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Genome-wide identification and expression analysis of the WRKY gene family in *Mikania micrantha*

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Abstract

Background WRKY transcription factors (TFs) regulate plant responses to environmental stimuli and development, including flowering. Despite extensive research on different species, their role in the invasive plant *Mikania micrantha* remains to be explored. The aim of this study was to identify and analyze WRKY genes in *M. micrantha* to understand their function in flowering and adaptation mechanisms.

Results By analysing the whole genome of *M. micrantha*, a total of 77 *M. micrantha WRKY* (*MmWRKY*) genes were identified. Based on phylogenetic relationships, sequence alignment, and structural domain diversity, the *MmWRKY* gene family was preliminarily classified into three major groups and five subgroups: Group I, Group II (II-a, II-b, II-c, II-d, II-e), and Group III. Expression profiles showed tissue-specific expression patterns, with many *WRKY* genes highly expressed in flowers, indicating potential roles in floral development. Real-time quantitative PCR confirmed that the selected 11 genes were highly expressed in floral tissues, supporting their functional significance in flowering.

Conclusion In this study, 77 *WRKY* genes were identified in *M micrantha*, and their phylogenetic relationships, structural domains, and expression patterns across various tissues and organs were comprehensively analyzed. This work provides a foundation for future functional characterization of *WRKY* genes in *M. micrantha*, which may contribute to the development of more effective strategies to control its rapid spread.

Keywords WRKY, Expression profile, Genome-wide identification, Expression patterns, *Mikania micrantha*

Background

WRKY transcription factors are one of the largest families of transcription factors in plants. It is a component that regulates many biological signaling networks in plants. Since the first WRKY transcription factor was isolated from *Ipomoea batatas* in 1994, more and more *WRKY* genes have been identified and studied in other species [1]. WRKY transcription factors often contain a highly conserved region at the C-terminus, the WRKYGQK heptapeptide region, and a C2H2 or C2HC-type zinc finger structural domain [2]. Furthermore, based on the number of structural domains and zinc-finger motif, WRKY proteins can be categorized into three distinct groups: those containing two WRKY domains with one C2H2 zinc-finger motif, Group II, and Group

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III contains one WRKY domain with a C2HC zinc-finger motif [3]. WRKY proteins positively or negatively regulate gene expression by binding to the cis-acting DNA element W-BOX in the promoter region of target genes, affecting different developmental programs [4, 5].

WRKY TFs have been confirmed to participate extensively in various pathways involved in plant responses to different biotic, abiotic, and hormonal signals, thereby influencing plant growth and development [6]. In rice, *OsWRKY45* regulates the salicylic acid signaling pathway by binding to the W-box of the gene promoter [7]. *AtWRKY25*, *AtWRKY26*, and *AtWRKY33* could enhance the tolerance of transgenic *Arabidopsis thaliana* to high-temperature stress by regulating the expression of the *Hsp101* and *Zat10* genes [8]. WRKY TFs are essential for the resistance of chrysanthemum to salt stress. *CmWRKY17* may function as a transcription factor to negatively regulate salt stress in transgenic plants [9]. Biotic stresses include pathogen infestation and herbivorous insect feeding, in which WRKY TFs play an essential role. Furthermore, WRKY TFs are also vital for several signal transduction processes mediated by phytohormones such as abscisic acid (ABA) and ethylene [10, 11]. *AtWRKY23* can modulate root growth by regulating the distribution patterns of growth hormones [12]. When plants respond to complex environmental changes, they often regulate flowering and reproductive capacity to cope with environmental stress. In plants, flowering time is determined by the complex interaction of endogenous and exogenous signals [13]. In previous studies, many transcription factors have been shown to be involved in flowering-related processes in plants, such as *AP1*, *MADs*-BOX, *FLOWERING LOCUS C* (*FLC*), *WRKY* [14–16]. Among them, WRKY proteins also play crucial roles in plant growth and flowering [17]. In Arabidopsis, *WRKY71* was shown to bind to W-box motifs within both the *FT* and *LFY* promoters, both in vivo and in vitro, facilitating flowering through their interaction [18]. *ATWRKY12* and *ATWRKY13* inversely regulate flowering time under short-day(SD) conditions [19]. The overexpression of *CpWRKY71* induced premature flowering and early leaf senescence in Arabidopsis [20]. *ATWRKY63* is a significant flowering regulator that regulates vernalization-induced flowering through transcriptional activation of *COOLAIR* and *COLDAIR* [21]. In *Reaumuria trigyna*, *RtWRKY23* not only increases salt stress tolerance by maintaining the balance of ROS and osmosis in the plant but also participates in the regulation of flowering under salt stress [22].

Although WRKY TFs have been characterized in Arabidopsis [23], their roles in *M. micrantha* Kunth (the Asteraceae family) are still unknown. *M. micrantha* is a noxious invasive plant known as the "mile-a-minute weed" [24]. It is a rapidly fast-growing perennial vine native to tropical America and has widely spread to Asia [25]. *M. micrantha* was listed as one of the 100 worst invasive plants by the International Union for Conservation of Nature (IUCN) in 2000 [26]. It is invasive in tropical Asia, parts of Papua New Guinea, the Indian Ocean, and the Pacific Islands, where it can destroy ecosystems by twinning and smothering other plants, preventing photosynthesis in cash crops and commercial forests [27]. *M. micrantha* shows high morphological plasticity and adaptability to different environments [28]. Moreover, *M. micrantha* has a strong allelopathic effect on other plants, releasing chemicals into the environment and thus affecting the growth and development of other species in the vicinity [29]. Rapid invasion of *M. micrantha* may seriously affects local biodiversity, nutrient cycling, and soil structure [30]. In the past 20 years, *M. micrantha* has been controlled mainly by different means, such as mechanical, chemical, biological and ecological methods [31–34]. However, these ways have not fundamentally prevented weeds. The primary reason was that *M. micrantha* has a large number of flowers and can propagate sexually through thousands of lightweight seeds [35, 36]. Therefore, it is essential to investigate the flowering mechanism of *M. micrantha* to determine ways to prevent it. Recently, the multi-organ transcriptome data of *M. micrantha* have been released [37], providing a valuable resource for analyzing the flowering-related genes in *M. micrantha*. Since the *WRKY* gene family plays a significant regulatory role in all stages of plant growth, especially flowering, a systematic analysis of the *WRKY* gene family in *M. micrantha* would be a meaningful research direction. In this study, we performed the first comprehensive analysis of the *WRKY* gene family in *M. micrantha*. A total of 77 *MmWRKY* gene family members were identified using the *M. micrantha* genome and bioinformatics analyses for phylogenetic inference, gene structure, motif composition, chromosomal location, Cis-acting elements of the promoter and covariance. We also investigated the expression patterns of *MmWRKY* genes in various tissues and organs. In addition, the expression pattern of the *MmWRKY* gene in inflorescence tissues at different times was determined by qRT-PCR (Quantitative Real-time PCR) to identify the genes with the highest expression in floral organs. This study provides valuable clues for the functional identification of *M. micrantha WRKY* gene family members, laying the foundation for future studies on the relationship between *WRKY* genes and flowering function. In addition, understanding the ecological consequences of *M. micrantha* invasion and the mechanism of its rapid growth is crucial for developing effective management strategies and restoring affected ecosystems.

Results

Identification and characterization of *WRKY* genes in *M. micrantha*

This study identified 81 *WRKY* candidate genes during the characterization process based on the annotation files of the *M. micrantha* genome. Genome annotation files were further checked using available *M. micrantha* transcriptome data, and four candidate *MmWRKY* genes with incomplete structural domains were eliminated. Finally, 77 *MmWRKY* transcription factors with intact structural WRKY domains were selected. They were renamed *MmWRKY1* through *MmWRKY77* to reflect their position on the chromosome.

Based on the complete amino acid sequences of these 77 MmWRKY proteins, all MmWRKY proteins were analyzed for CDS length, protein molecular weight (MW), isoelectric point (pI), and other genetic features (Additional file 1). Among the 77 MmWRKY proteins examined, MmWRKY20 was the most abundant protein, comprising 174 amino acids (aa), while MmWRKY15 was the largest protein, with 725 amino acids (aa). The molecular weights (MWs) ranged from 20.0 kDa to 81.4 kDa, and their pIs ranged from 4.95 to 9.95. Predicted subcellular localization analysis indicated that 73 MmWRKY proteins were localized in the nucleus.

Construction of a phylogenetic tree and multiple sequence alignment of *MmWRKY*

To determine the subfamily classification of the WRKY family members of *M. micrantha*, a neighbor-joining phylogenetic tree was constructed based on multiple alignments of the conserved regions of Arabidopsis, *H. annuus*, *L. sativa* and *M. micrantha* (Fig. 1). Number of structural domains and phylogenetic analysis revealed that all the MmWRKY proteins could be categorized into three subgroups: group I, II, and III. Group I had 17 members, Group II had 49 members, and Group III had 11 members. Group II proteins can be further categorized into II-a, II-b, II-c, and II-d, of which II-c had the largest number of members [4].

Multiple sequence alignment of the structural domains of the MmWRKY protein revealed that a representative structure, the WRKYGQK conserved region, is present in most WRKY proteins (Additional file 3). This structural domain can interact with the W-box (TTGACC /T) and is involved in many physiological processes and plant immune responses. Among the 77 MmWRKY proteins, 74 members had a conserved "WRKYGQK" structural domain, and two members had variations in the conserved heptapeptide structural domain. These variations were observed in MmWRKY11 and MmWRKY50, which contained "WRKYGEK" instead. Two zinc finger elements, C2H2-type (C-X4-C-X22-23-H-X-H) and C2HC-type (C-X7-C-X23-H-X-C) were contained in the *M. micrantha* WRKY protein. Among the three subfamilies, group I contains two intact WRKY structural domains and a C2H2-type zinc finger motif, group II proteins contain a WRKY structural domain, and a C2H2-type zinc finger structural domain, and a WRKY structural domain and a C2HC-type zinc finger structural domain were found in group III.

Analysis of the gene structure, conserved motifs, and cis-acting elements of *MmWRKY*s

77 MmWRKY proteins were identified by motif identification and analyzed for motif composition using the MEME online website. The analysis revealed that members of the same subfamily exhibited similar conserved motifs (Fig. 2A, Additional file 4). In group I, family members contained two WRKY structural domains, whereas the Group II and Group III subfamilies contained only one WRKY structural domain (Fig. 2B). In addition, group II-d also contains the plant zinc structural domain. All members of group I contained motifs 3 and 5, with motif eight being present only in groups II-a and II-b, suggesting that these two subfamilies may share similar functions. In addition to II-b, II-d also contains motif 9, with members of subfamily II-e containing a unique motif 10. Motif two is present in all family members, suggesting that it may be a component of the WRKY structural domain.

In this study, we explored the potential biological roles of the *MmWRKY* genes by analyzing cis-acting elements in the 2000 bp upstream promoter regions. Analysis shows that most members of the *MmWRKY* gene family have a light-cycle response element (G-box), with 2,220 such elements identified (Fig. 3). Additionally, elements related to biotic and abiotic stress response were detected, including responsiveness elements for low temperatures, anaerobic induction elements, and wound-responsive elements. MeJA-responsiveness elements (TGACG-motif) were detected in 62 family members, and 41 family members contained growth hormone response elements. 16 *MmWRKY* genes contained plant development-related response elements, such as seed-specific regulation (CATGCATG-motif), root specific regulation (gGTACGTGGCG-motif), and circadian control elements (CAAAGATATC-motif).

Chromosome localization and synteny analysis of *MmWRKY*s

To determine the distribution of the *MmWRKY* genes in the whole genome of *M. micrantha*, the chromosomal localization of 77 *MmWRKY* genes was visualized using TBtools software (Fig. 4A). Based on their positions on chromosomes 1-18, they were named

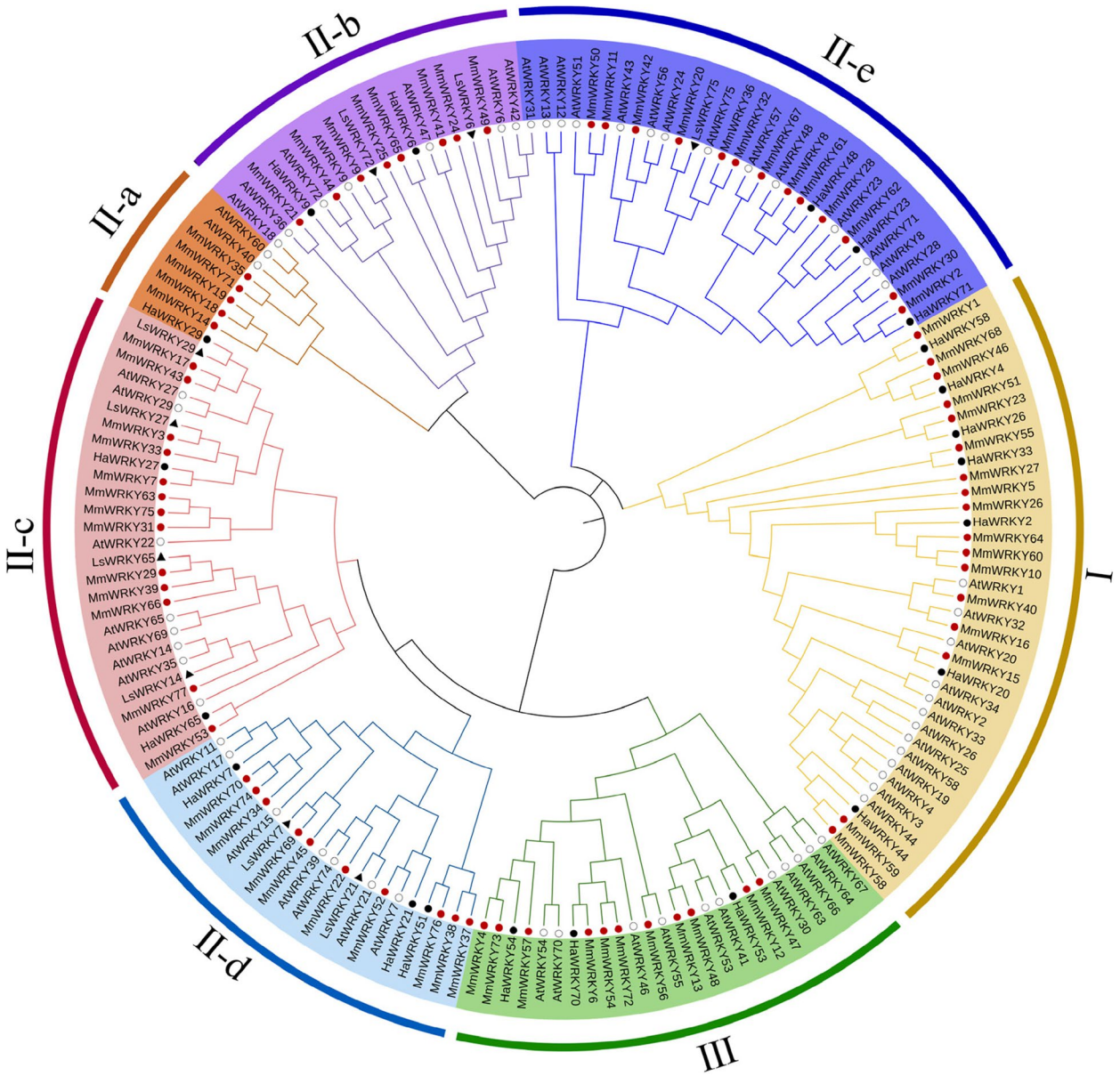

Fig. 1 A rootless phylogenetic tree of *M. micrantha*, *H. annuus*, *L. sativa* and *A. thaliana* WRKY proteins. MEGA 11.0 software was used to construct the unrooted neighbor-joining phylogenetic tree, and the bootstrap test replicate was set as 1000 times. Different colored blocks indicate different subfamilies of WRKY structural domains. The red circles represent the members of the WRKY proteins of *M. micrantha*. The white circles are from Arabidopsis WRKY proteins, the black circles are from sunflower WRKY proteins, and the triangles are from lettuce WRKY proteins

MmWRKY1-77, and *MmWRKY* was unevenly distributed on each chromosome, with the number of genes on each chromosome not correlated with chromosome length. Chromosome 7 had the largest number of *MmWRKY* genes, with eight, of which there were two in the *MmWRKY* gene subfamily I, and six in family II, including two members in subfamily I and six members in subfamily II. Chromosome 15 contained two *WRKY* genes, whereas only one member was distributed on chromosome 10. *MmWRKY72*, *73*, *74*, *75*, *76*, and *77* were not assigned to any of the chromosomes.

Among the 77 *MmWRKY* genes, collinearity analysis showed that they were unevenly distributed on 18 chromosomes except LG 15 of *M. micrantha* (Fig. 5, Additional file 5). Collinearity analysis revealed 18 duplication events in the *MmWRKY* gene family, with chromosome 7 showing the largest number of duplication events. Among them, there were more genes distributed on LG7,

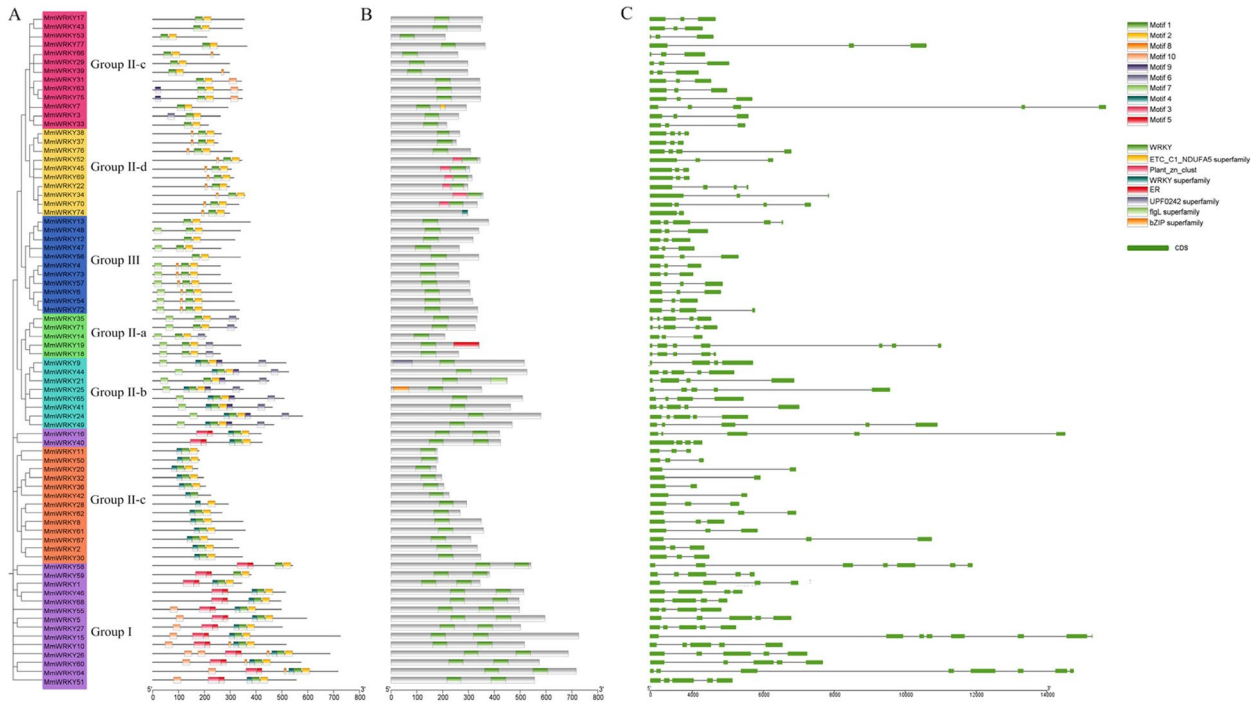

Fig. 2 Conserved protein motif structure and gene structure of the *M. micrantha WRKY* gene. **A** Conserved motifs of 77 MmWRKY proteins, with different colored boxes indicating motifs 1–10, and the conserved motifs were predicted using TBtools software. **B** MmWRKY structural domains, with WRKY structural domains in green. **C** Intron-exon structures of 77 members of the *M. micrantha* MmWRKY protein. Exon-intron structures were determined using TBtools software. Green squares indicate exons of genes. The Intron structures are not shown in the figure because introns are not labelled in the annotation file

while there were few genes on LG11, 12,14,4 and only one gene.

To further analyze the phylogenetic mechanism of the *WRKY* gene family in *M. micrantha*, we constructed a species collinear relationship between *M. micrantha* and *A. thaliana* (Fig. 4B, Additional file 6). The results showed that 46 *MmWRKY*s exhibited a syntenic relationship with *AtWRKY*s. For example, *AT2G23320.1* showed a covalent relationship with *MmWRKY1* and *MmWRKY69*, and *AT5G56270.1* showed a syntonic relationship with *MmWRKY10* and *MmWRKY26*.

1197 gene pairs were identified in the *MmWRKY* gene family using DupGen-Finder software to characterize the gene duplication events in *M. micrantha*. To examine and assess the evolutionary selection pressure of the *MmWRKY* genes, we calculated the Ka, Ks, and Ka/Ks ratios between duplicated gene pairs (Additional file 7). The results showed that the Ks values between all *MmWRKY* gene pairs ranged from 0.03 to 3, and all members had a Ka/Ks < 1.

Tissue-specific expression patterns of *MmWRKY*s

In order to compare the expression profiles of *WRKY* genes among different tissues of *M. micrantha*, the expression patterns of 77 *MmWRKY* genes were investigated in data from the transcriptomes of different organs of *M. micrantha* (flowers, roots, stems, and leaves). In this study, the TPM value of each *MmWRKY* gene was calculated to indicate its expression level in different organs. The results showed that the expression of most *MmWRKY* genes were higher in stems than in other tissues. There was a significant trend in the expression of some genes in different organs (Fig. 6A) 76 genes were expressed in at least one tissue, and *MmWRKY56* was not expressed (COUNT < 0).

MmWRKY71 and *MmWRKY12* were highly expressed in four tissues, indicating that they may play a role in the growth and development of *M. micrantha*. Among these genes, *MmWRKY37* was highly expressed in flowers, while its expression in other organs was not significant, suggesting that it may play a part in the flowering stage of the plant. *MmWRKY49* and *MmWRKY59* had low expression in all organs, and they may be expressed in other organs or only when subjected to stress. In all tissues, the fewest *MmWRKY* members were expressed in stems, and the most were expressed in flowers and roots. For genes of different subfamilies, only subfamily II-a was expressed in all tissues. These results suggest that *MmWRKY* has different expression levels in the

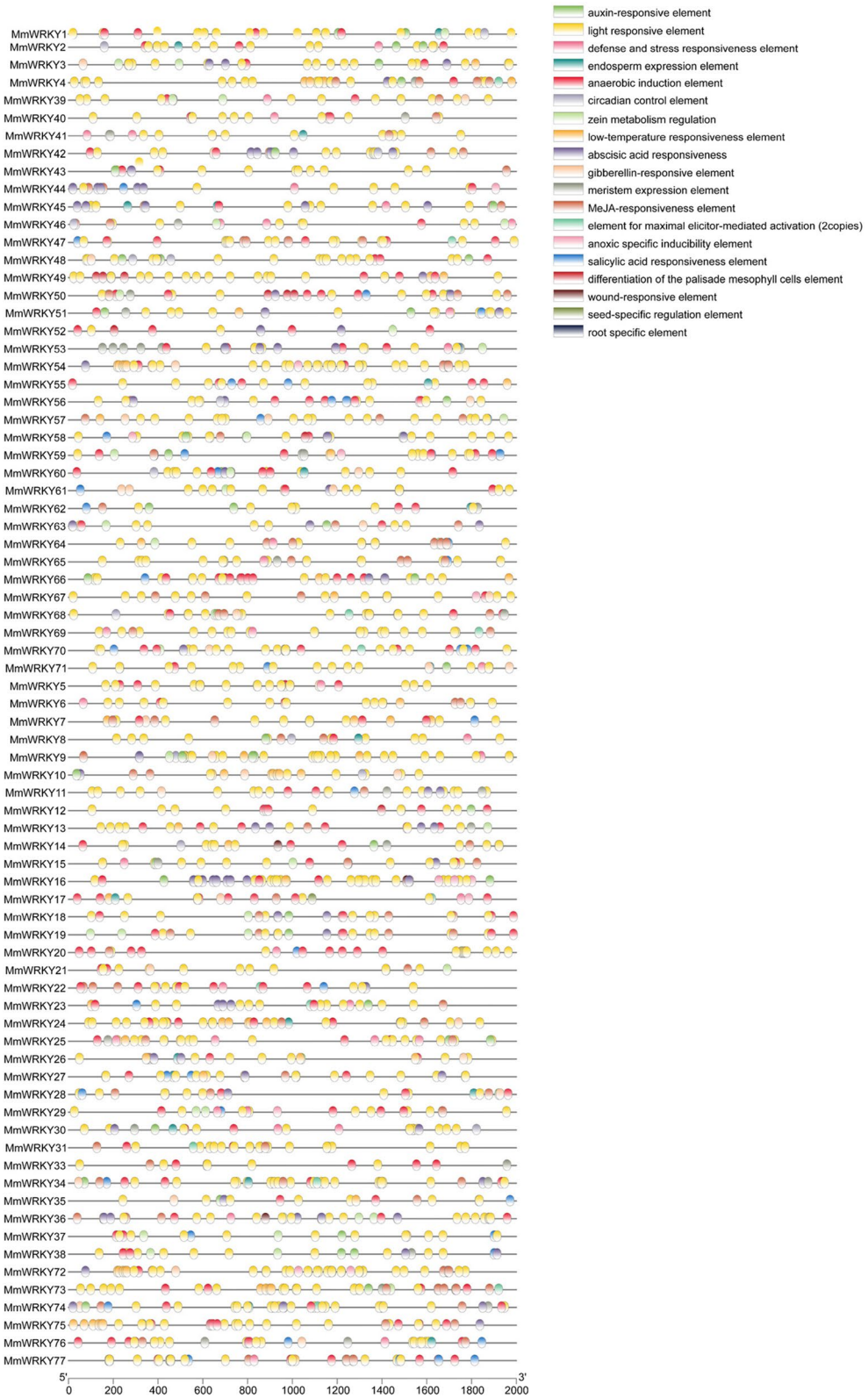

Fig. 3 Cis-acting elements of the promoter sequence of 77 *MmWRKY* genes in *M. micrantha*. Scale bar indicates gene length (bp). The cis-regulatory elements commonly found in WRKY genes are represented by differently colored squares

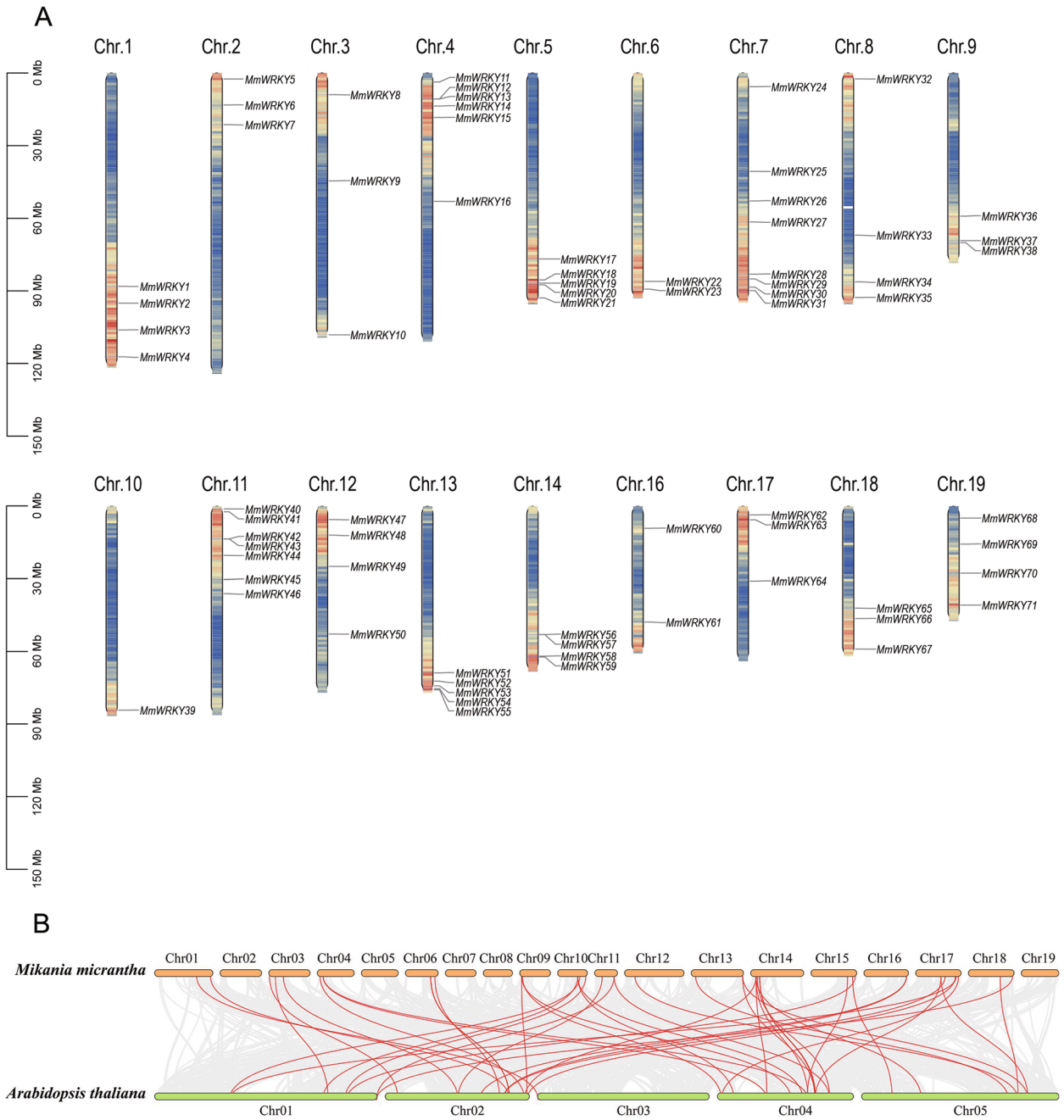

Fig. 4 Chromosomal distribution of the *M. micrantha WRKY* gene family. **A** Different colors represent the density of genes on the chromosome. The putative *WRKY* genes from *MmWRKY1-MmWRKY71* were renamed according to their chromosomal location. Six genes were not assigned to any chromosome. Chromosome numbers are given at the top of each chromosome, and the left side of each chromosome is related to the approximate physical location of each *WRKY* gene. The higher the gene density, the redder the chromosome color. **B** *A. thaliana* and *M. micrantha WRKY* covariance analysis. Red lines indicate collinearity relationship between the *M. micrantha* and *A. thaliana WRKY* genes

same organs and that genes of the same subfamily may also have different expression profiles.

To investigate the tissue-specific expression patterns of *MmWRKY* genes, we carefully selected 11 of the 77 *M. micrantha WRKY* genes. Our selection criteria aimed to include genes with relatively high mRNA levels across different tissues and genes that are highly expressed in all flower organs. The expression patterns of *MmWRKY* genes in four tissues (root, stem, leaf, and flower) were further examined by qRT-PCR

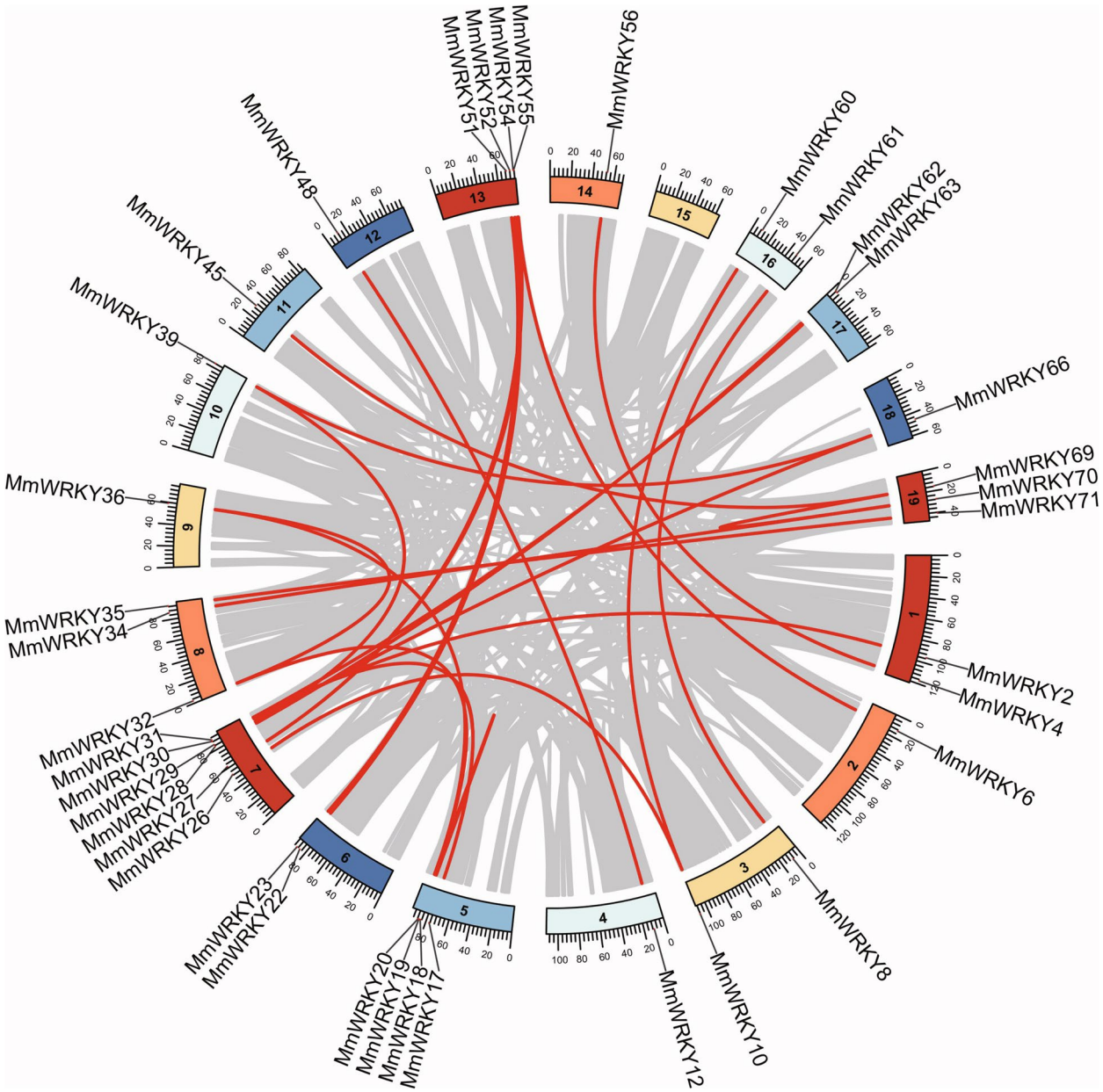

Fig. 5 Duplication analysis of *MmWRKY* genes on 18 chromosomes. The gray lines indicate all synteny blocks in the *M. micrantha* genome, and the red lines link *MmWRKY* genes in a collinear relationship

(Fig. 6B). qRT-PCR analysis revealed that many of the *MmWRKY* genes are highly expressed in floral tissues, suggesting their critical roles in flower development, flowering regulation, and reproductive processes. *MmWRKY37* showed significant expression in leaves, indicating a potential role in leaves germination. And the results were broadly consistent with the transcriptome results.

(See figure on next page.)

Fig. 6 Tissue-specific expression of the *MmWRKY* gene in five organs of *M. micrantha*. **A** Transcriptomic data examined the expression levels of *MmWRKY*s in root, stem, leave, and flower. The TPM values were transformed to log2 (value + 1). The color scale is shown at the right, and higher expression levels are shown in red, the lower expression levels are shown in green. **B** The relative expression of 11 *MmWRKY* genes was analysed by qRT-PCR in four tissues (root, stem, leaf and flower). Data were normalised to the relative expression of the genes in the roots, and the vertical bars indicate the standard deviation. Asterisk indicates a statistically significant difference compared to the control group (* $p < 0.05$, ** $p < 0.01$)

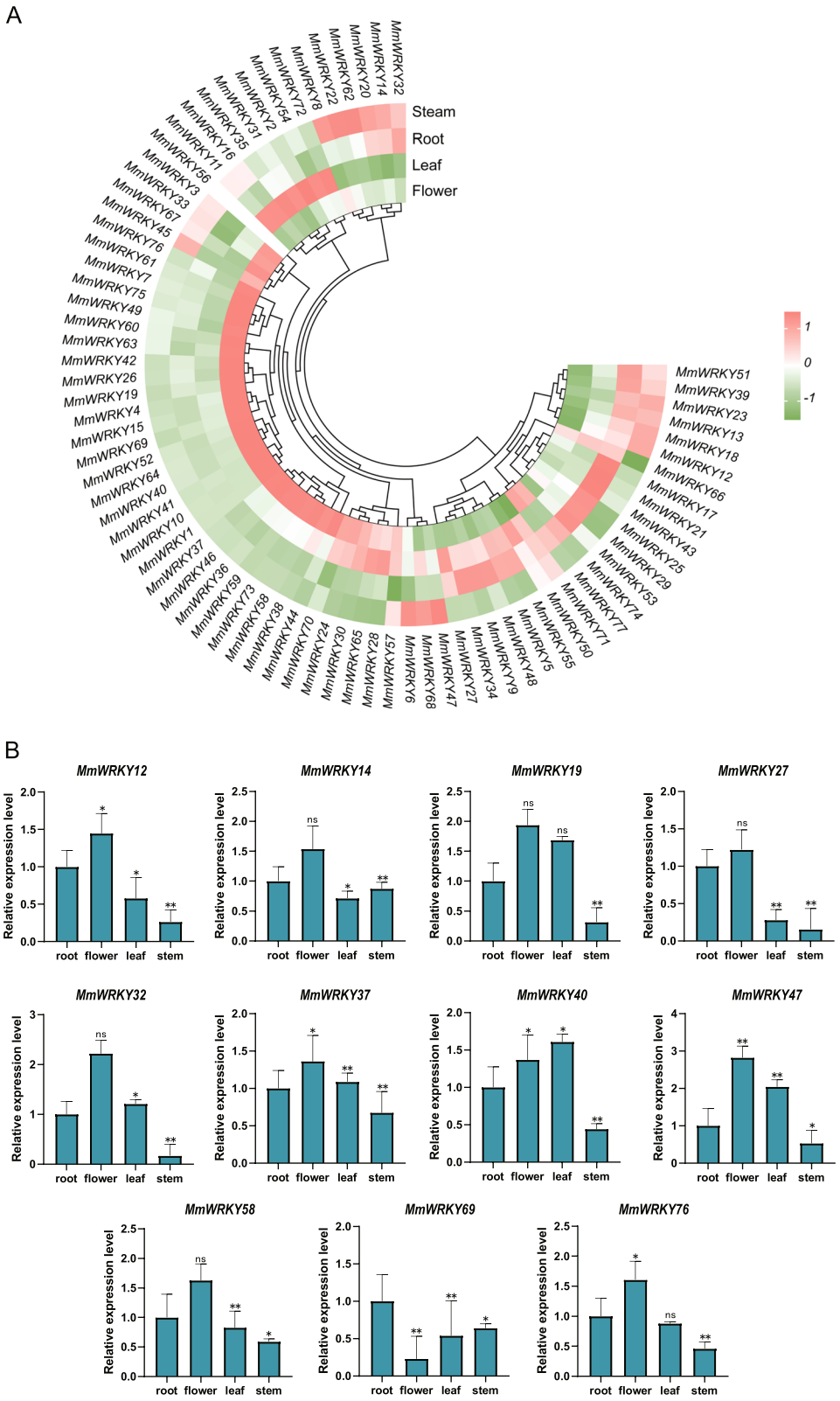

Fig. 6 (See legend on previous page.)

Discussion

Throughout evolution, the WRKY transcription factor family has been extensively implicated in various biological processes in plants, playing pivotal roles in plant growth, development, and secondary metabolism. In previous studies, WRKY TFs have been extensively characterized at the genome-wide level in various plant species, including rice [23], asparagus [38], and strawberry [39]. In this study, 81 members were identified, of which incomplete gene sequences were removed, resulting in 77 members of the *MmWRKY* gene family, and renamed *MmWRKY1-MmWRKY77* according to their position on the chromosome (Fig. 4A).

Different subfamilies within the WRKY transcription factor family often exhibit diverse functions in response to plant stress, which may be related to the number of WRKY structural domains and the type of zinc finger motifs [3]. In this study, the *MmWRKY* family was categorized into three subfamilies, with 17 members in subfamily I, 49 in subfamily II, and 11 in subfamily III. Group II was divided into II-a, II-b, II-c, II-d, and II-e based on the primary amino acid structure. However, subsequent studies have shown that among higher plants, the *WRKY* gene family is more accurately classified as Group I, II a +II b, II c, II d+ II e, and III. The Group II genes are not monophyletic [40]. The number of gene members of the subfamily II is usually found to be the highest in most studies. And the least of them, subfamily II-a, seems to play a crucial role in the regulation of biotic and abiotic stress responses [3]. In Arabidopsis, *WRKY40* interacts with *GSH-MPK3* and co-regulates the stress response in Arabidopsis under low temperature stress [41]. These suggest that subfamily II-a of *M. micrantha* may also be involved in the response to external stresses.

The results of protein sequence alignment in this study showed that five members from group II had sequence variation in the WRKY structural domain (*MmWRKY11*, *37*, *38*, *50*, *76*, Additional file 2). Through multiple sequence alignment, it was found that the WRKYGQK motif in *WRKY37*, *38*, and *76* had mutated to WKKYGEK, while in *WRKY11* and *WRKY50*, it had mutated to WRKYGKK. These variations in the WRKY domain may lead to functional differences among them. Previous research has indicated that this variation could potentially impact the normal interaction between *WRKY* genes and their downstream target genes [42]. Deletions in the WRKY domain have been identified in various plant species, with the WRKYGKK sequence being the predominant type of variation [43, 44]. Studies have demonstrated that the tobacco *NtWRKY12* gene with the WRKYGKK variant cannot interact with the canonical W-box sequence but instead recognizes and binds to an alternative "TTTTCCAC" sequence [45]. Wu [46] identified seven *WRKY* gene family members in the rice genome that harbor WRKYGKK variants. These variants were postulated to have arisen from evolutionary variations in the ancestral WRKY transcription factors in rice over an extended period. Furthermore, the zinc finger structural regions in specific *MmWRKY* genes, such as *MmWRKY38* and *MmWRKY74*, exhibit deletions in some structural domains. Previous studies have indicated that mutations occurring at the N-terminal end of zinc finger-like motifs can potentially diminish or altogether abolish DNA-binding activity [47]. Therefore, further studies on the variation of the WRKYGQK and WRKYGKK heptapeptide and zinc finger motifs in the variant *MmWRKY* genes and their function in regulating downstream gene targets and their expression patterns are necessary.

The diversity of intron-exon structures plays a crucial role in gene evolution [48]. In this study, the intron count in *M. micrantha* ranged from 2 to 8 (Fig. 2C). While Group I exhibited an average of five introns, Group II-b and Group III had averages of four and three introns, respectively. These findings suggest that group II and III may have originated from ancestral group I during evolution. Analyzing the promoters of *M. micrantha* can provide insights into the mechanisms regulating gene expression (Fig. 3). The prediction of cis-acting elements in the *MmWRKY* promoter regions revealed four significant types of responsive elements: light-responsive, hormone-responsive, growth, development-responsive, and temperature-inducible elements. Light and hormone-responsive elements were the most abundant, with 2220 and 660 elements, respectively. Notably, *MmWRKY2*, *4*, *17*, *46*, *50*, *53*, *and 27* contained elements related to seed development and root growth, suggesting that they may be directly or indirectly involved in the process of seed germination. Additionally, *MmWRKY71*, *24*, *26*, and *34* contained endosperm expression elements, indicate potential roles in seed endosperm development.

M. micrantha exhibits a clustering pattern of *WRKY* genes on its chromosomes, similar to the findings in *A. thaliana* and rice. Previous studies have proposed that gene clusters represent groups of genes residing within a 2 Mb nucleotide unit [49]. Notably, the largest gene cluster within the *MmWRKY* gene family encompasses five genes scattered across chromosomes 4, 5, and 11. This observation suggests a potential hotspot for *WRKY* gene clustering on these specific chromosomes, implying a significant occurrence of large-scale gene duplication at this locus during the long-term evolutionary process. Consequently, this phenomenon may be attributed to such a high density of *WRKY* genes in this genomic region. Gene duplication events serve as significant drivers of plant evolution, contributing to genome rearrangements

and amplifications, including whole genome duplication (WGD), tandem duplication (TD), proximal duplication (PD), transposed duplication (TRD), and dispersed duplication (DSD) [50]. These events facilitate the rapid adaptation of plants to diverse environmental changes and facilitate the acquisition of novel functional traits through the allocation of genetic material [49, 51]. Fragment duplications have been reported to be crucial in the expansion of the WRKY family in species such as *Scutellaria baicalensis* [52], *Neolamarckia cadamba* [53], and *Camellia oleifera* [54]. The analysis revealed 18 segmental duplication events among the 77 *MmWRKY* genes, indicating that a substantial portion of the *M. micrantha* (Fig. 5). *WRKY* gene family originated from chromosomal regions or whole chromosome duplications. These homologous gene pairs may have originated before species divergence, suggesting a conserved functional role. Furthermore, the Ka/Ks ratios of all homologous *MmWRKY* genes were consistently less than 1, suggesting evolutionary solid selection pressure. It has been shown that gene duplication events cause species to specialize in different tissues or stages of development (Additional file 7). Duplicated genes are more likely to have different expression profiles between genomes than single-copy genes. This suggests that in *M. micrantha*, genes that undergo duplication events may be involved in different functional regulatory mechanisms and may be involved in regulating gene expression at different stages of *M. micrantha* growth.

It has been shown that *WRKY* genes are usually explicitly expressed in various tissues and regulate plant growth and developmental processes [55]. Investigating gene expression patterns in different tissues is essential for mining functional genes. In this study, we performed RNA-seq analysis to investigate the expression patterns of 77 *MmWRKY* genes in different tissues of *M. micrantha*, including roots, stems, leaves, and flowers (Fig. 6A). Our results revealed a strong correlation between *MmWRKY* genes and the growth and developmental processes of *M. micrantha*, as most of these genes were expressed in all four tissues. Using phylogenetic and homology analyses and a comparative study with known *WRKY* genes in model plant and related species, we elucidated the potential biological functions regulated by *WRKY* genes in *M. micrantha* growth and development. For example, *AtWRKY41* induces early flowering as a positive regulator in *A. thaliana* [56]. In contrast, its homologous gene, *MmWRKY47*, is highly expressed in flowers, suggesting the potential value of *MmWRKY47* in flowering regulation in *M. micrantha*. *WRKY71* is most highly expressed in flowers and senescent leaves, and its heterologous expression leads to premature leaf senescence and early flowering in plants [18], whereas *MmWRKY37*, which is phylogenetically closely related to this gene, may have a similar function. Overexpression of *WRKY51* increased the seed germination rate, root length, photosynthetic rate, and chlorophyll content in plants subjected to salt stress [57], suggesting that *MmWRKY37*, a homologous gene of *WRKY51*, may be involved in the plant growth and development. According to previous studies, heterologous overexpression of *WRKY* genes usually leads to abnormal plant phenotypes or affects the reproductive process, such as Miscanthus *MIWRKY12* [58], wintersweet *CpWRKY75* [59], and cotton *GhWRKY27* [60]. Similar examples can also be found in other species; for example, strawberry *FvWRKY71* [61] overexpression caused early flowering of plants while overexpressing *VvWRKY37* [62] in poplar plants retarded germination. Overexpression or knockout experiments can be performed in subsequent studies to further investigate the effects of these genes on flowering and reproductive development in *M. micrantha*.

In addition, some *MmWRKY*s were expressed in almost all four tissues, so we selected 11 *WRKY* genes for qRT-PCR experiments. The results showed that all 10 *MmWRKY* genes, except *MmWRKY69*, were highly expressed in flowers (Fig. 6B). It can be inferred that genes with high expression levels in the roots may contribute to the ability of invasive plants to adapt to different ecological niches. Similarly, high expression in flowers indicates that these *MmWRKY* genes may play a role in the formation, flowering and senescence of floral organs, thereby enhancing the efficiency of *M. micrantha* in rapid reproduction and accelerating the rapid spread of its seeds. This study provides the first comprehensive genomic analysis of *WRKY* genes in the invasive species *M. micrantha* and reveals the tissue-specific expression patterns of *WRKY* genes in an invasive environment. The identification of *MmWRKY* genes that are highly expressed in floral organs ultimately provides new insights into the analysis of *M. micrantha*'s role in environmental adaptation. These findings provide the basis for targeted genetic or molecular interventions to better manage *M. micrantha* invasions.

Conclusion

In this study, 77 *MmWRKY* genes were identified from the genome of *M. micrantha*, which were classified into seven subfamilies based on phylogenetic analysis and unevenly distributed on 18 chromosomes. Gene structure, conserved motifs, collinearity analysis, and promoter cis-acting elements were analyzed, revealing that *MmWRKY* genes possess highly conserved structures, with members of the same subfamily often sharing similar gene structures. The analysis of promoter cis-element showed that the promoter of *MmWRKY* contains a large

number of light-responsive elements and hormone-responsive elements. In addition, the expression pattern of *M. micrantha* was analyzed in five different organs, and different *MmWRKY* genes were highly expressed in specific tissues. We selected 11 genes were highly expressed in flowers for qRT-PCR analysis to infer the potential roles of *MmWRKY* genes in floral organs. In order to better understand the diversity of *MmWRKY* gene functions, subsequent studies can also use overexpression or knockout transgenic plants to further verify growth or flowering-related functions. Our study will help elucidate the role of *MmWRKY* genes in growth and development and identify a new method for preventing and controlling *M. micrantha*.

Materials & methods

Identification of the WRKY proteins in *M. micrantha*

Whole-genome data of *M. micrantha* (GCA_009363875.1) were obtained from NCBI (https://www.ncbi.nlm.nih.gov/). Arabidopsis *WRKY* sequences were downloaded from the Arabidopsis Information Resource (TAIR) (https://www.arabidopsis.org/) Database. Using the TBtools program (v2.019), CDSs, and protein sequences were extracted from the whole-genome sequence of *M. micrantha*. The Arabidopsis *WRKY* nucleic acid sequence was used as a query sequence to identify the homologous *MmWRKY* sequence using the TBtools program. BLAST search of the obtained *M. micrantha WRKY* sequences in the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed using BLASTp. To identify all potential sequences of *MmWRKY* genes, we downloaded the HMM profile of the domain from the Pfam database with an e-value < 0.00 and other uses the default parameters. Submission of candidate MmWRKY protein sequences to the NCBI Web CD-Search Tool (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi), Pfam database (http://pfam-legacy.xfam.org/), and SMART (https://smart.embl.de/smart/batch.pl) was performed to search for and identify conserved WRKY protein structural domains (All settings are default parameters). After excluding *WRKY* genes that did not contain the WRKYGQK and zinc finger motif domains, the remaining WRKY sequences were placed into the ExPASy (https://www.expasy.org/) database for analysis, to estimate physicochemical parameters such as molecular weight (MW), isoelectric point (PI) and number of amino acids for each gene product. Subcellular localisation of *WRKY* genes performed using the WoLF PSORT (https://wolfpsort.hgc.jp/) online website.

Multiple sequence alignment and phylogenetic analysis of *MmWRKY* genes

A total of 77 *MmWRKY* genes containing complete structural domains were identified and sequentially numbered according to their location on the scaffold multiple sequence alignment of *WRKY* transcription factors between Arabidopsis and *M. micrantha* using ClustalW (https://www.genome.jp/tools-bin/clustalw). The protein sequences of related species of *M. micrantha WRKY* gene were also obtained by BLAST-p comparison in NCBI. Subsequently, phylogenetic trees were constructed using MEGA11 with default parameters, and 1000 bootstrap replicates were constructed using the neighbor-joining (NJ) method. Finally, NJ phylogenetic trees were constructed for the full-length sequences of WRKY proteins from *M. micrantha* and Arabidopsis, *Helianthus annuus*, and *Lactuca sativa*.

Analysis of gene structure conserved motifs, and cis-acting elements of *MmWRKY*s

Motif analysis of *MmWRKY* genes were performed using MEME (https://meme-suite.org/meme/tools/meme). The optimized parameters were as follows: Maximum number of motifs was 10, and each sequence occurs zero or one occurrence per sequence. The conserved structural domains of the *MmWRKY* genes were subsequently visualized using TBtools. Cis-acting elements within the 2,000 bp promoter sequences upstream of 77 *MmWRKY* genes were predicted using the PlantCare online website (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/), and visualized using TBtools software.

Chromosomal localization and co-expression network analysis of the *MmWRKY* genes

The chromosomal distribution of the *MmWRKY* genes was determined based on the annotation file (GFF3) and genome file (FA) and visualized by TBtools software. Gene duplication analysis was performed using Multiple Collinear Scanning toolkits (MCScanX) and visualized by Circos in TBtools software (with default parameters). Use KaKs_Calculator 2.0 to calculate synonymous (ks) and non-synonymous (ka) substitutions between each duplicate *MmWRKY* genes.

Analysis of the specific expression pattern of *MmWRKY* in different tissues

The expression profiles of the *MmWRKY* gene in flowers (SRR10596654), stems (SRR10596656), leaves (SRR10596655), and roots (SRR10596657) were generated based on full-length transcriptome data from multiple organs of *M. micrantha* [37]. The TPM values of all *MmWRKY* transcripts were evaluated, and heatmaps were generated based on the TPM values by TBtools.

Plant material, cDNA synthesis and qRT-PCR expression analysis

M. micrantha seeds were harvested from Dehong, Yunnan Province in 2022. *M. micrantha* was placed in light

culture under long-day conditions (14 h L/10 hD), with light/dark at 25 °C and 50% humidity. In October, tissue samples of *M. micrantha* roots, stems, leaves, and flowers were collected in Ruili, Yunnan Province. Total RNA was extracted from different organs of *M. micrantha*, including roots, stems, leaves, and flowers, using the TaKaRa MiniBEST Universal RNA Extraction Kit. Total RNA was reverse transcribed into cDNA by the RT mix with a DNase kit (UE). Three independent experiments were conducted for each sample. RNA integrity was assessed using 1.5% (v/v) agarose gel electrophoresis, and the RNA purity of different materials was quantified using a Nanodrop ND-1000 spectrophotometer. A kit was used according to the instructions to synthesize first strand of cDNA from 1 ug of total RNA. cDNA was diluted to 100 ng with RNase-free water and used as a template for qRT-PCR analysis. qRT-PCR was performed using a Roche Light Cycler® 480 instrument with SYBR Green chemistry. The *M. micrantha* actin gene was used as an internal control. The reaction was performed as follows: 30 s, 95 °C for 5 s, 60 °C for 20 s, and 40 cycles. Each reaction had three biological and technical replicates, and the real-time PCR amplification data were analysed using the $2^{-\Delta\Delta Ct}$ method. The primer sequences used in this study are shown in the Additional file 8.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-024-11187-0.

Supplementary Material 1.
Supplementary Material 2.
Supplementary Material 3.
Supplementary Material 4.
Supplementary Material 5.
Supplementary Material 6.
Supplementary Material 7
Supplementary Material 8.

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Authors' contributions
Z.Z. and N.Z. designed the study, provided financial support, supervised the research and revised the first draft. Z.Z. wrote the first draft and W.S. participated in the visualization. . S.Z., B.Y. and M.J. contributed to study design and supervision of the study. All authors have read and agreed to the published version of the manuscript.

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Data availability
All data generated or analysed during this study are included in this article and can be found in Additional file.

Declarations

Ethics approval and consent to participate
The *M. micrantha* used in the experiment came from Ruili, Yunnan Province, China. Before sampling *M. micrantha* in this area, we have obtained permission from the Yunnan Forestry and Grassland Pest Control and Quarantine Bureau. In addition, the specimen of *M. micrantha* has been preserved in the specimen Library of Southwest Forestry University, and its specimen number is SWFU20181006089. Otherwise, the corresponding authors (Bin Yang and Ning Zhao) belong to the Key Laboratory of Forest Disaster Warning and Control of Yunnan Province, Southwest Forestry University. This laboratory is specialized in the prevention and control of harmful forest organisms and qualifies the collection samples. The authors confirm that all experimental research and field studies on *M. micrantha*, including the collection of *M. micrantha*, must comply with relevant institutional, national, and international guidelines and legislation.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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