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Research article

Genome-wide characterization and expression and co-expression analysis suggested diverse functions of *WOX* genes in bread wheat

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ABSTRACT

WUSCHEL-related homeobox (*WOX*) genes belong to the homeobox superfamily, are plant-specific and play vital functions in the growth and development. Herein, we identified a total of 43 *TaWOX* genes in the allohexaploid (AABBDD) genome of *Triticum aestivum* L. These genes were distributed on the various chromosomes of each subgenome (A, B and D). The phylogenetic analysis showed the clustering of TaWOXs into three clades: ancient, intermediate and modern or WUS. The gene and protein structures including exon/intron organization, intron phases, and domain and motif distribution were found to be conserved in each phylogenetic clade. The subcellular localization was predicted as nuclear. The Ka/Ks analyses suggested the purifying selection of paralogous genes. The differential expression profiling of various *TaWOXs* in numerous tissue developmental stages and different layers of grains suggested their role in growth and development. Moreover, a few genes exhibited modulated expression during abiotic and biotic stress conditions, which revealed their roles in stress response. The occurrence of various *cis*-acting regulatory elements further confirmed their role in plant development and stress tolerance. The co-expression analyses suggested the interactions of these genes with other genes, involved in various processes including plant development, signalling and stress responses. The present study reported several characteristic features of *TaWOXs* genes that can be useful for further characterization in future studies.

1. Introduction

Cell division, cell differentiation and morphogenesis are the most important developmental processes in the multi-cellular organisms. Plants growth and developments are regulated by numerous important genes and factors (Li et al., 2019a; Ajdanian et al., 2019). The homeobox superfamily comprises various important groups of genes that play critical roles in growth and development (Gehring et al., 1994). It consisted of 14 gene families including *KNOX*, *BEL*, *WOX*, *HD-ZIP I-IV*, and *SAWADEE etc* (Mukherjee et al., 2009). This classification was performed based on conserved regions of the homeodomain (HD) along with other characteristic domains and motifs (Mukherjee et al., 2009). The nomenclature of these families was according to the combination of specific domains and motifs.

The WUSCHEL-related homeobox (*WOX*) gene family was firstly discovered in *Arabidopsis thaliana* (Laux et al., 1996). These genes are known to be involved in the plant growth and organ developmental processes (van der Graaff et al., 2009). They also regulate the proliferation of shoot and root apical meristems along with the stem cell homeostasis (Endrizzi et al., 1996; Haecker, 2004). The presence of *WOX* genes in the quiescent centre and organising centre of root and shoot meristems suggested their role in deciding the fate of cell differentiation in these organs, respectively (Sarkar et al., 2007; Forzani et al., 2014). It was reported that an *Arabidopsis WOX* mutant, *wus* was failed to develop shoot meristem (Endrizzi et al., 1996).

The *WOX* genes have been classified into three clades; ancient, intermediate and modern, during the evolution (van der Graaff et al., 2009). The ancient clade was termed due to their emergence in lower plants. The intermediate clade includes those *WOX* genes, which emerged in the ferns and are related to the vascular plants. The most recent clade i.e. the modern clade, also known as *WUS* clade, is responsible for the formation of seeds. This clade is exclusively identified in the seed-bearing gymnosperms and angiosperms (van der Graaff et al., 2009; Lian et al., 2014). However, it has been suggested that all the three clades are equally ancient depending upon their functional and selective adaptation (Wu et al., 2019). The number of *WOX* genes is independent of the genome size of plants. For instance, *Oryza sativa* consisted of 13 *WOX* genes, while *A. thaliana* has 15 *WOX* genes (Zhang et al., 2010). Moreover, a variable number of *WOX* genes in different plants usually depend upon the level of ploidy and duplication events (Wu et al., 2019;

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Wang et al., 2019; Li et al., 2019b). It has been observed that higher number of *WOX* genes are present in more evolved plants as compared to algae, which may be attributed to the neo-functionality of genes (Wu et al., 2019; Li et al., 2019b). The largest *WOX* gene family has been reported in the tetraploid genome of *Brassica napus* with 52 members (Li et al., 2019b), while only five *WOX* genes are present in *Phyllostachys edulis* (moso bamboo) (Xu et al., 2019). These genes have been found to be involved in various developmental processes including stem cell maintenance, embryo patterning, organ formation, flowering, *etc*. (Laux et al., 1996; Zuo et al., 2002; Haecker, 2004; Breuninger et al., 2008; Zhang et al., 2011; Nakata et al., 2012). The *WOX* gene family has been identified in several plant species including potato, tomato, pea, cotton, rice, maize, sorghum, *etc* (He et al., 2019; Zhang et al., 2010). In a previous study, WOX5 has been isolated and characterized in common wheat (Zhao et al., 2014).

Herein, we performed genome-wide identification and comprehensive characterization of the *TaWOX* gene family in bread wheat (*Triticum aestivum* L.). They were used for the analysis of gene and protein structure, the occurrence of conserved motifs and domains and their phylogenetic relationship. Further, Ka/Ks ratio, *cis*-acting regulatory elements, expression and co-expression analyses were carried out during various tissues developmental stages and in the presence of biotic and abiotic stress conditions. While our manuscript writing was in progress, Li et al. (2020) has also reported *TaWOX* genes. Moreover, they performed expression analysis for only a few genes and in the limited tissues. They lack several analyses including rigorous phylogenetic analysis, expression analyses in embryo developmental stages, stress conditions and co-expression analyses. The co-expression analysis suggested their interaction with a wide range of other genes involved in the diverse molecular processes. In the current study, we explored various characteristic features of *WOX* genes that will be helpful for their functional characterization in future studies.

2. Materials and methods

2.1. Identification of TaWOX proteins

Identification of TaWOX proteins was carried out by the BLASTp search using the available WOX protein sequences of *A. thaliana* against the *T. aestivum* protein model sequences downloaded from the Ensembl Plants (http://plants.ensembl.org/index.html), at e-value 10^{-10}. The HMMER search and the Pfam BLAST were used to further confirm the identified sequences (https://pfam.xfam.org/) (Finn et al., 2014). To validate the presence of signature homeodomain, the Simpler Modular Architecture Research Tool (SMART) (Letunic et al., 2015) and the NCBI CDD search were used (Marchlerbauer et al., 2017).

2.2. Chromosomal distribution and nomenclature

The EnsemblePlants (http://plants.ensemble.org/Triticumaestivum/) was used to find out the chromosomal localization of each identified gene. A bidirectional BLAST search was performed to find the homeologous *TaWOX* genes having sequence similarity ≥90%. The diagrammatic representation of the chromosomal distribution of the *TaWOX* genes was created using the MapInspect software (http://www.plantbreeding.wur.nl/uk/software_mapinspect.html.2012). For the nomenclature of *TaWOX*s, we followed the standard procedure prescribed as per the international rules for gene symbolization in *T. aestivum* (http://wheat.pw.usda.gov/ggpages/wgc/98/Intro.htm) as performed in earlier studies (Kaur et al., 2020).

2.3. Identification of duplication events

The prediction of duplication events was based on ≥80% sequence similarity, which was conducted through the bidirectional BLAST search at e-value 10^{-10}. Tandem and segmental gene duplication events were classified based on distance between paralogous gene pairs as suggested in the earlier studies (Shumayla et al., 2016, 2019; Sharma et al., 2020).

2.4. Multiple sequence alignment and phylogenetic analysis

Multiple sequence alignment of the WOX proteins was performed using the MultAlin and the MUSCLE programs at default parameters to get the conserved residues (Edgar, 2004). Phylogenetic relatedness of the TaWOX protein sequences with the other plants such as *A. thaliana, O. sativa, Sorghum bicolour* and *Zea mays* was studied through an evolutionary tree. A phylogenetic tree was constructed with the full length protein sequences using the Molecular Evolutionary Genetics Analysis (MEGA) 7 software following the Neighbor-joining method with 1000 bootstrap replicates (Kumar et al., 2016).

2.5. Gene structure analysis

The occurrence of exon and intron in the *TaWOX* genes was analysed by comparing the CDS and their corresponding genomic sequence as done in earlier studies (Taneja et al., 2016; Taneja and Upadhyay, 2018). The Gene Structure Display Server (GSDS 2.0) was used for the intron phase analysis and pictorial presentation of gene architecture (Hu et al., 2014).

2.6. Physicochemical properties of TaWOX proteins

The physicochemical properties of TaWOX proteins such as peptide length, molecular weight (MW) and isoelectric point (pI) were computed using the ExPasy tool (web.expasy.org/compute pi/) (Gasteiger et al., 2005). Subcellular localization was analysed using the CELLOv.2.5 (http://cello.life.nctu.edu.tw/cello2go/) and the Protcomp9.0 (http://www.softberry.com/berry.phtml?topic=protcomppl&group=programs&subgroup=proloc). The transmembrane regions were predicted using the TMHMMv.2.0, Phobius and Topcons tools (Krogh et al., 2001; Käll et al., 2004; Bernsel et al., 2009). The conserved motifs were determined using the Multiple EM for Motif Elicitation (MEME) Suite version 4.11.2 (Bailey et al., 2009).

2.7. Ka/Ks analysis

The ClustalOmega server (https://www.ebi.ac.uk/Tools/msa/clustalo) was used to align the nucleotide and protein sequences of the duplicated *TaWOX* genes. For the calculation of non-synonymous substitution per non-synonymous site (Ka), synonymous substitution per synonymous site (Ks), and the Ka/Ks ratio, we used PAL2NAL server (Suyama et al., 2006). To know the divergence time of each duplicated *TaWOX* gene pair, we used formula T = Ks/2r where divergence time and rate were denoted by T and r, respectively. The value of divergence rate was kept as 6.5×10^{-9} as reported for cereals and used in earlier studies (Gaut et al., 1996, Tyagi et al., 2020a, 2021).

2.8. Cis-acting regulatory elements

The *cis*-acting regulatory elements were analysed in 1500 bp upstream sequence from the translation start site of each *TaWOX* gene. These sequences were retrieved and search at the PlantCARE database for the analysis of the occurrence of *cis*-acting regulatory elements following the standard procedure (Lescot et al., 2002).

2.9. Expression analysis in tissue development stages

For the expression analysis, publicly available high throughput RNA sequence (RNA seq) data of *T. aestivum* was downloaded from the Expression ATLAS for different tissue developmental stages of leaf, stem, root, spike, grain and embryo (Papatheodorou et al., 2018; Choulet et al., 2014; Pingault et al., 2015). The expression value was calculated in term

of the fragment per kilobase per million (FPKM) using the Trinity package (Haas et al., 2013). Expression analysis of each gene was also carried out by using the data available at the WheatExp server (Pearce et al., 2015) (http://wheat.pw.usda.gov/WheatExp/; accession number: ERP004714) from similar stages.

2.10. Expression analysis during biotic and abiotic stress conditions

Expression analysis under biotic stress was carried out using publicly available high throughput RNA-seq data (PRJNA243835) generated in triplicates after 24, 48, and 72 h post-inoculation of *Puccinia striiformis f.* sp. *tritici* (Pst) and *Blumeria graminis f.* sp. *tritici* (Bgt), separately (Zhang et al., 2014). Moreover, RNA-seq data (accession number SRP062745) generated by Zhang et al. (2016) from the salt (150 mM NaCl) treated root tissue were used for the expression profiling of *TaWOX* genes under salt stress conditions after 6, 12, 24, and 48 h of treatment. Effect of heat (HS), drought (DS) and combined heat drought (HD) stresses were studied using the publicly available RNA-seq data (accession number SRP045409) generated after one and six hours of treatment (Liu et al., 2015). The differential expression analysis was performed using the Trinity package at FDR <0.05 with 0.001 p-value (Haas et al., 2013). The Hierarchical clustering Explorer tool (HCE.3.5) was used to generate heat maps (Seo et al., 2006).

2.11. Co-expression analysis

We used the CoExpress v.1.5 to identify the *TaWOX* genes co-expressing with the other genes in the genome of *T. aestivum* (Nazarov et al., 2010) at the following parameters: correlation power 1, threshold filter >0.9 with Pearson correlation coefficient. The co-expression analysis was performed using the expression data from tissue developmental stages, and various abiotic and biotic stress conditions. The functional annotation and gene ontology (GO) mapping of co-expressed transcripts were performed using the Blast2GO tool (Conesa and Götz, 2008; Nazarov et al., 2010). The interaction network was visualized through Gephi 0.91 (Bastian et al., 2009).

3. Results and discussion

3.1. Identification and chromosomal distribution TaWOX genes

A total of 43 *TaWOX* genes were identified in the genome of *T. aestivum* after an extensive BLASTp search (Additional file 1). In the allohexaploid genome of *T. aestivum*, these genes were found to be distributed on all the chromosomes except chromosome number six and seven (Figure 1, Table 1). Majority of *TaWOX* genes were localized on the chromosome group three. The chromosome group four carried only one gene in each A, B and D subgenome. Three genes were present on the 2A chromosome, while two genes were found on the 2B and 2D chromosomes. Rest of the chromosomes were found to be carrying an equal number of genes. Furthermore, the location of *TaWOX*-8Un gene was unknown. This may be due to the unavailability of complete genomic information. A total of 15, 13, and 14 *TaWOX* genes were distributed on the A, B, and D subgenomes, respectively. The results suggested an even distribution of the *TaWOX* genes on the A, B, and D subgenomes (Figure 1). A similar result was found by Li et al. (2020), but they found only two genes on the chromosome 2A while we found three genes (*TaWOX3, TaWOX4* and *TaWOX5*). In the case of diploid genome of *O. sativa* and *A. thaliana,* 13 and 15 *WOX* genes have been reported, respectively (Bi et al., 2016). The higher number of *TaWOX* genes could be due to the allohexaploid nature of the *T. aestivum* genome. Further, number of genes at each A, B and D subgenome was comparable to the diploid genome. The results suggested a direct correlation of the number of gene to the ploidy level of the genome.

3.2. Homeologous and paralogous TaWOX genes

TaWOX genes were clustered into 14 homeologous groups, which were equally contributed by all the three subgenomes expect *TaWOX-4*. It was only found on the chromosome 2 of A subgenome. The results indicated that the other homeologs from B and D subgenomes might have lost during evolution.

A total of five pair of paralogous *TaWOX* genes were predicted. All the duplication events among *TaWOX* genes were found to be tandemly duplicated (Figure 1; Additional file 2). The paralogous genes *TaWOX9-A, TaWOX10-A1* and *TaWOX10-A2* were found on the chromosome 3A with high degree of similarity, and in close proximity as tandem duplicated genes. Similarly, tandem duplication events were predicted among *TaWOX9-D, TaWOX10-D1* and *TaWOX10-D2,* as well. Further, *TaWOX9-B* and *TaWOX10-B* tandem duplicated genes were present on the chromosome 3B (Figure 1). Interestingly, all the duplication events were confined to the WUS clade and chromosome group 3 during evolution. No duplication events were traced from the ancient and intermediate clades. Survival of duplicated genes suggested their advantageous selection. These might have been kept for acquiring new functions such as for producing higher protein, required more in different tissues with different functions.

3.3. Ka/Ks analysis

We estimated the Ka/Ks ratio for the above five pairs of paralogous genes. The Ka/Ks computation is used for calculating type of selection pressure on the protein. Here, the resulted ratio can be < 1, $= 1$ or >1 where less than 1 means the functional constraint with negative or purifying selection of the genes. However, more than one indicates accelerated evolution with the positive selection (Nekrutenko et al., 2002).

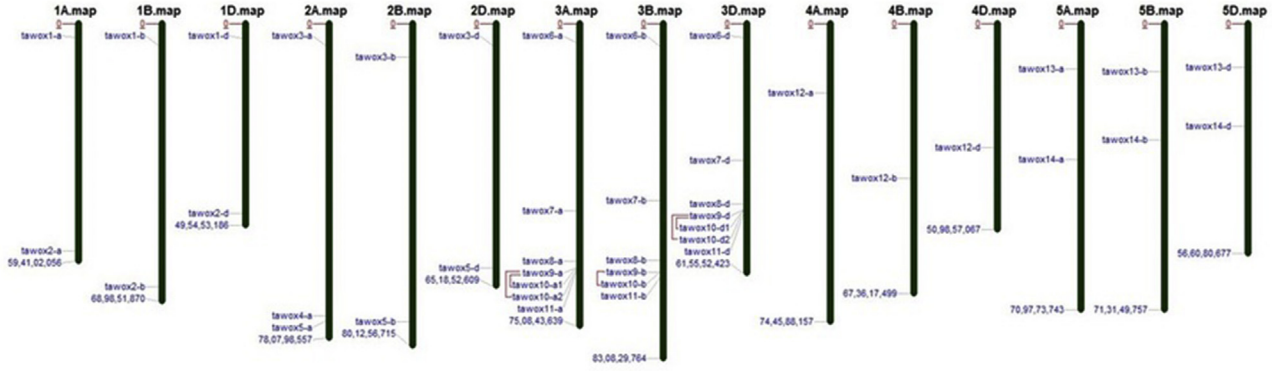

Figure 1. Chromosomal distribution of *TaWOX* genes. Chromosomes are represented by green vertical bars on which *TaWOX* genes are represented. Tandem duplication events are highlighted by red lines. The chromosome mapping was done by the MapInspect (http://mapinspect.software.informer.com/).

Table 1. Various characteristic features of *TaWOX* genes and proteins of *T. aestivum.*

GENE ID	Gene name	Chromosome	Exon	Protein length (AAs)	Isoelectric point	Mol.Wt. (kDa)	Subcellular localization
Ancient Clade							
TraesCS3A02G341700.1	TaWOX8-A	3A	3	265	6.0964	29.5	Nuclear
TraesCS3B02G373800.1	TaWOX8-B	3B	4	261	6.0964	29.2	Nuclear
TraesCS3D02G335500.1	TaWOX8-D	3D	2	263	6.8294	29.4	Nuclear
TraesCSU02G204800.1	TaWOX8-Un	Un	2	156	7.4367	17.7	Nuclear
Intermediate Clade							
TraesCS1A02G399400.1	TaWOX2-A	1A	4	486	7.7375	50.6	Nuclear
TraesCS1B02G427400.1	TaWOX2-B	1B	4	485	7.7252	50.6	Nuclear
TraesCS1D02G406900.1	TaWOX2-D	1D	4	486	7.881	50.8	Nuclear
TraesCS2A02G100700.1	TaWOX3-A	2A	3	265	6.7135	27.8	Nuclear
TraesCS2B02G117900.1	TaWOX3-B	2B	3	261	6.7135	27.4	Nuclear
TraesCS2D02G100200.1	TaWOX3-D	2D	3	264	6.8625	27.6	Nuclear
TraesCS3A02G073500.1	TaWOX6-A	3A	3	260	6.8407	27.9	Nuclear
TraesCS3B02G087800.1	TaWOX6-B	3B	3	261	6.7444	28	Nuclear
TraesCS3D02G073300.1	TaWOX6-D	3D	3	261	7.0197	27.9	Nuclear
TraesCS3A02G247200.1	TaWOX7-A	3A	3	515	7.3893	53.7	Nuclear
TraesCS3B02G272200.1	TaWOX7-B	3B	3	515	7.674	53.6	Nuclear
TraesCS3D02G244300.1	TaWOX7-D	3D	3	513	7.8833	53.4	Nuclear
TraesCS4A02G130200.1	TaWOX12-A	4A	3	307	7.8699	31.5	Nuclear
TraesCS4B02G174400.1	TaWOX12-B	4B	3	309	8.2579	31.8	Nuclear
TraesCS4D02G176400.1	TaWOX12-D	4D	3	306	7.7696	31.4	Nuclear
WUS Clade							
TraesCS1A02G052000.1	TaWOX1-A	1A	2	263	8.1704	28.5	Nuclear
TraesCS1B02G069000.1	TaWOX1-B	1B	2	264	8.1153	28.6	Nuclear
TraesCS1D02G054000.1	TaWOX1-D	1D	2	267	8.3764	29.1	Nuclear
TraesCS2A02G491900.1	TaWOX4-A	2A	3	308	8.2945	32.7	Nuclear
TraesCS2A02G514000.1	TaWOX5-A	2A	3	234	9.5634	25.3	Nuclear
TraesCS2B02G542600.1	TaWOX5-B	2B	3	237	9.529	25.8	Nuclear
TraesCS2D02G515600.1	TaWOX5-D	2D	3	237	9.9605	25.6	Nuclear
TraesCS3A02G358100.1	TaWOX9-A	3A	2	301	8.8572	33.4	Nuclear
TraesCS3B02G391100.1	TaWOX9-B	3B	2	299	9.409	33	Nuclear
TraesCS3D02G352500.1	TaWOX9-D	3D	2	298	8.4395	33.3	Nuclear
TraesCS3A02G358200.1	TaWOX10-A1	3A	2	288	10.048	31.9	Nuclear
TraesCS3A02G358400.1	TaWOX10-A2	3A	2	290	9.5964	32.3	Nuclear
TraesCS3B02G391200.1	TaWOX10-B	3B	2	290	10.0961	32.3	Nuclear
TraesCS3D02G352600.1	TaWOX10-D1	3D	2	285	9.5715	31.6	Nuclear
TraesCS3D02G352700.1	TaWOX10-D2	3D	2	291	9.0153	31.9	Nuclear
TraesCS3A02G368100.1	TaWOX11-A	3A	2	212	7.6866	24.5	Nuclear
TraesCS3B02G399800.1	TaWOX11-B	3B	2	209	7.7325	24.1	Nuclear
TraesCS3D02G361100.1	TaWOX11-D	3D	2	210	7.6866	24.2	Nuclear
TraesCS5A02G085000.1	TaWOX13-A	5A	2	318	10.3524	34.2	Nuclear
TraesCS5B02G091000.1	TaWOX13-B	5B	2	321	10.916	34.5	Nuclear
TraesCS5D02G097400.1	TaWOX13-D	5D	2	322	10.6937	34.4	Nuclear
TraesCS5A02G157300.1	TaWOX14-A	5A	2	241	8.8212	25.8	Nuclear
TraesCS5B02G156400.1	TaWOX14-B	5B	2	241	8.8212	25.8	Nuclear
TraesCS5D02G162600.1	TaWOX14-D	5D	2	242	8.3999	25.9	Nuclear

Table 2. The Ka/Ks ratio and divergence time of duplicated *TaWOX* gene pairs.

Gene A	Gene B	Ka	Ks	Ka/Ks	T = Ks/2r	Selection pressure
TaWOX9-A	*TaWOX10-A1*	0.1418	0.3323	0.4268	25.5	Purifying
TaWOX9-A	*TaWOX10-A2*	0.2054	0.4300	0.4777	33.1	Purifying
TaWOX9-B	*TaWOX10-B*	0.1860	0.3939	0.4723	30.3	Purifying
TaWOX9-D	*TaWOX10-D1*	0.0950	0.2825	0.3362	21.7	Purifying
TaWOX9-D	*TaWOX10-D2*	0.1954	0.3856	0.5066	29.6	Purifying

Ka-non-synonymous substitutions per non-synonymous site, **Ks-**synonymous substitutions per synonymous site, **T-**Divergence time and **r-** Divergence rate.

The occurrence of Ka/Ks ratio less than 1 for each *TaWOX* paralogous gene pair suggested the negative selection pressure or strong purifying selection. Further, the occurrence of these duplication events was predicted in the range of 21–33 Million years ago (MYA) (Table 2).

3.4. Phylogenetic analysis

We constructed a phylogenetic tree to understand the evolutionary relatedness among the *A. thaliana, O. sativa, S. bicolor, T. aestivum* and *Zea mays* WOX proteins. We found three distinct clades (Figure 2), as reported in previous literature (Zhang et al., 2010; Lian et al., 2014; Li et al., 2018; Wang et al., 2018, 2019; Chang et al., 2020). The ancient clade consisted of 13 WOXs which are orthologous to *Arabidopsis* WOX10, WOX13 and WOX14, out of which four WOXs belong to *T. aestivum*. The intermediate clade contained 34 WOXs, 15 TaWOXs orthologous to *Arabidopsis* WOX8, WOX9, WOX11 and WOX12. However, the modern or WUS clade consisted of 55 WOXs including eight from *Arabidopsis* (WOX1, WOX2, WOX3, WOX4, WOX5, WOX6, WOX7 and WUS) and 24 from *T. aestivum*. The ancient clade carried the lowest number of genes, while WUS clade comprised of nearly half of all sequences, making it the largest clade, as reported in other plants (Li et al., 2019b). Further, the homeologous and paralogous TaWOXs were tightly clustered, which suggested their structural homology. The paralogous genes might have maintained their conserved structure even after the duplication events. Our analysis supported the notion that the *WOX* gene family is monophyletic in origin and suggested their conserved role (Lian et al., 2014). Further observation suggested that the gene duplication events had occurred to expand the *TaWOX* gene family.

3.5. Gene structure analysis

We analysed the structural features of *TaWOX* genes including the exon-intron organization and the intron phase distribution. In the *TaWOX* genes, the number of exons ranged from one to four. In the ancient clade, three and four exons were found in *TaWOX8-A* and *TaWOX8-B,* respectively. While *TaWOX8-D* and *TaWOX8-Un* carried only two exons. All the introns were predicted to present in phase 1 in the ancient clade genes. In the intermediate clade, only *TaWOX2* group genes carried four exons, while resting all *TaWOX3, TaWOX6, TaWOX7* and *TaWOX12* group genes carried three exons (Figure 3; Additional file 3). Moreover, in this clade the majority of introns were found in phase 1 followed by phase 0. In the WUS clade, *TaWOX1, TaWOX9, TaWOX10, TaWOX11, TaWOX13* and *TaWOX14* consisted of two exons while only two genes had 3 exons (Figure 3, Additional file 3). In addition to it, the maximum number of introns was predicted to be in phase 1. Further, we found conserved exon-intron structure in all three clades supporting our phylogenetic tree. On the other hand, the phenomenon of exon loss in WUS clade could also be seen in *TaWOX* genes as reported previously in

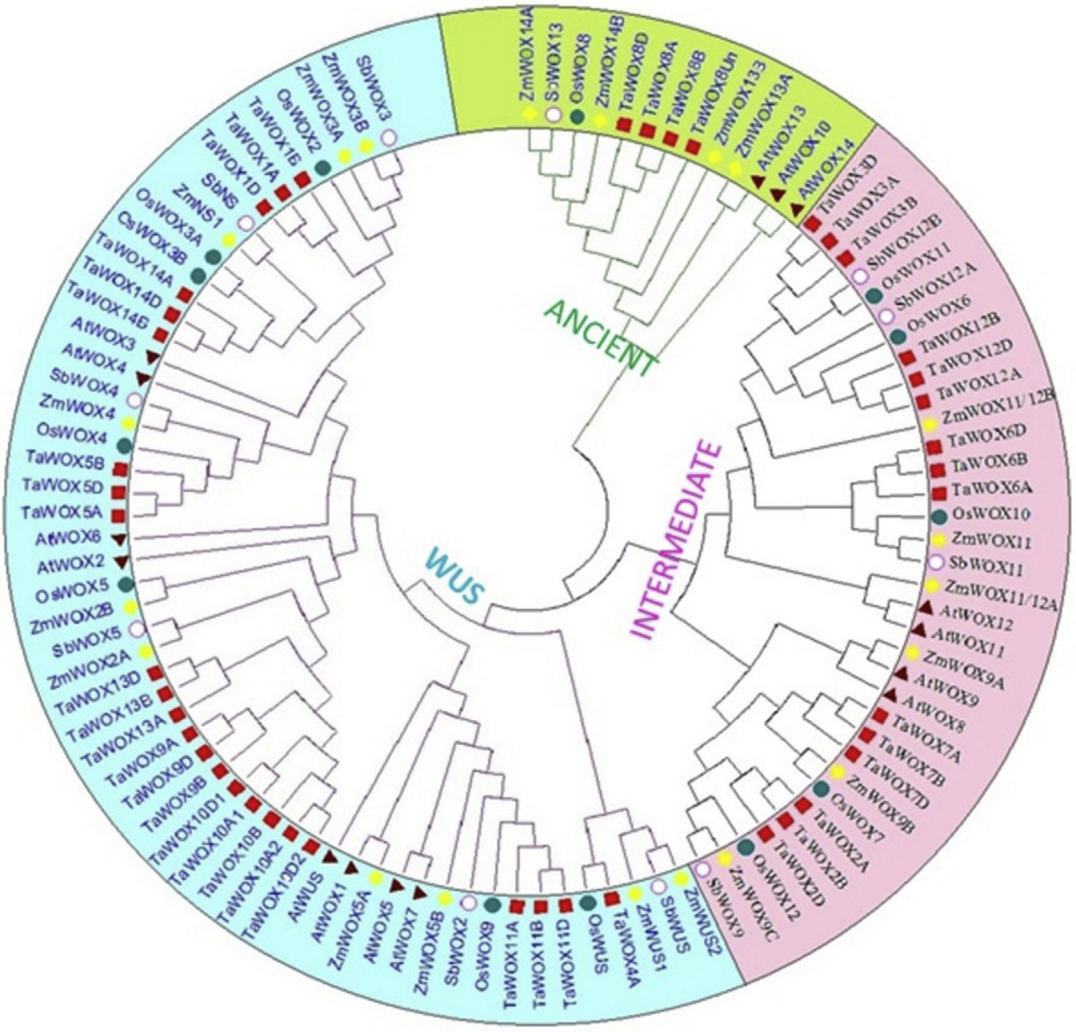

Figure 2. Phylogenetic analysis of the WOX proteins of wheat, *Arabidopsis*, rice, maize and sorghum. Green, pink and aqua blue colours shades represent the ancient, intermediate and WUS or modern clades, respectively.

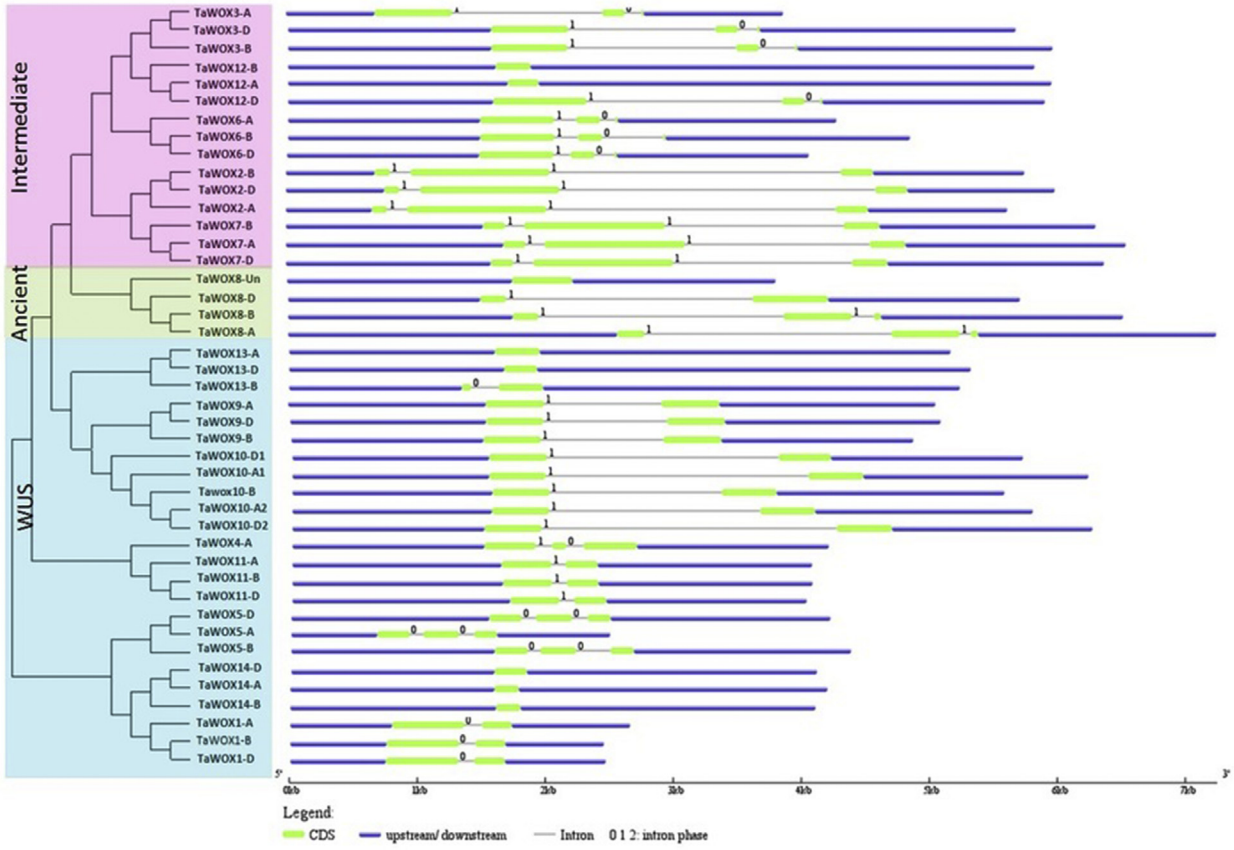

Figure 3. The exon-intron organization and intron-phase distribution of *TaWOX* genes. Ancient, intermediate and WUS clades are shown in green, pink and aqua blue shades, respectively.

rice and soybean *WOX* gene family (Hao et al., 2019). Gene structure variation in the members of ancient clade suggesting that the ancient genes might be associated with the process of rapid evolution (Wang et al., 2019).

3.6. Physicochemical characterization of TaWOX proteins

We analysed various physicochemical properties of WOX proteins including peptide length, MW and pI (Additional file 3, Table 1). The majority of TaWOX proteins consisted of 212–301 amino acid residues (aa), except TaWOX2 and TaWOX7 group proteins from the intermediate clade, which were of ~486 and ~515 aa, respectively. The longest and the heaviest protein was TaWOX7 (MW 53.7 kDa) while TaWOX11 was the smallest and the lightest one (MW 24.1 kDa). The average molecular weight of the ancient, intermediate and modern clades was 29.3, 38.2 and 29.5 kDa, respectively. We found great variation in the isoelectric point of TaWOX proteins (6.09–10.91).

By knowing the subcellular localization of WOX protein, we could correlate between its position and targeted function. All the TaWOX proteins were predicted to be localized in the nuclear region. Hence implying their specific function confined in nucleus and suggesting them as transcription factors. Similar features of WOX proteins have been reported from other plant species (Chang et al., 2020; Li et al., 2018; Wang et al., 2018).

3.7. Motifs and domain analyses

Multiple sequence alignment (MSA) gives intuitive format to concisely constraint evolutionary relationship between residues of protein for their characterization and in predicting function. We constructed MSA using the MultAlin with 43 fulllength TaWOX protein sequences. We found that the signature motif of the homeodomain with the helix-turn-helix-loop-helix was conserved in all the 43 TaWOX protein sequences (Figure 4). It was further confirmed using the SMART server (Additional file 4). We found 15 highly conserved amino acid residues within the homeodomain. In the first helix, five amino acid residues i.e. R, W, P, Q and L at 97, 98, 100, 104 and 107 position were found to be highly conserved. In the second helix, P and l at 120 and 132 positions were most conserved residues. However, in the third helix N, V, W, F, Q and N were the six conserved amino acid residues (Figure 4). Similar conservation of amino acid residues in the homeodomain with slight variation have been reported in various plant species (Haecker, 2004; Li et al., 2018). We found that the G is the most conserved amino acid residue present in both turn and loop.

Earlier studies in *Arabidopsis* reported the presence of other functional domains known as the WUS box and the Ear-like motif, which are present in WUS or modern clade, and responsible for transcription inhibition of some genes. We could also find conserved WUS box sequences in all the 24 TaWOX proteins of WUS clade (Figure 4). We observed TLELFPL as conserved motif in the majority of sequences, with slight variations. Such as the T was replaced by the L in the TaWOX9, TaWOX10 and TaWOX13 group proteins, while the last L was replaced by the Q or T in certain sequences. Similar variations are reported in other plants also (Zhang et al., 2010; Li et al., 2018).

We found amino acid residues 'PLELRLC' in TaWOX11-A, TaWOX11-B, and TaWOX11-D, and 'GTSPELTLR' in TaWOX4-A as an Ear-like motif beside the occurrence of WUS box (Figure 4) as reported in the many other plant species as well (Zhang et al., 2010; Li et al., 2018).

To understand the conserved structure of TaWOX proteins, we further analysed the occurrence of 15 conserved motifs. We found that

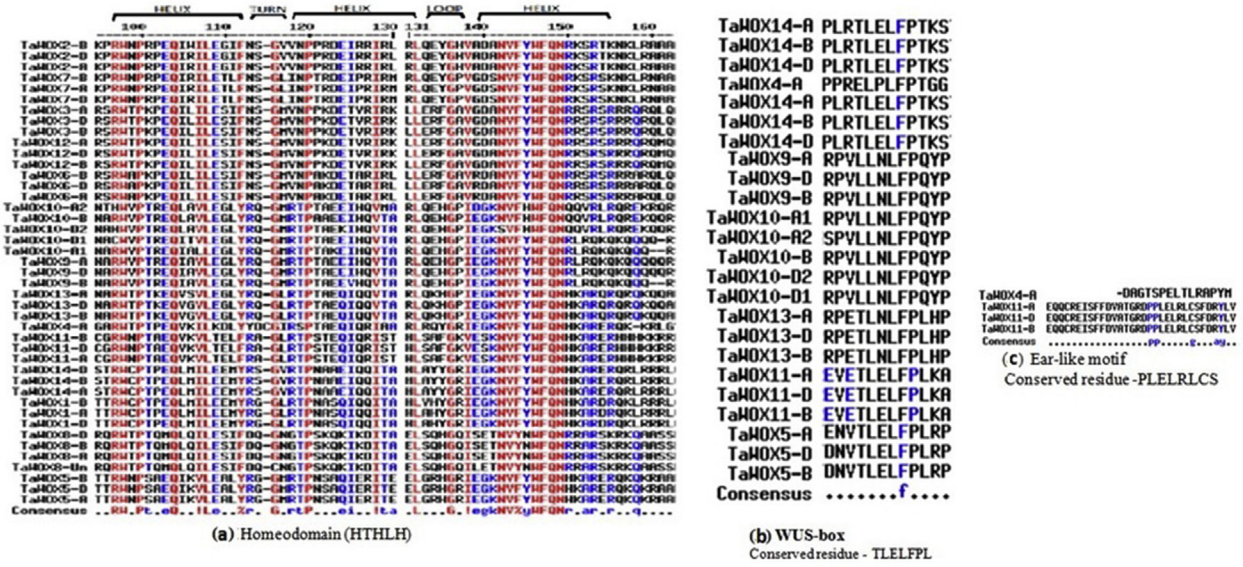

Figure 4. Multiple sequence alignment of TaWOX proteins. (a) Alignment of the homeodomain region of TaWOX proteins, which contain a typical helix-loop-helix-turn-helix structure. The highly conserved residues are shown in red and blue coloured font. (b) Alignment of the WUS-box (TLELFPL) that is located downstream of the homeodomain in TaWOX proteins. (c) Alignment of the EAR-like domain of certain TaWOX proteins.

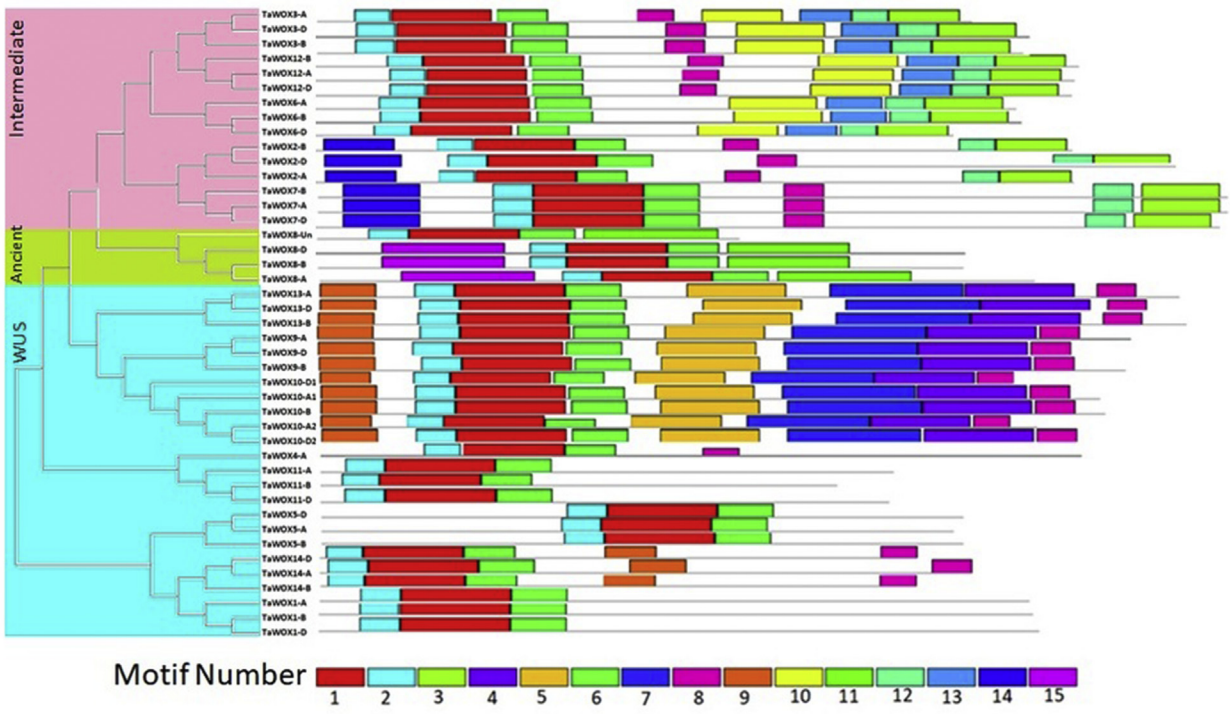

Figure 5. Schematic distributions of the conserved motifs present in the ancient, intermediate and WUS clades of TaWOX proteins.

the motifs one, two and six were the most conserved motifs present in the all 43 TaWOX proteins (Figure 5). The motif two and motif six corresponded to the alpha-helix 1 and the alpha-helix 3, respectively. However, motif 1 starting from the helix 1, ends in the helix 3, by accommodating both turn and loop as well. Uniformity in the motifs alignment of all the clades confirmed their close relatedness in the phylogeny. The ancient clade of wheat comprised of TaWOX8 group proteins clustered together with the presence of motif number 15 at the N-terminus, which was completely absent in other clades. It might have possible role in the early period of evolution. Genes of the intermediate clade had the motifs 10, 11, 12 and 13 in common (Figure 5). Furthermore, motifs 4, 5, 7 and 9 were only present in the WUS clade, suggesting introduction of new motifs during duplication and diversification process for the neo-functionalization particularly in the seed plants (Jain et al., 2008; Li et al., 2019b). Conserved motifs distribution in each clade implies conserved phylogenetic relatedness. The results further suggested that the ancient and intermediate clades have motifs responsible for transcriptional activation activities and thereafter developed WUS-motifs responsible for transcriptional repressive activities in the WUS clade (Zhou et al., 2018).

3.8. Cis-acting regulatory elements

The *cis*-acting regulatory elements in the 41 *TaWOX* genes were identified using the PlantCARE database (Lescot et al., 2002). All the identified elements were broadly categorized into four groups such as (1) light response (2) hormone (3) stress and (4) growth and development related (Additional file 5). The *cis*-regulatory elements ACE, G-Box, Box 4, TCT-motif, MRE, Sp1,AE-box, ATTAAT were identified as the light responsive elements. Moreover, G-box (CACGTG) element also plays an important role in response to abscisic acid, anaerobiosis and methyl-jasmonate, and it has an active role in ethylene induction along with seed specific expression. ABA response element (ABRE in 34 Ta*WOX* genes) and ACGTG (in 29 Ta*WOX* genes) were the most prevalent hormone responsive *cis*-acting regulatory element as compared to other elements related to the hormone response such as P-box, TATC-box, gibberellin-response element (GARE-motif), Auxin response element (AuxRR-core), TGA-box. Under stress response elements, methyl jasmonate-response elements (CGTCA-motif in 30 *WOX* genes and TGACG-motif in 29 *WOX*s), anaerobic responsive elements having GC and GT motifs active during plant defence responses (ARE in 29 *WOX* genes), CAACTG, MBS and LTR were frequently detected. The growth and development related *cis*-regulatory elements CAT-box, GCCACT involved in meristem-specific activation (present in 20 *WOX* genes), O2 site confers role in zein metabolism and circadian motif (present in 18 *WOX* genes), RY-elements specific for seed and shoot development (in 15 *WOX* genes; CATGCATG, MSA-like were predicted in the majority of *WOX* genes. The results revealed that the *TaWOX* genes have diverse *cis*-regulating elements that could response to a variety of stress, hormone and development-related factors that further suggested their roles in various biological pathways. Similar results are reported in tea and soybean (Wang et al., 2019; Hao et al., 2019).

3.9. Tissue specific expression analysis

TaWOX genes exhibited differential expression in various developmental stages of root, stem, leaf, spike, grain and embryo tissues. In-total 35 *TaWOX* genes showed expression in different tissues of wheat (Figure 6a,b). The majority of genes were highly expressed in spike and grain tissues, which suggested their role in the reproductive tissue and embryo development. For instance, genes belonging to WUS clade, *TaWOX1* group genes were highly expressed in spike tissue as compared to other tissues, while *TaWOX10* group genes showed significant expression in grain tissue. Similarly, *Arabidopsis AtWOX2* is involved in embryo and cotyledon boundary formation (Haecker, 2004). Further, several genes including *TaWOX11* and *TaWOX*5 group genes were highly expressed in the root and stem developmental stages. Phylogenetically these genes were grouped with *AtWUS* and *AtWOX4*, where *AtWUS* plays a key role in the development of shoot and floral meristems, and *AtWOX4* is responsible for lateral growth in *Arabidopsis*. Further, *TaWOX3* of the intermediate clade showed expression for root development which is very similar with the result of *OsWOX11* of rice (Zhao et al., 2009). These results suggested conventional roles for these similar genes in wheat (Laux et al., 1996).

To get further insight into the role of *TaWOX*, we analysed their expression in the endosperm, aleurone layer and transfer cell. A total of 12 *TaWOX* genes showed expression in these tissues. We observed developmental stage specific expression of *TaWOX* genes in the endosperm, which suggested specific roles of each *TaWOX* gene. For instance, *TaWOX8-A* of the ancient clade could express in all the endosperm developmental stages but specifically at the central region. Similar results are reported for the *Arabidopsis* (*AtWOX13*) ancient clade genes (Deveaux et al., 2008). *TaWOX10-A1, TaWOX10-A2* and *TaWOX10-D1* of the WUS clade were highly expressed at the later developmental stages of the endosperm and aleurone layer (Figure 6b) coinciding results with

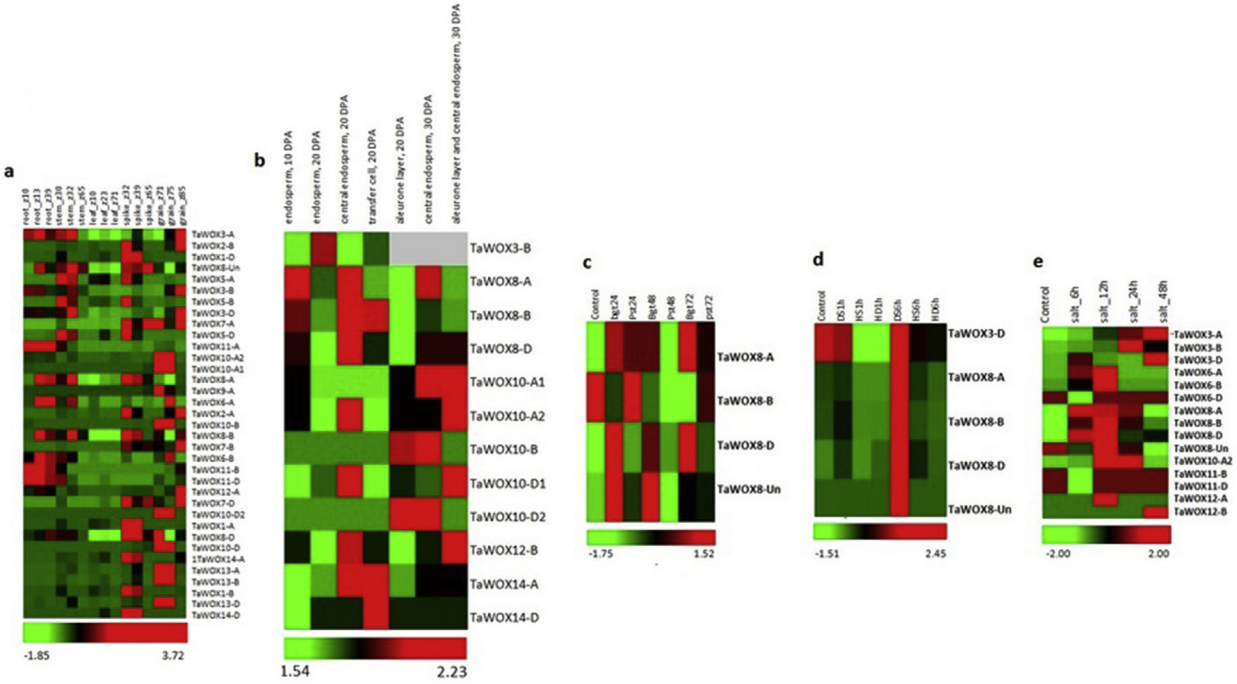

Figure 6. Relative expression profiles of *TaWOX* genes in various tissue development stages and in the presence of biotic and abiotic stress conditions. Figures 6a and b show expression profiling in various tissue development stages. Figure 6c shows expression under biotic (Bgt and Pst) stress conditions, figure 6d under heat, drought and their combination stresses, while figure 6e under salt stress.

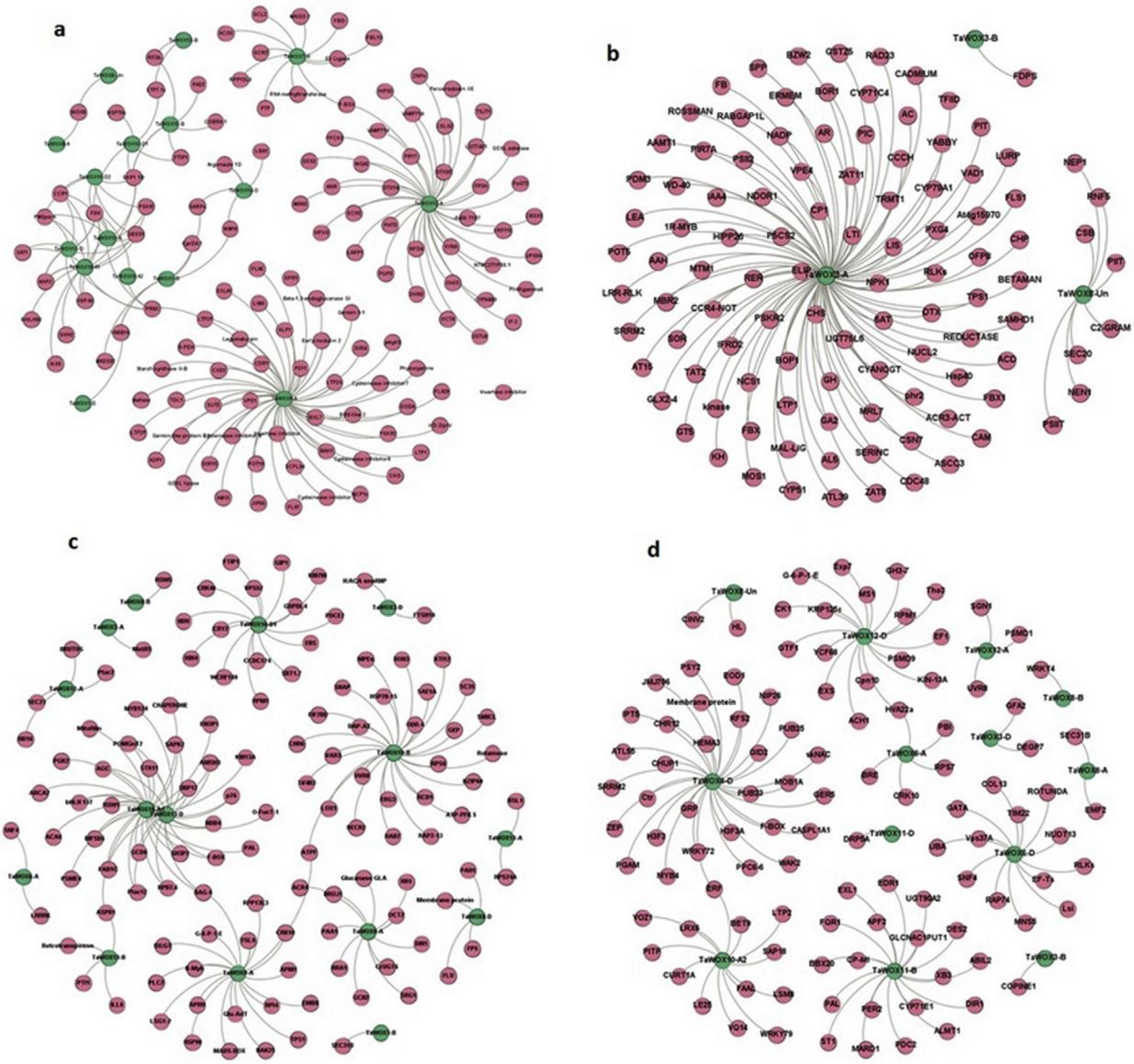

Figure 7. Interaction networks of the *TaWOX* genes in (a)various tissue development stages,(b) in the presence of biotic stress, (c) in the presence of heat and drought stress and (d) in the presence of salt stress. The interaction network was generated using the Gephi 0.9.1 tool.

AtWOX2 of the modern clade (Haecker, 2004). However, leaf developmental stages were deprived of significant expression of the majority of *TaWOX* genes.

TaWOX8 group genes belonging to the ancient clade also exhibited expression in all tissues, which suggested their role throughout the life cycle of plant. Broad expression pattern of the ancient clade genes reflected their conserved functions during evolution (Wang et al., 2019). Moreover, no such trend was found in the other clades. These assorted expressions of various *TaWOX* genes suggested their precise roles in various tissue developmental stages. Variable expression of *WOX* genes in different tissue has also been observed in other plants (Li et al., 2018, 2019; Rahman et al., 2017; Ramkumar et al., 2018).

3.10. Expression analysis under biotic stress

To study the expression pattern of *TaWOX* genes under biotic stress, we used the publicly available RNA seq data generated in response to fungal infection of triplicates after 24, 48, and 72 h post-inoculation of *Puccinia striiformis f.* sp. *tritici* (Pst) and *Blumeria graminis f.* sp. *tritici* (Bgt), separately (Zhang et al., 2014). Under biotic stress, only the ancient clade genes (*TaWOX8*) exhibited differential expression. The results indicated that the other genes might not have any relevant function in biotic stress response. All the *TaWOX8* group genes were upregulated after both Bgt and Pst infection, except *TaWOX8-B.* Moreover, the level of upregulation was higher in case of Bgt as compared to Pst infection (Figure 6c). Such differential expression of ancient clade genes suggested their function in biotic stress response which needs to be further established in future studies.

3.11. Expression analysis under abiotic stress

In case of abiotic stress, the expression of *TaWOX* genes was analysed under heat (HS), drought (DS) and combined heat drought (HD) stress after one and six hours of treatments (Liu et al., 2015), and under salt stress conditions after 6, 12, 24, and 48 h of treatment (Zhang et al., 2016). Out of 43 genes, only five *TaWOX* genes showed differential expression in heat and drought stresses; four genes of them belong to the ancient clade (Figure 6d). All the ancient clade genes were significantly upregulated after 6 h of DS, while all the genes were downregulated at other treatments. Similar differential expression pattern of

GmWOXs genes are reported under abiotic stress conditions (Hao et al., 2019).

Salt stress is a major threat to the plants that adversely affects the development and growth of plants. Hence, the expression profiling of *TaWOX* genes were performed to understand their putative role during salt stress. *TaWOX3* group genes of the intermediate clade were upregulated with the increase in the hours of salt treatment (Figure 6e). While the ancient clade *TaWOX*8 group genes were highly upregulated at initial hour of treatment while normalized at 48 h that might be due to adaptation of plants at the later hours. Similarly, variable expression of other *TaWOX* genes such as *TaWOX6* and *TaWOX12* of the intermediate clade and *TaWOX11* of the WUS clade was also observed. These results of differential expression of *WOX* genes suggested revisiting the function of these genes. They are not only related to the development, but might also be an important player in stress management, which need to be validated in future studies.

3.12. Co-expression and interaction analysis

To understand the interaction network of *TaWOX* genes, co-expression analysis was carried out in tissue developmental stages, and under abiotic and biotic stress conditions as well. In tissue developmental stages, 18 *TaWOX* genes exhibited co-expression with a total of 391 transcripts. Among them, *TaWOX12-A* of the intermediate clade was found to be co-expressed with 77 transcripts (Figure 7a; Additional file 6). These co-expressed transcripts encode the proteins related to the membrane integrity, fruit development, polarity specification, shoot apical meristem, transmembrane transport, DNA binding, Hydrolase activity, etc., which depicted their putative role in tissue development and signalling pathways (Figure 7a; Additional file 6).

In biotic stress, *TaWOX3-A* and *B* of the intermediate clade and and *TaWOX8-Un* of the ancient clade exhibited co-expression with a total of 169 other transcripts. These transcripts encoded for various receptor-like kinases, transcription factors, chalcone synthase, flavonoid pathway related and chloroplastic genes, which are related to transferase activity, regulation of transcription, oxidation and reduction process etc. The results suggested their involvement in various physiological and signal cascade mechanisms (Figure 7b; Additional file 7).

In case of heat and drought stresses, a total of 15 *TaWOX* genes showed co-expression with 157 different transcripts (Figure 7c; Additional file 8). These transcripts belong to various metabolic processes, transmembrane transport, post embryonic development, regulation of transcription, transmembrane transport activity related proteins, which depicted their role in defence and signalling pathways.

In salt stress, 13 *TaWOX* genes were found co-expressing with 167 other transcripts, in which *TaWOX8-D* of the ancient clade exhibited co-expression with 47 transcripts (Figure 7d; Additional file S9). The GO mapping of co-expressed transcripts suggested their role in water deprivation (GO:0009414), oxidation-reduction process (GO:0055114), regulation of transcription (GO:0006355), salt stress response (GO:0005634), peroxidase activity (GO:0004601), metal ion binding (GO:0046872) etc.

These results suggested diverse roles of *TaWOX* genes in plant development, stress responses, signalling cascade, and various physiological processes. Moreover, the functional validation of each candidate gene needs to be done in future studies.

4. Conclusions

The WUSCHELrelated homeobox (*WOX*) genes are known to play vital function during development in plants. Here, we performed identification of 43 *TaWOX* genes in an important crop plant *T. aestivum*. Phylogenetically, TaWOXs were clustered into three major groups termed as the intermediate, ancient and WUS or modern clades, confirmed by conserved exon-intron structure and motif composition in each clade. Tandem duplication events suggested that this phenomenon has contributed for the expansion of *WOX* gene family in wheat. Promoter analysis revealed the occurrence of *cis*-acting regulatory elements involved in growth and development as well as in stress response. The continuous expression of certain genes in all tissues reflects their conventional role as housekeeping genes in wheat. Moreover, the high expression of some *TaWOX* genes in various tissues developmental stages including spike, grain, endosperms etc. suggested their specific involvement in the growth and development of those tissues or organs. The differential expression of a few *TaWOX* genes under biotic and abiotic stress conditions revealed their roles in stress response. Co-expression analyses further suggested the involvement of *TaWOX* genes in the growth and development, and stress signalling. Moreover, the functional validation of each gene is necessary in the future studies. The current study would help in selecting the candidate *TaWOX* genes for functional validation in wheat to get improved crop variety.

Declarations

Author contribution statement

M. Rathour and A. Sharma: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

A. Kaur: Performed the experiments; Analyzed and interpreted the data.

S.K. Upadhyay: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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