AcademicPres

Available online: https://www.notulaebotanicae.ro

Print ISSN 0255-965X; Electronic 1842-4309

Not Bot Horti Agrobo, 2019, 47(4):1100-1115. DOI:10.15835/nbha47411651

Notulae Botanicae Horti Agrobotanici Cluj-Napoca

Original Article

# Genome-Wide Identification of WRKY Family Genes and Analysis of Their Expression in Response to Abiotic Stress in *Ginkgo biloba* L.

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**Abstract**

*Ginkgo biloba* is widely planted, and the extracts of leaves contain flavonoids, terpene esters and other medicinal active ingredients. WRKY proteins are a large transcription factor family in plants, which play an important role in the regulation of plant secondary metabolism and development, as well as the response to biotic and abiotic stress. In our study, we identified 40 genes with conserved WRKY motifs in the *G. biloba* genome and classified into groups I (groups I-N and -C), II (groups IIa, b, c, d, and e), and III, which include 12, 26, and 2 *GbWRKY* genes, respectively. Meanwhile, the expression patterns of 10 *GbWRKY* (*GbWRKY2*, *GbWRKY3*, *GbWRKY5*, *GbWRKY7*, *GbWRKY11*, *GbWRKY15*, *GbWRKY23*, *GbWRKY29*, *GbWRKY31*, *GbWRKY32*) under different tissue and abiotic stress conditions were analyzed. Under stress treatment, the expression patterns of 10 *WRKY* genes were changed. 10 ginkgo WRKY transcription factors were induced by ETH and SA, but there are two different induced response modes. The expression of 10 *WRKY* genes was inhibited under low temperature, high temperature and MeJA hormone induction. Most *WRKY* genes were up-regulated under the induction of high salt and ABA. G*bWRKYs* were differentially expressed in various tissues after abiotic stress and plant hormone treatments, thereby indicating their possible roles in biological processes and abiotic stress tolerance and adaptation. Our results provided insight into the genome-wide identification of GbWRKYs, as well as their differential responses to stresses and hormones. These data can also be utilized to identify potential molecular targets to confer tolerance to various stresses in *G. biloba*.

***Keywords:*** genome-wide; *Ginkgo biloba* L.; phytohormone; stress response; WRKY

## Introduction

Plants constantly encounter adverse conditions, such as water deficiency, salinity, threshold temperatures, nutrient starvation, and variable light conditions. Plants have evolved with intricate mechanisms at multiple levels to adapt to these conditions. At the molecular level, plant devotes a large portion of their genome capacity to transcription. Transcription factors are composed of a gene family of regulatory proteins in plant and exhibit a very diverse expression range. A total of 320370 transcription factors from 165 species are identified thus far (Jin *et al*., 2017). Among these transcription factors, WRKY proteins play important regulatory roles in many biological processes, such as plant development, metabolism, and responses to biotic and abiotic stresses (Rushton *et al*., 2010). For example, *Arabidopsis thaliana* WRKY 6 (AtWRKY6) is influenced by several external and internal signals that are often involved in triggering senescence processes and plant defense responses (Li *et al*., 2017). AtWRKY18 can bind directly to different W-boxes at the AtWRKY53 promoter region and repress the expression of AtWRKY53, which is an important senescence regulator of the AtWRKY family (Potschin *et al*., 2014). From a developmental perspective, AtWRKY6 is involved in the growth of lateral roots (Stetter *et al*., 2017). OsWRKY74 modulates tolerance to phosphate starvation in rice (Dai *et al*., 2016). The WRKY

*Received: 30 Aug 2019. Received in revised form: 15 Sep 2019. Accepted: 08 Nov 2019. Published online: 12 Nov 2019.*

genes also play an important role in secondary metabolism (Han *et al*., 2014; Spyropoulou *et al*., 2014). The physiological responses modulated by these WRKYs also include some signaling substances, such as salicylic acid (SA) (Li *et al*., 2004), jasmonic acid (JA) (Wei, 2010), and abscisic acid (ABA) (Sun *et al*., 2014). Numerous studies have also shown that many *WRKY* genes are involved in response to drought (Zhang *et al*., 2017), heat (Wang *et al*., 2017), and cold (Luo *et al*., 2017). A single *WRKY* gene often shows transcription activity in response to several stresses, thereby indicating that it has different regulatory function in diverse stress responses (Wang *et al*., 2017). Thus, a genome-wide analysis of *G. biloba WRKY* genes should help reveal the underlying complex molecular mechanisms of WRKY proteins in response to various stresses.

The WRKY proteins is one of the largest transcription factor superfamilies in plant and defined by a 60 amino acid-conserved sequence named WRKY domain (WD), which contains WRKYGQK motif at the N-terminal and a Zn finger-like motif at the C-terminal (Agarwal *et al*., 2011). In some WRKY proteins, the heptapeptide WRKYGQK motif shows slight variations, which have been replaced by WRKYGKK and WRKYGEK (Chen *et al*., 2010). The WRKY transcription factors in *A. thaliana* can be classified into three groups on the basis of the number of WDs and the nature of their Zn-finger motif (Eulgem *et al*., 2000). Group I members contain two WRKY conserved domains, whereas the other groups of proteins are composed of a single WD. Both groups I and II have a C2H2-type Zn finger motif (i.e., C-X4-5-C-X22-23-H-X1-H). Group II is divided into five subgroups (i.e., IIa-e) on the basis of the additional amino acid motifs present outside the WD. Group III differs from groups I and II in altering C2HC Zn finger motif (i.e., C-X7-C-X23-H-X-C) (Rushton *et al*., 2010).

*Ginkgo biloba* L. is a living fossil that has remained essentially unchanged in terms of gross morphology for more than 200 million years (Lin *et al*., 2011). *G. biloba* has become widespread and popular worldwide due to its outstanding resistance or tolerance to both herbivores and pathogens (Guan *et al*., 2016). Ginkgolides and bilobalide, which are collectively called terpene trilactones, are the most unique components of *G. biloba* extracts and have been linked to resistance to fungi (Pan *et al*., 2016) and possibly to endophytic bacteria (Yang *et al*., 2015). The biosynthesis of bilobalide was highly correlated with the mevalonic acid pathway, and the biosynthesis of ginkgolides was highly correlated with the methylerythritol 4-phosphate pathway (Chen *et al*., 2015). Most structural genes of the terpene trilactone biosynthetic pathway in *G. biloba*, including *DXS* (Gong *et al*., 2006; Kim *et al*., 2006a), *DXR* (Gong *et al*., 2005), *MECT* (Kim *et al*., 2006b), *CMK* (Kim *et al*., 2008a), *MECS* (Kim *et al*., 2006c), *HDS* (Kim and Kim, 2009), *HDR* (Kim *et al*., 2008b; Lu *et al*., 2008), *FPS* (Wang *et al*., 2004), *GGPPS* (Liao *et al*., 2004), *LPS* (Schepmann *et al*., 2001), *HMGR* (Shen *et al*., 2016), *MVD* (Pang *et al*., 2006), *AACT* and *MVK* (Chen *et al*., 2017), have been isolated. WRKY binding sites are found in the promoter regions of several ginkgo terpene trilactone biosynthetic gene, such as *GbLPS* (Kim *et al*., 2012), *GbDXS* (Xu *et al*., 2013), *GbGGPPS* (Xu *et al*., 2013), *GbHMGR* (Liao *et al*., 2015a), *GbMVD* (Liao *et al*., 2015b), *GbIDS* (Kang *et al*., 2013), *GbMECP* and *GbMECT* (Cheng *et al*., 2015). However, knowledge on the function of ginkgo *WRKY* genes is limited because on the limited works that was previously reported (Liao *et al*., 2015c; Liao *et al*., 2016). A draft of the genome (Guan *et al*., 2016) and numerous transcriptomes was reported. In the current study, we performed a genome-wide (Wang *et al*., 2010; Han *et al*., 2005; He *et al*., 2015; Du *et al*., 2016) identification of the *WRKY* gene family in *G. biloba*. The ginkgo WRKY transcription factor family has 40 members. Detailed analyses including gene classification, gene phylogeny, conserved motif composition, and expression pattern were performed. A total of 10 *GbWRKY* genes were further investigated for the alterations of expression patterns in response to abiotic stresses via real-time quantitative RT-PCR. Our results will aid in understanding the roles of the *WRKY* genes in ginkgo development, defense responses, and terpene trilactone biosynthesis. The results may provide valuable information for further identification of the function of this gene family in *G. biloba*.

## Materials and Methods

### *Identification of WRKY gene family members in G. biloba*

*G. biloba* unannotated genome sequences were downloaded from GigaDB (http://gigadb.org/) (Guan *et al*., 2016). HMM profile of WRKY DNA-binding domain (PF03106) downloaded from Pfam protein family database (http://pfam.sanger.ac.uk/) was exploited for the identification of *WRKY* genes from ginkgo genome using HMMER 3.1 (http://hmmer.org/). The default parameters were employed. All non-redundant gene sequences searched from the ginkgo genome data were collected and then compared with each other. The transcriptome of *G. biloba* was sequenced using four organs, including leaves of male and female tree, stamens, and gynoecia (our unpublished data). Subsequently, the key word "WRKY" was used as queries to search against the annotated unigenes. Three published *G. biloba* WRKY (GbWRKY) protein sequences (GbWRKY2 with ID: ALC78712.1, GbWRKY11 with ID: ALC78713 and GbWRKY31 with ID: AQU43027) were downloaded from GeneBank (https://www.ncbi.nlm.nih.gov/genbank/). Sequences from genome and transcriptome and published GbWRKY proteins that shared >95% matches were considered redundant. Furthermore, multiple alignments among those these putative WRKY proteins were performed to avoid repetition and the WRKY domain was searched using the SMART program (http://smart.embl-heidelberg.de/). Candidate proteins with an incomplete WRKY domain were removed (Gb_28473, Gb_41027, Gb_07810 and Gb_02625) and not used in subsequent analysis, and the final identified *GbWRKY* genes were named *GbWRKY1* to *GbWRKY40* (Table 1).

Table 1. Identification of the *WRKY* gene in the ginkgo genome and transcriptome

| Gene ID | Gene symbol | Subgroup | Related publications | Length (bp) |
|---|---|---|---|---|
| Gb_31953 | GbWRKY1 | I | | 659 |
| Gb_25118 | GbWRKY2 | I | (Liao *et al*., 2015c) | 775 |
| Gb_32055 | GbWRKY3 | I | | 775 |
| Gb_36273 | GbWRKY4 | I | | 548 |
| Gb_01391 | GbWRKY5 | I | | 861 |
| Gb_36184 | GbWRKY6 | I | | 679 |
| T1_20451 | GbWRKY7 | I | | 673 |
| Gb_02351 | GbWRKY8 | IIb | | 526 |
| Gb_39366 | GbWRKY9 | IIb | | 707 |
| Gb_23334 | GbWRKY10 | IIb | | 687 |
| Gb_15478 | GbWRKY11 | IId | (Liao *et al*., 2016) | 402 |
| T1_5610 | GbWRKY12 | IIb | | 261 |
| T1_7872 | GbWRKY13 | IIb | | 300 |
| Gb_40261 | GbWRKY14 | IIc | | 226 |
| Gb_26412 | GbWRKY15 | IIc | | 190 |
| Gb_26413 | GbWRKY16 | IIc | | 248 |
| Gb_40257 | GbWRKY17 | IIc | | 240 |
| Gb_26411 | GbWRKY18 | IIc | | 248 |
| Gb_40207 | GbWRKY19 | IIc | | 266 |
| Gb_16917 | GbWRKY20 | IIc | | 273 |
| Gb_05024 | GbWRKY21 | IIc | | 233 |
| Gb_05026 | GbWRKY22 | IIc | | 388 |
| Gb_17074 | GbWRKY23 | IIc | | 467 |
| Gb_01873 | GbWRKY24 | IIc | | 485 |
| Gb_26894 | GbWRKY25 | IIc | | 305 |
| CL3411 | GbWRKY26 | IIc | | 509 |
| CL6024 | GbWRKY27 | IIc | | 446 |
| Gb_16513 | GbWRKY28 | IId | | 360 |
| Gb_01527 | GbWRKY29 | IId | | 367 |
| Gb_12539 | GbWRKY30 | IId | | 252 |
| Gb_06156 | GbWRKY31 | IIb | (Li *et al*., 2016) | 427 |
| T1_13300 | GbWRKY32 | IId | | 356 |
| CL7159 | GbWRKY33 | IId | | 362 |
| Gb_25547 | GbWRKY34 | IIe | | 383 |
| Gb_05176 | GbWRKY35 | IIe | | 497 |
| Gb_25334 | GbWRKY36 | IIe | | 274 |
| Gb_03346 | GbWRKY37 | IIe | | 608 |
| Gb_17623 | GbWRKY38 | IIc | | 131 |
| Gb_00547 | GbWRKY39 | III | | 579 |
| Gb_00545 | GbWRKY40 | III | | 481 |

*Sequence analysis*

For the analysis of WDs of *G. biloba*, 6 AtWRKY protein sequences from different groups/subgroups were downloaded from The Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org/). The 65 amino acid spanning the WRKY core domain of all GbWRKY proteins and selected AtWRKY proteins (AtWRKY2, AtWRKY6, AtWRKY7, AtWRKY8, AtWRKY14, and AtWRKY30) were used to create multiple protein sequence alignments by using Clustal X2 with default settings. Motif detection was performed with MEME 4.10.1 (http://meme-suite.org/tools/meme). The parameters are as follows: any number of repetitions, a maximum of 20 motifs, and an optimum motif width between 6 and 50 residues. A structural motif annotation was also performed using the Pfam (Finn *et al*., 2016) and SMART (Letunic *et al*., 2015). The exon-intron organization of *G. biloba* WRKY genes was determined by comparing the predicted coding sequences with their corresponding full-length sequences by using the online program Gene Structure Display Server (http://gsds.cbi.pku.edu.cn) (Guo *et al*., 2007). Analysis results include the number and location of exons and introns.

*Phylogenetic analysis of WRKY proteins*

To analyze the subgroups of the putative ginkgo WRKY family, we downloaded 71 AtWRKY proteins from TAIR. The amino acid sequences of *Hevea brasiliensis* HbWRKY1

(ADF45433.1), *Coptis japonica var. dissecta* CjWRKY1 (BAF41990.1), *Taxus wallichiana var. chinensis* TwWRKY (AEW91476.1), *Gossypium arboretum* GaWRKY1 (AAR98818.1), Artemisia annua AaWRKY1 (ACJ12926.1), *Panax quinquefolius* PqWRKY1 (AES92925.1), and *Catharanthus roseus* CrWRKY1 (ADT82685.1), which were involved in biological synthesis of secondary metabolites, were downloaded from GenBank. A neighbor-joining tree was constructed by MEGA 6.0 with the following parameters: Poisson correction, complete deletion, and bootstrap analysis with 1000 replicates. The sequences of the 40 GbWRKYs, 71 AtWRKYs, HbWRKY1, CjWRKY1, TwWRKY, GaWRKY1, AaWRKY1, PqWRKY1, and CrWRKY1 used for tree building are listed in Table S1.

*Expression profile of GbWRKY under the hormones and stress treatments*

The 14-year-old grafts of *G. biloba* were grown in a garden at Yangtze University in Hubei Province, China. To investigate the tissue expression of *GbWRKY* genes, we collected the leaves, stems, roots, male and female strobili of *G. biloba* grafts, immediately frozen in liquid nitrogen, and kept at -80 °C prior to RNA extraction. The suspended callus lines, which were prepared according to the method provided by Liao (Liao *et al*., 2015a), were used as materials in the abiotic stresses and hormonal treatments to maintain consistency. The callus lines were mixed with 100 μmol $L^{-1}$ MeJA, 100 μmol $L^{-1}$ABA, 100 μmol $L^{-1}$ SA, 40 μmol $L^{-1}$ ETH, and 200 μmol $L^{-1}$ NaCl by using ginkgo callus without any treatment as control. The chilling and heat treatments were performed by placing the callus lines in a 4 °C and 40 °C growth room, respectively. The control was in a 25 °C growth room. The samples were harvested at 3, 6, 12, 24, 48, and 72 h after treatment, immediately frozen in liquid nitrogen, and kept at -80 °C until use.

*RNA extraction and quantitative RT-PCR analysis*

The total RNAs of roots, stems, leaves, male flowers, female flowers, and different stress-treated samples of *G. biloba* were extracted according to the instructions of the MiniBEST Plant RNA Extraction kit. cDNA was synthesized by reverse transcription polymerase chain reaction using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany). qRT-PCR technique was used to detect the expression level of WRKY genes, and glyceraldehyde-3-phosphate dehydrogenase gene (*GbGAPDH*, GenBank accession no. L26924) was selected as the reference gene. On the basis of the random selection of 10 *WRKY* transcription factor unigene fragments and *GbGAPDH* sequences, we designed the qRT-PCR primers according to the AceQ® qPCR SYBR® Green Master Mix (Without ROX) kit's real-time PCR primers (Table 2). qRT-PCR was conducted using a Bio-Rad Mini Opticon™ Real-time PCR Mini Cycler (BioRad, Hercules, CA, USA) with SYBR® Premix Ex Taq™ II Kit (Dalian TaKaRa) according to the method of Xu *et al.* (2014). The reaction system was 20 μL, and the following qPCR conditions recommended by the manufacturer were used: 40 cycles of 95 °C for 30 s, 95 °C for 5 s, and 60 °C for 30 s with the addition of a dissolution curve. Three biological replicates were performed for each gene under different tissue and stress conditions by using ultrapure water as a negative control. The $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008) was used to analyze the expression level of the WRKY gene. qRT-PCR data were technically replicated with error bars representing mean ± SD (n = 3).

*Statistical analysis*

Data were analysed with one-way ANOVA by using SPSS 11.0 for Windows (SPSS Inc., Chicago, IL, USA), and mean differences were compared with the lowest SD (LSD) test by using SPSS 17.0 software. The $p$ value of <0.05 was considered statistically significant.

## Results

A total of 40 WRKY proteins were obtained in *G. biloba* genome and transcriptome. The obtained GbWRKY sequences are shown in Table S1. The GbWRKY proteins contained 131 (GbWRKY38) to 861 (GbWRKY5) amino acid residues. The conserved WD was approximately 60 amino acid residues long. Multiple alignments were performed to clarify the sequence features of 40 predicted GbWRKY domain sequences. The 47 conserved WDs of GbWRKYs are presented in Fig. 1. In GbWRKY4N and GbWRKY20, the WRKYGQK motifs in the WDs were replaced by WRKYGKK. In GbWRKY21 and GbWRKY22, the WRKYGQK motifs in the WDs were replaced by WRKYGRK. In GbWRKY1, the WRKYGQK motifs in the WDs were replaced by WRKYAQK. In GbWRKY18, GbWRKY17,

Table 2. Primers for qRT-PCR

| Gene | Primer forward sequence (5'-3') | Primer reverse sequence (5'-3') |
|---|---|---|
| GAPDH | GGTGCCAAAAAGGTGGTCAT | CAACAACGAACATGGGAGCAT |
| GbWRKY2 | GGTCTTGGGGTGTTTGGTCT | GGAACTTCTGTTTGCTGCCTCT |
| GbWRKY29 | AGTGCCAGCAATCAGCGTT | CCCTTGATGGGTTTCTGTCC |
| GbWRKY7 | AGACGATCATCCTATGCCAAGA | CAGTATGTATCCTGTCCCCTTCA |
| GbWRKY3 | AGCCTTGGAATAGCTGGTGC | CAAGGAAAGATTGAACCGAAAC |
| GbWRKY11 | TGTAAGAGGTTGCCCTGCTAGA | TGGTTATGTTCTCCCTCGTATGT |
| GbWRKY15 | ACTACCCATTCTTCTACCGAACTTG | TTGATGAACTGTCGGTGGTGG |
| GbWRKY32 | ATCAGTCCACAAGCAGACCTCA | TTGTCATTAGCAACACTGCCATC |
| GbWRKY31 | CTGCGGTTTCTCAACAGTCTC | CCAGAAACTTGGAATCCTTCACAC |
| GbWRKY23 | GCAATGGCGGATTTGAAGTC | GCCCAGAATACAGACTTGCTTGA |
| GbWRKY5 | GTGAGAAACGACAGACAGCAGAAG | GCCCAAAATACGAGTGGATAGG |

GbWRKY15, GbWRKY16, GbWRKY14, and GbWRKY19, the WRKYGQK motifs in the WDs were replaced by WRKYGEK, which is one of the most common variants of motifs.

As described in the Introduction, almost all WRKY transcription factors in all plant species can be divided into three categories, namely, groups I, II, and III, on the basis of the number of WDs and the features of the C-terminal Zn finger structures. The WRKY proteins in group II can be further divided into five subgroups, namely, groups IIa, b, c, d, and e. In our analysis, 40 *Ginkgo* WRKY transcription factors were divided into three categories (Fig. 1). Out of these 40 WRKY transcription factors, 12 belonged to group I; their group I-N and I-C were clustered with groups I-N and I-C of A. thaliana, respectively. The Zn finger structure type was C-X4-5-C-X22-23-H-X1-H (C2H2). Most *G. biloba* WRKY transcription factors (26 in total) belonged to group II. These transcription factors were clustered together with four subgroups, namely, groups IIb, c, d, and e, of *A. thaliana*. The number of *G. biloba* WRKY transcription factors belonging to group IIc was the highest (13), whereas groups IIb, d, and e only had 4 transcription factors each. No *G. biloba* WRKY transcription factor belonged to group IIa, and GbWRKY1 showed a CYH2-type Zn finger domain. This Zn finger domain has not been reported in the WRKY family of other plants. Only two *GbWRKY* genes were clustered into group III.

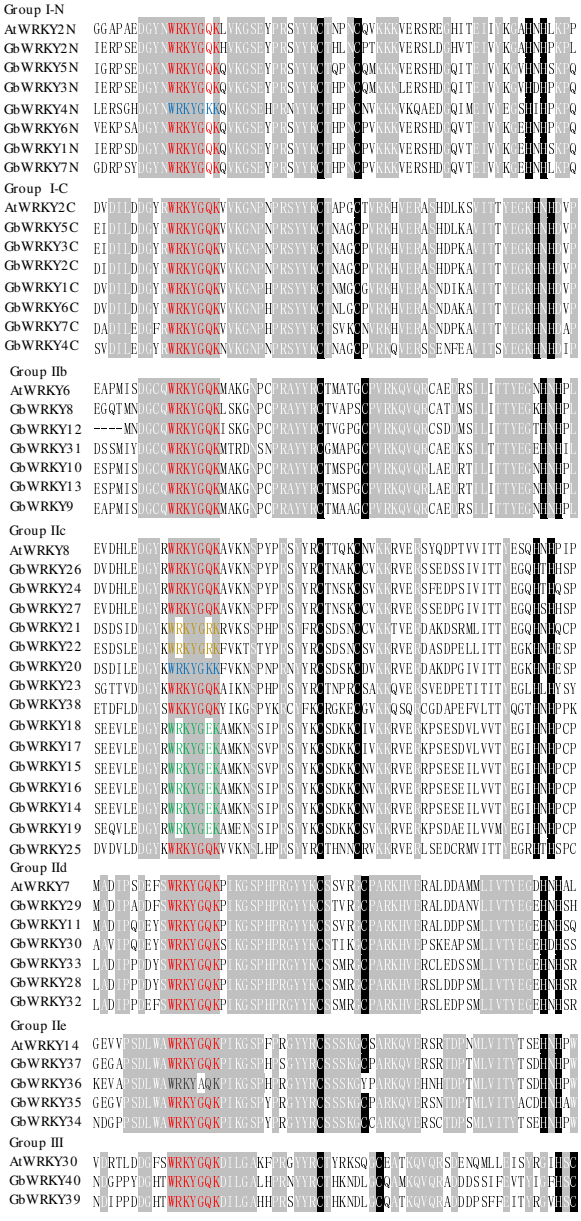


Fig. 1. Alignment of multiple GbWRKY and selected AtWRKY domain sequences. The suffix "N" or "C" indicates the N- or C-terminal WRKY domains of a specific WRKY protein. The different groups were analyzed each. The amino acids forming the zinc-finger motif are highlighted in black. The conserved WRKY amino acid signature is highlighted in red and other different colors

*Phylogenetic analysis of G. biloba WRKYs*

The WRKY transcription factor phylogenetic tree (Fig. 2) was constructed by combining the WRKY transcription factor protein sequences obtained from *A. thaliana*, *H. brasiliensis*, *G. arboretum*, and *T. chinensis var. mairei* and the GbWRKY transcription factor protein sequence in *G. biloba* were identified. The phylogenetic tree showed the evident clustering of WRKY transcription factors. Among these transcription factors, groups I-N, I-C, and III all belonged to a single lineage group; group II is divided into four small branches, namely, groups IIc, a + b, d, and e. Rushton *et al.* (2015) studied the phylogenetic relationships of *WRKY* genes in plants and found that groups IIa and b were clustered on one large branch, while groups IId and e were clustered on the other major branch. This result is consistent with the phylogenetic tree constructed in *G. biloba* WD analysis. At the same time, in this phylogenetic tree, the classification of GbWRKY found in *G. biloba* was essentially the same as that of *A. thaliana* and *H. brasiliensis*, which verifies the correctness of GbWRKY transcription factor classification and phylogenetic tree construction in *G. biloba* individual species. This result also showed that the classification of GbWRKY transcription factors in ginkgo was completely similar to the classification of WRKY transcription factors in other species. Fig. 2 shows that WRKY transcription factors from *G. biloba*, *A. thaliana*, and other species were distributed in the branches of the evolutionary tree. This result indicated that the homology of orthologous WRKY transcription factors is higher than that the homology of paralogous ones. The gene amplification events of GbWRKY transcription factors have evolved before the differentiation of these species. Groups IIc, IIa + b, and I-C were clustered on one large branch, thereby suggesting that they may have a common origin.

In the phylogenetic tree, WRKY transcription factors clustered together may have similar functions. To determine whether the *G. biloba* WRKY proteins regulate the terpene trilactone synthesis, we clustered them with WRKY proteins of other plants that regulate terpene trilactone synthesis. The results showed that these proteins do not have a close relationship. These GbWRKY proteins may have other functions, or the WRKY proteins of other plants of other plants we selected may be excessively few to be representative. AtWRKY3 and AtWRKY4 in group I-N had a similar mechanism of resistance regulation against *Botrytis cinereal* (Lai *et al*., 2008). AtWRKY38 and AtWRKY62 (Kim *et al*., 2008c) in group III both regulated the defense mechanisms of plants against Pseudomonas syringae. Similarly, AtWRKY18 and AtWRKY60 (Chen *et al*., 2010) clustered in group IId in phylogenetic tree played similar functions in response to salt and osmotic stresses. AtWRKY11 and AtWRKY17 clustered together in the group IId can modulate plant defense against *P. syringae* (Journot-Catalino *et al*., 2006). A similar situation can be analogous to *G. biloba*, where the clustered GbWRKY transcription factors possessed high homology and may also have similar functions. The function of WRKY transcription factors clustered in a group from different species may also possess certain similarity.

*Structural and conserved motif analysis*

WRKY proteins are featured by the WD, which is a 60-amino acid stretch containing a conserved amino acid sequence of WRKYGQK together with a Zn finger-like motif (Eulgem *et al*., 2000). Either one or two WDs were present in each protein. Some members of the family have Leu zippers, which contain several repeats of seven hydrophobic amino acids with Leu at every seventh position, toward the N terminus (Eulgem *et al*., 1999). Unfortunately, Leu zippers were absent in the *G. biloba* WRKY protein.

To fully understand the conservation and diversity of the WRKY protein domain of *G. biloba*, we used the online software MEME 5.0.11 for analysis. A total of 20 motifs were obtained to describe the details of the WRKY protein structure in *Ginkgo* (Fig. 3). As shown in Fig. 4, motifs 1, 2, and 4 corresponded to the WD distributed in almost all GbWRKY proteins. The WRKYGQK short peptide in the WRKY core motif was much conserved, and only a small number of variant WRKYGQK short peptides, such as WRKYGKK, WRKYGEK, WRKYGRK, and WRKYAQK, was found in groups I-N, IIc, and IIe. Certain group- or subgroup-specific motifs were identified as follows: motif 3 was unique to the N-terminal WDs of group I; motif 6 existed only in the WDs with GbWRKY15, GbWRKY16 and GbWRKY18, and they all belonged to group IIc; motifs 16, 17, 18, 4, 1, and 2 were found in GbWRKY28, GbWRKY11, GbWRKY29, GbWRKY30, GBWRKY32, GBWRKY33, and AtWRKY7, and these WRKY proteins belonged to group IId; motifs 16 and 17 were only present in group IId. GbWRKY2, GbWRKY3, GbWRKY5 and GbWRKY2 proteins also had common motifs. Motifs 14, 13, and 10 were only distributed at their C-terminus, thereby indicating the possibility that these four WRKY proteins have biological function of these. The analysis of the type and number of motifs among different groups of G. biloba WRKY proteins will contribute for further study of the structure and function of WRKY proteins in *G. biloba* and other plants.

Exon and intron structures can also provide information in analysing phylogenetic relationships and gene function in gene families. In this study, we identified 40 *GbWRKYs* in combination with ginkgo genome and transcriptome databases. Among *40 GbWRKYs*, 33 were identified from the genome. Therefore, we examined the exon–intron organization of 33 *GbWRKY* genes to gain in-depth understanding of the evolution of the WRKY family in *G. biloba*. As shown in Fig. 5A and 5B, all *GbWRKY* genes possessed 2 (*GbWRKY38*)-17 (*GbWRKY6*) exons. Each gene possessed at least two exons, no gene only had one exon. Genes from the same group or subgroup showed similar intron structures and numbers. All group IId members possessed three exons and two introns, and all group III members possessed four exons and three introns. In summary, the similar gene structure of WRKY members in the same group and the phylogenetic analysis results, further illustrate the accuracy of the classification of the *G. biloba WRKY* gene family.

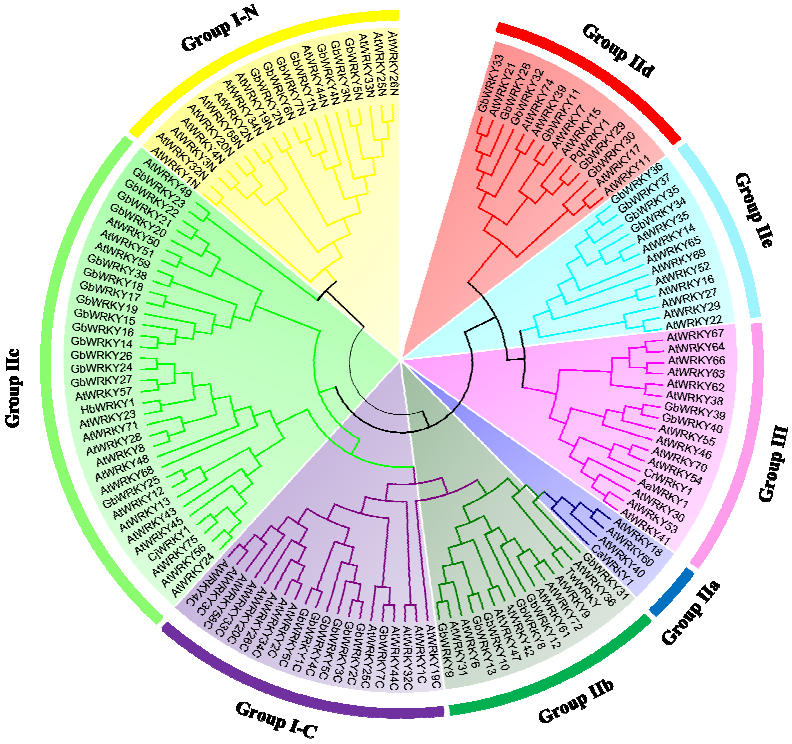


Fig. 2. Phylogenetic tree representing relationship among WRKY domains of *G. biloba*, *Arabidopsis thaliana* and other plants. The amino acid sequences of the WRKY domain of 40 GbWRKY, 71 AtWRKY proteins and 7 other plants were aligned with ClustalX and the phylogenetic tree was constructed using the neighbor-joining method in MEGA 6.0 with 1000 bootstrap replicates. Group I proteins with the suffix "N" or "C" indicates the N-terminal WRKY domains or the C-terminal WRKY domains. GI-C WRKY domain of Group I proteins (dark purple); GI-N WRKY domain of Group I proteins (yellow); group IIa proteins (dark blue); group IIb proteins (dark green); group IIc proteins (light green); group IId proteins (red); group IIe proteins(skyblue); group III proteins (roseo)

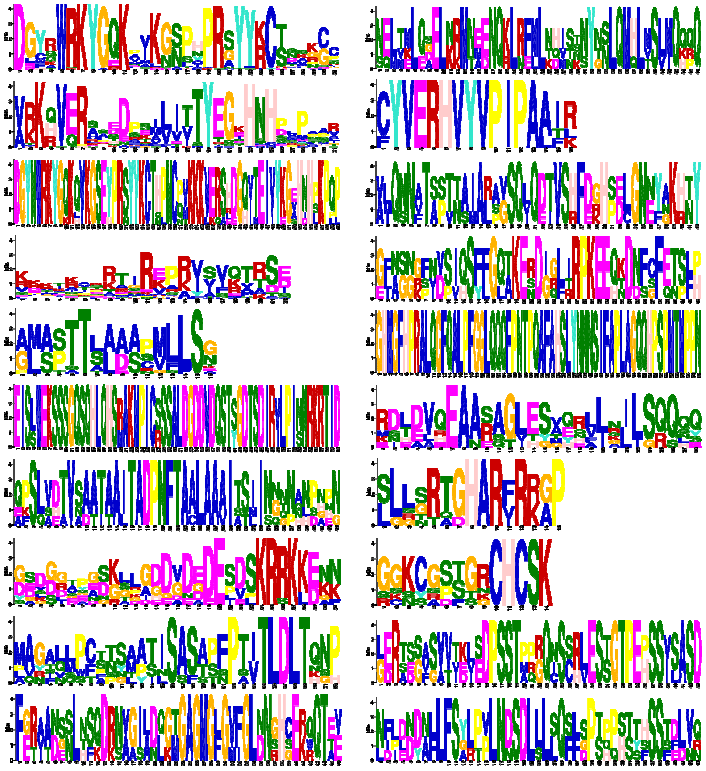

Fig. 3. Sequence information of each motif

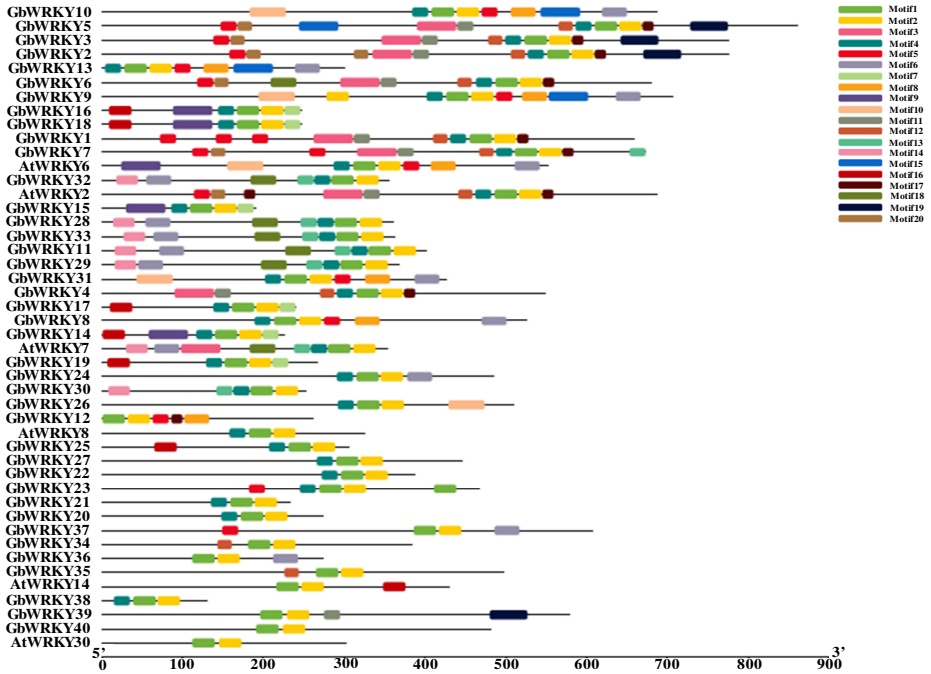


Fig. 4. Conserved motifs in WRKY protein sequences. The motif composition of 40 *G. biloba* WRKY proteins and 6 *Arabidopsis* WRKY protein is shown on the right. Different motifs are displayed in different coloured boxes, with a total of 1-20 numbers. The sequence information for each motif is provided in Table S1

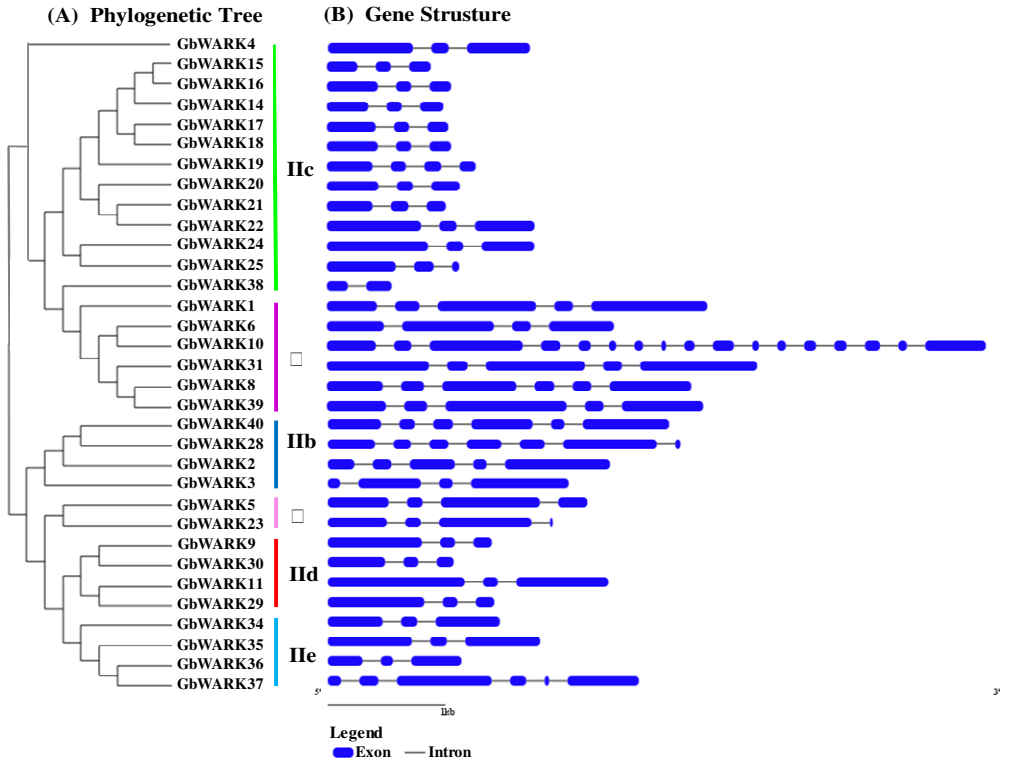


Fig. 5. Phylogenetic relationships and gene structure of conserved protein motifs in *WRKY* genes from *G. biloba*. (A) The phylogenetic tree was constructed based on the full-length sequences of *G. biloba* WRKY proteins using MEGA 6.0 software. (B) Intron and exon structures of *WRKY* genes in *G. biloba*. Blue boxes indicate exons; black lines indicate intron

*Differential expression profiles of GbWRKY in various G. biloba tissues*

As shown in Fig. 6, 10 *GbWRKY* genes were constitutively expressed in all tested *G. biloba* tissues. However, the expression levels were relatively different and showed organ specificity. The expression level of four *GbWRKYs* (*GBWRKY7*, *GbWRKY15*, *GbWRKY31*, and *GbWRKY5*) were significantly higher in leaves than in other tissues. *GbWRKY2* expression was the highest in male and female flowers, followed by leaves; *GbWRKY29* had the highest expression in stems, followed by female flowers; *GbWRKY3* and *GbWRKY11* had the highest expression levels in female flowers among the tested tissues; *GbWRKY23* had higher expression levels in leaves and female flowers than other tissues. *GBWRKY32* had similar expression levels in leaves, stems, and female flowers but significantly higher than in roots and male flowers. Hence, *GbWRKY4*, *GbWRKY15*, *GbWRKY31*, and *GbWRKY5* are genes with leaf-specific expression; *GbWRKY2* is a gene with male and female flower-specific expression; *GbWRKY29* is a gene with stem-specific expression.

*GbWRKY expression level under stress treatments in G. biloba*

A time course expression analysis of the *GbWRKY* genes was performed under ABA, SA, ETH, MeJA, NaCl, chilling, and heat treatments. Given that the expression of *GbWRKYs* has organ-specificity, to eliminate the errors caused by inconsistent materials, the same batch of *G. biloba* callus with the same growth tendency was selected as the stress material. As shown in Fig. 7A, the relative expression levels of 10 *GbWRKYs* were compared with the control within 72 h after 100 μmol $L^{-1}$ ABA-treated *G. biloba* suspension calli, the expression of 7 *GbWRKYs* (i.e., *GbWRKY2*, *GbWRKY29*, *GBWRKY7*, *GbWRKY3*, *GbWRKY11*, *GbWRKY15*, and *GbWRKY5*) were upregulated by ABA-induced. The remaining three *GbWRKYs* showed downregulated expression. Among these genes, *GbWRKY29* can respond quickly to ABA induction. The expression level of the gene reached its maximum at 3 h after treatment and was significantly higher than that of the control within 48 h after treatment. The expression level decreased to below the control level at 72 h after treatment. The gene expression levels of *GbWRKY2*, *GBWRKY7*, *GbWRKY11*, *GbWRKY15*, and *GbWRKY5* reached the maximum within 6-24 h after treatment. The expression level of *GbWRKY3* decreased rapidly at 3 h after treatment, rapidly increased to a maximum at 6 h after treatment, and then rapidly decreased below its expression level in the control group. The gene expression levels of *GbWRKY23*, *GbWRKY31*, and *GBWRKY32* during the entire treatment period were significantly lower those in the control. The results indicated that 7 of the 10 GbWRKY transcription factors were induced by ABA, and 3 genes were inhibited by ABA.

As shown in Fig. 7B, the relative expression of 10 *GbWRKYs* was determined within 72 h after callus induction with 100 μmol $L^{-1}$ SA. 6 *GbWRKYs* (*GbWRKY2*, *GbWRKY3*, *GbWRKY15*, *GbWRKY31*, *GBWRKY32*, and *GbWRKY5*) showed increased expression level compared with the control. After reaching the maximum, the expression gradually decreased below the control level as the treatment time increased. The expression levels of the remaining 4 *GbWRKYs* decreased at the early stage of treatment but rapidly increased within 24-48 h and reached a maximum value at 24 h. At the end of the experiment, the expression level decreased below the control level at 72 h after treatment. This result indicated that 10 GbWRKY transcription factors were all induced by SA, but two different patterns of induction response were observed.

As shown in Fig. 7C, seven *GbWRKYs* (i.e., *GbWRKY29*, *GBWRKY7*, *GbWRKY3*, *GbWRKY11*, *GbWRKY15*, *GbWRKY23*, and *GbWRKY5*) were observed after the treatment of *G. biloba* callus with 40μmol $L^{-1}$ ETH. The expression levels at 3-12 h of treatment were significantly higher than that of the control and decreased to the control level or lower at 24-72 h after treatment. The expression levels of the remaining three genes decreased at the early stage of treatment, suddenly reached the maximum at 12 h, and then quickly dropped below the control level. Hence, 10 GbWRKY transcription factors were induced by ETH, but two different patterns of induction response were observed.

As shown in Fig. 7D, after treatment with *G. biloba* callus with 100 μmol $L^{-1}$ MeJA, the expression of 8 *GbWRKYs* (*GbWRKY29*, *GBWRKY7*, *GbWRKY3*, *GbWRKY11*, *GbWRKY23*, *GbWRKY31*, *GBWRKY32*, and *GbWRKY5*) was downregulated compared with that of the control. Among these genes, *GbWRKY23*, *GbWRKY31*, and *GBWRKY32* were strongly inhibited by MeJA; at 3 h after treatment, the expression levels of *GbWRKY15* and *GbWRKY2* rapidly increased to their maximum values and then rapidly decreased below the control level. The results showed that *GbWRKY15* and *GbWRKY2* rapidly responded to MeJA induction in the 10 GbWRKY transcription factors, and the expression of the remaining 8 GbWRKYs were inhibited by MeJA.

As shown in Fig. 7E, 200 μmol $L^{-1}$ NaCl was used to treat *G. biloba* callus for 72 h, and the expression levels of 10 *GbWRKYs* were analyzed. The results showed that the expression levels of four *GbWRKYs* (i.e., *GbWRKY29*, *GBWRKY7*, *GbWRKY3*, and *GbWRKY11*) were downregulated. The expression levels of the remaining 6 *GbWRKYs* at 3 h after treatment increased rapidly to the maximum and then quickly decreased below the control level. This result indicated that the expression of four *GbWRKYs* of the ten *G. biloba* WRKY transcription factors was inhibited by high salinity, and six GbWRKYs were induced by high salinity.

As shown in Fig. 7F, *G. biloba* callus was treated with low temperature for 72 h to analyze the expression levels of 10 *GbWRKY* genes. The expression levels of the majority of the genes were lower than the control level, thereby indicating that the expression of these genes was inhibited by chilling treatment. Meanwhile, the expression of *GbWRKY32* at the initial stage under low temperature stress initially reached the lowest level, gradually increased, reached the maximum within 12-24 h, and then decreased

rapidly but remained above the control level before the end of the experiment. The *GbWRKY32* expression was induced by low temperature. The relative expression levels of 10 *GbWRKYs* were all downregulated compared with the control within 72 h after *G. biloba* callus heat treatment (Fig. 7G). The results showed that 10 *G. biloba* WRKY transcription factor genes were all expressed at high temperature.

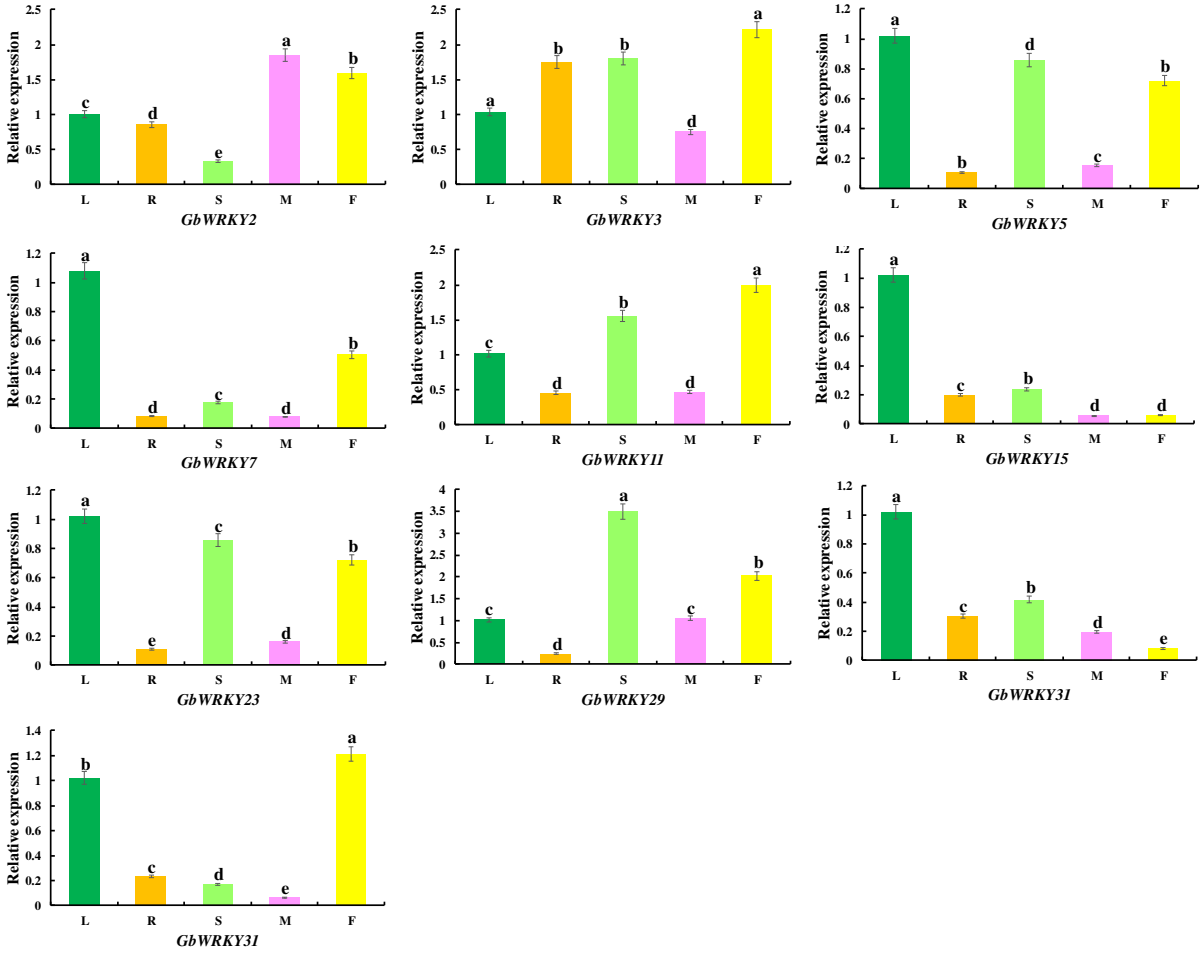


Fig. 6. Expression profiles of *GbWRKY* in *G. biloba* different tissue. L: leaf; R: root; S: stem; M: male flower; F: female flower. The gene expression level of *GbWRKY* in leaf was set to 1. Data are mean ± SD from triplicate experiments (n = 3)

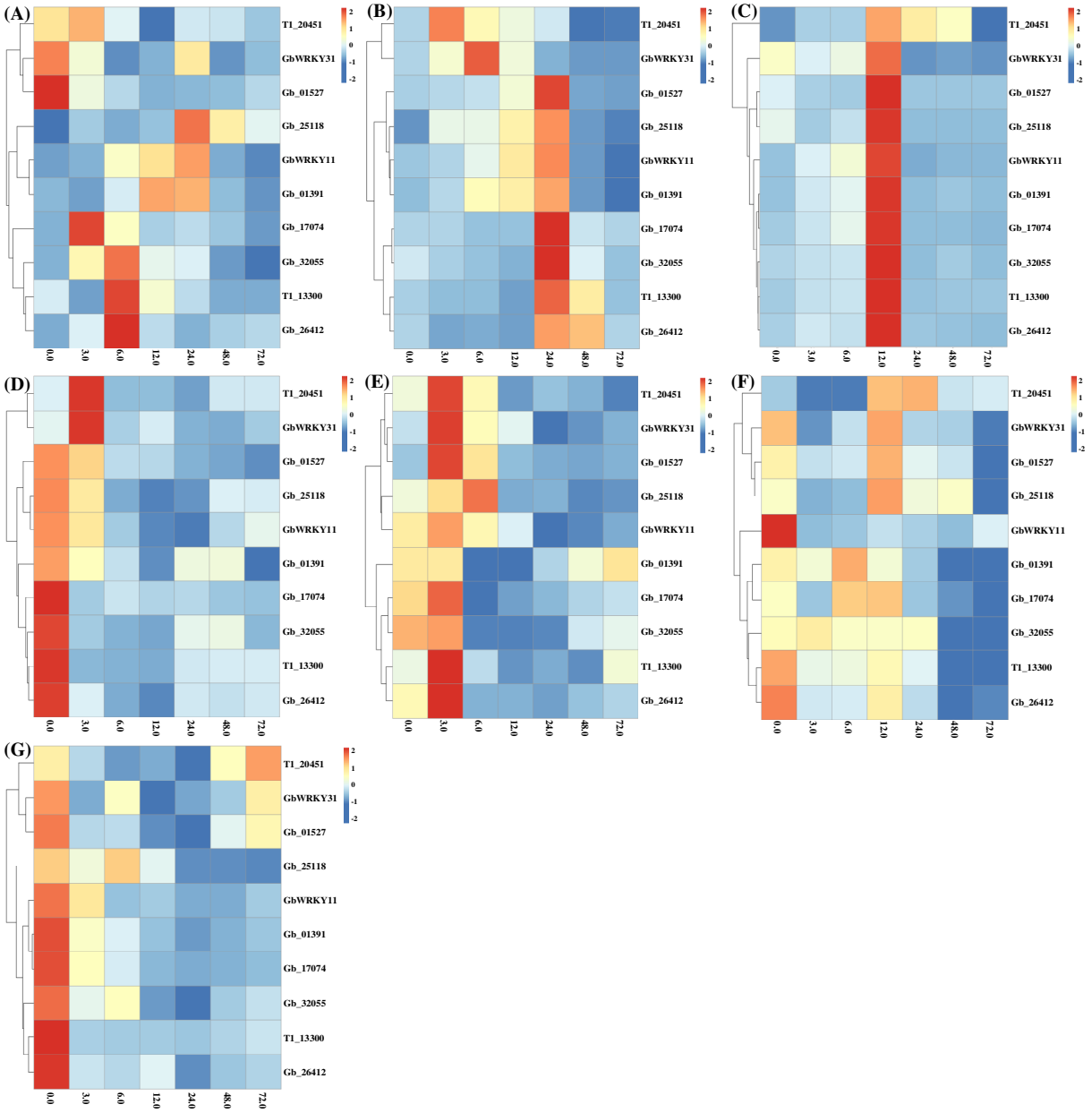


Fig. 7. Heatmap analysis of *GbWRKY* expression level in *G. biloba* under the induction of ABA, SA, ETH, MeJA, Salt, Cold, Heat. (A) Expression profiles of *GbWRKY* in response to ABA treatment; (B) Expression profiles of *GbWRKY* in response to SA treatment; (C) Expression profiles of *GbWRKY* in response to ETH treatment; (D) Expression profiles of *GbWRKY* in response to MeJA treatment; (E) Expression profiles of *GbWRKY* in response to salt treatment; (F) Expression profiles of *GbWRKY* in response to cold treatment; (G) Expression profiles of *GbWRKY* in response to heat treatment. Each column represents the time of stress treatment, whereas each row represents a gene. Expression differences are observed in different colors. The time of the stress treatment is shown at the top. The expression levels were normalized to *GbGAPDH* gene and shown as log10

## Discussion

The first plant *WRKY* gene, that is, *SWEET POTATO FACTOR1*, was cloned from sweet potato on the basis of its ability to bind specifically to the SP8 DNA sequence motif TTGACCT, which is known as the W box (Ishiguro and Nakamura, 1994). Subsequently, a large number of plant WRKY proteins was investigated in numerous plants. To date, 14549 WRKY sequences are available in the plant TF database (http://planttfdb.cbi.pku.edu.cn/). Since the release of a large number of publicly available sequences and even complete whole-genome sequences in some plants, the genome-wide analyses of the *WRKY* gene family have been performed. A total of 90 *WRKY* family members were observed in *A. thaliana*, 104 in *Oryza barthii*, 161 in *Zea mays*, 50 in *Picea abies* (Jin *et al*., 2017), and 21 in *Pinus taeda*. To date, *Glycine max* has the largest number of WRKY family members among the sequenced plants (Yin *et al*., 2013). The variation in the number of WRKY family members from different plant species may be due to genomic duplication events or environmental adaption, thereby indicating the specificity after divergence from the last come ancestor (Liu *et al*., 2017).

With the increasing number of identified and discovered *WRKY* genes, group I and II a + b separate hypotheses have been proposed. The former indicates that all WRKY genes evolved from the C-terminal domain of group I, whereas the latter claims that groups IIa and b genes may have evolved from a single domain ancestral algal *WRKY* gene (Rinerson *et al*., 2015). The analysis of the *WRKY* genes of the complete genomic sequence of several flowering plants divided WRKY transcription factors into the seven major subfamilies, namely, groups I, IIa, IIb, IIc, IId, IIe, and III (Eulgem *et al*., 2000). On the basis of the evolutionary analysis of WDs, conserved domains, and intron locations, Zhang and Wang (2000) proposed another model that classifies WRKY proteins into five groups, as follows: groups I, IIa + b, IIc, IId + e, and III. They divided WRKY transcription factors into two categories on the basis of the insertion site of introns (Zhang and Wang, 2000). The first class includes the *WRKY* gene with an intron of type R, whose splice site is between the two Gs arginine in codon AGG. The other class includes the V-type intron WRKY, where the splice site is located in front of the valine codon. WRKY transcription factors can activate or inhibit transcription in physiological processes (Xie *et al*., 2005). In addition to WDs and Zn finger structures, most WRKY transcription factors have nuclear localization signals, serine/threonine rich regions, glutamine-rich regions, proline-rich regions, kinase domain, and TIR-NBS-LRR. The presence of these structures makes WRKY transcription factors possess different transcriptional regulatory functions (Zhang and Wang, 2000). In our phylogenetic tree, the grouping of GbWRKY is consistent with those of the previous studies, where groups IIa and b are clustered together to form II a + b and closely related to group I-C. The relationship among groups III, IIe, and IId is also closer.

*GbWRKY* genes are commonly expressed in various tissues, but the expression levels are relatively different and possessed organ specificity. *GBWRKY7*, GbWRKY15, *GbWRKY31*, and *GbWRKY5* genes were specifically expressed in leaves; *GbWRKY3* and *GbWRKY11* genes were highly expressed in female flowers; *GbWRKY29* was highly expressed in stems; and *GbWRKY2* was highly expressed in male and female flowers. Genes that are specifically expressed in a particular tissue may have important regulatory effects on the development and metabolism of the tissues in which they are located.

WD binds to a W-box (TTGACC/T) in the promoters of the target genes (Rushton *et al*., 1996; Ciolkowski *et al*., 2008). W-Boxes can be found in the promoters of the systemic acquired resistance-related genes, including isochorismate synthase 1, non-expressor of *PR* genes 1, and pathogenesis-related 1 (Zheng *et al*., 2013). Determining whether a promoter contains multiple W-boxes and even contains motif clusters occasionally is difficult. W-Boxes are also found in the promoter of *WRKY* genes, thereby suggesting a potentially strong transcriptional networking among WRKY proteins (Llorca *et al*., 2014). The binding of WRKY proteins to W-boxes is a feature of both biotic and abiotic stress responses. A total of 26 *AtWRKY* genes that responded to salt stress and upregulated expression were observed in *A. thaliana* (Jiang and Deyholos, 2006). Out of 64 *GmWRKY* genes, 25 changed their expression levels in response to at least one abiotic stress (Zhou *et al*., 2008). A total of 49 *AtWRKY* genes also responded to bacterial infection and SA induction (Ramamoorthy *et al*., 2008). Our study found that the tested 10 *GbWRKY* genes can rapidly change their expression levels under stress. The expression of most genes was upregulated under SA, ETH, ABA, and high salt treatments and downregulated due to MeJA, chilling, and heat treatments. Each gene responded to at least one stress induction. Some *GbWRKY* genes rapidly responded to stress at the early stage of stress induction, and the expression level rapidly increased and then decreased sharply, thereby showing a rapid and transient response (e.g., *GbWRKY2*, *GbWRKY15*, *GbWRKY23*, *GbWRKY23*, *GBWRKY32*, and *GbWRKY5* under salt stress; *GbWRKY2* and *GbWRKY15* under MeJA stress). Some *GbWRKY* genes also played a role in late stress response (e.g., *GBWRKY32* in cold stress; *GbWRKY29*, *GBWRKY7*, *GbWRKY11*, and *GbWRKY23* in SA stress). Even a part of *GbWRKY* was continuously expressed during stress (e.g., *GbWRKY31* under cold stress and *GbWRKY15* under SA stress). In other plants, there are also related research reports. For example, the *OsWRKY11* of rice can activate the expression of some genes related to raffinose synthesis to promote the accumulation of raffinose in plants, thereby improving the drought resistance of rice. The expression of the *OsWRKY11* gene was also induced by high temperature, and the expression of *OsWRKY11* gene initiated by HSP101 promoter increased the heat tolerance of rice (Wu *et al*., 2009). WRKY protein can also directly regulate the expression of drought-resistant genes and improve the drought resistance of plants. STZ encodes a drought-tolerant protein with a Cys2/His2 zinc finger structure. Its expression level is induced by drought, high salt, cold damage and ABA treatment. It can increase the drought resistance of plants when overexpressed in Arabidopsis and rice (Sakamoto *et al*., 2004). The

*AtWRKY39* gene of *Arabidopsis* can promote high temperature stress response through signaling pathways mediated by SA and JA (Li *et al*., 2010). In addition to directly regulating the expression pattern of the target gene, some WRKY proteins indirectly regulate the expression level of the functional gene by interacting with other transcription factors. The *TcWRKY53* gene of *Thlaspi caerulescens* encodes a negative regulator involved in osmotic stress, and its expression is strongly induced by drought, NaCl and low temperature (Wei *et al*., 2008).

Most of the genes in eukaryotes are composed of exons and introns. Exons are mainly responsible for encoding proteins, while introns are characterized by self-splicing. By selecting splicing, a gene can produce different mature mRNAs, which can be translated into functionally distinct proteins, and even produce distinct proteins, sometimes the function is even the opposite (Howe and Ares, 1997). This regulatory mechanism allows certain genes to produce specific proteins in different cell tissues or at different stages of development to meet the metabolic needs of the organism (Blencowe *et al*., 2006). In this study, we analyzed the gene structure of different *GbWRKY* genes, different genes have different numbers of introns and exons, and the selected 10 *GbWRKY* genes have tissue expression specificity, which may be determined by gene structure. At present, regulatory elements of gene expression have been found in introns of many genes, and some genes have introns in response to stress response elements to assist in the inducible expression of genes (Balvay *et al*., 1992). Responding to stress is a complex process. Therefore, whether the induced expression of *GbWRKY* under different stress treatments is caused by the response to stress response elements in its introns requires further verification. The above-mentioned results indicated that the *GbWRKY* genes have a relatively complex expression pattern, thereby suggesting that the GbWRKY transcription factors have complex and variable regulation during the stress resistance of *G. biloba* to adapt to this series of complex physiological changes. Therefore, in order to thoroughly understand the regulation mechanism of the *GbWRKY* gene, a large amount of research is needed in the later stage.

## Conclusions

In our study, 40 *GbWRKYs* were identified and classified into groups I (groups I-N and -C), II (groups IIa, b, c, d, and e), and III, which include 12, 26, and 2 *GbWRKY* genes, respectively. The WRKYGQK core motif was highly conserved, with only a few common variants found in group I-N and II-c (WRKYGKK, WRKYGEK, and WRKYGRK). Zn finger structures differed remarkably in different GbWRKY groups. The sequence of the Zn finger structure of group I-N was C-X4-C-X22-H-X1-H. For group I-C and IIc, the sequence was C-X4-C-X23-H-X1-H. For groups IIb, d, and e, the sequence was C-X5-C-X23-H-X1-H. Through the qRT-PCR technique, 10 candidate *GbWRKY* genes were randomly selected for tissue expression. The results showed that these genes showed differential expression patterns in various tissues with organ specificity. Some genes had similar expression levels; hence, they may have similar functions. The *GbWRKY* gene expression levels in *G. biloba* callus under different elicitor treatments verified the crucial roles of the *GbWRKY* genes in stress response. However, whether all WRKY transcription factors play a role in plant physiology and whether the function is repeated are still unclear. The mechanism underlying the crosstalk between the WRKY transcription factor self-regulation pattern and the signal transduction pathway involving WRKY transcription factors are also unclear, and further research is needed. Our research information will aid in revealing the role of the WRKR transcription factors in plant stress responses and will be useful for further study of the function of the *WRKY* genes. However, in-depth research is needed to determine the function and role of each ginkgo *WRKY* gene.

## Acknowledgements

This work was supported by the National Science Foundation of China (No. 31370680).

## Conflict of Interest

The authors declare that there are no conflicts of interest related to this article.

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