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Genome-wide characterization of the WRKY gene family in *Juglans mandshurica* and expression profiling of *JmWRKY51* under drought stress

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Abstract

WRKY transcription factors are plant-specific regulators involved in plant growth, development, and responses to environmental stresses. However, the WRKY gene family and its potential roles in drought responses remain insufficiently characterized in *Juglans mandshurica*. In this study, a genome-wide analysis identified 89 non-redundant JmWRKY genes, which were systematically characterized based on phylogenetic relationships, conserved motifs, gene structures, chromosomal distributions, promoter cis-acting elements, and gene duplication patterns. Phylogenetic analysis classified JmWRKY proteins into three major groups, while collinearity and selection-pressure analyses indicated that segmental duplication contributed substantially to JmWRKY family expansion and that duplicated gene pairs were predominantly subjected to purifying selection. Transcriptome analysis and quantitative real-time polymerase chain reaction validation identified several drought-responsive JmWRKY genes. Among them, *JmWRKY51* was significantly induced by drought treatment and contained multiple stress- and hormone-responsive cis-acting elements in its promoter. A β-glucuronidase reporter assay further showed enhanced *JmWRKY51* promoter activity under drought stress. Protein-protein interaction prediction suggested that *JmWRKY51* may participate in a drought-associated regulatory network. These findings provide a comprehensive characterization of the WRKY gene family in *J. mandshurica* and identify *JmWRKY51* as a promising candidate for further investigation of drought stress tolerance mechanisms.

Keywords *Juglans mandshurica*; WRKY transcription factor; genome-wide analysis; drought stress; gene expression; abiotic stress

1 Introduction

Transcription factors (TFs) are sequence-specific DNA-binding proteins that orchestrate diverse biological processes-including cell differentiation, development, metabolism, and stress adaptation-through transcriptional regulation of target genes (Singh et al. 2002). Among these, the WRKY family is distinguished by a highly conserved DNA-binding domain comprising the signature WRKYGQK heptapeptide and a zinc-finger motif. Based on the number of WRKY domains and the configuration of the zinc-finger structure, WRKY proteins are phylogenetically classified into three major groups (I, II, and III), which collectively participate in developmental programming and environmental stress responses across the plant kingdom (Chen CJ et al. 2025; Chen X et al. 2025; Eulgem et al. 2000; Khoso et al. 2022).

Under drought conditions, WRKY transcription factors enhance plant tolerance by binding to stress-responsive cis-acting elements in target gene promoters, thereby modulating antioxidant enzyme activities and osmoprotectant accumulation. This regulatory paradigm has been documented in multiple species. In wheat (*Triticum aestivum*), transcriptome analysis identified 48 drought-responsive WRKY genes, among which *TaWRKY1* and *TaWRKY33* exhibited pronounced expression changes and

harbored ABRE and DRE elements in their promoters (He et al. 2016). In maize (*Zea mays*), *ZmWRKY79* is strongly induced by water deficit and promotes ABA accumulation via activation of biosynthetic genes such as *AAO3* , leading to enhanced stomatal closure, reduced water loss, and

improved antioxidant capacity (Gulzar et al. 2021). In Myrothamnus flabellifolia , *MfWRKY17* regulates ABA biosynthesis and stress-related gene expression, controlling stomatal aperture to sustain relative water content under desiccation (Huang et al. 2020). Overexpression of *MaWRKYIIc7* in mulberry (*Morus alba*) confers drought tolerance through coordinated regulation of antioxidant gene expression and stomatal movement (Su et al. 2025).

The application of exogenous *Bacillus* to mitigate drought stress has recently garnered considerable research interest. *Bacillus* species improve the rhizosphere microenvironment through secretion of secondary metabolites and exhibit significant potential for alleviating water deficit (Kuramshina and Khairullin 2023; Wang et al. 2022). Studies have demonstrated that *Bacillus* enhances plant performance under drought conditions by promoting growth. For instance, under water limitation, *Bacillus* inoculation significantly increased fresh weight, dry weight, root length, and plant height in flax (*Linum usitatissimum*) by 80.6%, 103.77%, 56.0%, and 35.1%, respectively (Rukh et al. 2025) *Bacillus subtilis* enhances cotton yield under drought stress by optimizing rhizosphere microbial communities, thereby improving soil water retention, nitrogen fertilizer utilization efficiency, photosynthesis, and water use efficiency (Ren et al. 2025). *Bacillus megaterium GEB3* upregulates antioxidant gene expression by activating the jasmonic acid signaling pathway, reducing reactive oxygen species accumulation and enhancing drought resistance in pepper (*Capsicum annuum*) (Park et al. 2025). *Bacillus pacificus G124*, isolated from arid soil, exhibits multiple growth-promoting properties including IAA and ACC deaminase production; following inoculation, growth indices, antioxidant enzyme activities, and osmotic adjustment substances significantly increased in *Arabidopsis thaliana* and *Medicago sativa*, while MDA and ROS levels decreased (Ma et al. 2024).

Juglans mandshurica is a Class II national protected tree species in China with important medicinal and ecological values. However, it is highly sensitive to water deficit, and drought has become a key factor limiting its population expansion and natural regeneration (Li WW et al. 2024; Wu et al. 2025). To date, systematic characterization of the WRKY gene family in *J. mandshurica* has not been undertaken. Furthermore, although the role of *Bacillus* in regulating plant drought resistance has received extensive attention, its effect on WRKY gene expression in *J. mandshurica* has not been systematically reported. Therefore, based on genomic data of *J. mandshurica*, this study comprehensively employed bioinformatic approaches to perform genome-wide identification, chromosomal mapping, phylogenetic analysis, domain and gene structure characterization, promoter cis-acting element prediction, collinearity analysis, and protein interaction network construction for the *J. mandshurica* WRKY family. The objectives were to screen core candidate genes involved in drought responses and provide a theoretical basis for drought-resistant molecular breeding of *J. mandshurica*.

2 Materials and Methods

2.1 Plant Material

Seedlings used for drought stress experiments were raised from seeds collected from elite *Juglans mandshurica* individuals in the seed orchard of Fushun County, Liaoning Province, China. Seedlings with relatively uniform growth were selected and pre-cultivated in 1/2 Hoagland nutrient solution for 2 days prior to stress treatment. For the time-course expression analysis, young leaves were collected at 0, 3, 6, 9, 12, and 24 h after the initiation of stress treatment, wrapped in aluminum foil, immediately frozen in liquid nitrogen, and stored at -80 ℃ until RNA extraction. For tissue-specific expression analysis, female flower buds, male flower buds, stems, leaves, and fruits were harvested from mature

elite individuals in 2024, frozen in liquid nitrogen, and stored at -80 ℃. Three biological replicates were collected for each sample.

2.2 RNA Extraction and cDNA Synthesis

Total RNA was extracted using the RNA Extraction Kit (vazyme, Nanjing, China) according to the manufacturer's protocol. RNA integrity was assessed by 1% agarose gel electrophoresis, and concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). First-strand cDNA was synthesized from 1 μg of total RNA using the HiScript III RT SuperMix for qPCR Kit (vazyme, Nanjing, China) following the manufacturer's instructions.

2.3 Genome-Wide Identification and Chromosomal Mapping of *WRKY* Genes in *J. mandshurica*

The whole-genome sequence and gene annotation files of *J. mandshurica* were obtained from our research group's sequencing data. *Arabidopsis thaliana* WRKY gene data, including whole-genome sequences, protein sequences, and annotation files, were retrieved from The *Arabidopsis* Information Resource (TAIR) database (https://www.arabidopsis.org/; (Philippe et al. 2012)). To identify WRKY family members in *J. mandshurica*, the full-length amino acid sequence of AtWRKY4 (AT1G13960.1) was used as the query in BLASTP searches implemented in TBtools (Chen et al. 2020), yielding an initial set of candidate sequences. The hidden Markov model (HMM) profile for the WRKY domain (PF03106) was downloaded from the Pfam database (https://pfam.xfam.org/; (Finn et al. 2016)). Candidate proteins were further screened using the HMM-Search module of TBtools with the thresholds E-value $\leq 1 \times 10^{-10}$ and bits ≥ 85. The resulting non-redundant *JmWRKY* genes were designated *JmWRKY1* through *JmWRKY88* based on their sequential chromosomal positions and visualized on chromosomes using TBtools.

2.4 Physicochemical Properties of the *JmWRKY* Gene Family

Physicochemical properties of the deduced JmWRKY proteins, including molecular weight, isoelectric point (pI), and grand average of hydropathicity (GRAVY), were computed using TBtools based on the *J. mandshurica* genome annotation file (Chen et al. 2020). Subcellular localization was predicted using the WoLF PSORT online tool (https://wolfpsort.hgc.jp/; (Horton et al. 2007)).

2.5 Phylogenetic Analysis of the *JmWRKY* Gene Family

Multiple sequence alignment of full-length WRKY protein sequences from *J. mandshurica* and *A. thaliana* was performed using MUSCLE (Edgar 2004) with default parameters, and ambiguously aligned regions were trimmed using trimAl (Capella-Gutiérrez et al. 2009). The best-fit amino acid substitution model was selected using ModelFinder implemented in IQ-TREE v2.0.6 (Kalyaanamoorthy et al. 2017; Nguyen et al. 2015). A maximum likelihood (ML) phylogenetic tree was subsequently constructed using IQ-TREE with 1000 ultrafast bootstrap replicates. The resulting tree was visualized and annotated using iTOL v6 (https://itol.embl.de/; (Letunic and Bork 2021)). JmWRKY proteins were classified into groups and subgroups according to their clustering relationships with the well-characterized *A. thaliana* WRKY family members (Eulgem et al. 2000).

2.6 Gene Structure, Promoter Cis-Acting Elements, and Collinearity Analysis of the *JmWRKY* Gene Family

Exon-intron structures of *JmWRKY* genes were characterized by comparing coding sequences wi th corresponding genomic sequences using TBtools (Chen et al. 2020). Conserved motifs within JmWRKY protein sequences were identified using the MEME Suite (https://meme-suite.org/me me/index.html; Bailey et al. 2015), allowing up to 10 motifs with widths ranging from 6 to 15 0 amino acid residues. Conserved domains were annotated using the NCBI Conserved Domain Database (CDD; Marchler-Bauer et al. 2017) and visualized in combination with the phylogenet ic tree, motif distribution, and gene structures using TBtools.

For promoter cis-acting element analysis, the 2000 bp genomic sequences upstream of the trans lation start codon of each JmWRKY gene were extracted using TBtools. Cis-acting regulatory elements were predicted using the PlantCARE database (https://bioinformatics.psb.ugent.be/webto ols/plantcare/html/; (Lescot et al. 2002)), and elements related to stress and hormone responses were catalogued for each gene.

Collinearity analysis of WRKY gene pairs within the *J. mandshurica* genome and between *J. mandshurica* and A. thaliana was performed using MCScanX (Wang et al. 2012). Gene pairs separated by five or fewer intervening genes on the same chromosome were classified as tandem duplications, while those located on conserved syntenic blocks were defined as segmental duplications. Non-synonymous (Ka) and synonymous (Ks) substitution rates for duplicated gene pairs were calculated using the Nei-Gojobori method implemented in TBtools, and Ka/Ks ratios were used to infer selective pressure.

2.7 Protein-Protein Interaction Network Analysis of *JmWRKY* Genes

The protein-protein interaction (PPI) network of JmWRKY proteins was predicted using the STRING database v11.5 (https://string-db.org/; (Szklarczyk et al. 2021)) with *A. thaliana* AtWRKY proteins as query templates. Orthologous relationships between *A. thaliana* AtWRKY and *J. mandshurica* JmWRKY proteins were established through sequence homology alignment. Interaction pairs with a combined confidence score ≥ 0.700 were retained for network construction. The resulting PPI network was imported into Cytoscape v3.10.0 (Shannon et al. 2003) for visualization, with node size scaled to degree centrality and edge thickness proportional to interaction confidence scores.

2.8 Differential Expression Patterns of the *JmWRKY* Gene Family and Response Characteristics of Core Genes under Drought Stress

Transcriptome data of *J. mandshurica* under drought stress were obtained from our research group's prior sequencing efforts. Expression levels of *JmWRKY* genes, expressed as fragments per kilobase of transcript per million mapped reads (FPKM), were extracted from the transcriptome dataset. Differentially expressed genes (DEGs) were identified using DESeq2 (Love et al. 2014) with the thresholds $|\log_2 \text{fold-change}| \geq 1$ and false discovery rate (FDR) adjusted P-value < 0.05. A heatmap of JmWRKY expression across treatment groups was generated using TBtools with Z-score normalization and hierarchical clustering based on Pearson correlation with average linkage (Chen et al. 2020).

For qRT-PCR validation, the *J. mandshurica Actin* gene was used as the internal reference. Gene-specific primers were designed using Primer Premier 5.0 and are listed in Supplementary Table S1. qRT-PCR reactions were performed on a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific) using SYBR Green qPCR Master Mix (Biotech, Beijing, China). The thermal cycling program consisted of an initial pre-denaturation at 95°C for 30 s, followed by 40 cycles of denaturation

at 95°C for 5 s and annealing/extension at 60°C for 30 s. Three technical replicates were performed for each of the three biological replicates. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Data were plotted and statistically analyzed using GraphPad Prism v9.0.

2.9 Promoter activity analysis using GUS reporter assay

The promoter activity of *JmWRKY51* under drought stress was evaluated using a β-glucuronidase (GUS) reporter assay. The promoter region of *JmWRKY51* was cloned and fused upstream of the GUS reporter gene to generate the *JmWRKY51*pro::pCAMBIA1301 construct. The recombinant construct was introduced into plant materials, and the resulting materials were subjected to drought stress treatment and corresponding control conditions.

After treatment, leaves were collected for histochemical GUS staining. Samples were incubated in GUS staining solution under appropriate conditions until blue coloration was clearly visible. Following staining, the samples were decolorized and photographed under identical imaging conditions. The intensity and distribution of blue coloration were compared between drought-treated and control samples to evaluate the transcriptional responsiveness of the *JmWRKY51* promoter to drought stress.

3 Results

3.1 Genome-wide identification and characterization of the WRKY gene family in *Juglans mandshurica*

To identify WRKY family members in *Juglans mandshurica*, the full-length amino acid sequence of *AtWRKY4* from *Arabidopsis thaliana* was employed as a query in BLAST searches against the *J. mandshurica* genome. An initial set of 637 candidate sequences was retrieved, which was subsequently refined through hidden Markov model (HMM) profiling using the WRKY domain (PF03106) and conserved domain validation. This pipeline yielded 89 non-redundant *JmWRKY* genes. Chromosomal mapping anchored 88 of these loci to 16 chromosomes, while one gene remained unplaced on the current assembly. The mapped genes were systematically designated *JmWRKY1* through *JmWRKY88* based on their sequential chromosomal positions. The distribution of *JmWRKY* loci across chromosomes was notably uneven: chromosome 1, the longest linkage group, carried the highest complement with nine genes, whereas chromosomes 14 and 16 each harbored only a single member. Discernible gene clusters were observed on chromosomes 1, 2, 5, 6, 7, 8, and 12. This asymmetric genomic distribution suggests that segmental duplication events have contributed substantially to the expansion and architectural organization of the *JmWRKY* repertoire in *J. mandshurica*.

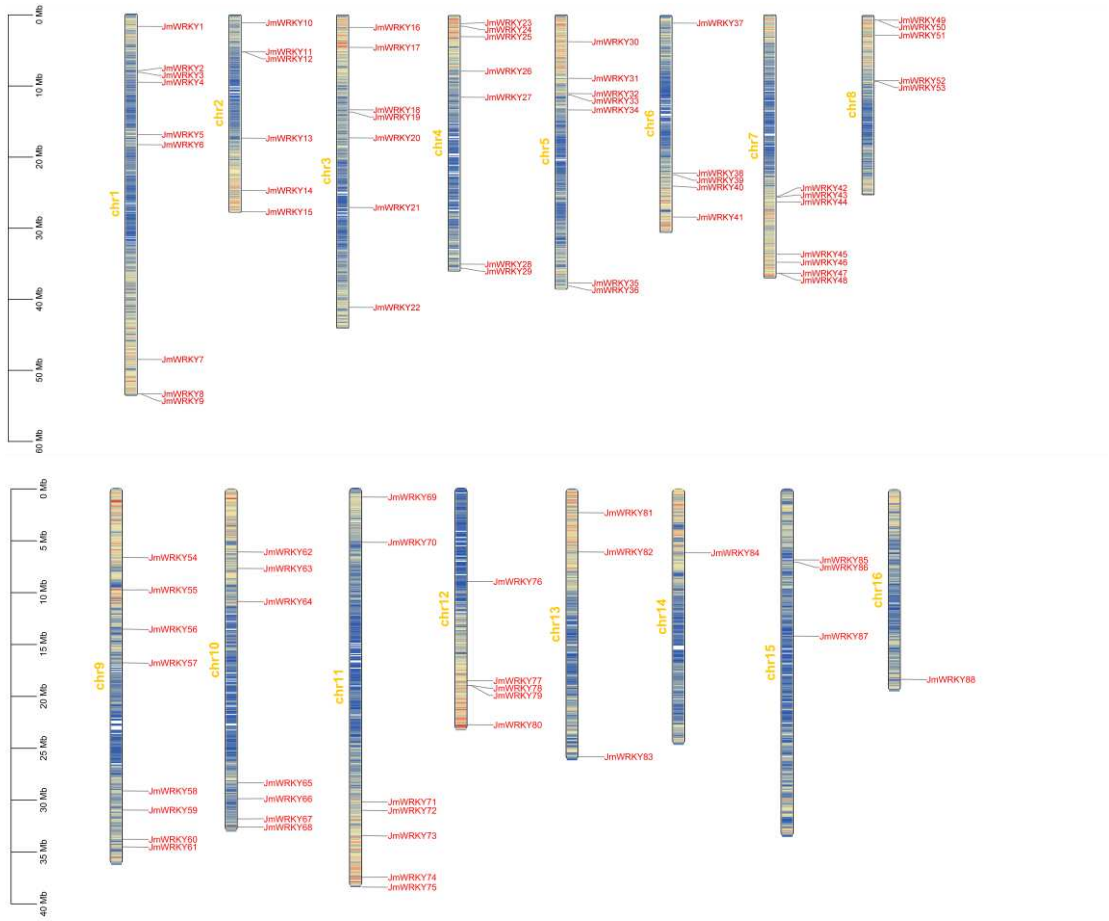


Fig. 1 Chromosomal distribution of JmWRKY genes in *Juglans mandshurica*. The 88 anchored JmWRKY loci are depicted across the 16 chromosomes. Chromosome numbers are indicated at the top of each bar, and gene clusters are marked with colored brackets.

3.2 Physicochemical Properties of the *JmWRKY* Gene Family

Physicochemical characterization of the deduced JmWRKY proteins revealed substantial diversity in primary sequence properties across the family. The predicted polypeptide lengths varied considerably, spanning from 152 amino acids (JmWRKY69) to 747 amino acids (*JmWRKY75*). Correspondingly, molecular weights ranged from 17.49 kDa (*JmWRKY83*) to 80.37 kDa (*JmWRKY75*). Isoelectric point (pI) values, which reflect the net charge state of proteins under physiological conditions, ranged from acidic (4.90 for *JmWRKY87*) to basic (10.01 for *JmWRKY78*). Despite this physicochemical heterogeneity, subcellular localization prediction using WoLF PSORT consistently assigned all 89 JmWRKY proteins to the nuclear compartment, consistent with their canonical function as transcription factors. Detailed parameters for each family member are provided in Table S2.

3.3 Phylogenetic Analysis of the *JmWRKY* Gene Family

To resolve the evolutionary relationships among WRKY proteins in *J. mandshurica* and to establish a standardized nomenclature framework, we constructed a maximum likelihood (ML) phylogenetic tree incorporating the full-length WRKY protein sequences from both *J. mandshurica* (88 sequences) and *Arabidopsis thaliana* (71 sequences). The resulting topology recapitulated the canonical tripartite classification established for plant WRKY families. Specifically, the 88 JmWRKY proteins included in

the phylogenetic reconstruction (one sequence was excluded due to incomplete domain architecture) were distributed among Groups I, II, and III (Fig. 2). Group II constituted the largest assemblage, comprising 60 members, and was further resolved into five well-supported subgroups (IIa-IIe) in accordance with the *Arabidopsis* classification scheme. Subgroup IIc contained the greatest number of members (22), followed by IIa and IIb. Group I and Group III accounted for 15 and 13 members, respectively. The co-clustering of *JmWRKY* and *AtWRKY* sequences within each subgroup indicates strong orthologous relationships and implies functional conservation between the corresponding WRKY lineages of these two species.

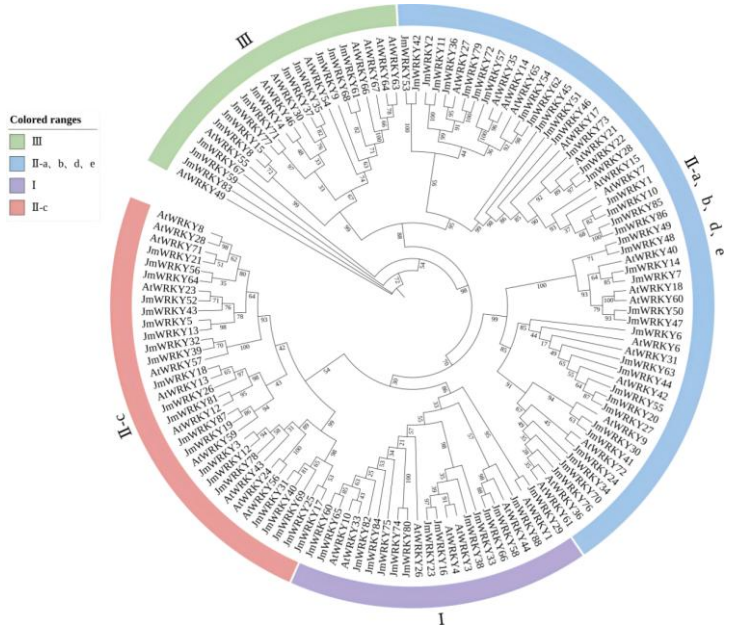


Fig. 2 Phylogenetic classification of the JmWRKY family. The maximum likelihood tree was constructed using full-length WRKY protein sequences from *Juglans mandshurica* (88 sequences) and *Arabidopsis thaliana* (71 sequences). Branches are colored according to group assignment: Group I (red), Group II (blue, with subgroup shading), and Group III (green). Bootstrap support values (≥50%) are indicated at major nodes. The outer ring displays the corresponding subgroup designations (IIa-IIe).

3.4 Analysis of Motifs, Domains, and Gene Structures of the *JmWRKY* Gene Family

To gain further insight into the structural diversification of the JmWRKY family, we characterized conserved protein motifs, domain architectures, and exon-intron configurations across the 88 phylogenetically clustered members (Fig. 3).

Motif analysis using MEME identified ten conserved sequence blocks, ranging from 21 to 50 amino acid residues in length. The distribution of these motifs exhibited strong group-specific patterning. For instance, motif 5 was detected exclusively in Group I members, whereas motif 4 was restricted to Group III, suggesting that these sequence signatures may contribute to lineage-specific regulatory or biochemical functions. Notably, motif 2-corresponding to the canonical WRKYGQK heptapeptide-was universally present across nearly all JmWRKY proteins, reaffirming its indispensability for DNA-binding activity. Conserved domain interrogation via the NCBI Conserved Domain Database further corroborated the presence of the WRKY domain as the predominant structural module, with additional auxiliary domains-including ADIP superfamily, Atg16-CDD superfamily, and UPF0242 superfamily-detected in a subset of proteins.

Exon-intron structural analysis revealed that *JmWRKY* genes contain between one and eight exons, with the majority (37.5%; 33 of 88 genes) possessing a characteristic two-exon architecture interrupted by a single intron. Members clustering within the same phylogenetic group or subgroup displayed remarkably similar exon-intron organizations with respect to exon number, length, and intron phasing, whereas pronounced structural divergence was evident among the three major groups. This congruence between gene structure and phylogenetic topology reinforces the monophyletic origins of each WRKY subgroup and implies that structural conservation may underpin functional redundancy among closely related paralogs.

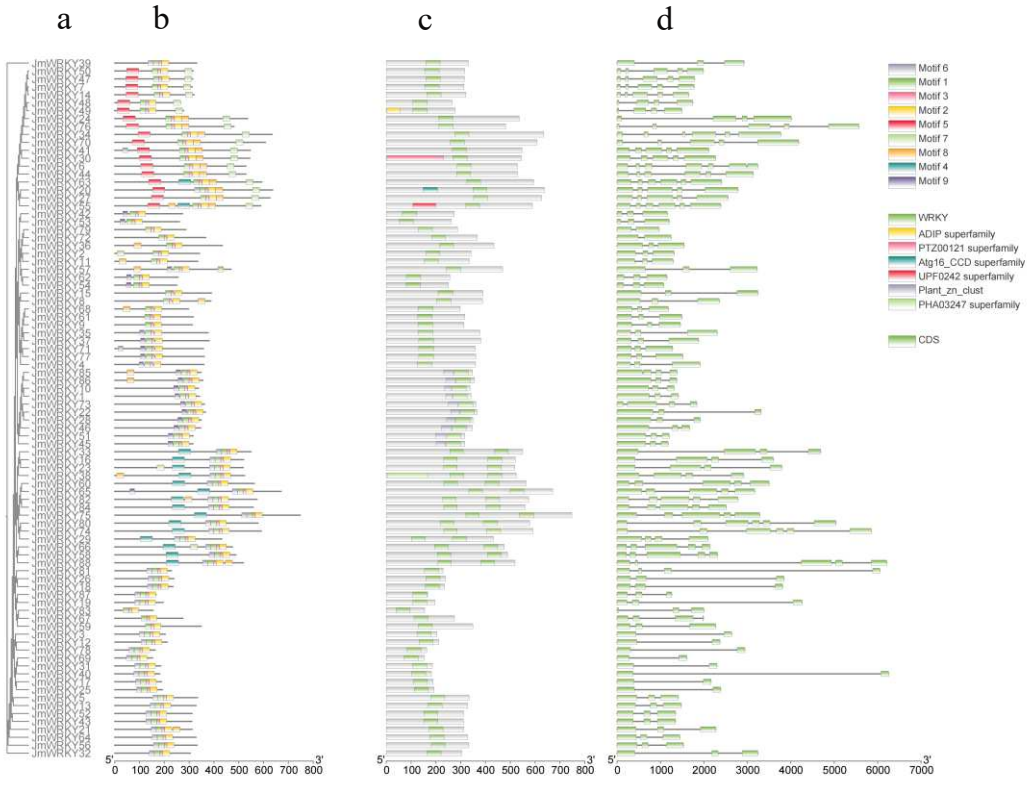


Fig. 3 Structural characterization of the JmWRKY family. **a** Phylogenetic tree of 88 JmWRKY proteins constructed using IQ-TREE with the maximum likelihood method. **b** Distribution of ten conserved motifs identified by MEME analysis. Each colored box represents a distinct motif. **c** Conserved domain architectures predicted using the NCBI Conserved Domain Database. The WRKY domain (green) is universally present, with additional auxiliary domains shown in distinct colors. **d** Exon-intron structures of *JmWRKY* genes. Exons are represented by green boxes, introns by black lines. Scale bar at bottom indicates gene length in kilobases (kb).

3.5 Analysis of Promoter Cis-Acting Elements of the *JmWRKY* Gene Family

To assess the regulatory potential of *JmWRKY* genes in abiotic stress signaling, we retrieved the 2000 bp genomic regions upstream of the translation start site for each locus and interrogated these sequences for known cis-regulatory elements using the PlantCARE database. A diverse repertoire of 41 distinct cis-acting elements was identified across the JmWRKY promoter panel. Among these, 22 elements were associated with phytohormone responsiveness and environmental stress perception, while the remainder corresponded to light responsiveness, tissue-specific expression, and

developmental regulation. Stress-related motifs included those linked to drought inducibility (e.g., ABRE-ABA-responsive element, MBS-MYB binding site involved in drought signaling), wound responsiveness (WUN-motif), and anaerobic induction (ARE, GC-motif).

Quantitative comparison revealed that the promoter regions of *JmWRKY17*, *JmWRKY85*, *JmWRKY52*, and *JmWRKY62* harbored the highest densities of stress-associated elements, implying that these genes may function as primary sensors or early responders in stress perception cascades. Of particular interest, the promoter of *JmWRKY51* contained a canonical ABRE motif, which serves as a cognate binding site for *bZIP* transcription factors in the ABA-dependent drought signaling pathway. The presence of this element, together with the transcriptional behavior described below, positions *JmWRKY51* as a plausible candidate for mediating ABA-regulated drought adaptation in *J. mandshurica*.

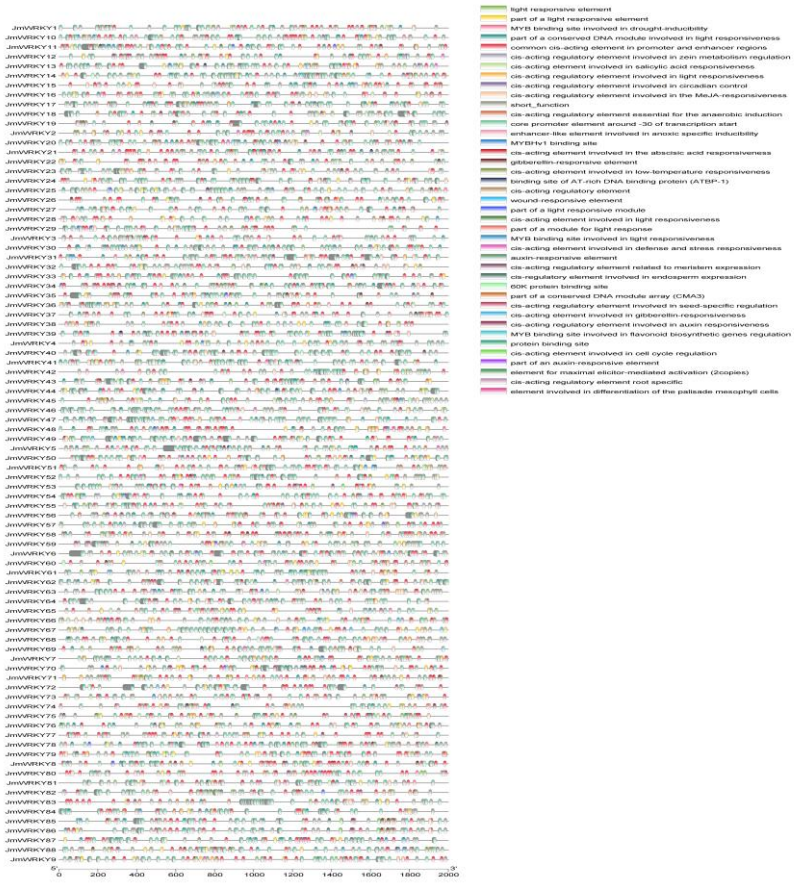


Fig. 4 Cis-regulatory element profiling of JmWRKY promoters. Heatmap representation of stress-responsive and hormone-related cis-acting elements identified within the 2000 bp upstream regions of *JmWRKY* genes. Element counts per promoter are normalized and color-coded from low (blue) to high (red). The complete list of identified elements and their genomic coordinates is provided in Supplementary Table S3. ABRE, ABA-responsive element; MBS, MYB binding site; ARE, anaerobic response element; WUN, wound-responsive motif.

3.6 Collinearity Analysis of the *JmWRKY* Gene Family

Gene duplication is a principal mechanism driving the expansion and functional diversification of transcription factor families. To elucidate the duplication history of the JmWRKY repertoire, we performed collinearity analysis within the *J. mandshurica* genome and between *J. mandshurica* and *Arabidopsis thaliana* using MCScanX.

Within the *J. mandshurica* genome, we identified 85 duplicated *JmWRKY* gene pairs. The overwhelming majority (75 pairs; 88.2%) were classified as segmental duplications, while the remaining 10 pairs (11.8%) represented tandem duplications. Nine JmWRKY loci participated in multiple duplication events, suggesting recurrent recruitment of these genomic segments during family expansion. To infer the selective pressures acting on duplicated *JmWRKY* genes, we calculated the ratio of non-synonymous (Ka) to synonymous (Ks) substitution rates for each paralogous pair. All duplicated gene pairs exhibited Ka/Ks ratios significantly below 1 (Supplementary Table S4), indicating that purifying selection has been the predominant evolutionary force shaping the JmWRKY family, with functional divergence constrained by strong selective pressure against deleterious amino acid substitutions.

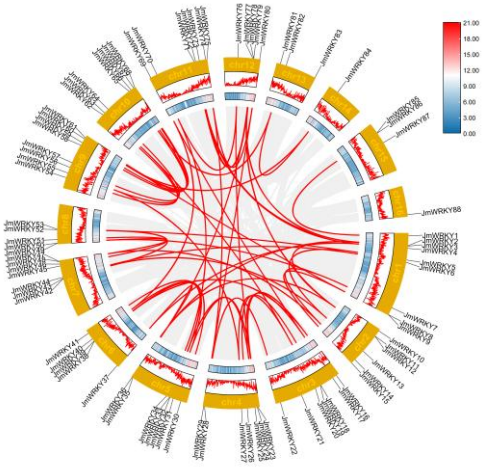


Fig. 5 Syntenic relationships among *JmWRKY* genes in *Juglans mandshurica*. Colored connecting lines denote segmentally duplicated *JmWRKY* gene pairs across the 16 chromosomes. Chromosomes are depicted as colored bars with gene density indicated by the inner histogram. Tandemly duplicated gene pairs are marked with red asterisks adjacent to the respective chromosomal positions.

Inter-species collinearity analysis between *J. mandshurica* and *A. thaliana* identified 57 syntenic *WRKY* gene pairs distributed across the respective genomes. The extensive collinear blocks conserved between these distantly related eudicot species underscore the deep evolutionary ancestry of the WRKY family and the retention of ancestral genomic architecture. Notably, several *J. mandshurica* chromosomes displayed syntenic relationships with multiple *A. thaliana* chromosomes, indicative of lineage-specific chromosomal rearrangements and segmental reshuffling subsequent to the divergence of the Juglandaceae and Brassicaceae lineages. These orthologous relationships provide a valuable framework for the functional inference of uncharacterized *JmWRKY* genes based on their well-studied *Arabidopsis* counterparts.

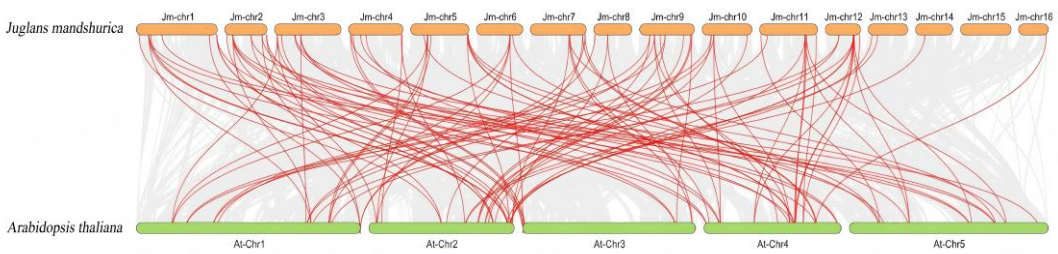


Fig. 6 Cross-species synteny of *WRKY* genes between *Juglans mandshurica* and *Arabidopsis thaliana*. Gray lines in the background represent genome-wide collinear blocks, while colored connecting lines highlight syntenic *WRKY* gene pairs. Chromosomes of *J. mandshurica* (Jm01-Jm16) and *A. thaliana* (At1-At5) are arranged along the respective axes.

3.7 Protein-Protein Interaction Network Analysis of *JmWRKY* Genes

To explore the potential functional associations of JmWRKY proteins within regulatory networks, we constructed a predicted protein-protein interaction (PPI) network based on orthologous relationships with the well-curated *Arabidopsis thaliana* interactome.The resulting network architecture indicated that *JmWRKY51* showed relatively high connectivity with multiple WRKY partners based on predicted associations (Fig. 7).Among the highly connected predicted partners of *JmWRKY51*, *JmWRKY62*, *JmWRKY57*, *JmWRKY15*, and *JmWRKY29* showed relatively stronger associations, with JmWRKY62 and JmWRKY57 displaying higher connectivity.

The central placement of *JmWRKY51* within this predicted interactome, coupled with its strong connectivity to multiple Group II and Group III members, suggests that it may function as a signaling integrator or transcriptional co-regulator in stress-responsive pathways. Such hub-topology positioning is characteristic of transcription factors that coordinate multi-gene regulatory modules in response to environmental perturbation. The predicted interactions, particularly the tight clustering of *JmWRKY51* with *JmWRKY62* and *JmWRKY57*, imply the existence of a cooperative regulatory subnetwork that may be mobilized under drought conditions. While these predictions provide valuable hypotheses, experimental validation will be required to substantiate these physical associations in *J. mandshurica*.

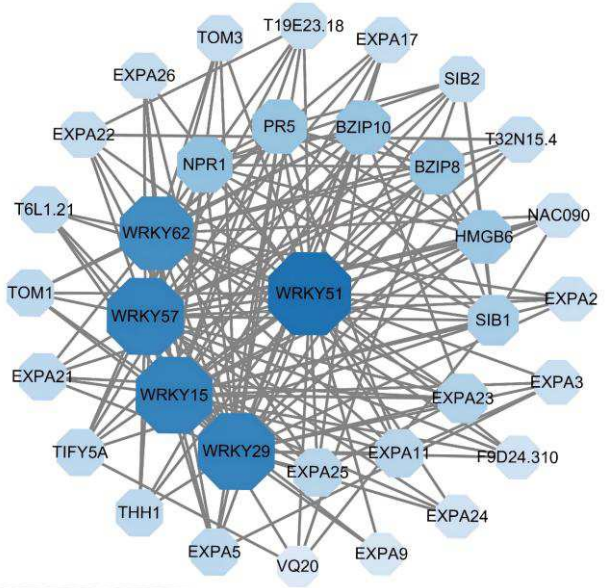


Fig. 7 Predicted protein-protein interaction network of JmWRKY proteins. Network nodes represent individual JmWRKY proteins, with edges indicating predicted functional associations. Node size is proportional to the number of interacting partners (degree centrality). *JmWRKY51 (*red) is positioned as the central hub, with its strongest interactors-*JmWRKY62*, *JmWRKY57*, *JmWRKY15*, and *JmWRKY29*-highlighted in orange. Edge thickness corresponds to interaction confidence scores. Group assignments are indicated by node border colors following the scheme established in Fig. 2.

3.8 Differential Expression Patterns of the *JmWRKY* Gene Family and Response Characteristics of Core Genes under Drought Stress

To characterize the transcriptional dynamics of *JmWRKY* genes under water deficit, we analyzed RNA-sequencing data derived from *J. mandshurica* seedlings subjected to a progressive drought regime, encompassing control conditions (CK), mild drought (T), moderate drought (S), and severe drought (D). In parallel, we evaluated the effect of rhizobacterial inoculation by including *Bacillus* sp.-treated cohorts under equivalent stress intensities (YT, YS, YD).

Transcriptome-wide profiling revealed a clear dose-dependent activation of the JmWRKY family in response to escalating drought severity. Relative to the well-watered control, the number of differentially expressed JmWRKY genes increased progressively from 23 under mild stress (15 up-regulated, 8 down-regulated) to 41 under moderate stress (29 up-regulated, 12 down-regulated), reaching 57 under severe stress (38 up-regulated, 19 down-regulated). This graded transcriptional response underscores the sensitivity of WRKY-mediated signaling to the magnitude of water limitation.

Within the drought-responsive subset, several *JmWRKY* genes exhibited pronounced and sustained induction patterns. *JmWRKY17*, *JmWRKY24*, *JmWRKY40*, *JmWRKY49*, *JmWRKY51*, *JmWRKY67*, and *JmWRKY74* displayed incremental upregulation across the stress gradient. Among these, *JmWRKY51* was particularly responsive, with FPKM values increasing 3.7-fold under mild stress, 12.2-fold under moderate stress, and 22.2-fold under severe stress relative to control conditions (Fig. 8; Supplementary Table S5). In contrast, a smaller subset of genes-including *JmWRKY29*, *JmWRKY32*, and *JmWRKY55*-exhibited progressive downregulation as drought intensified, suggesting divergent regulatory roles or negative feedback mechanisms within the family.

The application of exogenous *Bacillus* sp. modulated the drought-induced expression landscape in a stress intensity-dependent manner. Under mild drought, *Bacillus* treatment (YT) was associated with a 60% increase in the number of up-regulated *JmWRKY* genes compared with the corresponding drought-only group (T). Conversely, under moderate (YS) and severe (YD) stress conditions, *Bacillus* inoculation attenuated the magnitude of transcriptional activation, with the number of up-regulated genes decreasing by 31% and 21%, respectively. Notably, the drought-induced hyperactivation of J*mWRKY51* was partially mitigated by *Bacillus* treatment under both moderate and severe stress, with FPKM values reduced by 39.5% and 18.4%, respectively, compared with the corresponding uninoculated drought groups. These observations suggest that rhizobacterial colonization may buffer extreme transcriptional responses, potentially contributing to stress acclimation.

Collectively, these transcriptomic data identify *JmWRKY51* as a highly drought-responsive member of the *J. mandshurica* WRKY family, whose expression is tightly coupled to the severity of water deficit and is subject to modulation by rhizosphere-associated microbiota.

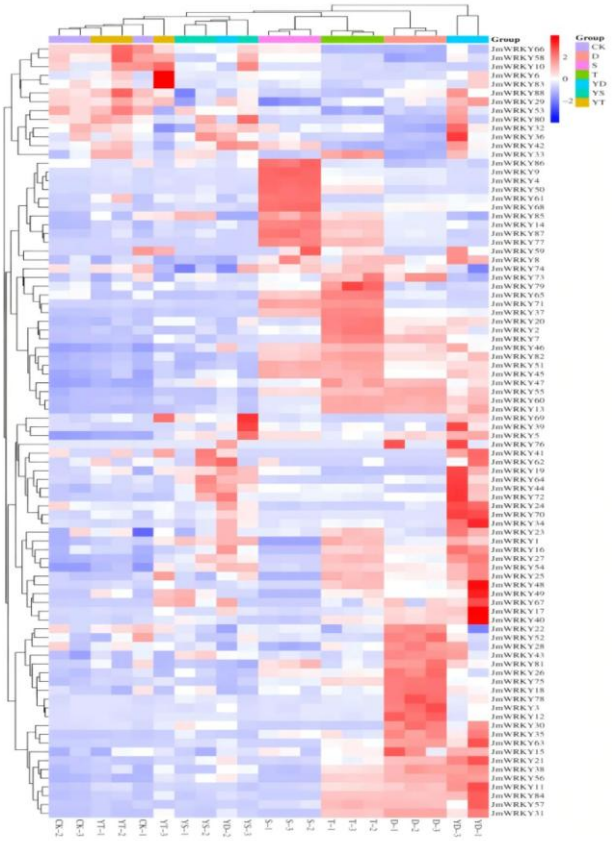


Fig. 8 Transcriptional profiling of *JmWRKY* genes under progressive drought stress. Heatmap depicting FPKM-normalized expression values for 88 *JmWRKY* genes across seven treatment conditions: CK (control), T (mild drought), S (moderate drought), D (severe drought), YT (mild drought + *Bacillus*), YS (moderate drought + *Bacillus*), and YD (severe drought + *Bacillus*). Expression levels are Z-score normalized and color-coded from low (blue) to high (red). Hierarchical clustering was performed using Pearson correlation with average linkage. The complete FPKM matrix is provided in Supplementary Table S5.

3.9 Expression Verification of the Core Gene *JmWRKY51* under Drought Stress

Based on the convergent evidence from transcriptome profiling and PPI network analysis, *JmWRKY51* was prioritized for independent experimental validation using quantitative real-time PCR (qRT-PCR).

Tissue-specific expression profiling revealed that *JmWRKY51* transcripts accumulated to markedly different levels across the five organs examined. Expression was most abundant in stems, where transcript levels significantly exceeded those in all other tissues examined (Fig. 9). Male flower buds exhibited the second highest expression, followed by substantially lower levels in female flower buds, leaves, and fruits. The pronounced expression in vascular-rich tissues (stems) and male reproductive organs suggests a potential role for *JmWRKY51* in water transport regulation and reproductive development under optimal growth conditions.

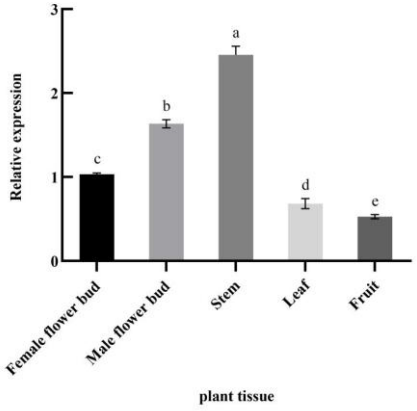


Fig. 9 Tissue-specific expression profile of *JmWRKY51* in *Juglans mandshurica*. Relative transcript abundance was determined by qRT-PCR in female flower buds, male flower buds, stems, leaves, and fruits. Expression values were normalized to *JmActin* and are presented relative to the level in leaves (set to 1.0). Data represent mean ± SD from three biological replicates. Different lowercase letters above bars denote statistically significant differences among tissues (one-way ANOVA followed by Tukey's HSD test, $P < 0.05$).

To recapitulate the drought-responsive expression dynamics observed in the transcriptome dataset under controlled conditions, we monitored JmWRKY51 transcript abundance in seedlings exposed to PEG 6000-simulated osmotic stress across a range of concentrations (10%, 15%, and 20% w/v) over a 24 h time course (Fig. 10). *JmWRKY51* displayed concentration-dependent induction kinetics. Under mild osmotic stress (10% (w/v) PEG 6000), transcript levels rose gradually, peaking at 9 h post-treatment before declining by 12 h and returning to near-basal levels at 24 h. A similar temporal profile was observed under 15% (w/v) PEG 6000, albeit with a more pronounced early induction phase evident at 6 h. In contrast, exposure to severe osmotic stress (20% (w/v) PEG 6000) elicited a rapid and robust transcriptional response, with maximal expression achieved as early as 6 h, followed by a progressive decline at subsequent time points.

Comparative analysis across the three stress intensities revealed that during the early phase of treatment (3-6 h), *JmWRKY51* expression levels were significantly higher under 20% PEG than under the lower concentrations, indicative of accelerated stress perception and signal transduction under severe water deficit. At later time points (12-24 h), transcript abundance declined across all treatments, converging toward baseline levels irrespective of initial stress intensity. Throughout the experimental period, control seedlings maintained consistently low basal expression. Collectively, these results demonstrate that *JmWRKY51* is rapidly and transiently induced by osmotic stress in a concentration-responsive manner, with higher stress intensities eliciting both accelerated and amplified transcriptional activation.

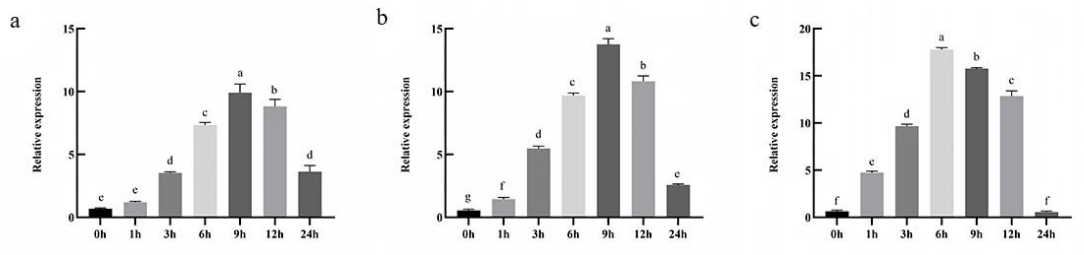


Fig. 10 Time-course expression analysis of *JmWRKY51* under PEG 6000-simulated osmotic stress. **a** Seedlings were treated with 10% (w/v) PEG 6000. **b** 15% (w/v) PEG 6000. **c** 20% (w/v) PEG 6000. Samples were harvested at 0, 3, 6, 9, 12, and 24 h post-treatment. Relative expression levels were normalized to *JmActin* and are expressed as fold change relative to the 0 h control. Data represent mean ± SD from three biological replicates. Asterisks indicate significant differences from the corresponding control at each time point (Student's t-test: $P < 0.05$).

3.10 *JmWRKY51* promoter activity is enhanced by drought stress

To further investigate whether the *JmWRKY51* promoter is responsive to drought stress, a GUS reporter assay was performed using the *JmWRKY51*pro::pCAMBIA1301 construct. Under normal growth conditions, leaves showed relatively weak and diffuse blue GUS staining, with the staining mainly distributed along the vascular tissues and parts of the leaf blade (Fig. 11c).

Following drought stress treatment, GUS staining became visibly stronger and more extensive throughout the leaf tissues, with particularly pronounced blue coloration along the vascular regions and leaf veins (Fig. 11d). Compared with the control condition, the drought-treated leaves exhibited an obvious increase in GUS staining intensity, indicating enhanced activity of the *JmWRKY51* promoter in response to drought stress.

These results are consistent with the transcriptional expression patterns of *JmWRKY51* observed in the transcriptome and qRT-PCR analyses, in which *JmWRKY51* was strongly induced under drought or PEG-simulated osmotic stress. Together with the presence of drought- and hormone-responsive cis-acting elements, particularly the ABRE motif, in the promoter region, the GUS assay provides additional evidence that the *JmWRKY51* promoter is transcriptionally responsive to drought stress.

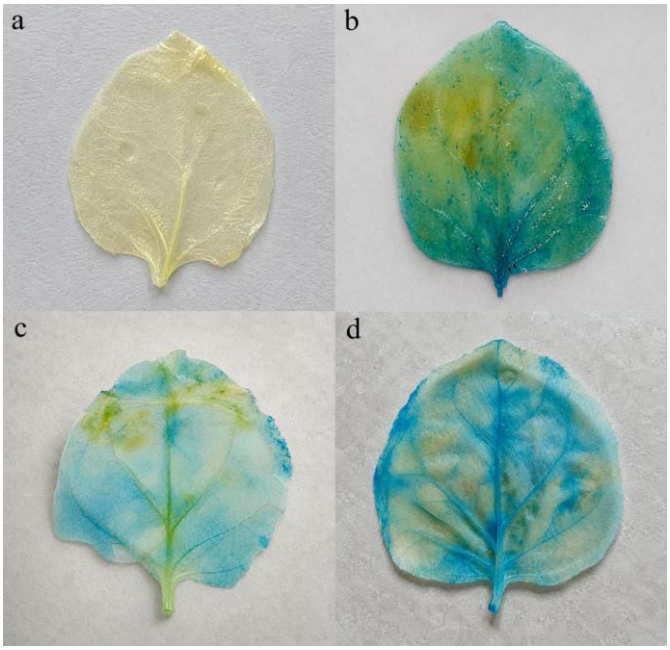


Fig 11. Histochemical analysis of *JmWRKY51* promoter activity under drought stress.**a**, Blank control; **b**, positive control; **c**, GUS staining of the *JmWRKY51* promoter (*JmWRKY51*pro::pCAMBIA1301) under normal conditions; **d**, GUS staining of JmWRKY51pro::pCAMBIA1301 after drought stress treatment.

4 Discussion

The WRKY transcription factor family represents one of the most extensively characterized regulatory gene families in plants, playing important roles in developmental regulation and environmental adaptation (Singh et al. 2002; Eulgem et al. 2000). In this study, we performed a systematic genome-wide characterization of the WRKY repertoire in *Juglans mandshurica*, identifying 89 non-redundant *JmWRKY* genes distributed across 16 chromosomes. This complement is comparable in size to those reported for *Arabidopsis thaliana (*74 members; (Wei et al. 2016)), *Carya cathayensis* (89 members; (Zhu et al. 2025)), and slightly fewer than that of *Juglans regia (*104 members; (Hao et al. 2021)). The relatively conserved WRKY family size among *Juglandaceae* species and the model eudicot *Arabidopsis* suggests that the ancestral WRKY repertoire has been maintained during lineage diversification, with gene gain and loss occurring in a relatively balanced manner (Huang et al. 2019). This evolutionary pattern differs from that observed in species that have experienced recent whole-genome duplication events. For example, tetraploid alfalfa (*Medicago sativa*) contains 198 MsWRKY members, reflecting extensive WRKY family expansion associated with polyploidization (Li XY et al. 2024). The moderate number of WRKY members in *J. mandshurica* may therefore be related to its diploid genome background and evolutionary history, in which the retention and modification of existing regulatory components may have been favored over extensive gene family expansion.

Gene duplication represents an important mechanism contributing to the expansion and diversification of gene families. Within the JmWRKY family, we identified 85 duplicated gene pairs, most of which (88.2%) were classified as segmental duplications, whereas only a small proportion represented tandem duplications. This predominance of segmental duplication is consistent with previous observations in several plant species. In Chinese rose (*Rosa hybrida*), all identified duplication events involving RcWRKY members were attributed to segmental duplication (Yan et al. 2024). Similarly, segmental duplication events were more abundant than tandem duplications in poplar (*Populus* spp.) and apricot (*Prunus armeniaca*) WRKY families (Ma et al. 2025; Jing et al. 2023). These findings support the important contribution of segmental duplication to WRKY family expansion across diverse plant lineages.Furthermore, all duplicated *JmWRKY* gene pairs exhibited Ka/Ks ratios below unity, indicating that purifying selection has played a major role in maintaining the conservation of duplicated genes. This evolutionary constraint suggests that duplicated *JmWRKY* genes may have retained important ancestral functions or undergone partial functional differentiation during evolution.

Promoter cis-regulatory element analysis provides useful information for predicting potential transcriptional regulation patterns (Zhang and Yu 2010). In rubber tree (*Hevea brasiliensis*), stress-responsive gene expression has been associated with the enrichment of light- and hormone-responsive elements in promoter regions (Yang J et al. 2025). Similarly, stress-related motifs identified in the promoter regions of potato genes have provided clues regarding their potential roles in environmental responses (Geng et al. 2025). Functional studies in *Arabidopsis* have further demonstrated that specific promoter elements, including ABRE and MBS motifs, can contribute to drought-responsive transcriptional regulation (Tang et al. 2023).In our study, promoter analysis identified 22 types of hormone- and stress-associated cis-acting elements within JmWRKY regulatory regions. Several genes, including *JmWRKY17*, *JmWRKY85*, *JmWRKY52*, and *JmWRKY62*, exhibited

relatively high densities of stress-responsive elements, suggesting their potential responsiveness to environmental signals. Notably, the promoter region of *JmWRKY51* contained an ABRE motif, which is commonly associated with ABA-responsive regulation (Huang et al. 2023). Together with the drought-induced expression pattern observed in transcriptome and qRT-PCR analyses, these results suggest that *JmWRKY51* may participate in ABA-associated drought response pathways. However, the functional contribution of individual cis-elements requires further experimental validation.

Expression profiling across tissues and environmental conditions provides important evidence for predicting gene functions (Sakuma et al. 2002). Genes displaying tissue-specific expression patterns are often associated with specialized physiological processes. Previous studies have shown that WRKY and other stress-related genes exhibit distinct expression profiles among different plant organs (Dong et al. 2025; Yang S et al. 2025; Gong et al. 2025).In *J. mandshurica*, *JmWRKY51* transcripts were detected in all five examined tissues, including female flower buds, male flower buds, stems, leaves, and fruits, although transcript abundance varied among tissues. The relatively higher expression level observed in stems may indicate a potential association with stem-related physiological processes, including water transport. Combined with its drought-responsive expression pattern and promoter characteristics, these results suggest that *JmWRKY51* may participate in developmental regulation and environmental response.

Dynamic transcriptional responses to abiotic stress are important characteristics of regulatory gene families. Previous studies have identified drought-responsive WRKY members in various plant species. For example, drought-induced expression patterns of *PpWRKY* genes in *Poncirus polyandra* were confirmed by qRT-PCR analysis (Zhang et al. 2025), while *GhWRKY44* showed enhanced expression under PEG and ABA treatments in cotton (Wang et al. 2024).In the present study, transcriptome analysis under different drought intensities identified *JmWRKY51* as a strongly responsive WRKY member, with transcript abundance increasing under drought treatment. qRT-PCR validation further confirmed its induction under PEG-simulated osmotic stress. The concentration-dependent increase in expression suggests that *JmWRKY51* is responsive to drought-related signals. The transient induction pattern observed during stress treatment may reflect dynamic transcriptional regulation during stress adaptation.

The application of exogenous plant growth-promoting rhizobacteria (PGPR), particularly *Bacillus* species, has been widely investigated as a potential approach for improving plant performance under drought conditions (Kuramshina and Khairullin 2023; James and Richards 2006). Previous studies have shown that inoculation with *Bacillus* strains can enhance drought-related physiological responses in different plant species. In maize, inoculation with *Bacillus* strains R60 and R9-1 improved seedling performance and drought tolerance under water-deficit conditions (Li et al. 2021) while fermentation broths of *Bacillus* sp. Y2 and P1 promoted drought resistance and biomass accumulation (Jia et al. 2017). Similarly, *Bacillus subtilis* B26 was reported to alleviate drought stress in timothy grass (*Phleum pratense*) through effects on osmotic adjustment in roots and shoots (Gagné-Bourque et al. 2016).

In the present study, we observed that exogenous *Bacillus* treatment influenced drought-responsive expression patterns of the JmWRKY family in a stress intensity-dependent manner. Under mild drought conditions, *Bacillus* inoculation was associated with increased expression of several *JmWRKY* genes, whereas under moderate and severe drought conditions, the expression levels of some drought-responsive genes, including *JmWRKY51*, were relatively reduced compared with drought treatment alone. These results suggest that *Bacillus* treatment may influence WRKY-associated

transcriptional responses under different drought conditions. The observed expression patterns may reflect differences in stress perception or downstream regulatory responses following microbial treatment; however, the underlying mechanisms remain unclear.

Several possibilities may contribute to the observed transcriptional changes. For example, *Bacillus* treatment may affect phytohormone-related signaling, including pathways associated with auxin and ABA responses, or influence plant physiological status under drought conditions. In addition, PGPR-induced systemic responses may interact with transcriptional regulatory networks involving *WRKY* genes. Further investigations, including hormone profiling, physiological measurements, and functional analysis of candidate genes, will be required to clarify the relationship between *Bacillus* treatment, drought responses, and JmWRKY expression dynamics.

5 Conclusion

In this study, we performed a genome-wide identification and characterization of the WRKY gene family in *Juglans mandshurica*. A total of 89 *JmWRKY* genes were identified and systematically analyzed based on their phylogenetic relationships, conserved motifs, gene structures, chromosomal distributions, promoter cis-acting elements, and duplication patterns. Evolutionary analyses indicated that segmental duplication contributed to the expansion of the JmWRKY family, while purifying selection played an important role in maintaining the conservation of duplicated genes.

Transcriptome analysis and qRT-PCR validation revealed that several *JmWRKY* genes exhibited drought-responsive expression patterns. Among them, *JmWRKY51* showed significant induction under drought conditions and possessed multiple stress- and hormone-responsive cis-elements, suggesting its potential involvement in drought-related regulatory processes. Although further functional validation is required, *JmWRKY51* represents a promising candidate gene for future studies investigating drought adaptation mechanisms in *J. mandshurica.*

Overall, this study provides a comprehensive resource for understanding the composition and evolutionary characteristics of the WRKY gene family in *J. mandshurica* and establishes a foundation for further functional characterization of WRKY-mediated stress responses in woody plants.

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Author Contributions: Conceptualization, C.L., L.Z. and Q.D.; formal analysis, Q.D. and J.L.; investigation, Q.D., X.D. and J.L.; data curation, Q.D.; writing-original draft preparation, Q.D. and J.L. All authors have read and agreed to the published version of the manuscript.

Data availability

The genome sequence and annotation files generated and analyzed during the current study are currently subject to data management and ownership restrictions and are therefore not publicly available. The transcriptomic datasets generated and analyzed in this study are currently being prepared for deposition in an appropriate public repository. Accession numbers and links to the publicly available datasets will be provided upon completion of the data deposition process. Upon reasonable request, additional genome-related information and data may be obtained from the corresponding author.

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: The study was conducted in accordance with relevant government guidelines and regulations. No endangered or protected species were involved in this study. Sampling did not require specific permissions for the study location

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