Özgen et al., 1998) supplemented with 2 mg · $L^{-1}$ 2,4-D. The cultures were maintained at 26 °C in the dark. Twelve days after initiating the culture, the calli were classified as embryogenic callus (EC) or non-embryogenic callus (NEC) according to previously described criteria (Nabors et al., 1983). EC and NEC were transferred to embryo differentiation medium supplemented with 1 mg/L 1-naphthoxyacetic acid (NAA), 1 mg/L 6-BA, 1 mg/L zeatin, and 1 mg/L kinetin, and the cultures were maintained at 26 °C in the presence of light. The number of explants differentiating into somatic embryos was recorded 5 and 20 days after initiating the culture.

### 2.2. DNA and RNA isolation

Genomic DNA was isolated from the fresh leaves of the plants mentioned above by using a modified CTAB procedure (Murray and Thompson, 1980). Total RNA was extracted from the following parts by using Trizol (Tiangen Biotech Co. Ltd., Beijing, China): callus grown in induced medium, at 3, 6, 9, and 12 days of growth; callus grown in differentiation medium, at 5, 10, 15, and 20 days; embryonic callus and non-embryonic callus induced for 12 days in induction medium; and the root, stem, leaf, spikelet, stamens, ovary, and seed of the common wheat cultivar Liangmai 4. First-strand cDNA was synthesized using the PrimeScript RT reagents kit with gDNA Eraser (TaKaRa Biotechnology Co. Ltd. Dalian, China).

### 2.3. Isolation of WOX5 genes in common wheat and its relatives

*WOX5* DNA sequences from maize, rice, and *Arabidopsis* were retrieved from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nim.nih.gov/BLAST/Nucleotide/EST/) and aligned using the ClustalW program (version 1.83). Based on the conserved sequences, we designed a pair of primers (HDF/R) to amplify the wheat *WOX5* gene open reading frame (ORF) (Table 2). The amplified PCR product was purified and cloned into a pMD19-T vector (TaKaRa Biotechnology Co. Ltd. Dalian, China), and the thermal asymmetric interlaced PCR (TAIL-PCR) method was utilized to isolate the wheat *WOX5* 5′ sequence (Liu and Whittier, 1995). Three gene-specific primers (P1, P2, and P3) were designed to amplify the HD and 3′ terminal sequences by EST assembly (Table 2). On the basis of the 5′ sequences identified using TAIL-PCR, we generated primers (WOX5F/R) to amplify full-length *WOX5* from wheat and wheat-related species (Table 2). The final DNA sequence for each *WOX5* ORF was constructed using the sequencing results for 3 independent clones.

### 2.4. Sequence and phylogenetic analyses

Sequence alignment and protein prediction were performed using the DNAMAN software (V5.2.10; Lynnon Biosoft). To determine the evolutionary relationship of the wheat *WOX5* gene with other plants, the *WOX5* nucleotide sequences from *Oryza sativa* L. (GenBank: NM_001051372), *Zea mays* (GenBank: AM490239, AM490238), *Glycine max* (GenBank: XM_003518358), *Arabidopsis* (GenBank: NM_111961), *Vitis vinifera* (GenBank: XM_002275972), *Pisum sativum* (GenBank: JN603580), and *Nymphaea jamesoniana* (GenBank: FM882235) were used to construct a phylogenetic tree. Multiple alignments of the DNA and protein sequences were performed using the ClustalW program (version 1.83) (Thompson et al., 1994). The MEGA 4.0 program was used to create a neighbor-joining (NJ) tree by using the substitute model of maximum composite likelihood (Tamura et al., 2007). In the NJ analysis, gaps were treated as missing data. The bootstrap values were calculated based on 1000 replicates to estimate the topological robustness.

### 2.5. Bacterial expression of cloned TaWOX5 ORFs

PCR mutagenesis was used to express the TaWOX5 ORF in bacteria. The PET30a-F1 and PET30a-R1 primer sets were designed to introduce the two restriction enzyme sites *Nde*I and *Eco*RI into the ORF of *WOX5*. The PCR conditions used for amplifying the ORF were identical to those described above except that the template was plasmid DNA purified from the respective clones. The modified ORFs were cloned into the pET30a expression vector (Novagen) and then expressed in BL21 *Escherichia coli* (DE3) cells. To induce bacterial expression, the cells were treated with 0.5 mM IPTG for 3–5 h. The samples were then boiled in 1% SDS, 5% 2-mercaptoethanol, and 0.25 M Tris–HC1, pH 6.8 (Santambrogio et al., 1993); subsequently, they were run on a 15% SDS-PAGE gel, which was then stained with Coomassie Blue.