Genome-wide Identification and Expression Profiling Analysis of WOX Family Proteins Encoded Genes in Triticeae Plant Species

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Abstract

Background: Genotype dependence of plant regeneration is an important factor restricting the genetic improvement of Triticeae plant species. The WUSCHEL-related homeobox (WOX) is a group of plant specific transcription factor, which play an important role in plant growth and development, and cell division and differentiation. Recent studies revealed that the application of regeneration-related genes such as WOX and BABY BOOM (BBM) could improve plant regeneration. The application of WOX genes is one of the ways to improve the genetic transformation system of Triticeae and other species, but there are rare studies in this area.

Results: From the available genome sequence database, in total 136 WOX transcripts were identified for the Triticeae plants, including 43 in Triticum aestivum, 30 in Triticum turgidum, 25 in Triticum dicoccoides, 17 in Hordeum vulgare, 13 in Aegilops tauschii, and 8 in Triticum urartu. All of the WOX family genes were distributed on the chromosomes of homologous groups 1 to 5 in the six Triticeae species, part of which were confirmed by their specific PCR markers using a set of T. durum-T. aestivum genome D substitution lines. All of the WOX proteins in the six Triticeae species could be grouped into three clades, similar to those in rice (Oryza sativa L.) and Arabidopsis. WOX family members were conserved among these Triticeae plants, all of them contained the conserved HOX DNA-binding homeodomain, and WUS clade members contained the characteristic WUS motif, while only TaWUS and TaWOX9 in all the six Triticeae plant species contained the ERF-associated amphiphilic repression (EAR) motif. The expression profiles of TaWOX genes by quantitative real-time PCR (qPCR) showed obvious difference among WOX family members.

Conclusions: Totally 130 WOX genes were identified in the six Triticeae plant species. The WOX family genes were located on the chromosomes in the five homologous groups except groups 6 and 7 in the Triticeae species, and their expression profiles were different in different tissues, indicating that each of them had diverse function. The findings in this study could provide a basis for evolution and functional investigation and practical application of the WOX family genes in Triticeae plant species.

Background

Triticeae tribe belongs to the Poacea family, and is made up of more than 350 plant species in 30 genera around. In Triticeae plants, a series of species such as Triticum aestivum (bread wheat), Hordeum vulgare (barley), Secale cereale (rye), Triticum urartu, Triticum dicoccoides, and Triticum turgidum have been cultivated as crops and provides necessary nutrition for more than two billion people in the world [1, 2]. For a long time, people are interested in understanding the origination, genetic basis and evolution of Triticeae plants for their better improvement. Clear interpretation on genetic information of Triticeae plants may bring us closer to achieve the aforementioned objective. The assembling of wheat genome is a milestone in interpreting the genetic information of Triticeae plants. However, due to the large genome size of up to 16 Gb, the genomic study on wheat is legged behind of rice and maize [3]. The application of modern biotechnology tools such as transgene and gene editing in plant breeding can help us to increase yield, improve quality, and enhance biotic or abiotic resistance of major crops, but the realization of these aims depends on genetic transformation. The ability of regenerating new plantlets from in vitro tissues is a big limitation that restricts the application of genetic transformation and gene editing systems [4, 5].

Regeneration ability is one of important genetically physiological traits for most plants, which enables plants recover from wound tissues and form new organs. For modifying plants using genetic-engineering strategy, shoot or somatic embryo production from isolated tissues or cells is an indispensable step to achieve transgenic plants. But, it is still difficult to obtain regenerated plants in the process of genetic transformation from most genotypes (especially the extensively commercial varieties) of wheat and other Triticeae species [5–7]. During plant regeneration, a series of genes express in an orderly manner under the regulation of auxin and cytokinin. These regeneration-related genes
include \textit{WUSCHEL-RELATED HOMEBOX (WOX)}, \textit{AUXIN RESPONSE FACTOR (ARF)}, \textit{BABY BOOM (BBM)}, \textit{SCARECROW (SCR)}, \textit{SHORT ROOT (SHR)}, \textit{PLETHORA (PLT)}, \textit{CUP-SHAPED COTYLEDON (CUC)}, and \textit{YUCCA (YUC)}, which express during the progress of embryonic patterning, somatic embryogenesis, cell differentiation, wound repairation, and epigenetic reprogramming [5, 8–12]. An in-depth understanding of regeneration-related genes in molecular level will make it possible to break through the bottleneck in genetic transformation and build a more efficient transformation system with less genotype-dependent. The application of regeneration-associated genes including \textit{WUS} and \textit{BBM} in crop transformation has achieved a great success, by which various maize inbred lines and tissues, and recalcitrant genotypes of \textit{Indica} rice, sugarcane, and sorghum can be efficiently transformed for getting transgenic plants [13, 14].

The WOX family is a group of plant specific transcription factors and belongs to the homeobox (HB) transcription factor family [15]. All the identified \textit{WOX} genes contain a conserved sequence of amino acids (60–66 residues), which is called as homeodomain (HD) encoded by the \textit{HB} DNA sequence [16, 17]. The distinctive WUS-box motif forms as TL-X-L-F-P-X-X(TL-[DEQP]-L-F-P-[GITVL]-[GSKNTCV]), of which the consensus structure is TLELFPLH [15]. These homolog sequences fold into a DNA-binding domain. Update published data suggests that \textit{WOX} genes act as pivotal regulators during the progress of embryonic development and polarization, plant growth and development, stem cell differentiation, embryo patterning, and flower development [18–22]. There are 15 \textit{WOX} genes in \textit{Arabidopsis thaliana}, 13 in rice, and 21 in maize [15, 23, 24]. In \textit{Arabidopsis}, as a stem cell regulator, \textit{AtWUS} expresses in the organizing-center (OC) cells in the shoot apical meristem and regulates plant growth and shoot stem cell maintaining [25, 26]. Ectopic overexpression of \textit{WUS} genes promotes cell dedifferentiation in shoot meristem, somatic embryo formation, adventitious shoot and lateral leaf origination [26–28].

It is found that \textit{AtWOX1} possibly regulates the activity of S-adenosylmethionine decarboxylase polyamine homeostasis and/or the expression of \textit{CLAVATA3 (CLV3)}, and has an important function in meristem development in \textit{Arabidopsis}. Overexpression of \textit{AtWOX1} leads to abnormal meristem development and polyamine homeostasis [29]. Normally, \textit{AtWOX2} expresses in the zygote and early embryogenesis formation, and performs functions in correcting the apical domain development of the embryos [23]. \textit{AtWOX2} triggers the expression of \textit{PINFORMED1 (PIN1)}, which is an auxin transport and localizes auxin to the cotyledonary tips of early embryo and root pole [18]. \textit{AtWOX3 (PRESSED FLOWER1, PRST)} expresses in the peripheral layer of shoot meristem and regulates cells to form the lateral domain in vegetative and floral organs [30]. The expression of \textit{AtWOX2} and \textit{AtWOX3} are regulated by Leafy Cotyledon2 (LEC2), and \textit{AtWOX2} and \textit{AtWOX3} play essential roles in somatic embryogenesis [31]. \textit{AtWOX4} expresses in a narrow domain in cambial cells, and \textit{AtWOX4} coordinating with PHLOEM INTERCALATED WITH XYLEM (PXY) acts as a key regulator for cambium activity in the main stem [32]. \textit{AtWOX5} expresses in the QC of meristematic zone in root tips, regulates the columella stem cell (CSC) identity, and helps to maintain the root stem cell niche [33]. \textit{AtWOX6 (PRETTY FEW SEEDS2, PFS2)} expresses in developing ovules and primordials and differentiating organs, regulates ovule development, and affects differentiation and maturation of leaves, outer integuments and floral primordial [34]. \textit{AtWOX7} expresses during all development stages of lateral root, but primarily involves in the initiation of lateral root [35]. \textit{AtWOX8 (STIMPY-LIKE)} and \textit{AtWOX9 (STIMPY)} are closely homologs [36, 37] and responsible for maintaining the normal development of both basal and apical embryo lineages at early development stage [18]. The expression of \textit{AtWOX8} is induced by \textit{AtWRKY2} in the basal cell lineage at the initiation stage of embryogenesis [38]. \textit{AtWOX11} plays a key role in the course of vascular cambium differentiation to new lateral root founder cells. \textit{AtWOX11} is strongly induced expressed in de novo root organogenesis, which is the same as its homologous \textit{AtWOX12} [39, 40]. \textit{AtWOX13} expresses mainly in meristematic tissues to promote replum development and orchestrate fruit patterning [41]. \textit{AtWOX14} is regulated by the \textit{CLAVATA3/ESRLIKE41/PHLOEM INTERCALATED WITH XYLEM (CLE41/PXY)} pair, expresses in the procambium during stem maturation, and promotes xylem differentiation, vascular cell differentiation and lignification in inflorescence stems [42, 43].
Based on the phylogenetic analysis in *Arabidopsis*, plant WOX proteins are naturally divided into three clades: WUS and WOX1 to WOX7 in the WUS clade; WOX8, 9, 11, and 12 in the intermediate clade; and WOX10, 13, and 14 in the ancient clade [15]. But, the WOX genes in *Triticeae* plant species have not been fully identified and characterized yet. Therefore, the objectives of this study are, (1) identifying WOX genes in the six *Triticeae* plant species including *T. aestivum*, *T. turgidum*, *T. dicoccoides*, *H. vulgare*, *A. tauschii*, and *T. urartu*, and aligning them onto chromosomes; (2) dividing all of the WOX proteins in the six *Triticeae* species into groups by phylogenetic analysis using deduced protein sequences from all the WOX genes and the sequences of OsWOX genes from rice and AtWOX genes from *Arabidopsis*; and (3) analyzing the differential expression of TaWOX genes in different tissues by RNA sequencing (RNA-seq) and quantitative real-time PCR (qPCR). Our results would provide insights for further understanding the functions and evolution clarification of WOX family genes in *Triticeae* plants, and facilitate their application in gene transformation for the improvement of *Triticeae* plants.

**Results**

**Identification of WOX genes in *Triticeae* plant species**

Totally, 43 TaWOX transcripts were obtained using the recently released IWGSC wheat genome [3], and there were still 6 pseudo gene copies (Table 1). Specifically, 15 WOX transcripts in *H. vulgare* (Table 2), 13 WOX transcripts in *A. tauschii* (Table S1), 23 WOX transcripts in *T. dicoccoides* (Table S2), 28 WOX transcripts in *T. turgidum* (Table S3), and 8 WOX transcripts in *T. urartu* (Table S4) were identified from IWGSC genome database, respectively. Some homologous alleles of WOX genes were not annotated as transcripts in the database, but were also collected and listed in the tables. For example, *TaWUSb* and *TaWUSd* were located on chromosomes 2B and 2D in *T. aestivum*, respectively (Table 1). The WUS genes in other five *Triticeae* plant species were also located on their group 2 chromosomes (Table 2, Table S1-S4). *TdWOX12a, TdWOX12b, TdWOX7b* and *TdWOX13b* were located on chromosomes 1A, 1B, and 3B in *T. dicoccoides*, respectively (Table S2).
Table 1
Characteristics of TaWOX gene family members in T. aestivum.

| Gene    | Gene locus       | Chromosome | Gene stretch region                  | mRNA length (bp) | Protein sequence length (aa) | UniProt ID                  |
|---------|-----------------|------------|--------------------------------------|-----------------|-----------------------------|-----------------------------|
| TaWOX2a | TraesCS1A02G052000 | 1A         | 33,397,501 – 33,398,955:-1           | 1314            | 263                         | A0A1D5S1T3                  |
| TaWOX12a| TraesCS1A02G399400 | 1A         | 563,818,671 – 563,823,103:1          | 1854            | 486                         | A0A1D5RPD4                  |
| TaWOX2b | TraesCS1B02G069000 | 1B         | 53,364,615 – 53,365,864:-1           | 1119            | 264                         | A0A1B1XWM5 W5ABBS5          |
| TaWOX12b| TraesCS1B02G427400 | 1B         | 652,781,930 – 652,786,496:1          | 1983            | 485                         | A0A1D5SDQ8                  |
| TaWOX2d | TraesCS1D02G054000 | 1D         | 35,059,826 – 35,061,088:-1           | 1138            | 267                         | W5ANF9                      |
| TaWOX12d| TraesCS1D02G406900 | 1D         | 470,219,711 – 470,224,514:1          | 2028            | 486                         | A0A1D5SWV6                  |
| TaWUS   | TraesCS2A02G491900 | 2A         | 724,513,458–724,514,647:1            | 927             | 308                         | A0A1D5TC72                  |
| TaWOX4a | TraesCS2A02G514000 | 2A         | 738,371,677–738,372,966:1            | 1061            | 234                         | A0A1D5TF70                  |
| TaWOX11a| TraesCS2A02G100700 | 2A         | 53,782,606 – 53,785,288:1            | 1380            | 265                         | A0A1D5TJVO                  |
| TaWOX11b| TraesCS2B02G117900 | 2B         | 81,755,546 – 81,758,516:1            | 1366            | 261                         | A0A1D5U6K9                  |
| TaWOX4b | TraesCS2B02G542600 | 2B         | 740,320,190–740,321,561:1            | 1002            | 237                         | W5BBK8                      |
| TaWOX11d| TraesCS2D02G100200 | 2D         | 52,227,203 – 52,229,885:1            | 1379            | 264                         | A0A1D5TJV1 A0A1D5V0E6       |
| TaWOX4d | TraesCS2D02G515600 | 2D         | 606,709,221–606,710,431:1            | 979             | 237                         | A0A1D5UH04                  |
|         |                 | 2D         | 590,146287–590,147498:1              |                 |                             |                             |
| TaWOX10a| TraesCS3A02G073500 | 3A         | 45,776,166 – 45,777,448:1            | 992             | 260                         | A0A1D5VKG7                  |
| TaWOX7a | TraesCS3A02G247200 | 3A         | 465,225,214–465,228,773:1            | 1968            | 515                         | A0A1D5V4S9                  |
| TaWOX8a | TraesCS3A02G341700 | 3A         | 588,932,808 – 588,937,056:1          | 2230            | 265                         | A0A077RTA5 A0A1D5VD81 A0A1D5VQY0 A0A1D5WIZ6 A0A1D6RQB3 A0A1D6RQB4 A0A1D6RQB5 W5CGX8 |
| Gene          | Gene locus         | Chromosome | Gene stretch region     | mRNA length (bp) | Protein sequence length (aa) | UniProt ID   |
|--------------|--------------------|------------|-------------------------|------------------|-------------------------------|--------------|
| TaWOX14.1a   | TraesCS3A02G358200 | 3A         | 606,515,981 – 606,519,197:1 | 1162             | 288                           | A0A1D5VFV1  |
| TaWOX13a     | TraesCS3A02G358100 | 3A         | 606,444,775 – 606,446,830:1 | 1138             | 301                           | A0A1D5VA42  |
| TaWOX14.2a   | TraesCS3A02G358400 | 3A         | 606,573,438 – 606,576,220:1 | 1133             | 290                           | A0A1D6RQ92  |
| TaWOX9a      | TraesCS3A02G368100 | 3A         | 617,060,395 – 617,061,453:1 | 949              | 212                           | T1WFN3      |
| TaWOX10b     | TraesCS3B02G087800 | 3B         | 56,055,903 – 56,057,760:1  | 1196             | 261                           | A0A1D5VWS6  |
| TaWOX7b      | TraesCS3B02G272200 | 3B         | 438,378,936 – 438,382,259:1 | 1776             | 515                           | A0A077RSZ6  |
| TaWOX8b      | TraesCS3B02G373800 | 3B         | 586,694,870 – 586,698,391:1 | 1216             | 261                           | A0A077S168  |
| TaWOX13b     | TraesCS3B02G391100 | 3B         | 616,425,121 – 616,426,978:1 | 900              | 299                           | A0A1D5VST7  |
| TaWOX14b     | TraesCS3B02G391200 | 3B         | 616,645,332 – 616,647,892:1 | 1216             | 290                           | A0A1D5WB93  |
| TaWOX9b      | TraesCS3B02G399800 | 3B         | 631,036,656 – 631,037,718:1 | 948              | 209                           | D8L9N7      |
| TaWOX10d     | TraesCS3D02G073300 | 3D         | 33,294,918 – 33,295,992:1  | 786              | 261                           | A0A077RHZG9 |
| TaWOX7d      | TraesCS3D02G244300 | 3D         | 339,473,290 – 339,476,679:1 | 1834             | 513                           | A0A1D5WHW6  |
| TaWOX8d      | TraesCS3D02G335500 | 3D         | 447,560,283 – 447,562,999:1 | 792              | 263                           | A0A1D5VD82  |
| TaWOX13d     | TraesCS3D02G352500 | 3D         | 463,197,196 – 463,199,275:1 | 1112             | 298                           | A0A1D5WMN9  |
| TaWOX14.1d   | TraesCS3D02G352600 | 3D         | 463,227,796 – 463,230,501:1 | 895              | 285                           | A0A1D5WPP9  |
| TaWOX14.2d   | TraesCS3D02G352700 | 3D         | 463,378,560 – 463,381,808:1 | 942              | 291                           | A0A1D5WNX7  |
| TaWOX9d      | TraesCS3D02G361100 | 3D         | 474,614,857 – 474,615,873:1 | 901              | 210                           | T1WGQ3      |
| TaWOX6a      | TraesCS4A02G130200 | 4A         | 170,708,103 – 170,711,065:1 | 1350             | 307                           | A0A341TSN5  |
| TaWOX6b      | TraesCS4B02G174400 | 4B         | 382,691,977 – 382,694,806:1 | 1254             | 309                           | A0A1D5XN16  |
| TaWOX6d      | TraesCS4D02G176400 | 4D         | 306,795,298 – 306,798,208:1 | 1262             | 306                           | A0A341UK30  |
| Gene     | Gene locus | Chromosome | Gene stretch region                  | mRNA length (bp) | Protein sequence length (aa) | UniProt ID       |
|----------|------------|------------|--------------------------------------|------------------|-------------------------------|-----------------|
| *TaWOX5a* | TraesCS5A02G085000 | 5A         | 111,588,730 – 111,590,895:1          | 1220             | 318                           | A0A341UT17      |
| *TaWOX3a* | TraesCS5A02G157300 | 5A         | 336,949,988 – 336,951,183:1          | 1060             | 241                           | A0A1D5YD57      |
| *TaWOX5b* | TraesCS5B02G091000 | 5B         | 118,451,983 – 118,454,221:1          | 1302             | 321                           | A0A1D5ZG91      |
|           |             |            |                                      |                  |                               | A0A1D6A0K9      |
| *TaWOX3b* | TraesCS5B02G156400 | 5B         | 288,891,901 – 288,893,003:1          | 968              | 241                           | W5F9A2          |
| *TaWOX5d* | TraesCS5D02G097400 | 5D         | 108,103,399 – 108,105,722:1          | 1381             | 322                           | W0Z680          |
| *TaWOX3d* | TraesCS5D02G162600 | 5D         | 254,023,305 – 254,024,410:1          | 1006             | 242                           | W5FQU4          |
| *TaWOX8u* | TraesCSU02G204800 | Un         | 304,503,012 – 304,503,827:1          | 617              | 156                           | A0A077RQB3      |
|           |             |            |                                      |                  |                               | A0A096UQ47      |
|           |             |            |                                      |                  |                               | A0A1D6RTL8      |
|           |             |            |                                      |                  |                               | A0A1D6RTL9      |
| *TaWUSb*  |             | 2B         | 714,777,526 – 714,778,733:1          | 921              | 306                           |                 |
| *TaWUSd*  |             | 2D         | 590,146,287 – 590,147,498:1          | 927              | 308                           |                 |
|           |             | 1D         | 6,219,571- 6,220,231:1               |                  |                               |                 |
|           |             | 3A         | 64,319,914 – 64,325,218:-1           |                  |                               |                 |
|           |             | 3B         | 83,465,544 – 83,470,232:-1           |                  |                               |                 |
|           |             | 3B         | 83,471,253 – 83,471,941:-1           |                  |                               |                 |
|           |             | 3D         | 52,801,752 – 52,812,298:-1           |                  |                               |                 |
|           |             | 3D         | 463,261,309 – 463,261,744:-1         |                  |                               |                 |
### Table 2  
Characteristics of HvWOX gene family members in *H. vulgare*

| Gene     | Gene locus            | Chromosome | Gene stretch region       | mRNA length (bp) | Protein sequence length (aa) | Uniprot ID |  |
|----------|-----------------------|------------|---------------------------|-----------------|------------------------------|------------|
| HvWOX2   | HORVU1Hr1G010580      | 1H         | 24,444,001–24,445,742:1   | 1742            | 279                          | A0A287ELV0|
| HvWOX12  | HORVU1Hr1G087940/50   | 1H         | 540,693,806–540,698,431:1 | 1470            | 489                          | A0A287GM87|
|          |                       |            |                           |                 |                              | A0A287GM65|
| HvWOX11  | HORVU2Hr1G017270      | 2H         | 40,107,707–40,111,565:1   | 927             | 308                          | A0A287H773|
| HvWOX4   | HORVU2Hr1G113820      | 2H         | 729,806,496–729,808,073:1 | 1151            | 228                          | A0A287JHP1|
| HvWOX10.1| HORVU3Hr1G013290      | 3H         | 28,673,837–28,674,948:1   | 786             | 261                          | M0Y8G7    |
| HvWOX10.2| HORVU3Hr1G013330      | 3H         | 28,785,048–28,786,156:1   | 815             | 261                          | A0A287K575|
| HvWOX7   | HORVU3Hr1G060950      | 3H         | 464,417,446–464,421,050:1 | 2027            | 516                          | A0A287L9L2|
| HvWOX8.1 | HORVU3Hr1G080660      | 3H         | 589,829,423–589,834,968:1 | 3229            | 267                          | M0 × 0 × 0|
| HvWOX8.2 | HORVU3Hr1G080690      | 3H         | 590,115,430–590,116,290:1 | 584             | 130                          | A0A287LWD8|
| HvWOX9   | HORVU3Hr1G085050      | 3H         | 610,834,437–610,835,788:1 | 1165            | 209                          | F2E473    |
| HvWOX14  | HORVU3Hr1G086430      | 3H         | 616,993,938–616,996,482:1 | 1216            | 283                          | M0XTJ6    |
| HvWOX13  | HORVU3Hr1G086450      | 3H         | 617,085,484–617,087,698:1 | 824             | 274                          | A0A287M365|
| HvWOX6   | HORVU4Hr1G051530      | 4H         | 423,508,136–423,511,456:1 | 1710            | 306                          | M0Y4Z0    |
| HvWOX5   | HORVU5Hr1G022120      | 5H         | 111,001,136–111,003,388:1 | 1046            | 276                          | A0A287QMF0|
| HvWOX3   | HORVU5Hr1G049190      | 5H         | 381,765,625–381,766,908:1 | 1126            | 186                          | A0A287R4V3|
| HvWUS    |                       | 2H         | 717,822,805–717,905,740:1 | 942             | 313                          |            |

**Identification of WUS homologous genes in Triticeae plant species**

In the six *Triticeae* plant species, only one transcript of *WUS* gene was annotated as *TaWUSa* on chromosome 2A in wheat in the database (Table 1). We found the homologous fragments of *TaWUSa* on chromosomes 2B and 2D in wheat (Table 1), 2D in *A. tauschii* (Table S1), 2A and 2B in *T. dicoccoides* and *T. turgidum* (Tables S2 and S3), and 2H in barley (Table 2). According to the results of multiple sequence alignment, the full length of the open reading frame (ORF) of these homologous genes can be achieved, and their deduced amino acid sequences were highly consistent.
with TaWUS (Fig. 1A). To understand if these genes can normally transcribe and express, promoter analysis was performed. It was showed that the promoter region of the WUS genes in the six Triticeae plant species all contained core promoter elements including transcription start TATA-box and AT ~ TATA-box, indicating they possessed potential transcriptional activity (Fig. 1B). In the promoter region of TaWUSa, TdWUSa, TtWUSa, and TuWUS, a fragment of GGTCAT was existed, which is a cis-acting regulatory element involved in auxin responsiveness. Nevertheless, this element was not detected in the promoter of AtaWUS, TaWUSb, TaWUSd, TdWUSb, and TtWUSb.

Chromosomal location of WOX genes in Triticeae plant species

In general, no WOX gene was found on homologous groups 6 and 7 for the genomes of the six Triticeae plant species, i.e., T. aestivum, T. turgidum, T. dicoccoides, H. vulgare, A. tauschii, and T. urartu, (Tables 1 and 2, and Tables S1-S4). In T. aestivum, except TaWUS, all the TaWOX genes had three copies of transcripts on its genomes A, B, and D. Three homologous alleles of TaWUS were located on chromosomes 2A, 2B, and 2D. The homologous genes of TaWOX2 or TaWOX12 were located on chromosomes 1A, 1B, and 1D. Three copies of TaWOX4 or TaWOX11 were located on chromosomes 2A, 2B, and 2D. The three homologous genes of TaWOX7 to TaWOX10, TaWOX13 and TaWOX14 were all located on chromosomes 3A, 3B, and 3D. The three alleles of TaWOX6 were located on chromosomes 4A, 4B, and 4D. The three alleles of TaWOX3 or TaWOX5 were located on chromosomes 5A, 5B, and 5D. Further investigation would be needed for the unknown chromosomal location of an incomplete transcript of TaWOX8. No WOX gene was found on homologous groups 6 and 7 (Table 1, Fig. 2A). The HvWOX genes in H. vulgare showed the similar chromosomal localization to the TaWOX genes in T. aestivum and AtaWOX genes in A. tauschii. HvWOX2 and HvWOX12 were located on chromosome 1H; HvWOX4 and HvWOX11 were located on chromosome 2H; HvWOX7 to HvWOX10, HvWOX13, and HvWOX14 were located on chromosome 3H; HvWOX6 was located on chromosome 4H, and HvWOX3 and HvWOX5 were located on chromosome 5H. (Table 2; Fig. 2B). There are additional copies of HvWOX8 and HvWOX10 on chromosome 3H. The HvWOX10.1 and HvWOX10.2 showed complete sequence consistency, but HvWOX8.2 was shortened compared with HvWOX8.1.

Similar situation was observed in A. tauschii. AtaWOX2 and AtaWOX12 were located on chromosome 1D. AtaWOX4 and AtaWOX11 were located on chromosome 2D. AtaWOX7 to AtaWOX10, AtaWOX13, and AtaWOX14 were all located on chromosome 3D. AtaWOX6 was located on chromosome 4D, AtaWOX3 and AtaWOX5 were located on chromosome 5D (Table S1, Fig. S1A). Similar results were also obtained in T. dicoccoides and T. turgidum. As expected, all the TdWOX and TtWOX genes were located on the corresponding chromosomes of their genomes A and B because the two species only have the two genomes (Table S2, Table S3, Fig. S1B, Fig. S1C). Additional copies of TdWOX8a and TtWOX14a were also existed on the corresponding chromosomes.

To verify the chromosomal locations of those WOX genes in the six Triticeae species, partial sequences of some of the WOX genes were amplified by their specific primers using a set of T. durum-T. aestivum genome D substitution lines (Fig. 3). The TaWUSa and its two homologs (named as TaWUSb and TaWUSd) were detected in T. aestivum L. cv CS (ABD genome), T. durum cv Langdon (AB genome), and other substitution lines except 2D(2A), indicating that the two copies TaWUSa and TdWUSa were located on chromosome 2A. TaWUSb was amplified in CS, Langdon, and other substitution lines except 2D(2B), indicating that TaWUSb was located on chromosome 2B. TaWUSd only appeared in CS, 2D(2A) and 2D(2B), indicating that it was located on chromosome 2D (Fig. 3). Similarly, WOX2a, WOX2b, WOX6a, and WOX6b were absent in 1D(1A), 1D(1B), 6D(6A), and 6D(6B), respectively. WOX2d and WOX6d were only detected in CS and the substitution lines which contain chromosome 1D or 4D (Fig. 3).

Evolution of WOX family proteins in Triticeae plant species
Phylogenetic trees of WOX family proteins in *Triticeae* species were constructed based on the deduced protein sequences. From the phylogenetic trees, it was suggested that WOX proteins in *Triticeae* plants were also divided into three clades, like those in many other plant species [44, 45]. However, the WOX protein classification in wheat was closer to that in rice in comparison with that in *Arabidopsis*. TaWUS, TaWOX2 to TaWOX5, TaWOX9, TaWOX13, and TaWOX14 were assigned to the same clade with the homologous proteins in rice, corresponding to *Arabidopsis* WUS clade (AtWUS and AtWOX1 to AtWOX7). TaWOX6, TaWOX7, and TaWOX10 to TaWOX12, and their homologous proteins from rice were classified into a clade, corresponding to an *Arabidopsis* intermediate clade (AtWOX8, 9, 11, and 12). TaWOX8 and OsWOX8 were clustered in separated branches, showing correspondence to an *Arabidopsis* ancient clade (AtWOX10, 13, and 14) (Fig. 4).

Barley WOX proteins were also divided into three clades: the first clade harbored HvWOX2, 3, 5, 9, 13 and 14; the second clade was for HvWOX8 only; and the third clade included HvWOX6, 7, and 10 to 12 (Fig. S2A). Similar to wheat, one branch in *A. tauschii* contained AtaWOX2 to AtaWOX5, 9, 13 and 14. AtaWOX6, 7, and 10 to 12 were clustered into the same branch, but AtaWOX8 was belonged to another branch alone (Fig. S2B). In *T. turgidum*, TtWOX proteins were also divided into three clades: TtWOX2 to TtWOX5, 9, 13 and 14 were in the first branch; TtWOX6, 7, and 10 to 12 were in the second branch; and the three copies of TtWOX8 were clustered into the same group with OsWOX8 (Fig. S2C). In *T. dicoccoides*, TdWOX2 to TdWOX5, 9, 13 and 14 were clustered in one branch, TdWOX8 was in other branch alone, and TdWOX6, 7, and 10 to 12 were in another branch (Fig. S2D). In *T. urartu*, only eight sequences coding WOX family proteins were retrieved because there was no complete genome information on *T. urartu* yet. The deduced protein sequences from gene sequences of TuWOX and OsWOX were used to construct a phylogenetic tree, in which TuWOX2, 5, and 9 were grouped together, and TuWOX10 and TuWOX6/11 were in the same branch, and the two homologous sequences of TuWOX8 were clustered together (Fig. S2E).

The phylogenetic tree of the WOX family proteins from the six *Triticeae* species was also constructed via maximum likelihood method (Fig. 5). Based on the tree, it was clearly seen that the WOX proteins with the same names from the six *Triticeae* species were clustered together (Fig. 5), indicating that the WOX proteins were conserved in these plant species.

**Analysis for the conserved motifs of WOX proteins in *Triticeae* species**

All the amino acid sequences of WOX proteins in the six *Triticeae* species were deduced from their transcripts mentioned above. Each member contained HOX homeodomain, which were the most noteworthy symbol and defining feature of this protein family (Fig. 6, Fig. S3). Sequences of HOX homeodomain of the three clades of WOX proteins were conserved in the six *Triticeae* species (Fig. 7A). The conserved WUS-box motif TLXLFPXX (TL-[DEQP]-LFP-[GITVL]-[GSKNTCV]) was found in TaWUS, WOX2 to WOX5, and WOX9 in these *Triticeae* species (Fig. 6A, Fig. 7B). While, there was one amino acid residue change in ELXLFPXX of TaWUS and LLXLFPXX of WOX13 and WOX14 in the *Triticeae* species (Fig. 7B). The carboxy-terminal ERF-associated amphiphilic repression (EAR) domain of L-[ED]-L-[RST]-L only exists in WUS and WOX9 (Fig. 6A), and EAR domain of WOX9 in these *Triticeae* species showed highly conserved (Fig. 7C).

**Expression patterns of TaWOX genes in different organs of wheat**

The *WOX* genes mainly expressed in the meristematic region, and played a regulatory role in the process of plant growth and tissue differentiation. We retrieved the data from expVIP website (http://wheat-expression.com), and sketched the contours of expression pattern of *TaWOX* genes. It is showed that *TaWUS* expressed in root during seedling stage, in spike during vegetative stage, and in spike and leaf/shoot during productive stage. Its expression level was higher in spike than other organs (Fig. S4A). All the three homologues of *TaWOX*2 to 4, 7, 8, and 12 showed...
higher expression level in developing spike than other organs, and even higher at vegetative stage than reproductive stage (Fig. S4B-D, G, H, and L). The expression level of TaWOX5 was higher in grain than that in other organs at reproductive stage (Fig. S4E). TaWOX6, 9 to 11 showed a high transcriptional activity in root (Fig. S4F, I-K). The transcripts of TaWOX10 and TaWOX11 mainly accumulated in root at seedling stage while the expression level of TaWOX9 was high in root at vegetative stage (Fig. S4I-K). The transcript levels of TaWOX6b and TaWOX6d in root were increased at productive stage compared with vegetative stage (Fig. S4F).

Further, we used wheat root, stem, leave, spike at booting stage, and anther at heading stage as well as immature embryo, callus derived from the immature embryos at proliferative and differential stages as materials to perform expression profiling analysis of TaWOX genes by qPCR assay. The results indicated that expression patterns of TaWOX genes changed greatly in different organs at different stages (Fig. 8). The expression levels of TaWUS and TaWOX6 to 8 were relative high in spike (Fig. 8A, B), and the expression levels of TaWOX9 and TaWOX11 were high in root (Fig. 8B, C). Additionally, TaWOX2 showed high activity in embryo, and TaWOX3 and TaWOX4 showed high expression levels in embryogenic callus and differential callus, respectively (Fig. 8A).

**Discussion**

In Triticeae plant species, wheat and barley are two important crops globally, which account for a large proportion of food production in the world. With the completion of assemble and annotation of the colossal wheat genome, a great progress on functional genomic study in Triticeae plants, especially in wheat, has been achieved [46–49]. It is well-known that wheat genome was originated from the natural hybridization of its three ancestor species. Therefore, wheat genome consisting of three genomes of A, B, and D has a large number of repeated gene sequences, and most wheat genes have three or more copies [50]. In present study, we identified 43 WOX gene copies in the genome of T. aestivum, 42 of which was consistent with the result reported by Li et al. [51], and a new locus of TaWOX8 was added to the results of TaWOX family. Particularly, we firstly identified 17 WOX genes in H. vulgare, 13 in A. tauschii, 30 in T. turgidum, 25 in T. dicoccoides, and 8 in T. urartu. There were still several duplicated copies of WOX gene such as TaWOX14a, TaWOX14d, HvWOX10, and TdWOX14. A few of WOX-like pseudo genes were found to be scattered over Triticeae genomes, which might be a duplication of WOX genes or the other genes losing transcriptional activity during their evolution progress.

WUS plays an indispensable role on the stem cell niche maintenance in shoot apical meristem (SAM), lateral primordia differentiation and other diverse cellular processes [26]. The deficiency of WUS gene will lead to the loss of function of SAM and terminated plant growth [25]. However, only the allele of TaWUS located on chromosome 2A was annotated as a transcript. TdWOX12a, TdWOX12b, TdWOX7b and TdWOX13b, which have a high sequence identity with their homologous genes from wheat, were also not annotated as transcripts in the database. The DNA sequences and deduced protein sequences of four genes TdWOX12a, TdWOX12b, TdWOX7b, and TdWOX13b were added into the WOX members in the six Triticeae species (Table S2). In barley, the annotation of HORVU1Hr1G087940 and HORVU1Hr1G087950 and their deduced protein sequences A0A287GM87 and A0A287GM65 are actually originated from HvWOX12 (Table 2).

In previous studies, the classification and naming of WOX genes in wheat were confused to some extent. This might be attributed to the different naming scheme of WOX genes in Arabidopsis and rice [15, 23, 24]. For example, the TaWOX5 reported by Zhao et al. [52] were regarded as TaWOX9 due to their highly similarity to OsWOX9, even though it showed a close similarity to AtWOX5 in all the WOX members in Arabidopsis (Fig. 4). Several reported TaWOX members such as TraesCS3A02G358100, TraesCS3B02G391100, TraesCS3D02G352500, TraesCS3A02G358200, TraesCS3A02G358400, TraesCS3B02G391200, TraesCS3D02G352600, and TraesCS3D02G352700 on chromosomes 3A, 3B, and 3D, respectively, were named as TaWOX13 and TaWOX14 [51] according to new nomination regulation. However, TaWOX13...
was not similar to AtWOX13 or OsWOX13, and AtWOX14 was also not similar to AtWOX14 in transcripts. While, TaWOX13 and TaWOX14 were similar to the homologs of TaWOX5 according to phylogenic analysis (Fig. 4). The WOX13 and WOX14 in other Triticeae species showed the similar phylogenetic relationship with WOX5 members (Fig. 5).

All the TaWOX genes in wheat have three or more copies. Due to their sequence similarity, it is difficult to distinguish the expression level of each copy of TaWOX genes. A feasible approach was applied to estimate the amount of mRNA by calculating transcript amount of each copy. Zhao et al. indicated that the transcriptional level of individual TaWOX5 allele was varied during the period of callus growth in wheat [52]. Based on the results in the present investigation, the expression profiles of other WOX alleles were also changed in different wheat organs, which need to be justified by further research.

Conclusions

To our knowledge, this is the first study on genome-wide and contrastive analysis on WOX family genes in Triticeae plant species. In total, 130 WOX genes were identified, including 43 in T. aestivum, 28 in T. turgidum, 23 in T. dicoccoides, 15 in H. vulgare, 13 in A. tauschii, and 8 in T. urartu. The homologous genes of TaWUSb, TaWUSd, and WUS in other five Triticeae species were annotated, which were predicted to express normally according to promoter element analysis. Four novel homologous alleles of TaWOX genes including TdWOX12a, TdWOX12b, TdWOX7b, and TdWOX13b were also identified in T. dicoccoides. All of these WOX members showed evolutionary conservation and same chromosomal location arrangement. Based on the RNA-seq data in wheat-expression database and qPCR array results, TaWOX genes were found to have tissue-specific expression feature. The results showed in this study would be helpful to further understand the molecular function and evolutionary relationship of WOX family genes in Triticeae plants, and potentially apply them in plant genetic transformation in the future.

Methods

Materials and cultivation conditions

Wheat line Chinese Spring (CS) stored in our laboratory was used as the plant material to conduct gene identification and expression analyses. A set of T. durum-T. aestivum genome D substitution lines and their genetic background Langdon (LD), which were kindly provided by Dr. Steven Xu at the Northern Plains Crop Science Laboratory of the USDA-ARS, North Dakota, USA, and genetically identified by Prof. Zhishan Lin at the institute of Crop Sciences (ICS), Chinese Academy of Agricultural Sciences (CAAS), Beijing, China, were used to verify the chromosomal localization of the WOX genes identified in this study. In each of these disomic substitution lines, a pair of A-genome or B-genome chromosome in the tetraploid wheat T. durum was replaced by a corresponding pair of D-genome chromosomes from T. aestivum. For example, in substitution line 1D(1A) chromosome 1D from T. aestivum D replaces the chromosome 1A in T. durum. Thirty seeds of those wheat materials were planted as a trail with 1 m in length and 20 cm in width in the experimental station of ICS, CAAS, Beijing, China, under natural soil conditions without stress.

Rna Isolation And Qpcr Analysis

The wheat samples for roots, stems, and leaves were collected at three-leaf stage, for young spikes at booting stage, and for anthers at heading stage. The immature embryo samples were collected 15 days post anthesis (DPA). Callus samples were induced from the immature embryos on MS medium containing 2,4-D 2.0 mg L⁻¹ under dark condition
and collected after cultured for one week and two weeks, respectively. The calli were cultured for differentiation on 1/2 MS medium containing 5.0 mg L\(^{-1}\) Zeatin in a photoperiod of 14 h-light and 10 h-darkness and sampled one week later.

Total RNA was extracted using TRIzol™ Reagent Kit (Invitrogen 15596026), and reverse transcription reaction was performed using the PrimeScript™ RT reagent (Takara) according to the manufacturer's protocol. The qPCR was performed on ABI7500 Thermal Cycler using 2 × RealStar Green Fast Mixture (with ROX II, Genestar). TaActin (Genbank: AB181991) was used as internal controls, and three biological replicates were adopted. Gene-specific primers were designed with premiere primer 6.0 (Table S5). Each qPCR reaction system (20 µL) contained 10 µL of 2 × RealStar Green Fast Mixture, 0.4 µL of forward primer (10 mM), 0.4 µL of reverse primer (10 mM) and 1 µL of diluted cDNA (200 ng µL\(^{-1}\)). The thermal cycling conditions were 95 °C for 5 min, 40 cycles of amplification (95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s), and 95 °C for 10 s at dissociation stage, followed by 65–95 °C with increments of 0.5 °C for 0.05 s.

Database used for searching WOX family genes in Triticeae plants

Twenty-six predicted WOX family protein sequences of wheat were obtained from Plant TFDB database (http://planttfdb.cbi.pku.edu.cn), and retrieved Genbank (https://www.ncbi.nlm.nih.gov/genbank) with AtWOX of Arabidopsis, OsWOX of rice, and ZmWOX of maize (Zea mays). Using all of the protein sequences above as queries to conduct TBLASTN search on Gramene (http://ensembl.gramene.org/Tools/Blast) and URGI (https://urgi.versailles.inra.fr) for the identification of WOX proteins encoded genes in wheat genomes. Then, BLASTN search with sequences of TaWOX genes was performed in the genomes of H. vulgare, T. urartu, T. dicoccoides, T. turgidum, and A. tauschii. All the genetic analysis was carried out using these protein sequences of the six Triticeae plants. Based on the BLAST results from Gramene and URGI, the WOX genes from the Triticeae plants were located on exact chromosomes. The location chart was made by MapGene2Chrom web v2.1 (http://mg2c.iask.in/mg2c_v2.1/).

Dna Isolation And Pcr Analysis

Wheat genomic DNA was isolated by NuClean Plant Genomic DNA kit (Cwbio, CW0531M) from the leaf samples at three-leaf stage. PCR reaction system (20 µL) contained 10 µL of 2 × Taq Master Mix (containing Mg\(^{2+}\) and dNTP, Vazyme, China), 0.5 µL of each forward primer and reverse primer (10 mM), and 1 µL of gDNA (1 µg µL\(^{-1}\)), adding ddH\(_2\)O up to 20 °C. Sequences of all the primers used for the detection were shown in Table S6. The thermal cycling conditions were 94 °C for 5 min, 35 cycles of 94 °C for 20 s, 60 °C for 20 s, 72 °C for 30 s, and then 72 °C for 10 min.

Phylogenetic Trees Construction

The full-length of the WOX proteins of Triticeae species were aligned by ClustalW algorithm. Phylogenetic analysis and phylogenetic tree construction were performed by the MEGA X program (https://www.megasoftware.net/) using maximum approach and 1000 bootstrap replicates. Sequences of TaWOX proteins were aligned with AtWOX and OsWOX proteins, and phylogenetic tree was constructed to confirm classification and phylogenetic relationship of the identified TaWOX members. Then taking OsWOX proteins as model, the phylogenetic trees were constructed between OsWOX and HvWOX, OsWOX and TdWOX, OsWOX and TtWOX, OsWOX and AtaWOX members to name and classify the WOX members in the six Triticeae species.

Conserved Protein Motif Analysis
The conserved domain HD, was identified by SMART software (http://smart.embl-heidelberg.de/). The distinctive WUS-box motif as TLXLFPXX(T-L-[DEQP]-L-F-P-[GITVL]-[GSKNTCV]) and the EAR domain as LXLXL(L-[ED]-L-[RST]-L) were both defined in a strict sense. TEXshade software was employed to perform the multiple sequence alignments for HD domains, WUS-box motifs, and EAR motifs. The logo diagrams were drawn by canonical conserved residues including HD domains, WUS-box motifs, and EAR motifs by SeqLOGO in TBTools.

Expression analysis of TaWOX genes using RNA-seq data

RNA-seq data of 43 TaWOX genes was downloaded from expVIP (http://wheat-expression.com/). The expression level in root and leaves/shoot at seedling stage, in root and leaves/shoot spike at vegetative stage, and in root, spike, grain at vegetative stage were analyzed and compared.

Statistical analysis

The SPSS 19.0 software package was employed to statistically analyze the expression data of the target genes achieved by qPCR. Statistical comparisons of multiple sets of data was carried out by Duncan's multiple range test. The histogram was made using the Excel software.

Abbreviations

ARF
Auxin response factor; BBM: BABY BOOM; CLE41: CLAVATA3/ESRLIKE41; CLV3: CLAVATA3; CS: Chinese Spring; CSC: columella stem cell; CUC: CUP-SHAPED COTYLEDON; DPA: days post anthesis; EAR: ERF-associated amphiphilic repression; HB: homeobox; HD: homeodomain; LEC2: LEAFY COTYLEDON2; OC: organizing-center; ORF: open reading frame; PFS2: PRETTY FEW SEEDS2; PIN1: PINFORMED1; PLT: PLETHORA; PRS1: PRESSED FLOWER1; PXY: PHLOEM INTERCALATED WITH XYLEM; qPCR: quantitative real-time PCR; RNA-seq: RNA sequencing; SAM: shoot apical meristem; SCR: SCARECROW; SHR: SHORT ROOT; WOX: WUSCHEL-related homeobox; YUC: YUCCA.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Availability of data and material

All data used or analyzed in this study are included in this published article and additional files. Twenty-six predicted WOX family protein sequences of wheat could be downloaded from Plant TFDB database (http://planttfdb.cbi.pku.edu.cn). Genome sequences and annotation of WOX genes in the six Triticeae species could be downloaded from Gramene (http://ensemb.l.gramene.org/Tools/Blast) and URGI (https://urgi.versailles.inra.fr). Transcriptome data used for gene expression analysis could be downloaded from expVIP (http://wheat-expression.com/).

Competing interests
The authors declare that they have no competing interests.

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Authors’ contributions

The experiment was conceived by XY and HL. LS and HL analyzed the data, KW and XS assisted with bioinformatics analysis. LS performed the PCR and qPCR experiments. The manuscript was drafted by LS, XY and HL, and corrected and approved by all authors.

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