A peer-reviewed version of this preprint was published in PeerJ on 4 May 2017.

Ning P, Liu C, Kang J, Lv J. 2017. Genome-wide analysis of WRKY transcription factors in wheat (*Triticum aestivum* L.) and differential expression under water deficit condition. PeerJ 5:e3232 https://doi.org/10.7717/peerj.3232

Genome-wide analysis of WRKY transcription factors in wheat (*Triticum aestivum* L.) and differential expression under water deficit treatment

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WRKY proteins, comprising one of the largest transcription factor families in plant kingdom, play crucial roles in the plant development and stress responses. Despite several studies on WRKYs in wheat (Triticum aestivum L.) were investigated, functional annotation information about wheat WRKYs was limited. Here, 171 TaWRKY transcription factors (TFs) were identified from the whole wheat genome and compared with proteins from other 19 species representing nine major plant lineages. A phylogenetic analysis, coupled with gene structure analysis and motif determination, divided these TaWRKYs into seven subgroups (Group I, IIa-e, III). Chromosomal location showed that the most TaWRKY genes were enriched on four chromosomes, especially on chromosome 3B, and 85 (49.7%) genes were either tandem (5) or segmental duplication (80), which suggested that though tandem duplication has contributed to the expansion of TaWRKY family, segmental duplication probably played a more pivotal role. The cis-acting elements analysis revealed putative functions of WRKYs in wheat during development as well as under numerous biotic and abiotic stresses. Finally, the expression of TaWRKY genes in flag leaves, glumes and lemmas under water stress were analyzed, and we found different TaWRKY genes preferentially express in specific tissue during the grain-filling stage. Our results provide a more extensive knowledge on TaWRKYs, which helps to complete the information of WRKY gene family in wheat, and also contribute to screen more candidate genes for further investigation on function characterization of WRKYs under various stresses.

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ABSTRACT

WRKY proteins, comprising one of the largest transcription factor families in plant kingdom, play crucial roles in the plant development and stress responses. Despite several studies on WRKYs in wheat (*Triticum aestivum* L.) were investigated, functional annotation information about wheat WRKYs was limited. Here, 171 TaWRKY transcription factors (TFs) were identified from the whole wheat genome and compared with proteins from other 19 species representing nine major plant lineages. A phylogenetic analysis, coupled with gene structure analysis and motif determination, divided these TaWRKYs into seven subgroups (Group I, IIa-e, III). Chromosomal location showed that the most *TaWRKY* genes were enriched on four chromosomes, especially on chromosome 3B, and 85 (49.7%) genes were either tandem (5) or segmental duplication (80), which suggested that though tandem duplication has contributed to the expansion of TaWRKY family, segmental duplication probably played a more pivotal role. The *cis*-acting elements analysis revealed putative functions of WRKYs in wheat during development as well as under numerous biotic and abiotic stresses. Finally, the expression of *TaWRKY* genes in flag leaves, glumes and lemmas under water stress were analyzed, and we found different *TaWRKY* genes preferentially express in specific tissue during the grain-filling stage. Our results provide a more extensive knowledge on TaWRKYs, which helps to complete the information of WRKY gene family in wheat, and also contribute to screen more candidate genes for further investigation on function characterization of WRKYs under various stresses.

Keywords: wheat, WRKY, water deficit, expression

INTRODUCTION

Plants have evolved a wide range of unique strategies to cope with various biotic and abiotic stresses through physical adaption, molecular and cellular changes (Ahuja et al., 2010; Knight & Knight, 2001). Transcription regulation of gene expression in response to developmental and environment changes, mediated by the DNA-binding transcription factors, is one of the most important regulatory mechanisms in plants (Ahuja et al., 2010; Buscaill & Rivas, 2014). WRKYs, one of the largest families of regulators, have been proven to play key roles in

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numerous stress responses and some development processes(Rushton et al., 2010). Since the first report of WRKY TFs identified in sweet potato(Ishiguro & Nakamura, 1994), WRKY proteins have been found throughout the plant lineage and also in some diplomonads, social amoebae, fungi incertae seais, and amoeboza in succession(Rinerson et al., 2015).

WRKY TFs were defined by the presence of one or two highly conserved WRKY domains (WDs) of 60 amino acid residues, including the almost invariant WRKYGQK heptapeptide at the N-terminus, followed by a C_2H_2 (C-X_{4-5}-C-X_{22-23}- H-X-H) or C_2HC (C-X_7-C-X_{23}-H-X-C) zinc-finger structure at the C-terminus (Eulgem et al., 2000; Rushton et al., 2010). The WRKY family members are classified into three groups (I, II and III) based on the number of WDs and the features of their zinc-finger-like motif (Eulgem et al., 2000; Rushton et al., 2010). Group I typically contains two WDs including a C_2H_2 zinc-finger structure, whereas Group II and III are characterized by a single WD, including a C_2H_2 and C_2HC zinc-finger motif, respectively. Group II can be further divided into five subgroups (IIa-IIe) based on phylogenetic analysis of the WDs (Eulgem et al., 2000; Rushton et al., 2010). Members of WRKY family regulate gene expression through exclusively binding to the W-box (TTGACC/T), a *cis*-element in the promoter region of target genes (Bakshi & Oelmüller 2014; Ulker & Somssich, 2004)

Recently, accumulating evidence has demonstrated that WRKY TFs, as important components of plant signaling web, regulate specific transcriptional programs during plant development, as well as in response to a variety of biotic and abiotic stimuli (Ahuja et al., 2010; Bakshi & Oelmüller, 2014; Rushton et al., 2012; Rushton et al., 2010). For example, 61 of the *PtrWRKY* genes in Populus are induced by biotic and abiotic treatments, such as Marssonina bruuea, salicylic acid (SA), methyl jasmonate (MeJA), wounding, cold and salinity (Jiang et al., 2014). In rice, expression of *OsWRKY*71 gene is induced by cold stress(Kim et al., 2016), while it also encodes a transcriptional repressor of GA signaling in aleurone cells (Zhang et al., 2004). Five transgenic broccoli lines over-expressing *BoWRKY6* demonstrated significant increased resistance to downy mildew, with resistant levels from low to very high(Jiang et al., 2016). In addition, WRKY TFs were also implicated to modulate plant development, such as seed development and germination (Raineri et al., 2016; Xie et al., 2007; Zhang & Wang, 2011), root growth (Ding et al., 2015; Ranjan & Sawant,

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2014) , stem elongation (Yu et al., 2012; Zhang et al., 2011), embryogenesis (Jimmy & Babu 2015; Lagace & Matton 2004), senescence (Ricachenevsky et al., 2010; Sakuraba et al., 2016), trichome development (Johnson, 2002).

Wheat (*Triticum aestivum* L.), one of the world's three main cereal with the highest monetary(Keating et al., 2014), is affected by multi-environment stresses, such as salinity, extreme temperature and especially drought, which is a principal constraint to global production of wheat (Mwadzingeni et al., 2016; Wang et al., 2015). However, the mechanism by which wheat responds to abiotic stress has been still poorly understood, which might due to its large genome (approximately 17GB). The identification and functional characterization of the WRKY family in wheat will contribute to clarify the mechanism of stress response. Several studies on wheat WRKY identification have been reported in succession. Forty-three and 92 putative TaWRKYs were early identified from publicly available EST by Niu et al. (2012) and Zhu et al. (2013), respectively. Okay et al. (2014) characterized 160 TaWRKYs and their expression profiling in RNA-Seq libraries. Recently, Zhang et al. (2016) identified 116 WRKYs, and 13 of them were characterized as senescence-associated genes. Here, we provide a more extensive knowledge on TaWRKYs based on the whole genome sequence of wheat. A total of 1113 WRKY TFs were identified in 20 plants representing the nine major evolutionary lineages to gain preliminary insight into the evolution of the WRKY family in Plantae. In addition, we identified 171 TaWRKYs from wheat, and detailed analysis, including gene classification, physical and chemical parameters prediction, phylogenetic analysis, chromosomal location, duplication events, conserved motif determination, exon-intron structure and *cis*-acting element analysis were employed. Finally, gene expression patterns of *TaWRKY* genes in flag leaf, glume and lemma tissues under water deficit treatment were further determined using qRT-PCR. These results will helps to complete the information of WRKY gene family in wheat**,** and also contribute to screen more candidate genes for future functional investigation of TaWRKYs under various stresses.

Materials AND Methods

Database search and identification of WRKYs

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The protein sequences of 20 plants from nine different major taxonomic lineages were downloaded from several public databases. All of the amino acid sequences were obtained from the following sources: the Eudicots *Arabidopsis thaliana* (At), *Populus trichocarpa* and the Monocots *Brachypodium distachyon*, *Oryza sativa*, *Sorghum bicolor*, *Triticum aestivum* (Ta, IWGSC1+popseq.31.pep) and *Zea mays*, and Basal Magnoliophyta *Amborella trichopoda* and the Bryophyte *Physcomitrella patens* and the Lycophyte *Selaginella moellendorffii* and the Chlorophytes *Ostreococcus lucimarinus* and the Rhodophytes *Cyanidioschyzon merolae* from the Ensembl Plants (http://plants.ensembl.org/info/website/ftp/index.html); the Eudicot *Cucumis sativus* and Chlorophytes *Coccomyxa subellipsoidea* C-169, *Micromonas pusilla* CCMP1545 and *Volvox carteri* and Glaucophyte *Cyanophora paradoxa* from Phytozome (http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism= PhytozomeV9); the Chlrophyte *Ostreococcus tauri*, Gymnosperms *Picea sitchensis* and Rhodophyte *Galdieria sulphuraria* from NCBI (http://www.ncbi.nlm.nih.gov/protein/). The evolutionary relationship of these 20 species were obtained from NCBI (https://www.ncbi.nlm.nih.gov/Taxonomy/CommonTree/wwwcmt.cgi), and visually displayed by phylogenetic tree using FigTree v1.4.3 program (http://tree.bio.ed.ac.uk/software/figtree/).

To identify the WRKY TFs in various species, the HMM profile of the WRKY domain (PF03106) downloaded from the Pfam database (http://pfam.xfam.org) (Finn et al., 2016) was applied as a query to search against the local protein database using HMMsearch program (HMMER3.0 software: http://hmmer.janelia.org/) (Finn et al., 2011) with an E-value cutoff of 1.0. The sequences obtained were then submitted to the Pfam database to detect the presence of WRKY domains. The protein sequences containing complete or partial WRKY domains, which may be pseudogenes, incomplete assemblies, sequencing errors or mispredictions (Rinerson et al., 2015), were both considered as putative WRKYs. The physical and chemical properties including number of amino acids (NA), molecular weight (MW), theoretical pI (pI) , grand average of hydropathicity (GRAVY), aliphatic index (AI) and instability index (II) of putative TaWRKY proteins were calculated using the online ExPASy-ProtParam tool (http://web.expasy.org/ protparam/).

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Phylogenetic analysis

MEGA7.0 program was employed to construct the unrooted phylogenetic tree of identified WRKY protein domains in *Triticum aestivum* L. and *Arabidopsis thaliana* L. using the maximum likelihood method(Kumar et al., 2016). The parameters of the constructed trees were: test of phylogeny: bootstrap (1000 replicates), gaps/missing data treatment: partial deletion, model/method LG model, rates among sites: gamma distributed with invariant sites (G). Only the value of bootstrap more than 60 could be displayed on the tree.

Chromosomal location of *TaWRKY* genes

To map the locations of *WRKY* gene transcripts in *Triticum aestivum* L., MapInspect software (http://www.softsea.com/download/ MapInspect. html) was employed to visualize the chromosomal distribution of deduced *TaWRKY* genes according to their initial position and length of chromosome. The chromosomal location information of *TaWRKYs* was obtained from Ensembl Plants website (http://archive.plants.ensembl.org/Triticum_aestivum/Info/ Index).

To detect the gene duplication, the CDS sequences of *WRKY* genes in wheat were blasted against each other (e-value<$1e^{-10}$, identity > 90%) (Song et al., 2014). Tandem duplicated *TaWRKY* genes were defined as two or more adjacent homologous genes located on a single chromosome, while homologous genes between different chromosomes were defined as segmental duplicated genes (Bi et al., 2016).

Characterization of gene structure, conserved motif and putative *cis*-acting elements

The exon-intron structures of TaWRKY genes were obtained by mapping the CDS to DNA sequences using the Gene Structure Display Server2.0 (http://gsds.cbi.pku.edu.cn/) (Hu et al., 2015). CDS (IWGSC1+popseq.31.cds) and genomic sequences (IWGSC1+popseq.31.dna) in *Triticum aestivum* L. were downloaded from Ensembl Plants website (ftp://ftp.Ensemblgenomes.org/pub/release-31/plants/fasta/triticum_aestivum/).

To discover motifs in TaWRKY protein sequences, the online tool MEME 4.11.2 (Multiple Expectation Maximization for Motif Elication: http://meme-suite.org/) was utilized to identify the conserved motifs in full length TaWRKYs (Bailey et al., 2009). The optimized

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parameters were as follows: distribution of motifs, zero or one occurrence per sequence; maximum number of motifs, 10; minimum sites, 6; maximum width 60.

The 1.5kb upstream of the transcription start site (-1) of all identified *TaWRKY* transcripts was extracted as promoter to predict *cis*-acting elements using the PlantCARE online (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot M, 2002). Then statistics derived from hits of various *cis-acting* elements in all TaWRKY transcripts were made, and displayed by diagram.

Plant materials, drought treatment and qRT-PCR

A hexaploid winter wheat (*Triticum aestivum* L.) cv. Zhengyin1 (St1472/506) was taken in our pot experiment, which was carried out from October, 2015 to June, 2016 in a greenhouse. Seeds were sown in each plastic pot filled with 7 kg of soil, earth-cumuli-orthic Anthrosols collected in northwest China. An equivalent of 0.447 g (urea)/kg^{-1} (soil) and 0.2 g (K_2HPO_3)/ kg^{-1} (soil) were mixed in soil with a net water content of 29.2% at the largest field water capacity. Water control was carried out from anthesis (April 17, 2016). Normal water supply and artificial soil desiccation were implemented with 70-75（control group）and 45-50% (moderate water stress) of the largest field capacity respectively. Spikes and flag leaves of wheat collected at 0, 1, 3, 5, 10, 15, and 25 days after anthesis (DAA), were immediately frozen in liquid nitrogen and then stored at -80℃ for subsequent analysis. Spikes were separated as glume, lemma, grain, palea, and rachis, and only glume and lemma were used in experiment.

Total RNA was extracted from wheat tissues using the Trizol reagent (Tiangen, China) following the manufacturer's instruction, and then digested with RNase-free DNaes I. The quantity and concentration of RNA was evaluated by UV spectrophotometry. The first strand cDNA synthesis was generated using PrimeScriptTM RT Reagent Kit (TaKaRa, China), and the synthesized cDNA products were diluted 1:9 with nuclease-free water to use in qRT-PCR. Primer Primer 5.0 and AllelelID 6.0 (http://www.premierbiosoft.com/index.html) were used to design gene-specific primers (Table S1). Wheat Tublin was taken as the reference gene.

The qRT-PCR was carried out using based on SYBR GreenSYBR® Premix Ex Taq™ (TaKaRa, Japan) according to the manufacturer's instructions with Bio Rad CFX96TM

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real-time PCR detection system (BioRad, USA). Reaction parameters for thermal cycling were: 95℃ for 30 s, followed by 39 cycles of 95℃ for 5 s and 60℃ for 30 s, and at last a melting curve (65-95℃, at increments of 0.5℃) generated to check the amplification. The gene expression levels were calculated with the $2^{-\Delta\Delta CT}$ methods (Livak & Schmittgen, 2001), and three biological replicates were used.

Results

Identification of WRKYs in wheat and comparative analysis

To comprehensively analyze and identify WRKY TFs in plants, 20 plants representing the nine major evolutionary lineages were chosen for analysis. After searched by HMMER and detected WRKY domains by Pfam database, a total of 1113 WRKY TFs were obtained (Table S2). The evolutionary relationships of various species and the number of WRKY TFs were shown in Fig.1. Most of terrestrial plants, including Monocots, Eudicots and Bryophytes, contained 86 to 171 WRKY proteins, while *Picea sitchenis* belonging to the Gymnosperm carried only eight WRKY TFs, which could be due to sequencing unfinished. However, six or less WRKY proteins were found in aquatic algaes, of which the number of WRKY TFs in Rhodophytes and Glaucophytes showed zero. In general, the number of WRKY TFs in many higher plants was more than that in lower plants, which suggested that the WRKY TFs may play an important role in the process of plant evolution. The number of WRKY proteins increased as plants evolved, possibly because of genome duplication.

In wheat, a total of 174 WRKY proteins were searched using HMM search program. Subsequently, all obtained sequences were verified by Pfam database, which resulted in the identification of 171 WRKY TFs (Table S3). There were 115 TaWRKYs named as TaWRKY1 to TaWRKY115 based on their chromosome location, and other 56 sequences called TaWRKY116 to TaWRKY171 as they were anchored in the scaffolds. Among the 171 TaWRKYs, manual inspection showed that some of them are partial sequences, in which WRKY domains or zinc-finger structures were incomplete or even did not exist.

The parameters used to describe the TaWRKY proteins were shown in Table S3. Molecular weight, theoretical pI and aliphatic index could not be computed in sequences containing several consecutive undefined amino acids. The lengths of TaWRKY proteins

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ranged from 44 (TaWRKY121) to 1,482 residues (TaWRKY78), whereas the PI ranged from 4.96 (TaWRKY164) to 10.73 (TaWRKY40). This suggested that different TaWRKYs might operate in various microenvironments (Wang et al., 2014). The values of grand average of hydropathicity were all negative, which indicated that TaWRKY proteins were all hydrophilic. Almost all TaWRKYs were defined as unstable proteins, and only 30 TaWRKYs with instability index less than 40 were considered to be stable proteins.

Classification and phylogenetic analysis of TaWRKYs

To categorize and investigate the evolutionary relationship of the TaWRKY proteins in detail, we constructed an unrooted maximum-likelihood phylogenetic tree with 262 putative WDs in *Arabidopsis* and wheat (Fig. 2). Based on the classification of AtWRKYs and primary amino acid structure feature of WRKY (Eulgem et al., 2000), TaWRKYs were classified into three major groups (Groups I, II and III). The 30 TaWRKYs possessing two WDs and C_2H_2-type zinc finger motifs (C-X_{3-4}-C-X_{22-23}- H-X_1 -H) were classified into group I. Group II was comprised of 95 sequences, and each protein contained a single WD and C_2H_2-type zinc finger structure (C-X_{4-5}-C-X_{23}-H-X_1-H). We further divided Group II into five subgroups, including IIa, IIb, IIc, IId and IIe with 11, 7, 50, 17 and 10 members, respectively. Finally, 45 TaWRKYs with a single WD were assigned to Group III because of their C_2HC zinc-finger structure (C-X_{6-7}-C-X_{23-28}-H-X_1-C).

As shown in Table S3, besides the highly conserved WRKYGQK motifs, we found three variants in TaWRKYs, namely WRKYGKK (10), WRKYGEK (11) and WSKYGQK (1), which were distributed in subgroup IIc, III and TaWRKY157, respectively. In addition, two zinc-finger form variants, C-X_6-P-X_{23}-H-X-C and C-X6-F-X23-H-X-C were identified in TaWRKY80 and TaWRKY166, respectively. The unique one in all putative TaWRKYs is TaWRKY157, which contained two WDs but with C_2HC-type zinc finger structure (C-X_7-C-X_{23}-H-X_1-C). The “Group I Hypothesis” sees all WRKY genes evolving from Group I C-terminal WRKY domains (Rinerson et al., 2015). Therefore, TaWRKY157 could be taken as an intermediate type of Group I-III member preliminary, though it was classified into Group III in phylogenetic tree.

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In this study, the Group II was found to be the largest group of WRKY TFs family in wheat. The members in Group II accounted for approximately 55.6% of all putative TaWRKYs, which was consistent with *Musa balbisiana* (Goel et al., 2016), pepper(Diao et al., 2016) and soybean(Song et al., 2016a). *S*ubgroups IIa and IIb were separated from one clade, and IId and IIe clustered to a branch, which is similar to previous study in wheat (Okay et al., 2014; Zhu et al., 2013)

Chromosomal location of *TaWRKY* genes

Among the 171 *TaWRKY* genes, 115 were mapped onto the 21 wheat chromosomes, and the other 56 were anchored in the scaffolds (*TaWRKY116-171*) (Table S3, Fig. 3). There were relatively more *TaWRKY* genes distributed in Chromosomes 3B (18, 15.7%), 5B (11, 9.57%), 2A (9, 7.8%) and 5D (9, 7.8%). In contrast, chromosome 6B, 7A and 7B contained only one *TaWRKY* genes (0.870%). In general, most identified *TaWRKY* genes were observed in the top and bottom sections of chromosomes and only less in central sections. It suggested that the *TaWRKY* genes were mapped on the all chromosomes with a significantly non-random and uneven distribution. The *TaWRKY* genes density in each chromosome was ranged from 0.004/Mb (7B) to 0.056/Mb (5D) (Fig. S1).

Duplication events of *WRKY* genes have been found universally in a number of plants, such as peanut (Song et al., 2016b), white pear (Huang et al., 2015) and *Brassica napus* (He et al., 2016). In this study, we identified 79 *TaWRKY* gene duplication pairs which corresponded to 85 genes (Table S4, Fig.3). It indicated that some of the *TaWRKY* genes have more than one duplicated gene, which could be due to the multiple rounds of whole genome duplication in wheat. As shown in Fig. 3, two WRKY tandem duplication clusters (*TaWRKY59*/*TaWRKY60*, *TaWRKY113*/*TaWRKY114*/*TaWRKY115*) were identified on chromosome 3B and 7D, respectively. In addition, there were 80 genes have undergone segmental duplication, which were paralogs of *WRKY* genes on different chromosomes (Bi et al., 2016).

Gene structure analysis of *WRKY* genes in wheat

The exon-intron distribution was analyzed to further detect structural features of *TaWRKY* genes. Figure S2 showed that the number of introns in TaWRKY family genes varied from 0

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to 5, while 0 to 8 in rice (Xie et al., 2005) and 0 to 22 in *Musa acuminate* (Goel et al., 2016), respectively. It suggested that *WRKY*s in wheat show lower gene structure diversity. A total of 72 (42.11%) *TaWRKY* genes with two introns accounted for the largest proportion, followed by 44 (25.73%), 23 (13.45%), 15 (8.77%), 14 (8.19%) and 3 (1.75%) genes, possessing 1, 3, 4, 0 and 5 introns, respectively. The distribution pattern of introns and exons was group-specific, which is similar to cassava (Wei et al., 2016) and carrot (Li et al., 2016), and *TaWRKY* gens belonging to the same subfamily shared similar exon-intron structure. For example, *TaWRKYs* in Group III contained 0-5 introns, approximately 91.11% (41/45) of which possessed 1-2 introns.

There were two types of introns (V-type and R-type) located in WRKY domain, which were characterized based on their splice site (Bi et al., 2016; Wang et al., 2014; Xie et al., 2005). V-type introns (phase 0) have a splice site before the V (Valine) residue in C_2H_2 zinc finger structure, and R-type introns (phase 2) on the R (Arginine) residue of the WRKY domain (Bi et al., 2016; Wang et al., 2014; Xie et al., 2005). In our study, all of the *TaWRKY* genes (17) in Group IIa and IIb only contained V-type introns except *TaWRKY165*, which had no intron. However, R-type introns were mostly observed in all the other groups (Group I, IIc, IId, IIe and III) (Fig. S2). It indicated that the intron phases were significantly conserved within the same group but remarkably different between groups (Chen, 2014). These results provided an additional evidence to support the phylogenetic groupings and *TaWRKYs* classification.

Motif composition analysis of TaWRKYs

The conserved motifs of WRKY proteins in wheat were predicted to explore the similarity and diversity of motif compositions. A total of 10 distinct motifs, named motif 1-10, were detected using MEME online program (Fig. 4). Of these 10 motifs, motif 1 and 4 contained a WRKYGQK sequence, which is basic feature of TaWRKYs. At least one of them contained in almost all deduced TaWRKYs, except several incomplete proteins, such as TaWRKY11, 43 and 162. Motif 1 was observed almost in all groups, whereas motif 4 dispersed in Group I mostly.

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As displayed schematically in Fig. 4, TaWRKYs within the same group or subgroup shared similar motif compositions. For instance, motif 6 and 10 were unique to Group I, whereas motif 7 is specific to Group III. The motif unique to a particular group is likely to be involved in specific biological process in plants. Therefore, it could be speculated that each family or subfamily of WRKY genes might be responsible for the specific biological process (Goel et al., 2016; Lippok, 2003). Furthermore, members in subgroup IIa and IId showed almost the identical motif distribution pattern, indicating functional similarity among them. Interestingly, these two subgroups were also clustered to a branch in phylogenetic tree. Likewise, the same phenomenon was also observed in subgroup IId and IIe. These results further validated the categorization of TaWRKYs and phylogenetic relationships.

Variety of *cis*-acting elements in the promoter regions of wheat *WRKY* genes

It was reported that *cis*-acting elements in the promoter of are crucial to gene expression, which is an en essential part of its function (Dehais, 1999; Lescot M, 2002). The 1.5kb upstream promoter regions of all *TaWRKYs* were used to predict *cis*-acting elements using the online database PlantCARE. Here we found various *cis*-acting elements in 142 out of 171 *TaWRKY* genes, while the rest of WRKYs could not be detected because of short sequence in their upstream regions (Table S5, Fig.5). A great deal of *cis*-acting elements were related to response of hormones and biotic stresses, including methyl jasmonate (MeJA), abscisic acid (ABA), salicylic acid (SA), gibberellins(GA), auxin (IAA), zein and fungus.

MeJA-responsive elements with largest portion were found in the promoter regions of 109 *TaWRKY* genes. Additionally, some elements involved in various abiotic stresses, such as light, wound, cold, heat, anaerobic induction and drought, were identified in a large number of *TaWRKY* genes. A total of 44 light-responsive elements were almost distributed in all of the *TaWRKY*s. There were also some elements observed in genes may regulate expression of different tissues (seed, root, shoot, leaf, phloem/xylem, endosperm and meristem) in wheat development. Interestingly, a total of 76 *TaWRKY*s contained W-box (TTGACC), which regulates gene expression by binding WRKY, indicating these genes may auto-regulated by itself or cross-regulated with others (Chi et al., 2013; Jiang et al., 2014). MBSI, a MYB binding site involved in flavonoid biosynthetic genes regulation, only existed in *TaWRKY87*

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and *TaWRKY142*, which suggested that these two genes may regulate flavonoid metabolism. There were two unique genes, *TaWRKY58* and *TaWRKY94*, which might response cold and drought stresses for containing a cold and dehydration responsive elements, C-repeat/DRE. Another special MYB binding site MBS, participated in drought response, were identified in 103 genes, indicating that most of *TaWRKY*s seem to be involved in drought stress response (Table S5). It was noteworthy that all members analyzed contained more than one *cis*-element. Our analysis and previous studies both suggested that *TaWRKY* genes are involved in transcriptional regulation of plant growth and stress responses (Bakshi & Oelmüller 2014; Ding et al., 2015; Raineri et al., 2016; Rushton et al., 2010).

Expression profiles of *TaWRKY* genes under drought stress.

With the exception of two shorter sequences (*TaWRKY122 and 169*), 12 out of 171 transcripts were selected as candidate drought responsive genes according to their orthologous *WRKYs* in Arabidopsis, which are involved in water deprivation, using the Biomart (http://plants.ensembl.org/index.html) (Table S6). The *AtWRKYs* responding to water stress were obtained based on function annotation in TAIR database (http://www.arabidopsis.org/index.jsp). To validate these candidate 12 drought-response genes, we determined their expression pattern in flag leaves, glumes and lemmas using qRT-PCR. In our study, expression of genes could be detected at the transcript level almost in all selected tissues during the grain-filling period except *TaWRKY8* (Table S7, Fig. 6).

As shown in Fig. 6, we found that *TaWRKY* genes in glumes and lemmas share more similar expression pattern compared with in flag leaves. A relatively large group of genes, including *TaWRKY1, 20, 31, 112, 123, 142,* and *149* were significantly up-regulated in flag leaves at 0, 3 or 5 DAA, which suggested that these genes were highly induced at the early grain-filling stage (0-8 DAA). Among them, some genes, like *TaWRKY123* and *142*, maintained slightly up-regulated initially and then were restrained followed by an increase in the last point of the drought treatment in glumes. In addition, peaks in the expression of several members (*TaWRKY1, 20, 123* and *142*) were mostly found at 5DAA in lemma, which lags behind other two tissues. Furthermore, water stress induced the most rapid upregulation of some genes, and showed differences in three tissues. For example, both *TaWRKY31* and

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TaWRKY149 were induced quickly in flag leaves after the onset of the drought treatment (0 DAA), approximately increasing up 6.67 and 8.22-fold, respectively. However, later induction was observed in glumes and lemmas. The immediate transcription response observed upon water stress appeared to be related to a more rapid perception of the drought (Eulgem et al., 2000; Rushton et al., 2010). Genes in another interesting cluster, composed of *TaWRKY90, 97, 120* and *133*, were down-regulated or slightly changed in glumes and lemmas during the early grain-filling stage, and induced during the middle (9-15 DAA) or late grain-filling stage (16-25 DAA) under drought stress. Whereas, two genes (*TaWRKY120, 133*) of them in flag leaves were strongly induced 3 days after water stress. It indicated that *TaWRKY120* and *TaWRKY 133* genes were predominantly expressed in flag leaves at the early-filling stage. Our data suggested that the tissues-specific expression of *TaWRKYs* was existed in wheat, and it appeared to be consistent with their role in tissues.

Discussion

WRKY TFs are one of the largest families of transcriptional regulators in plants, and form integral parts of signalling webs that regulate many plant process (Rushton et al., 2010). Although some investigation on wheat WRKYs have been reported in sucession, characterization and functional annotation information about TaWRKYs was still sufficient. Niu et al. (2012) identified 43 putative TaWRKYs, named TaWRKY1 to TaWRKY43, which were represented with the same names in another study carried out by Okay et al. (2014). Zhu et al. (2013) identified 92 TaWRKYs from the NCBI dbEST and/or the DFCI gene index, and constructed a phylogeny map. A total of 160 TaWRKYs were characterized in terms of their HMM profiles, conserved domains, distribution among WRKY groups and phylogenetic relationships, and discovered some drought responsive members validated in leaf and root tissues (Okay et al., 2014). Recently, Zhang et al. (2016) identified 116 WRKYs, and 13 of them were characterized as senescence-associated genes.

Our present study improves the information of WRKYs in wheat, and provides a more comprehensive knowledge based on the wheat genome. We identified WRKYs in 20 species and characterized 171 wheat WRKYs in terms of gene classification, physical and chemical parameters prediction, phylogenetic analysis, chromosomal location, duplication events,

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conserved motif determination, exon-intron structure and *cis*-acting element analysis, which might help to screen candidate stress-responsive genes in wheat for further study.

The whole genome duplication can result in divergence and formation of species over time, accompanied with retention or loss of some duplicated genes (Dehal P, 2005). To understand the evolution of WRKY transcription factors family, we identified a total of 1113 WRKY proteins in wheat and other 19 species representing the nine major plant lineages. Interestingly, the number of WRKY TFs in many higher plants was more than that in lower plants, which implied that the WRKY TFs might play significant roles during evolution from simpler unicellular to more complex multicellular forms. Thus, we could preliminary speculate that multiple rounds of duplication during evolution might be the main reason for the different numbers of the WRKY proteins in diverse species.

Compared with the species analyzed in this study, the wheat (*Triticum aestivum* L.) genome contained the highest number of WRKY TFs (171). The expansion of *WRKY* gene family in wheat might be due to the following reasons. (1) *Triticum aestivum* L. is an allohexaploid, and originated from two recent hybridizations between three diploid progenitors, donors of the A, B and D subgenomes (Glover et al., 2015). *Triticum aestivum* L. genome experienced the whole genome duplication events after two hybridizations at approximately 0.8 and 0.4 million years ago (MYA), respectively (Glover et al., 2015), which would produce a large number of paralogs (Conant & Wolfe 2008). (2) Likewise, small-scale gene duplication, including segmental and tandem duplication, might be also significant in the evolution of *WRKY* gene family in wheat. The origin of new genes during evolution was dependent on gene duplication. Gene duplication allows essential genes to undergo mutations in the duplicated copy, suggesting that similar genes would diverge over the long evolutionary time period, and then improving the expansion and evolution of gene family (Conant & Wolfe 2008; De et al., 2008). The current investigate showed that 85 of 171 (49.7%) *TaWRKY* genes evolved from either tandem duplication or segmental duplication. Interestingly, 80 of *WRKY* genes were segmentally duplicated and only five were tandemly duplicated, implying that high segmental and low tandem duplications existed in *TaWRKY* genes, consistent with white pear (Huang et al., 2015), grapevine (Wang et al., 2014) and soybean (Song et al., 2016a). Our results showed that number of duplicated genes was mainly

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determined by segmental events, because genes generated from segmental duplication have more chance to be retained due to subfunctionalization or neofunctionalization (Huang et al., 2015; Lynch, 2000; Moore & Purugganan, 2005; Wang et al., 2005). Therefore, it was inferred that though tandem duplication has contributed to the expansion of TaWRKY family, segmental duplication probably played a more pivotal role (Zhu Y, 2014). (3) The expansion of gene families along a specific lineage can be due to chance or the result of natural selection. Adaptive expansion of gene families occurs when natural selection would favor additional duplicated genes (Demuth & Hahn, 2009).

Increasing research suggests that the WRKY transcription factors are involved in various biological processes, including plant development, responses to biotic and abiotic stresses (Eulgem & Somssich, 2007; Liu et al., 2015; Luo et al., 2013; Rushton et al., 2010; Zhao et al., 2015). Plant hormones, as essential endogenous signal molecules within the plant, can regulate cellular processes, plant growth and development under severe stress conditions (Grove, 1979; Kermode, 2005; Ryu & Cho, 2015). Many evidence indicated that the expression of *WRKY* genes was affected after hormone treatment (Jiang et al., 2014; Yang et al., 2009). In wheat, a large amount of *cis*-acting elements responding to phytohormones, such as MeJA, ABA, SA, GA etc., were detected in *TaWRKY* genes. It suggested that these *WRKY* genes might regulate growth and development of wheat by functioning as key factors in regulating specific signalling pathways. In addition, WRKY transcription factors were involved in responses to abiotic stresses. For instance, *TaWRKY44* in transgenic tobacco confers multiple abiotic stress tolerances, including drought, salt and osmotic stress (Wang et al., 2015). Twelve *GmWRKY* genes were differentially expressed under salt stress (Song et al., 2016a). In this work, a large number of *TaWRKY* genes contained several *cis*-acting elements associated with abiotic stresses, such as light, wound, cold, heat, anaerobic induction and drought, implying that a number of *WRKY* genes in wheat participate in various abiotic stresses. In general, the results indicated that most of *TaWRKY* genes were involved in multiple biotic and abiotic stresses, which was consistent with previous study (Eulgem & Somssich, 2007; Jiang et al., 2014).

Drought is one of the most significant stresses resulting in reduction of wheat production (Keating et al., 2014). The grain-filling is mainly sustained by photosynthesis of flag leaves

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and spikes under drought treatment, and photosynthesis of spike is less sensitive to drought than that in flag leaves (Jia et al., 2015; Tambussi et al., 2005). However, the investigation on *TaWRKY* genes was mostly focus on root and leaves, and limited in spikes. In this study, we determined relative expression of *TaWRKY* genes in flag leaves, glumes and lemmas during the grain-filling period upon water deficit using qRT-PCR. Obvious differences of gene expression pattern between flag leaves and spikes (glumes and lemmas) were observed in wheat. For example, *TaWRKY142* were up regulated at 3 DAA in flag leaves, but induced in glumes and lemmas since the imposition of the water stress and maintained upregulation during the early grain-filling stage, which suggested the putative role of *TaWRKY142* gene in spike tissues. In addition, the majority of *TaWRKY*s in a group shared similar expression pattern, but some were not. For instance, *TaWRKY1, 120* and *133*, the orthologs to *AtWRKY3,* all belong to Group I. Both *TaWRKY120* and *TaWRKY133* were up regulated at 3DAA and the middle-late grain filling stage in flag leaves, while induction of TaWRKY1 were only observed at 3DAA. The structure analysis showed that *TaWRKY120* and *TaWRKY133* have similar exon-intron structure and same motif composition, while *TaWRKY1* showed difference with them, which may help to explain why *TaWRKYs* in a group do not necessarily shared the similar expression pattern. The similar phenomenon was also reported in *Salvia miltiorrhiza*. Five genes (*SmWRKY2, 24, 39, 54* and *55*), belonging to Group I, were predominantly expressed in roots, whereas the other Group I members, including *SmWRKY42, 13* and *60* were mainly expressed in stems, leaves and flowers, respectively (Li et al., 2015).

Conclusion

In this study, our results provide a more extensive knowledge on WRKYs in wheat. With the whole wheat genome sequence, 171 TaWRKYs were identified, and compared with proteins from other 19 species representing nine major plant lineages to gain preliminary insight into the evolution of the WRKY family in Plantae. A phylogenetic analysis, coupled with gene structure analysis and motif determination, divided these TaWRKYs into seven subgroups (Group I, IIa-e, III). Chromosomal location showed that the most *TaWRKY* genes were enriched on four chromosomes, especially on chromosome 3B, and 85 (49.7%) genes were either tandem or segmental duplication. The *cis*-acting elements analysis revealed putative

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functions of WRKYs in wheat during development as well as under numerous biotic and abiotic stresses. Finally, the differential expression of *TaWRKY* genes in flag leaves, glumes and lemmas under drought stress were analyzed, and we found different *TaWRKY* genes preferentially express in specific tissue during the grain-filling stage. Taken together, our results will help to complete the information of WRKY gene family in wheat, and also contribute to screen more candidate genes for further investigation on function characterization of WRKYs under various stresses.

ACKNOWLEDGEMENTS

We are grateful to Mr. Dangxiao Liu and Mr. Huawei Liu for providing valuable suggestions.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

This study was supported by the projects of the National Natural Science Foundation of China (grant number 31271624). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
National Natural Science Foundation of China: 31271624.

Competing financial interests

The authors declare no competing financial interests.

Author Contributions

- Pan Ning conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, prepared the figures and/or tables, reviewed drafts of the paper.
- Congcong Liu conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and /or tables.
- Jingquan Kang contributed materials, performed the experiments.
- Jinyin Lv conceived and designed the experiments, contributed regents, reviewed drafts of the paper.

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Data Availability

The following information was supplied regarding data availability:

The raw data has been supplied as Supplemental Information.

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Figure 1(on next page)

Evolutionary relationship of 20 species among nine lineages within the Plantae.

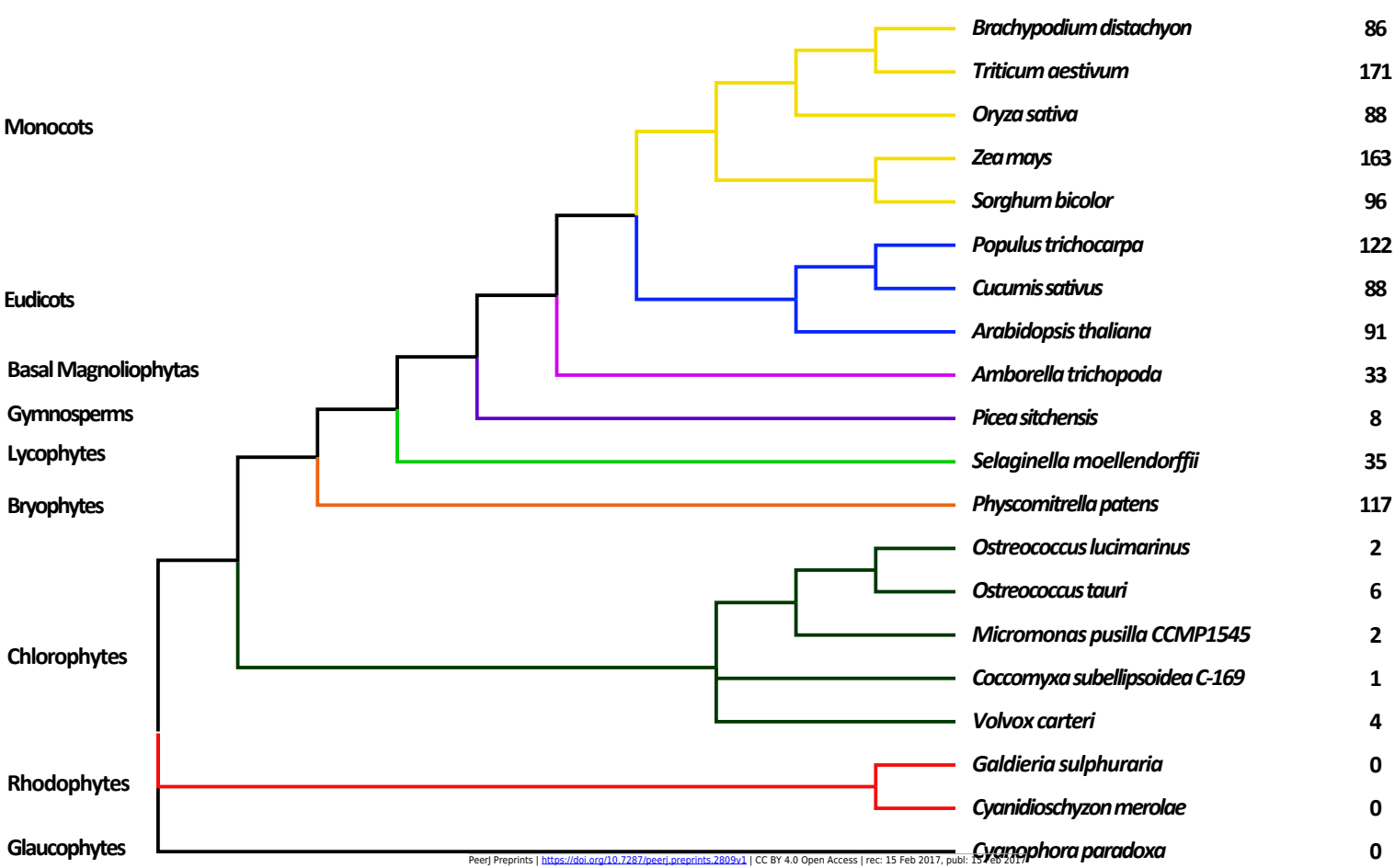

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Figure 2(on next page)

Phylogenetic tree of WRKY domains from wheat and *Arabidopsis*.

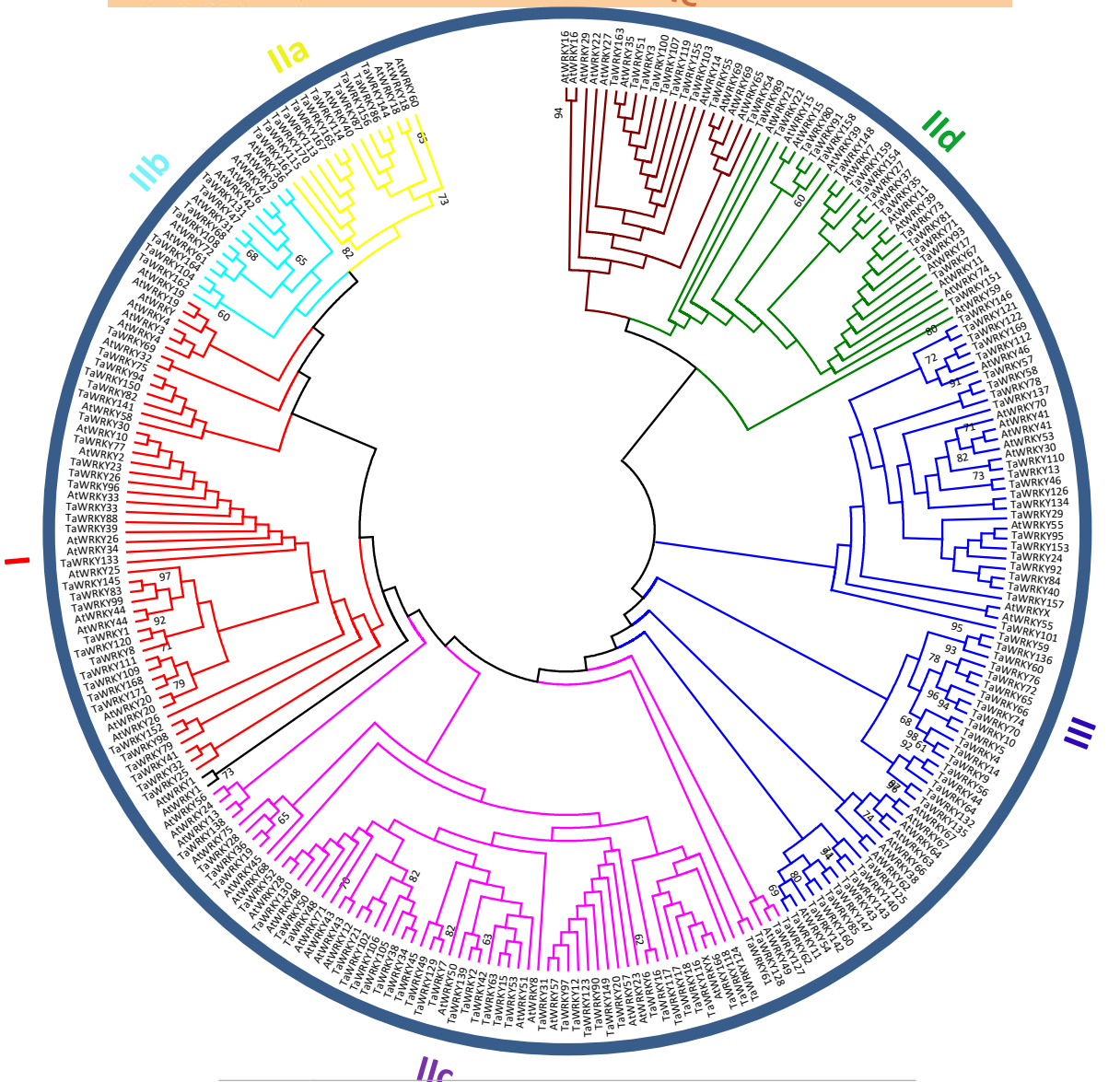
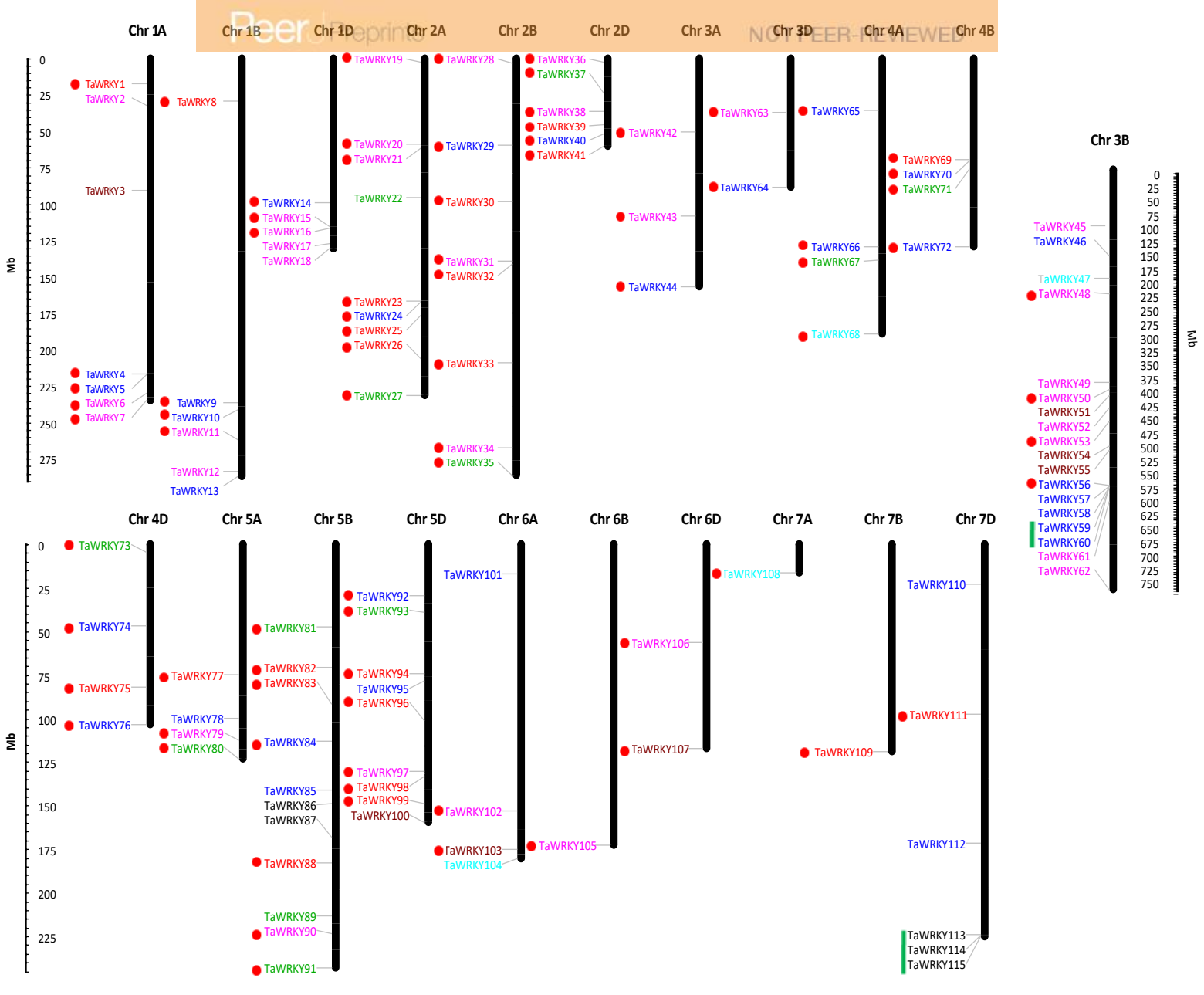


Figure 3(on next page)

Chromosome distribution of *TaWRKY* genes.

The chromosomal position of each *TaWRKY* was mapped according to the wheat genome. The chromosome numbers were shown at the top of each chromosome. Fifty six *TaWRKYs* on the scaffold (*TaWRKY115-171*) could not be anchored onto any specific chromosome. The location of each *WRKY* gene was pointed out by a line. The scale is in mega bases (Mb). The black boxes indicated the tandem duplication genes, and the segmental duplicated genes were shown with red dots.

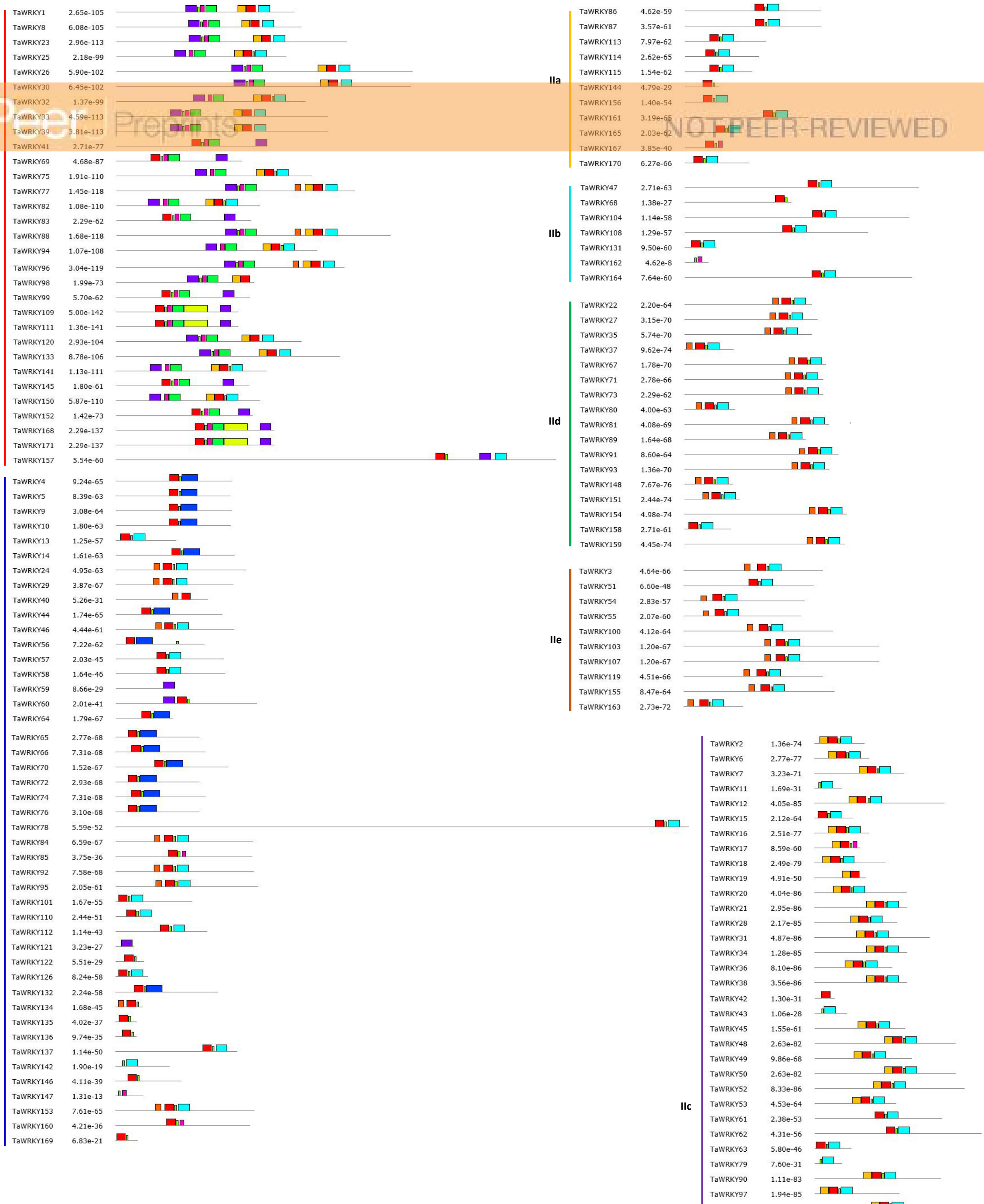


Group I Subgroup Ia Subgroup Ib Subgroup Ic Subgroup Id Subgroup IIe Group III

Figure 4(on next page)

Schematic diagram of conserved motifs in TaWRKYs.

(A)



(B)

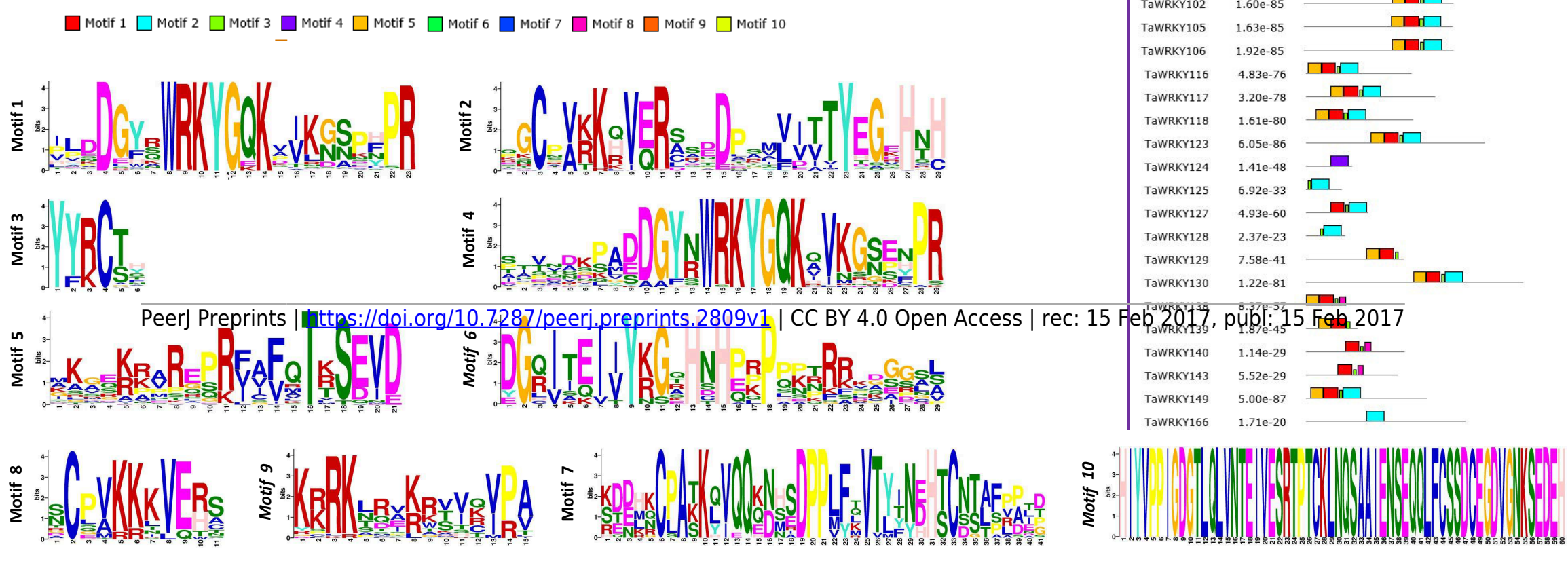


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Figure 5(on next page)

The number of *TaWRKY* genes containing various cis-acting elements.

The cis-acting elements were identified with the online PlantCARE program using the 1.5kb upstream from the transcription start site of *TaWRKY* genes. The graph was generated based on the presence of cis-acting element responsive to specific elicitors/conditions/processes (x-axis) in *WRKY* gene family members (y-axis).

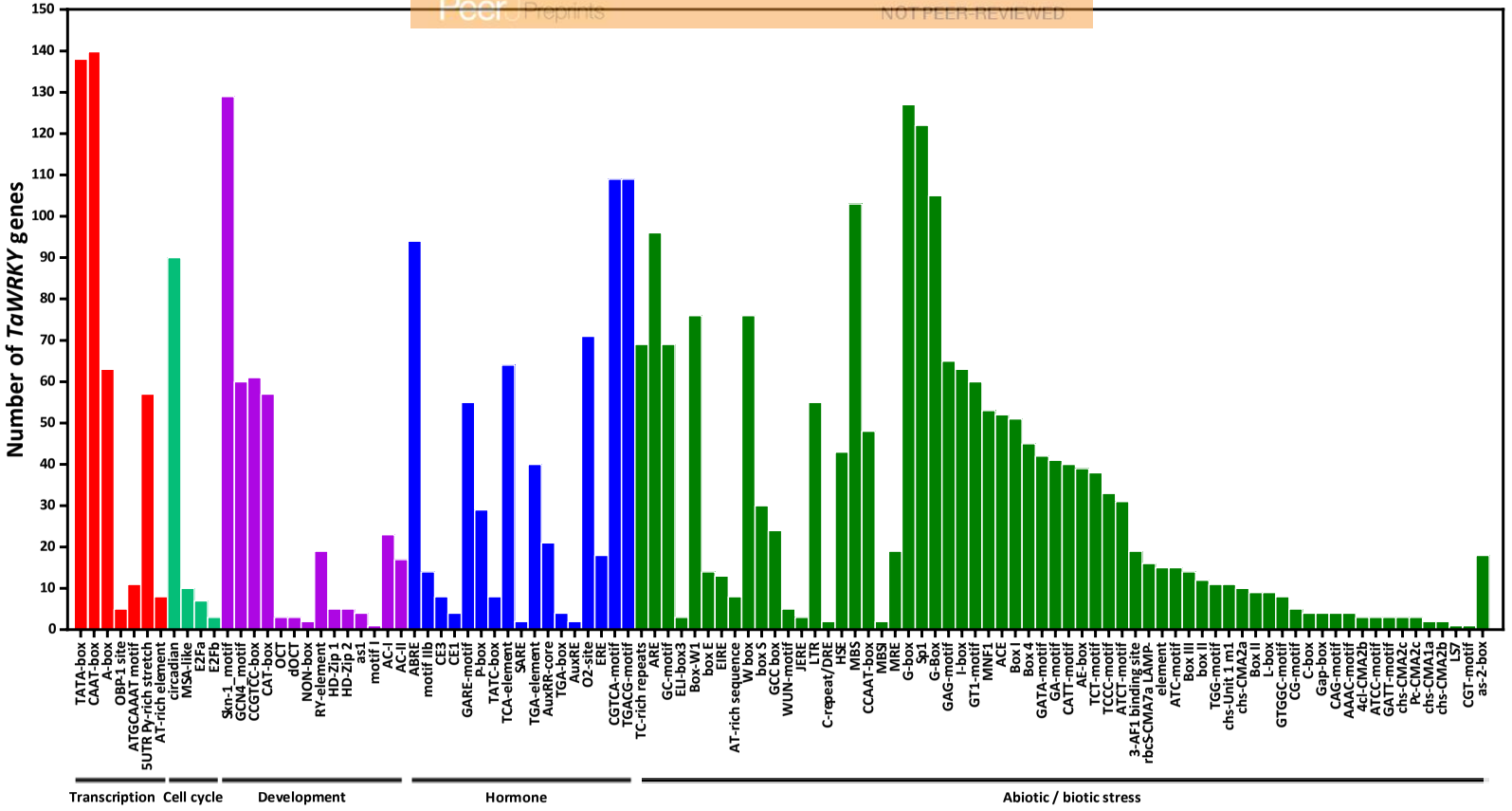

Fig. 6

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Figure 6(on next page)

The expression pattern of *TaWRKY* genes in flag leaves, glumes and lemmas during the grain filling stage under drought stress.

(A) Bar graphs showing the relative expression values of each *TaWRKY* genes after drought treatment. Samples were collected 0, 1, 3, 5, 10, 15, and 25 days after anthesis, from which water control was carried out. The mean ± SE of three biological replicates are presented. Relative fold changes were obtained by qRT-PCR using the $2^{-\Delta\Delta CT}$ method. (B) Heat map showing the expression profile of *TaWRKYs* in flag leaves, glumes and lemmas under drought stress. Heat map was generated based on log2 transformed count value from three replicates of qRT-PCR data using R language. Red and green boxes indicated high and low expression levels of genes, respectively. Blank indicated the gene was not detected.