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Genome-Wide Identification and Expression of the *AP2/ERF* Gene Family in *Morus notabilis*

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Abstract: The *AP2/ERF* gene family, referring to an exclusive class of transcription factors unique to plants, is involved in various biological processes, including plant growth and responses to environmental stresses like high salt and drought. In this study, the *AP2/ERF* gene in *M. notabilis* was comprehensively identified and bioinformatically analyzed based on the genomic data of *M. notabilis*. 106 members in the *MnAP2/ERF* gene family were identified in the *M. notabilis* genome and were categorized into five subfamilies: *ERF*, *AP2*, *DREB*, *RAV*, and *Soloist*, with the *ERF* subfamily representing 80.19% of the total. The *MnAP2/ERF* gene family was observed to be distributed on six chromosomes of *M. notabilis*. Members in the *MnAP2/ERF* gene family exhibited obvious differences in amino acid number, molecular weight, isoelectric point, and other properties. Approximately 68.87% of the MnAP2/ERF proteins were acidic, all exhibiting hydrophilic characteristics. Differences in conserved sequences and arrangement of AP2 domains were observed among distinct subfamilies, with genes in the same subfamily sharing similar conserved domain compositions. There were 47 genes without untranslated regions and 44 genes with two untranslated regions. The upstream functions of promoters were concentrated on light reactions and plant hormones. Evolutionarily, significant structural differences were identified, and 28 MnAP2/ERF gene family proteins could interact with each other. Moreover, 35 family genes were involved in 22 fragment repeat events, and 55 *MnAP2/ERF* and 84 *AtAP2/ERF* genes showed collinearity. The expression of the *MnAP2/ERF* gene family was significantly different in different parts, indicating that these gene family members were involved in different physiological activities. These results established a theoretical foundation for investigating the functional and evolutionary aspects of *AP2/ERF* gene family genes in *M. notabilis*, as well as exploring the root morphogenesis of *M. notabilis*. Additionally, this study contributes to a basis for the improvement of cultivar stress resistance of *M. notabilis*.

Keywords: *AP2/ERF* gene family; evolutionary analysis; gene expression; bioinformatics

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1. Introduction

Morus notabilis (*M. notabilis*), a deciduous tree or shrub belonging to the Moraceae Gaudich. family, is a perennial woody plant native to central China. It is widely distributed in Guangxi, Yunnan, and Hubei in China [1–3]. Renowned for its robust adaptability to various soils, *M. notabilis* is extensively utilized in afforestation and mountain reforestation efforts. It thrives in soils ranging from slightly acidic to slightly alkaline [4]. Beyond its ecological benefits, *M. notabilis* holds good economic benefits. Its fruit, a sweet and juicy mulberry, is praised by the medical community as "the best health fruit in the 21st century" [5]. Additionally, *M. notabilis* wood is dense, tough, and beautifully textured, making it a high-grade raw material for crafting agricultural tools and furniture [6,7]. Moreover, *M. notabilis* cuttings propagation has found its widespread application in mass propagation. This is attributed to the fact that it not only can fully respond to the rapid

construction and development requirements of mulberry production but also can maintain the good traits of the mother plant while avoiding degradation and producing better seed stocks faster [8–10]. In each stage of cutting propagation, how to improve the stress resistance of cutting roots is an urgent problem to be solved at present [11]. Furthermore, the survival rate of cuttings directly affects their overall quality, and there is a growing imperative to breed high-yielding and stress-resistant *M. notabilis* clones, facilitating their adaption to marginal land development and utilization.

Transcription factors can regulate the specific expression of target genes at particular times and in specific spatial locations, exerting a pivotal effect on gene expression [12–14]. They can enhance the ability of plants to withstand various stresses by modulating the level of target genes. Among all transcription factors, the *APETALA2/Ethylene-responsive factor* (*AP2/ERF*) family serves as the largest family in plants. It regulates the plant growth and development and responds crucially to environmental stresses. This family, a major player in plant transcription factors, participates in regulating diverse plant signaling pathways [15–44]. A key characteristic of the *AP2/ERF* gene family is the presence of at least one AP2 domain, consisting of 60–70 amino acids, in all its members [18–20]. Categorization of the *AP2/ERF* gene family is based on the number of AP2 domains contained and other conserved sequence features, resulting in four subfamilies: *AP2*, *ERF*, *RAV*, and *Soloist* [21]. In general, the *AP2* subfamily possesses two AP2 domains, while ERF, *RAV*, and *Soloist* have a single AP2 domain [22]. The *ERF* subfamily, in addition to the AP2 domain, features a B3 domain. Further division of the *ERF* family can be performed based on the DNA-binding domain, resulting in the *ERF* and *DREB* subfamilies [23]. The *Soloist* subfamily stands out due to substantial differences in its protein sequence characteristics and specific gene structures, warranting its classification as a distinct subfamily within the *AP2/ERF* family [24].

With the widespread adoption of genome sequencing in various plant species, the *AP2/ERF* gene family has found its extensive application in *Arabidopsis thaliana* [25], *Populus* L. [26], *Oryza sativa* L. [27], *Zea mays* L. [28], *Vigna radiata* (L.) R. Wilczek [29], and *Betula alba*. [30]. The *RAV* genes within the *AP2/ERF* gene family are responsive to hormones such as ethylene and brassinolide [31], and their overexpression has been demonstrated to enhance plant resistance to salt and drought stresses [32,33]. On the other hand, genes in the *ERF* subfamily primarily participate in the responses to both biotic and abiotic stresses, including pathogenic bacteria and disease stimuli [34,35], water stress [36], salt stress [37], as well as high and low-temperature stresses [38,39]. Furthermore, some *AP2/ERF* transcription factors in *A. thaliana* stimulate the expression of several crucial enzymes involved in proline synthesis, facilitating and promoting the synthesis of proline. This, in turn, elevates the content of free proline, thereby contributing to an enhancement in plant cold tolerance [40]. Some previous studies focusing on *AP2/ERF* gene families across different plant species have highlighted their significant role in the generation and development of adventitious roots [41]. For example, Ye et al. [42] found that *ABR1* and *ERF109* promoted the accumulation of auxin after injury treatment and actively participated in the regulation of adventitious root regeneration. Conversely, the *SPL2/10/11* transcription factor inhibited auxin synthesis after injury treatment, thereby weakening the ability of adventitious root regeneration. Yuka Kitomi et al. [43] identified the involvement of *CRL5* in priming rice crown root development by inducing the expression of *OsRR1* (a type A response regulator in cytokinin signaling). Moreover, Dossa et al. [44], in their analysis of *AP2/ERF* transcription factors in sesame seeds, noted that most *DREB* genes exhibited higher expressions in the roots, primarily functioning in response to drought stress. In addition, Swinka et al. [45] reported that certain ERF-VI proteins mediated the transcription of cytokinins, representing crucial factors in the production of adventitious roots. At present, while the whole-genome sequencing of the *M. notabilis* genome has been completed, a comprehensive genome-wide exploration of the structure, classification, and expression patterns of the *MnAP2/ERF* gene family remains unreported.

Here, we employed bioinformatics methods to delve into the *MnAP2/ERF* gene family, covering different aspects like chromosome localization, evolutionary analysis, protein physicochemical properties, conserved domains, gene structures, and species co-linearity. Meanwhile, expression patterns of genes in this family were examined at the protein tertiary structure level. This research aims to establish a foundational reference for further investigation of the transcription factor functions in *M. notabilis*. Additionally, it seeks to offer molecular insights into theories related to cutting propagation and stress resistance formation in *M. notabilis*.

2. Materials and Methods

2.1. Data Sources

The entire genome sequence and protein sequence data for *M. notabilis* employed in this research were acquired from the *M. notabilis* gap-free reference genome (BioProject number: PRJCA015883), as published by Ningjia He's team at Southwest University [46]. Additionally, the whole genome sequence and protein sequence data for *A. thaliana* were downloaded from the TAIR database (https://www.arabidopsis.org/, accessed on 18 October 2023).

2.2. Identification of Members of the MnAP2/ERF Gene Family

Firstly, the protein sequence data for *AtAP2/ERF* gene family members were retrieved from the plantTFDB database (http://planttfdb.gao-lab.org/, accessed on 18 October 2023). One hundred and seventy-five associated protein sequences were yielded. The local blast analysis (E value < 0.05) was then performed on 175 *AtAP2/ERF* gene family members using *M. notabilis* genome data to identify the homologous proteins in *A. thaliana*. Concurrently, the hidden Markov model (HMM) with the AP2 domain (PF00847) was downloaded from the InterPro database (https://www.ebi.ac.uk/interpro/, accessed on 18 October 2023). The *M. notabilis* whole protein database was searched using HMMER v3.4 (http://eddylab.org/software/hmmer/, accessed on 18 October 2023) software (E value < 0.05), generating proteins containing at least one AP2 domain. All protein sequences obtained by the above two methods underwent scrutiny using the online software NCBI-CDD (https://www.ncbi.nlm.nih.gov/cdd/, accessed on 18 October 2023) and SMART (https://smart.embl.de, accessed on 18 October 2023). Redundant sequences and proteins without the AP2 domain were excluded. Consequently, 106 complete AP2 domain protein sequences were retained, constituting the members of the *MnAP2/ERF* gene family in *M. notabilis*. Finally, these 106 members of the *MnAP2/ERF* gene family were named based on the aforementioned domain characteristics.

2.3. Chromosomal Mapping and Numbering of MnAP2/ERF Gene Family Members

Using TBtools v2.038 software [47], the *M. notabilis* genomic GFF file and the gene IDs corresponding to the previously mentioned 106 MnAP2/ERF proteins were simultaneously inputted. Subsequently, the positions of the MnAP2/ERF proteins on the chromosomes (Chr 1–Chr 6) were visualized and annotated according to their sequence order.

2.4. Analysis of Physicochemical Properties of MnAP2/ERF Gene Family Members

The physical and chemical properties of the 106 *MnAP2/ERF* gene family members, such as amino acid number, molecular weight, isoelectric point, and average hydrophobic index, were analyzed using the online software ExPASy-Compute pI/Mw tool (https://web.expasy.org/compute_pi/, accessed on 19 October 2023). In addition, WoLF PSORT (https://wolfpsort.hgc.jp/, accessed on 19 October 2023) was employed to predict the subcellular localization of these *MnAP2/ERF* gene family members.

2.5. Evolutionary Tree Construction of MnAP2/ERF Gene Family Members

To explore the evolutionary relationship between the *MnAP2/ERF* gene family and other plant *AP2/ERF* gene families, an evolutionary tree of *M. notabilis* and *A. thaliana* *AP2/ERF* gene family proteins was constructed using the neighbor-joining (NJ) method. The

process involved aligning a total of 281 protein sequences from *M. notabilis* and *A. thaliana* using ClustalW 2.0 software. The parameters were set to Gap Opening of 10, Extension of 0.2, Delay Divergent Sequences of 30, and DNA Transition Weight of 0.5. Subsequently, the alignment results were entered into MEGA 11 software (https://www.megasoftware.net/, accessed on 23 October 2023) to build the evolutionary tree based on 1000 Bootstrap samples, with other parameters set to their default values.

2.6. Motif Analysis and Gene Structure Analysis of MnAP2/ERF Gene Family

The motif sequences of the *MnAP2/ERF* gene family were examined using the online software MEME (https://meme-suite.org/meme/tools/meme, accessed on 23 October 2023), with the number of motifs set to 10. After that, the obtained motif sequences were visualized using TBtools v2.038 software [47]. Moving on to gene structure analysis, the *M. notabilis* genome CDS file was initially searched to retrieve the CDS sequence of the *MnAP2/ERF* gene family. Subsequently, the full-length gene file was obtained with the assistance of Activestate software (https://www.activestate.com/, accessed on 23 October 2023). Finally, both files were inputted into the online software GSDS 2.0 (http://gsds.gao-lab.org/, accessed on 23 October 2023) for visualization of the gene structure.

2.7. Promoter Cis-Acting Elements of the MnAP2/ERF Gene Family

To investigate the specific cis-acting elements influencing the gene expression in the promoter region of the *MnAP2/ERF* gene, DNA sequences from the promoter region of these gene family members were extracted from the *M. notabilis* genome. Then, predictive analysis was conducted using the PlantCARE database (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 25 October 2023). Next, the analysis was specifically focused on predicting the functional elements associated with light response, hormone response, stress response, and growth and development response within the promoter region. Finally, a visual analysis of the results was performed using TBtools v2.038 software.

2.8. Tertiary Structure and Protein–Protein Interaction (PPI) Analysis of MnAP2/ERF Gene Family Proteins

The online tool CPHmodels database (https://services.healthtech.dtu.dk/services/CPHmodels-3.2/, accessed on 25 October 2023) was employed to predict the tertiary structure of the identified MnAP2/ERF protein sequences by homology modeling. Subsequently, utilizing the MnAP2/ERF protein sequence as a basis, the STRING online platform (https://cn.string-db.org/, accessed on 25 October 2023) was utilized to elucidate the primary mode of action of homologous proteins in *A. thaliana* using 106 *MnAP2/ERF* gene family protein sequences as the reference species. The results were then visually analyzed using Cytoscape software (https://cytoscape.org/, accessed on 25 October 2023).

2.9. Gene Repeat Events and Collinearity Analysis of the MnAP2/ERF Gene Family

To comprehend the repeat events within the *MnAP2/ERF* gene family and their collinearity with the *AtAP2/ERF* gene family, a comparative analysis was conducted on protein sequences. Specifically, *M. notabilis* itself and AP2/ERF protein sequences of *A. thaliana* were compared by MCScanX v3-28-2013 software. Gene pairs within *M. notabilis* species and *A. thaliana* were extracted with the help of TBtools v2.038 software to construct a collinearity map for *M. notabilis* within its own genome and between *M. notabilis* and *A. thaliana AP2/ERF* genes (E value < −10).

2.10. Analysis of the Expression Pattern of the MnAP2/ERF Gene Family in Different Organs of M. notabilis

We downloaded the transcriptome data of *M. notabilis* from NCBI (accession number: PRJNA597172) [48] and used RSEM v1.3.3 software to calculate the gene expression level of each sample to derive the FPKM value [49], from which we extracted the *MnAP2/ERF* family genes of *M. notabilis*, organized them into a matrix file, and submitted them to the

local software TBtools v2.038 to draw a heatmap of the tissue expression of the *MnAP2/ERF* family genes in the *M. notabilis*.

3. Results and Analysis

3.1. Chromosomal Location and Nomenclature of MnAP2/ERF Gene Family Members

To investigate the distribution of MnAP2/ERF proteins on the *M. notabilis* chromosome, the localization of 106 *MnAP2/ERF* genes on the chromosome was visualized by TBtools v2.038 software, as illustrated in Figure 1. The results unveiled an uneven distribution on six chromosomes of *M. notabilis*, with the highest concentration on chromosome 1, comprising 31 family members. There are 23, 27, 16, and 6 family members on chromosomes 2, 3, 4, and 5, respectively. Notably, the least distribution was observed on chromosome 6, with only three family members. Then, the *MnAP2/ERF* gene family members were named based on their gene IDs, considering their order, spatial distribution on the chromosomes, and binding domains. This nomenclature provided a foundation framework for the subsequent exploration of the functional characteristics of the MnAP2/ERF proteins.

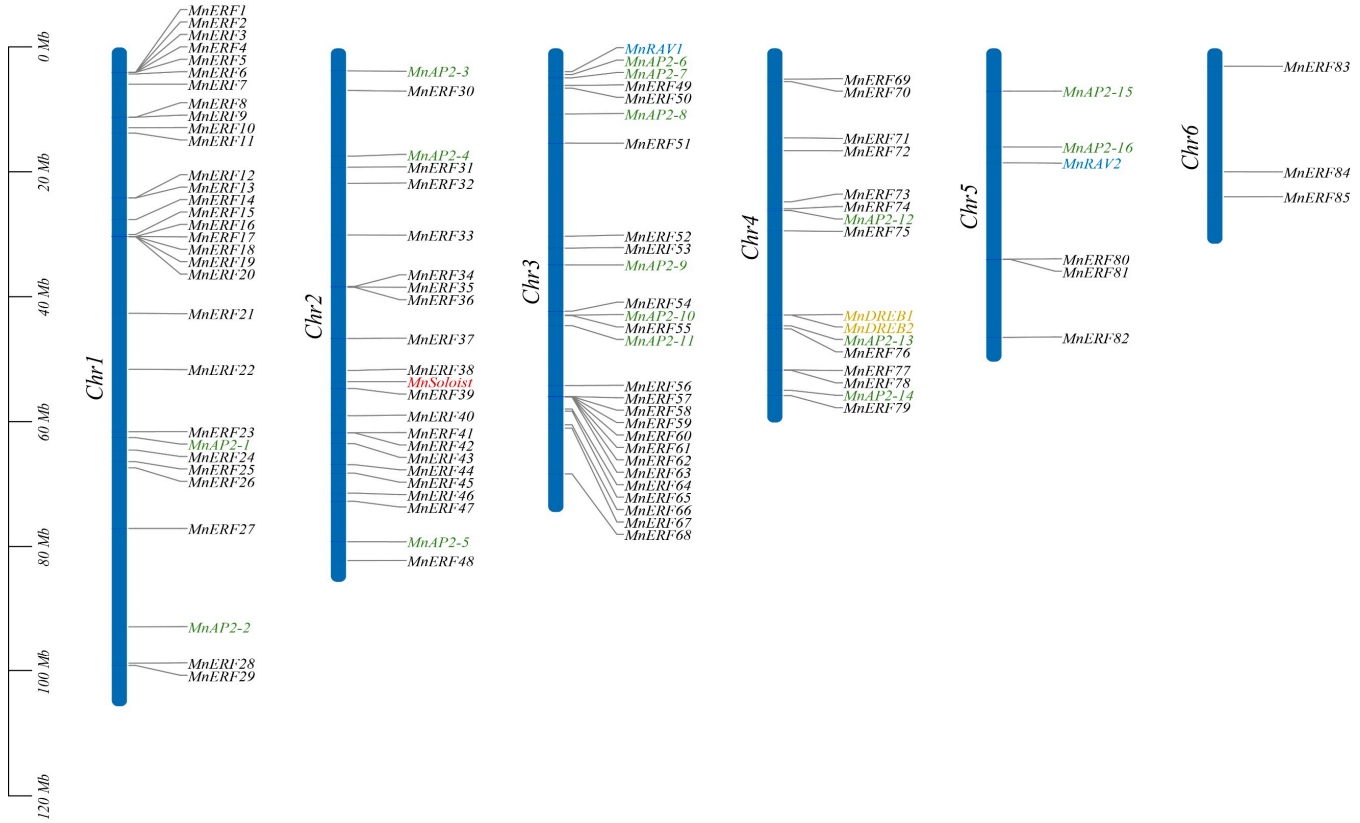

Figure 1. Chromosome arrangement of *MnAP2/ERF* gene family members in *M. notabilis*. Different colors indicate different subgroups: Black indicates the *ERF* subfamily; green indicates the *AP2* subfamily; yellow indicates the *DREB* subfamily; blue indicates the *RAV* subfamily; red indicates the *Soloist* subfamily.

3.2. Physicochemical Properties of MnAP2/ERF Gene Family Members

Based on *M. notabilis* genomic data, we identified a total of 85 *MnERF* members, 16 *MnAP2* members, 2 *MnRAV* members, 2 *MnDREB* members, and 1 *Soloist* member in this investigation. The physicochemical analysis of the protein properties of *MnAP2/ERF* gene family members, as outlined in Table S1, revealed notable differences in the number, molecular weight, isoelectric point, and other properties of amino acids. The protein lengths of the 106 *MnAP2/ERF* gene family members ranged from 129 aa (MnERF47) to 1119 aa (MnAP 2-15), which was slightly different within the same subfamily. Remarkably, the protein length of the MnAP2 subfamily proteins was significantly higher than that

of the other four subfamilies. The molecular weight of the protein exhibited a wide range, spanning from 14.6 kD (MnERF47) to 123.15 kD (MnAP2–MnAP15). Regarding the isoelectric point, 31.13% of the MnAP2/ERF family proteins had values greater than 7 with an average value of 6.67, while 68.87% had values less than 7, averaging 6.60. These data indicate that most of the MnAP2/ERF family proteins were acidic in nature. Furthermore, the average hydrophobicity index of the MnAP2/ERF family proteins was less than 0, signifying their hydrophilic nature. Subcellular localization bioinformatics analysis of MnAP2/ERF gene family proteins demonstrated that 77 proteins are located in the nucleus, 11 in chloroplasts, 2 in peroxisomes, 2 proteins in the lysosome, and 1 in the cytoskeleton.

3.3. Evolutionary Analysis of AP2/ERF Gene Family

An evolutionary tree was generated using protein sequences from the *AtAP2/ERF* gene family and the identified *MnAP2/ERF* gene family, with the results presented in Figure 2. Based on the evolutionary analysis, the 281 AP2/ERF proteins were categorized into five *ERF* (I–V) subfamilies, two *AP2* (I–II) subfamilies, one *DREB* subfamily, one *Soloist* subfamily, and one *RAV* subfamily. Among these subfamilies, *ERF-V* exhibited the most members, while the *Soloist* subfamily possessed the fewest members, with only one member. Through evolutionary tree and binding domain analyses of the tree, it was evident that the evolutionary structure of *ERF*, *AP2*, and *RAV* gene family in *M. notabilis* closely resembles that of *A. thaliana*. The gene homology between *MnAP2/ERF* genes and *AtAP2/ERF* genes indicates a relatively conserved evolutionary pattern within the *AP2/ERF* gene family across these species.

3.4. Conserved Motifs and Gene Structures of MnAP2/ERF Gene Family Members

The results of intron/exome analysis depicted in the structure of *MnAP2-ERF* genes in Figure 3 revealed that all 106 *MnAP2/ERF* genes contained exons. Notably, only one gene (*MnERF13*) displayed a distinctive structure with 18 exons, while the other remaining genes exhibited exon numbers of 1~11. The largest proportion of genes (62) had a single exon, followed by 20 genes with two exons. Moreover, there were 55 genes without introns and 21 genes with one intron, while there were 20 genes containing more than three exons and introns. Among the 106 *MnAP2-ERF* genes, 47 genes did not have untranslated regions, while 44 genes featured two untranslated regions, primarily located at both ends. Upon closer gene structure analysis, it was observed that the absence of both introns and untranslated regions was exclusive to the *ERF* subfamily, with 30 genes exhibiting this pattern. Conversely, most genes in the *AP2* subfamily showcased a higher number of introns and exons. In conclusion, the *MnAP2/ERF* genes demonstrated consistent gene structures within the same subfamily.

Utilizing the MEME tool to investigate the conserved motifs in MnAP2/ERF proteins, 10 conserved motifs were identified and listed in Table 1, with detailed characteristic information presented in Figure 3. The analysis unveiled noteworthy findings within the ERF subfamily, where motif 8 was observed in eight genes (*MnERF58*, *MnERF62*, *MnERF60*, *MnERF57*, *MnERF61*, *MnERF63*, *MnERF78*, and *MnERF77*) and motifs 4, 2, 1, 5, and 7 usually persisted consecutively. Motif 3 was identified in the *AP2* and *DREB* subfamilies, although the specific motif composition varied between the two subfamilies. Among the 106 genes analyzed, three genes (*MnRAV1*, *MnRAV2*, and *MnSoloist*) contained only two motifs, and the motif composition of *MnSoloist* (Motif1 and Motif5) appeared in 90% of MnAP2/ERF proteins.

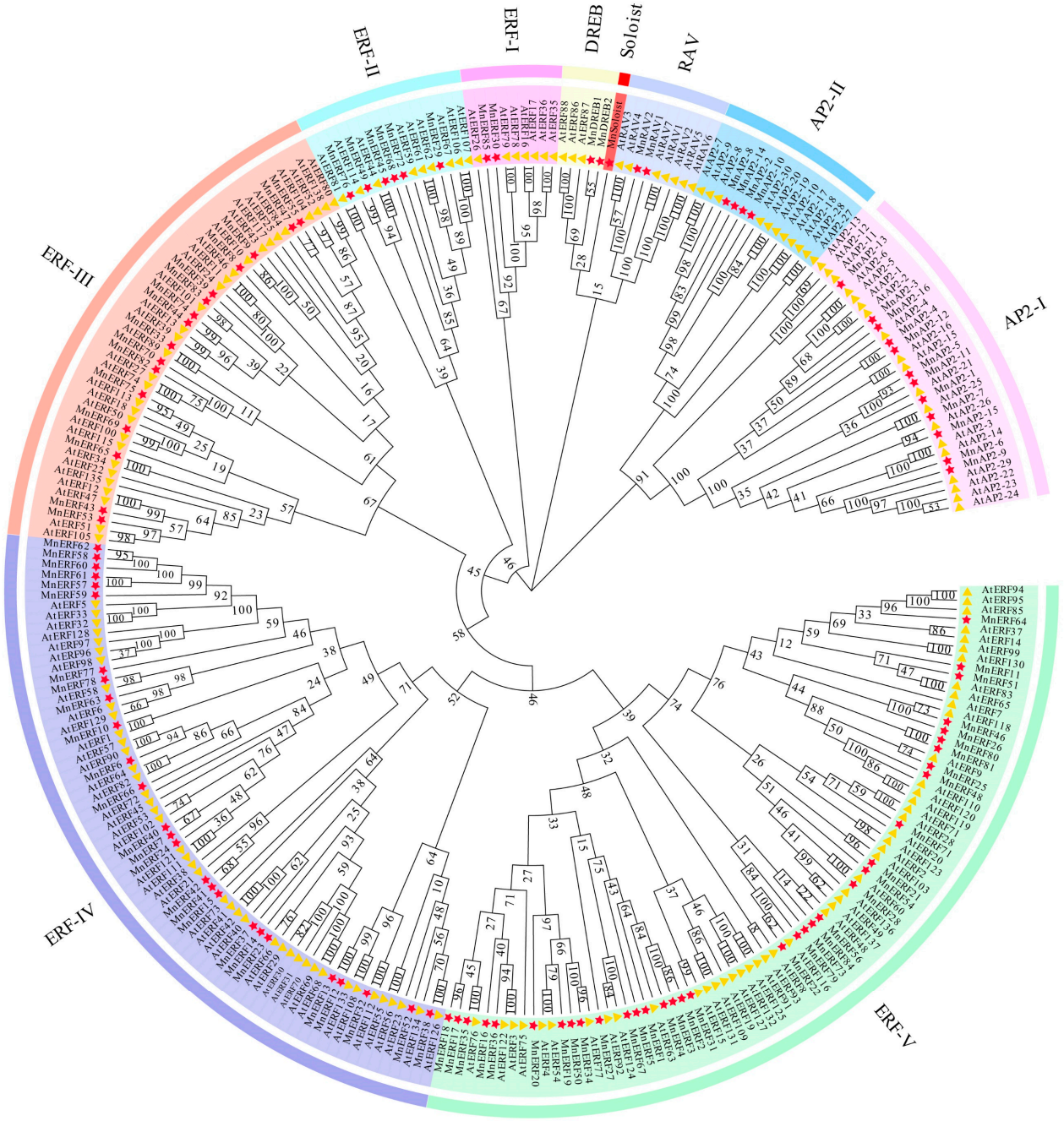

Figure 2. Evolutionary tree of *AP2/ERF* gene family in *M. notabilis* and *A. thaliana.* Red stars represent AP2/ERF family genes in M. notabilis; yellow triangles represent AP2/ERF family genes in *A. thaliana.*

Figure 3. Gene structure and protein conserved motifs of *MnAP2/ERF* gene family.

Table 1. Conserved motif sequence of *MnAP2/ERF* proteins.

Motif	Length/aa	Conserved Amino Acid Sequences
Motif 1	26	VWLGTFDTAEEAARAYDVAALKLRGS
Motif 2	11	RPWGKWVAEIR
Motif 3	50	RAYDLAALKYWGPSTTINFPLSBYEKELEEMKNMTKZEFVASLRRKSSGF
Motif 4	11	RRSSKYRGVRR
Motif 5	11	KAKLNFPELTY
Motif 6	29	HRWTGRYEAHLWDNSCRREGQSRKGRQGA
Motif 7	23	SLPRPASTDAKDIQAAAAEAAAA
Motif 8	41	EEEZRVFYVDEEEVFGMPSFFADLAEGLLLSPPPSYMSSDD
Motif 9	11	GASQYRGVTRH
Motif 10	17	VIELEYLDDKYLEELLD

3.5. Cis-Acting Elements in the Promoter Region of MnAP2/ERF

The promoter sequences, spanning 2000 bp upstream of the start site of the *MnAP2ERF* gene family, were subjected to analysis using Plant CARE, and the results are illustrated n in Figure 4. The findings revealed that 20 cis-acting elements were detected in the *MnAP2ERF* gene family in four types: light-responsive, hormone-responsive, stress-responsive, and developmentally responsive. Overall, there are a maximum of 34 cis-acting elements on *MnERF64* and a minimum of 6 cis-acting elements on *MnERF5*. With the exception of *MnERF74* and *MnERF73*, all Mn*AP2/ERF* genes encompassed multiple promoter cis-elements. Each *MnAP2/ERF* gene contained light-responsive elements, which accounted for 36.6% of the *AP2/ERF* gene family. In addition, several *MnAP2/ERF* family members included a large number of response elements associated with plant hormones and stress response. Notably, elements responding to the methyl jasmonate constituted the largest proportion at 15.0%, followed by the abscisic acid response elements at 14.7% and the anaerobic sensing elements at 8.1%. Additionally, a substantial number of elements were observed to be involved in abiotic stress and other related elements in the *MnAP2/ERF* family, such as cryogenic response, defense, and stress response elements. Among them, 61 genes contained low-temperature response elements, and 64 genes contained defense stress elements. This suggests a pivotal regulatory role of the *MnAP2/ERF* in response to various abiotic stress conditions.

3.6. Tertiary Structure and Protein–Protein Interaction Association of MnAP2/ERF Gene Family Proteins

In this study, we conducted homology modeling to derive the 3D structures for the 106 MnAP2/ERF transcription factors based on their amino acid sequences. The outcomes are visually presented in Figure 5. The analysis revealed a high degree of structural similarity among the 106 MnAP2/ERF proteins, indicating a generally uncomplicated structure for these transcription factors. Despite the overall structural uniformity, distinct coiling patterns were observed in different sequences, likely associated with the composition of α-helix, β-fold, corner, and random coil. Notably, the MnERF13 protein exhibited the highest counts, with 20 α-helices and 36 β-folds, while MnSoloist, MnERF70, and MnERF32 displayed the least structures, with one α-helix and two β-folds. Other proteins predominantly featured α-helix counts ranging from 8 to 17 and β-fold counts of 15~23. Among the 106 proteins, MnERF32 in the *ERF-V* subfamily and MnERF43 and MnERF65 in the *ERF-III* subfamily were similar in spatial structure. The resemblances or disparities between these three proteins and the remaining 103 proteins may underlie the functional similarities or differences.

To explore the protein–protein interactions (PPI) in the MnAP2/ERF family and their gene functions, a homology mapping method was employed to predict the PPI network, taking the protein interaction relationships in AtAP2/ERF as a reference. The results of this analysis highlight 28 MnAP2/ERF protein interaction relationships, as presented in Figure 6. Among them, there are many interactions between MnAP2/ERFARF family proteins, such as MnAP2-2, which can interact with MnERF59, MnERF58, MnERF34, MnERF82, MnERF57, MnERF77, MnERF47, MnERF17, MnERF26, MnERF56, MnERF31, and MnERF8. Additionally, MnAP2-2 demonstrated interactions with AtEIL1, AtEIN3, AtDREB3, and AtABF2 in the AtAP2/ERF family of proteins. The graphical representation indicates that MnAP2-2, MnERF21, MnERF77, and MnERF72 exhibit high connectivity within the PPI network. The functions associated with MnAP2-2 and MnERF77 appear to be related to stress, growth, and development. This observation suggests that these proteins may be crucial in the reproduction and stress resistance of *M. notabilis*, providing potential molecular insights for future research endeavors.

Figure 4. Cis-acting elements of the promoter of the *MnAP2/ERF* gene family.

Figure 5. Prediction for tertiary structure of MnAP2/ERF proteins.

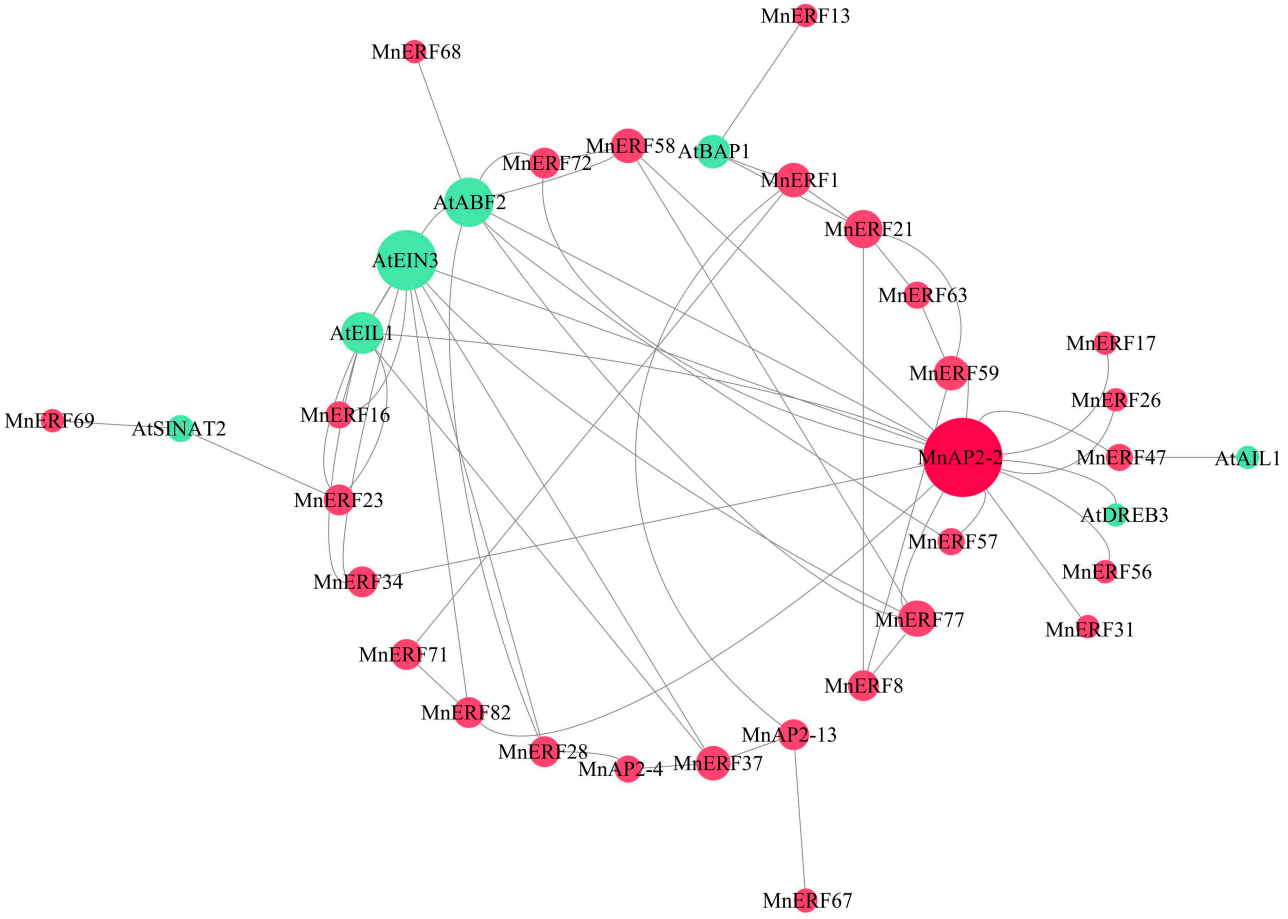

Figure 6. PPI of MnAP2/ERF and AtAP2/ERF proteins. Red represents the AP2/ERF gene family proteins of *M. notabilis*, and green represents the AP2/ERF gene family proteins of *A. thaliana.*

3.7. Gene Duplication Events and Species Collinearity of the MnAP2/ERF Gene Family

Figure 7A demonstrates the intraspecific collinearity analysis on *MnAP2/ERF* members. It revealed the involvement of 35 genes from the *MnAP2/ERF* gene family in 22 segmental duplication events, indicating the significant role of segmental duplication in shaping the *MnAP2/ERF* gene family. Specifically, *MnERF16*, *MnERF24*, M*MnAP2-2*, *MnERF34*, *MnERF40*, *MnERF50*, *MnAP2-8*, and *MnAP2-14* were all involved in the two fragment duplication events each, indicating a high degree of similarity with other genes. To delve deeper into the evolutionary dynamics of the *MnAP2/ERF* gene family, collinearity analysis was conducted between *A. thaliana* and *M. notabilis AP2/ERF* genes. The results, as demonstrated in Figure 7B, suggested the collinearity between 55 *MnAP2/ERF* and 84 *AtAP2/ERF*, revealing 99 collinearity events. This signifies that the gene family was highly conserved in the evolutionary process. However, some *MnAP2/ERF* and *AtAP2/ERF* displayed no collinearity relationship. This could be attributed to the emergence of new genes in the *AP2/ERF* gene family during later family evolution.

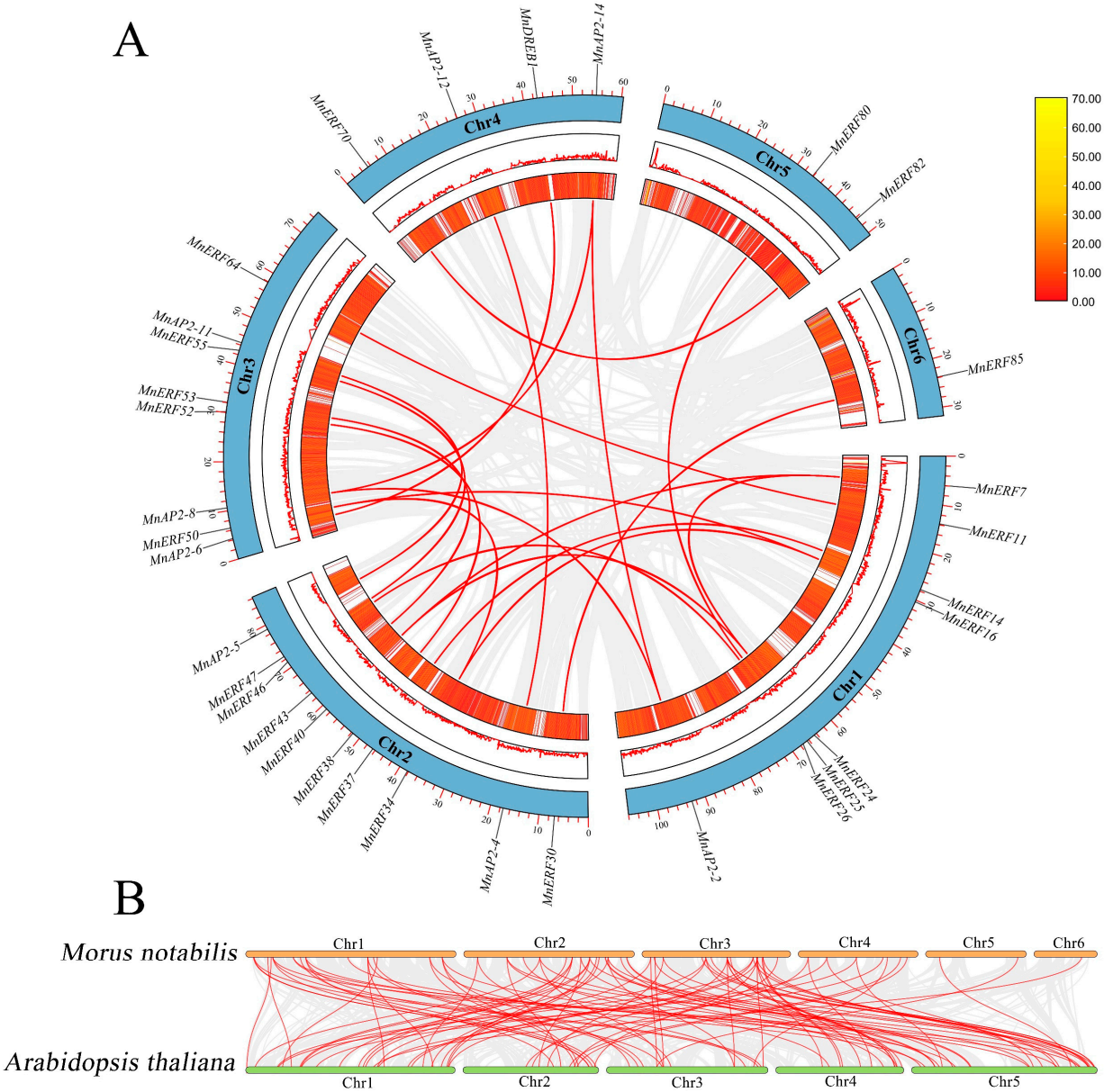

Figure 7. Collinearity analysis of *AP2/ERF* gene families in different species. (**A**) Collinearity analysis of the *MnAP2/ERF* gene families in *M. notabilis*; (**B**) collinearity analysis of the *AP2/ERF* gene families in *M. notabilis* and *A. thaliana.* Each red line represents a gene repetition event, the heat map and line graph in A represent the density of mulberry genes in each chromosome, and Chr represents the number of chromosomes.

3.8. Expression Analysis of MnAP2/ERF Gene Family in M. notabilis

Based on the transcriptome data of *M. notabilis*, the expressions of 106 *MnAP2/ERF* genes were obtained in different tissues of *M. notabilis*, and the results were visualized by using TBtools v2.038 software to draw heat maps (Figure 8). The results showed that most of the *MnAP2/ERF* genes were detected to be expressed and formed five specific expression clusters in five tissues. *MnERF6*, *MnERF7*, *MnERF12*, *MnERF13*, *MnERF16*, *MnERF19*, *MnERF22*, *MnERF23*, *MnERF24*, *MnERF30*, *MnERF35*, *MnERF38*, *MnERF41*, *MnERF44*, *MnERF47*, *MnERF51*, *MnERF52*, *MnERF63*, *MnERF65*, *MnERF79*, *MnAP2-2*, *MnAP2-16*, *MnAP2-7*, and *MnAP2-8* showed a high expression pattern in the root tissues, and a low expression in flowers and leaves. It is hypothesized that these genes may be involved in gene regulation in the underground. *MnERF3*, *MnERF33*, *MnERF50*, *MnERF80*, *MnERF81*, and *MnAP2-10*, *MnAP2-12*, and *MnAP2-14* showed significantly higher expression in male

flowers than in other parts of the body, and *MnERF1*, *MnERF9*, *MnERF10*, *MnERF25*, *MnERF48*, *MnERF53*, *MnERF57*, *MnERF58*, *MnERF61*, *MnERF62*, *MnERF64*, *MnERF69*, *MnERF71*, *MnERF73*, *MnERF74*, and *MnAP2-3* were expressed significantly higher in leaves than in other parts of the plant, *MnERF21*, *MnERF25*, *MnERF32*, *MnERF34*, *MnERF36*, *MnERF37*, *MnERF39*, *MnERF42*, *MnERF46*, *MnERF54*, *MnERF77*, *MnERF84*, *MnERF85*, *MnAP2-4*, *MnAP2-11*, *MnSoloist*, and *MnRAV1* were expressed in bark significantly higher than other sites, and *MnERF15*, *MnERF17*, *MnERF20*, *MnERF27*, *MnERF29*, *MnERF43*, *MnERF45*, *MnERF49*, *MnERF55*, *MnERF66*, *MnERF82*, *MnAP2-15*, *MnAP2-1*, and *MnAP2-9* were specifically expressed in the winter buds. *MnAP2-6*, *MnAP2-7*, *MnRAV1*, *MnERF18*, *MnERF37*, *MnERF35*, *MnERF5*, *MnERF78*, and *MnERF79* were very low or even not expressed in the five tissues, suggesting that these genes were relatively silent in the five tissues.

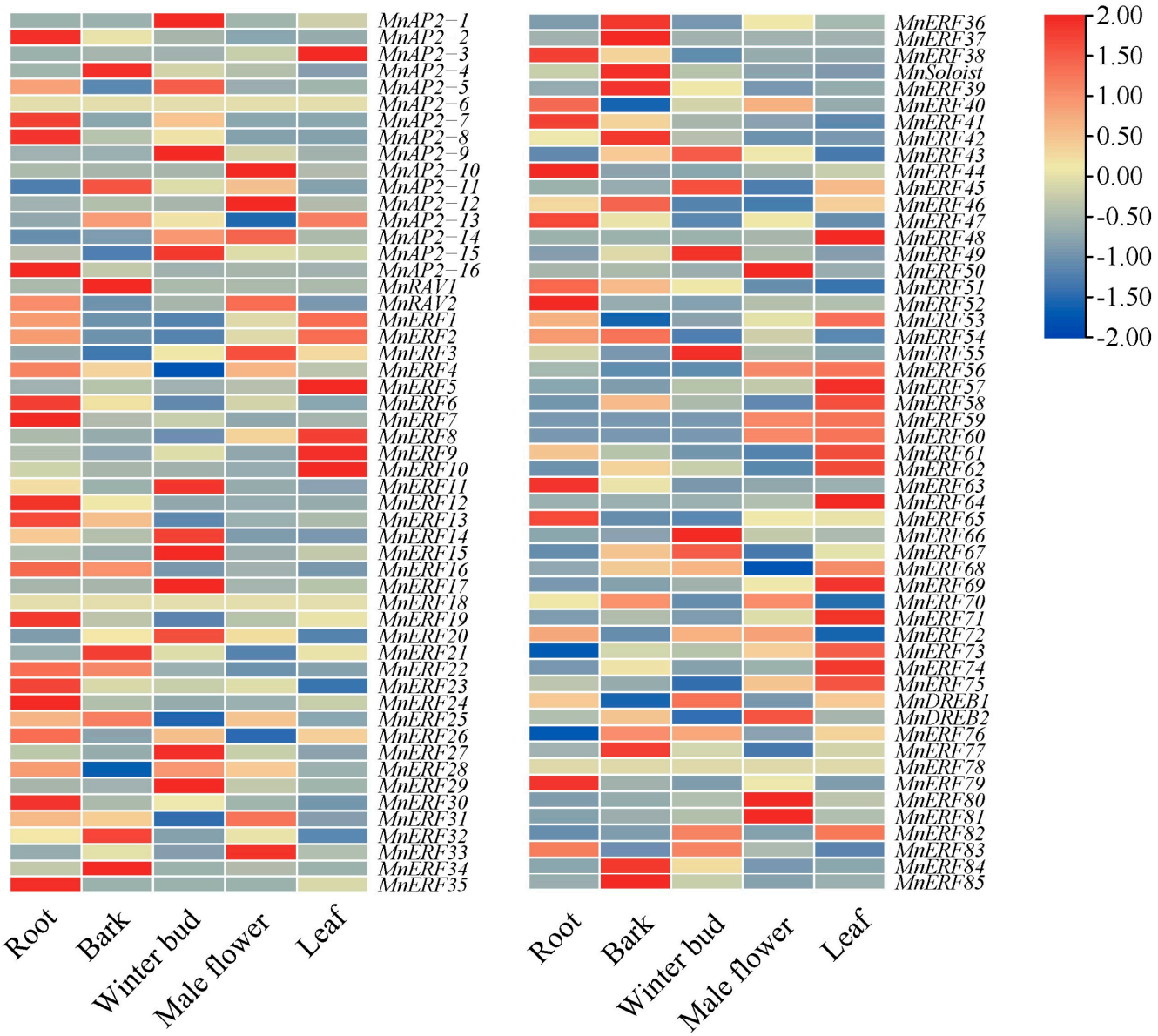

Figure 8. Expression analysis of the *MnAP2/ERF* gene family in five tissues.

4. Discussion

The *AP2/ERF* family, recognized as one of the largest transcription factor families in plants, is widely distributed across various pathways [16] and has been extensively investigated because of its high significance in gene breeding and stress resistance. In this

study, 106 members of the *MnERF* gene family were screened from the whole genome of *M. notabilis*, which was comparatively lower than that in other plants such as *Solanum lycopersicum* (112) [50], *A. thaliana* (147) [25], *Glycine max.* (148) [51], and *Solanum tuberosum* L. (181) [52]. According to the phylogenetic and domain characteristics, the 106 *MnAP2/ERF* gene family members were classified into five subfamilies: *ERF*, *AP2*, *DREB*, *RAV*, and *Soloist*. Among these, the *ERF* subfamily displayed the largest number of members (85), accounting for 80.19% of the total. This distribution is similar to the observation in other species, where the *ERF* subfamily comprised over 80% of the total gene families, including *A. thaliana*, tomato, and poplar.

The transgenic and overexpression studies involving *AP2/ERF* transcription factors have demonstrated their potential to enhance plant resistance to both biotic and abiotic stresses. For instance, the *DREB* subfamily transcription factor has been shown to interact with the cis-acting dehydration response elements to improve the plant response to stress, and the overexpression of the *AhDREB1* gene in tobacco can improve salt and drought resistance [53]. Similarly, the overexpression of *DREB19* in *A. thaliana* significantly enhances the ability of plants to withstand drought and high salinity stress without adversely affecting their phenotype [54]. The expression of the *DREB* transcription factor *VrDREB2A* of *Vigna radiata* is induced by high salt, drought, and abscisic acid treatment. Compared with wild-type *A. thaliana*, overexpression of this gene can activate the expression of downstream genes of transgenic *A. thaliana* and improve the tolerance of transgenic Arabidopsis to high salt and drought stress [55]. Wheat transcription factor *TaERF3* positively regulates adaptive responses to salt and drought stress by activating stress-related genes [56]. *DREBA-5* transcription factor *ScDREB5* in *Syntrichia caninervis* Mitt. can enhance reactive oxygen species (ROS), the salt-tolerance of transgenic *A. thaliana* can be improved by reactive oxygen species clearance, up-regulation of genes related to ion homeostasis, and jasmonic acid biosynthesis [57]. Meanwhile, they observed that inhibiting the expression of *ERF3* led to a great reduction in the number and length of root crowns and the root crown development was delayed or even inhibited through experimental observation. Consequently, *ERF* genes are critical in the formation and early development of root crowns. In *A. thaliana*, *AtRAP2.6* has been recognized as an important regulator of plant meristem regeneration and exerts a vital role in recovering cell damage. Moreover, the activity of the *RAP2.6* gene was inversely proportional to the concentration of auxin IAA, highlighting its inhibitory role in IAA synthesis [58]. Combined with the expression changes of the *MnAP2/ERF* gene family in five tissues, we found that most MnAP2/ERFs have some tissue expression specificity. The identification and analysis of *MnAP2/ERF* transcription factors serve as valuable genetic resources for cultivating *M. notabilis* with high resistance to biotic and abiotic stresses through transgenic culture.

5. Conclusions

In this study, the *MnAP2/ERF* gene family of *M. notabilis* was analyzed. Most of the MnAP2/ERF proteins were acidic proteins, accounting for 68.87% of all members, and exhibited hydrophilic characteristics. Evolutionary analysis of the protein in the *MnAP2/ERF* gene family suggested that the *ERF* subfamily can be categorized into five (ERF I~ERF V), while the *AP2* subfamily was composed of two classes (*AP2-I.~AP2-II.*). The other three subfamilies constituted separate classes, and proteins within the same class possessed similar properties. Furthermore, a comparison with *A. thaliana* revealed that the *MnAP2/ERF* genes experienced high conservation during the species evolution. An investigation on the conserved motifs and gene structure of the *MnAP2/ERF* gene family reported that the *MnAP2/ERF* genes had similar structures in the same subfamily, with 47 genes without untranslated regions and 44 with two, mainly at both ends of the genes. In addition, the functions of the upstream of the gene promoter were mainly concentrated in the photoreactive and plant hormone components, indicating that this gene family was involved in various physiological activities. Abundant elements involved in abiotic stress, such as hypothermia, defense, and stress response, were also identified in the *AP2/ERF*

family, emphasizing its vital regulatory role in the response to abiotic stress in *M. notabilis*. Significant differences in conserved sequences among different subfamilies were noted, with varying arrangements of AP2 domains. The analysis of the structure and function of family proteins indicated a notable diversity in the mode of action among the 106 proteins, with 28 MnAP2/ERF gene family proteins implicated. The collinearity analysis further unveiled that 35 family genes in the *MnAP2/ERF* gene family were involved in 22 fragment repeat events, and 55 *MnAP2/ERF* genes exhibited collinearity with 84 AtAP2/ERF genes, suggesting the generation of new genes in the *AP2/ERF* gene family during the evolution. Finally, The *MnAP2/ERF* gene family formed specific expression clusters in roots, staminate flowers, leaves, bark, and winter buds, which accounted for 76.4% of the gene family, suggesting that most of the *MnAP2/ERF* genes play a significant role in the growth and development of root, male flower, leaf, bark, and winter buds.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f15040697/s1, Table S1: Basic information of *MnAP2/ERF* gene family in *M. notabilis*.

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