Characterization of *WRKY* Gene Family in Whole-Genome and Exploration of Flowering Improvement Genes in *Chrysanthemum lavandulifolium*

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Chrysanthemum is a well-known ornamental plant with numerous uses. *WRKY* is a large family of transcription factors known for a variety of functions ranging from stress resistance to plant growth and development. Due to the limited research on the *WRKY* family in chrysanthemums, we examined them for the first time in *Chrysanthemum lavandulifolium*. A total of 138 *ClWRKY* genes were identified, which were classified into three groups. Group III in *C. lavandulifolium* contains 53 members, which is larger than group III of *Arabidopsis*. The number of introns varied from one to nine in the *ClWRKY* gene family. The "WRKYGQK" motif is conserved in 118 members, while other members showed slight variations. AuR and GRE responsive *cis*-acting elements were located in the promoter region of *WRKY* members, which are important for plant development and flowering induction. In addition, the W box was present in most genes; the recognition site for the *WRKY* gene may play a role in autoregulation and cross-regulation. The expression of the most variable 19 genes in terms of different parameters was observed at different stages. Among them, 10 genes were selected due to the presence of CpG islands, while nine genes were selected based on their close association with important *Arabidopsis* genes related to floral traits. *ClWRKY36* and *ClWRKY45* exhibit differential expression at flowering stages in the capitulum, while methylation is detected in three genes, including *ClWRKY31, ClWRKY100,* and *ClWRKY129.* Our results provide a basis for further exploration of *WRKY* members to find their functions in plant growth and development, especially in flowering traits.

Keywords: *WRKY* transcription factors, ornamental, motifs, *cis*-acting elements, CpG islands, methylation, flowering traits

INTRODUCTION

Chrysanthemum lavandulifolium is one of the original species of *Chrysanthemum × morifolium* and uses as a model plant. It is a major ornamental plant, originated in China, an important cut flower with diversified petal color and shape having a variety of flower structures, which makes it a highly valuable ornamental plant in the floral industry worldwide (Nguyen and Lim, 2019). Chrysanthemum is among the four most important ornamental plants in the world used for

beautification and aesthetic value (Shinoyama et al., 2012). The flower of chrysanthemum is also known for its health benefits, and it contains important antioxidants and phenolic compounds (Lin and Harnly, 2010; Yue et al., 2018).

Due to a simple diploid ($2n = 2x = 18$) genetic background, *C. lavandulifolium* dominates over *C. morifolium* (Huang et al., 2012; Dong-ru et al., 2019). The wild ancestors having desired qualities against biotic and abiotic cues can be used for the genetic improvement of cultivated varieties. Identification and incorporation of such genes into commercial varieties reduces pesticide use, improves plant health, and induces photoperiod-controlling flowering mechanisms (Wu et al., 2010; Fu et al., 2013; Yang et al., 2017).

Genetic architectural study is one of the major research directions in any plant. By narrowing the path further, transcription activation and silencing is the major field of concern. Many transcription factors (TFs) have been explored to switch on and off against certain environmental conditions. *WRKY* TF is one of the major families first identified in plants in 1994 by the name of DNA-binding protein in sweet potato, which is labeled as *SWEET POTATO FACTOR1 (SPF1)* (Ishiguro and Nakamura, 1994). *WRKY* TFs play important roles in resistance against biotic (fungal or bacterial pathogen) and abiotic stresses, growth, and development. Several *WRKY* TFs have been proven to provide resistance against biotic stresses exerted by fungal or bacterial pathogens by influencing other associated genes (Turck et al., 2004; Duan et al., 2007; Zhou et al., 2008, 2011; Li et al., 2009, 2011; Chen et al., 2010; Scarpeci et al., 2013; Phukan et al., 2016; Yokotani et al., 2018; Warmerdam et al., 2020). The name *WRKY* has been given to this family for their distinguishing characteristic, the *WRKY* domain, which is composed of highly conserved 60 amino acids and a zinc finger motif. *WRKY* TFs are classified into three groups based on the number of (WRKYGQK) domain, and zinc-finger motif. Group I has two domains, and groups II and III have one domain. Groups I and II have C2H2 type zinc finger motifs while group III has a C$_2$HC zinc finger motif. Members of group II were further classified into subgroups (Rushton et al., 2010).

WRKY TFs are extensively studied in *Arabidopsis* and most other plants. *WRKY*7 and *OsWRKY11* expression resulted in blooming delay in *Arabidopsis* and rice (Cai et al., 2014; Chen et al., 2019), *WRKY13* has an adverse effect on flowering time, and *WRKY*12, *GsWRKY20* and *CsWRKY50* promote flowering, possibly through the influence of gibberellic acid under short-day conditions in *Arabidopsis* (Luo et al., 2013; Kumar et al., 2016; Li et al., 2016), *GhWRKY22* regulating pollen development (Wang et al., 2019; Ramos et al., 2021). While different *WRKY* family TFs play an important role in chrysanthemum, they showed resistance to salt stress (Liu et al., 2013, 2014; Li et al., 2015b; Jaffar et al., 2016; Liang et al., 2017; Wang K. et al., 2017; He et al., 2018; Zhang et al., 2020), and *CmWRKY1* and *CmWRKY15* transgenic lines in *C. morifolium* interact with *SA* and *ABA* and altered plant development (Fan et al., 2015, 2016; Bi et al., 2021). *CmWRKY48* showed resistance against aphid infestation (Li et al., 2015a). They also play a regulatory role in the flowering stages. The complex network of *VQ20*, *WRKY2*, and *WRKY34* interaction influences the plant gametogenesis and plays a crucial part in

the development of pollen and pollen tube growth, modulating flowering (Lei et al., 2017; Ma et al., 2020).

Flowering time is a complex character regulated by a network of different external and internal signals. Different pathways have been identified, including photoperiod pathway, vernalization pathway, gibberellin-regulated pathways, and autonomous floral initiation pathway (Soares et al., 2020). Therefore, flowering induction studies are more crucial in ornamental plants, especially in chrysanthemum, where all the ornamental characteristics are associated with flowering, i.e., flower color, shape, and size. Chrysanthemum is a short-day plant and needs a specific photoperiod to induce flowering, which makes it relatively expensive as well as limits the annual production of chrysanthemum flowers. We explored the *WRKY* gene family in *C. lavandulifolium* for the first time, prior, the *WRKY* gene family has only been studied in the *C. morifolium* (Song A. et al., 2014). The size of the *WRKY* family in *C. morifolium* comprised 15 members, which were named from *CmWRKY1* to *CmWRKY15* (GenBank: KC615355–KC615369). As *WRKY* is a large family, influencing floral characteristics, we need to explore them deeply. Our present study was a part of such investigation in which we tried to explore the *WRKY* gene family in *C. lavandulifolium* comprised structural gene analysis, protein motifs, phylogenetic relatedness of *C. lavandulifolium* and *Arabidopsis thaliana*, *cis*-acting elements, recognition of CpG rich regions, the methylation status of the genes, and expression of 19 important genes in different tissues at different stages to check their role in flowering-related traits. This investigation will lead us to identify variation in transcription regions in the *WRKY* family for future chrysanthemum research.

MATERIALS AND METHODS

Plant Material and Treatment

The plant materials of *C. lavandulifolium* were obtained from Beijing Forestry University. The plants' G1 lines were grown under controlled environmental conditions in the growth chamber. All the plants used in this research came from the G1 line. The plants were propagated by cuttings and grown in pots. The plants were kept under long-day conditions (14 h L/10 h D) light/dark at a 25°C temperature. The photoperiod conditions were changed to short-day conditions (12 h L/12 h D) when the plants attained maturity (more than 14 leaves). The temperature was kept constant. The plants were watered every 7 days. Stem, root, leaf, buds, and capitulum samples for quantitative real-time polymerase chain reaction (qRT-PCR) were taken at different growth stages, including young, mature, and flowering. The plants were considered at a young stage when they attained eight leaves. The plants were called into the adult stage when the number of leaves was 14, and they were transferred to short-day conditions. The flower develops in short conditions, and samples were taken at different stages of flower development (**Supplementary Figure 1**). The little buds (1–3 mm in diameter) appeared after 7 days in short-day conditions and were taken for RNA extraction. The median buds (5–8 mm in diameter) were

taken after a few days before flower opening. The first flower samples were taken when the flowers had just opened.

Characterization of *WRKY* Gene Family in *Chrysanthemum lavandulifolium*

The *C. lavandulifolium* genome has been sequenced (Wen et al., 2022). All the protein and genomic sequences of *ClWRKY* members were obtained from PRJNA681093, National Center for Biotechnology Information (NCBI). The genomic information of *C. lavandulifolium* was completed by Henan University and Beijing Forestry University, and related papers have been published (Wen et al., 2022). The redundant sequences, which lack the *WRKY* domain, were removed after the sequence analysis using manual inspection in the Mega X software. A total of 138 definite *ClWRKY* sequences were taken for further study. The *ClWRKY* members were named from *ClWRKY1* to *ClWRKY138*, according to the source provider (**Supplementary Table 3**).

Classification of *ClWRKY* Proteins

Multiple protein sequence alignments of *C. lavandulifolium* and *A. thaliana WRKY* were made using ClustalX and MEGA-X version 10.2.6 (Molecular Evolutionary Genetics Analysis) (Kumar et al., 2018). The phylogenetic tree based on amino acid (aa) sequences of *ClWRKY* and *AtWRKY* conserved *WRKY* domains was constructed following the neighbor-joining method with 1,000 bootstraps to determine the close association of both gene families.

Gene Structure and Protein Motif Analysis

The gene structure (intron-exon) of *ClWRKY* members, full-length gene, and coding (CDS) sequences were analyzed in the online database, Gene Structure Display Server (GSDS)[1] as described (Hu et al., 2015). Analysis of the conserved protein motifs was performed using the NCBI CDD batch[2] and the TBtools software (Chen et al., 2020).

Cis-Acting Elements in the Promoter Region of *ClWRKY* Members

The promoter sequences of all 138 *ClWRKY* members were submitted to the PlantCARE software to analyze their promoter region for *cis*-acting elements (Lescot et al., 2002). For this purpose, a 2,000 bp sequence upstream of the gene was taken to identify *cis*-acting elements. The MEME suite software was used to identify *de novo* motifs and known enriched motifs (Bailey et al., 2015). The *de novo* motif analysis was performed to identify significant patterns (Bailey and Elkan, 1994).[3] The known enriched motifs were identified *via* simple enrichment analysis using the MEME software (Bailey and Grant, 2021).[4] Tomtom motif comparison tool was used to quantify the similarity between motifs (Gupta et al., 2007).

[1] http://gsds.gao-lab.org/

[2] https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi

[3] https://meme-suite.org/meme/tools/meme

[4] https://meme-suite.org/meme/tools/sea

CpG Island Analysis of the Promoter Region of *ClWRKY* Gene Family

For CpG analysis, promoter regions from the *WRKY* gene family were sequenced. For the exploration of CpG islands, the "Methyl Primer Express version 1.0" software was used (Methyl Primer Express Software v1.0 Quick Reference Card).[5] All the sequences were submitted to the software one by one to identify CpG islands or rich regions. The minimum length of the island was kept at 200 bp, while the maximum length was 2,000 bp. The criterion of "C + G/Total bases" was 50%. The CpG observed/CpG expected ratio was 0.6.

Genomic DNA Extraction and Bisulfite Treatment

The total DNA was extracted from different tissues of *C. lavandulifolium*. The tissues included root, stem, leaf, and capitulum at three different growth stages, i.e., young stage, adult stage, and flowering stage. Total genomic DNA was extracted using the "Plant Genomic DNA Kit" (TOLOBIO), according to the manufacturer's instructions. DNA was quantified using Thermo Scientific NanoDrop^{TM} 2000/2000c Spectrophotometers, and the quality of DNA was checked by 1.5% agarose gel electrophoresis.

Total genomic DNA from each sample was treated with sodium bisulfites using the "DNA Bisulfite Conversion Kit" (TIANGEN) according to the manufacturer's instructions. The conversion efficiency and quality were measured using Thermo Scientific NanoDrop^{TM} 2000/2000c Spectrophotometers.

Methylation-Specific Polymerase Chain Reaction

Ten genes were selected on the basis of CpG analysis. Primers for all ten genes were designed using the "Methyl primer express version 1.0" software. For this purpose, two types of primers (methylated and unmethylated) were designed to check the possible methylated sites. The primer length ranged from 18 to 22 bp. The PCR amplicon length was between 100 and 175 bp. Methylation-Specific Polymerase Chain Reaction (MSP) was conducted to analyze the CpG island among the ten selected genes after treatment with bisulfite for methylation status. The MSP conditions were as follows: pre-denaturation for 3 min at 95°C, followed by 34 cycles of the 30s at 95°C and 30 s at 55°C, 1 min at 72°C, and a final extension for 5 min at 72°C. PCR products were resolved onto 1.5% agarose gel and observed under UV transilluminator.

Quantitative Real-Time Polymerase Chain Reaction

Nineteen (Zhou et al., 2011) variable genes on the basis of CpG island and phylogenetic analysis were selected for gene expression patterns. Primers for all the 19 *ClWRKY* genes were designed using the "Primer Premier 5" software. RNA was extracted from different plant tissues, i.e., root, stem, and leaves at young, adult, and flowering stages, while the capitulum samples, which

[5] thermofisher.com

comprise little buds, median buds, and first flower (right at the time of flower opening), were taken at flowering stages. The RNA was extracted using the RNAprep Pure Plant Plus Kit (spin column) according to the manufacturer's instructions. The cDNA was synthesized using the "StarScript II First-strand cDNA Synthesis Mix with gDNA Remover." The expression of all the selected genes was observed in qRT-PCR. The qRT-PCR was carried out on light cycle "Quantageneq225 Fluorescent Quantitative PCR," using "2 × RealStar Green Fast Mixture." The conditions of PCR were as follows: denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 20 s, and extension at 65°C for 5 s. The actin gene of the chrysanthemum was used as an internal control. The data from qRT-PCR for relative expression were analyzed using the $2^{-\Delta \Delta Ct}$ method proposed by Livak and Schmittgen (2001).

RESULTS

Identification and Characterization of *WRKY* Gene Family in *Chrysanthemum lavandulifolium*

WRKY genes possess a conserved "WRKYGQK" domain. The "WRKYGQK" heptapeptide is the most defining characteristic of *WRKY* TFs. The *C. lavandulifolium WRKY* sequences were obtained from PRJNA681093, NCBI. A total of 153 *WRKY* gene members have been selected; among them, 138 were exhibited for sequence analysis, and 15 were considered defective. The *WRKY* members are named from *ClWRKY*1 to *ClWRKY*138 according to their location on the chromosome. Protein motif analysis showed homology in most of the conserved regions. Among 138 *ClWRKY* members, 85.51% of members have homology in the "WRKYGQK" core domain, and 19 members have two conserved *WRKY* domains. However, 14.5% of *WRKY* members showed variation in the conserved heptapeptide domain and had substitutions in the core domain. Out of 14.5% variants, 13 members (*ClWRKY*37, 38, 39, 49, 50, 51, 52, 53, 54, 74, 76, 77, and 98) have "WRKYGKK," substituting glutamine as a lysine aa. *ClWRKY*12 replaced histidine instead of lysine, two genes, *ClWRKY*20 and *ClWRKY*21, shared sixth and seventh as glutamine and asparagine substitutions. High diversity has been found in three genes, i.e., (*ClWRKY*27) "WKKYGEQK," (*ClWRKY*97) "WKKYGEKK," and (*ClWRKY*131) "WRKNGQN" *WRKY* domains, respectively (**Figure 1**).

For the determination of important properties such as aa length, molecular weight, and isoelectric point, we thoroughly analyzed the *WRKY* gene. The average length of *WRKY* proteins was 313 aa, while 74 aa was the minimum and 697 aa was the maximum length. The *WRKY* members with the least and highest lengths were *ClWRKY*58 and *ClWRKY*36, respectively. The molecular weight ranged from 0.02 kDa (*ClWRKY*55) to 76.26 kDa (*ClWRKY*36). The pIs (isoelectric point) ranged from 4.73 (*ClWRKY*75) to 10.63 (*ClWRKY*97). Among 138 *ClWRKY* members, 82 were acidic (having less than 7 pI), while 56 were

basic (having more than 7 pI). More details of *ClWRKY* features are available in **Supplementary Table 1**.

Phylogenetic Analysis and Classification of *ClWRKY* Proteins

A phylogenetic tree was constructed between *C. lavandulifolium* and *A. thaliana* to investigate the relatedness of different *WRKY* genes and to understand the classification of different groups among *WRKY* members. *Arabidopsis* is a model plant for genetic study and has been explored for the function of *WRKY* genes. The MEGA-X software was used for agglomerative construction following the neighbor-joining method to find the correlation between *WRKY* members of *C. lavandulifolium* and *A. thaliana*. The *WRKY* members are divided into three groups or seven subgroups. Group I has 27 members, and group II has 58 members with 9 members in IIa, 10 members in IIb, 10 members in IIc, 10 members in IId, and 19 members in IIe. Group III has 53 members and was found to be the largest group in this classification (**Figure 2**). The majority of the *ClWRKY* members clustered in the corresponding groups. Some of the *WRKY* members (*ClWRKY*20, 21, 55, 56, and 58) had one *WRKY* domain, but still, clustered in group I; by the rules, group I members should have two domains. These *WRKY* members may evolve from two *WRKY* domain genes and lose one domain in the process of evolution. Previously, such exceptional cases of *WRKY* members with one *WRKY* domain clustering in group I have been reported (Bi et al., 2016).

Some of the *ClWRKY* members clustered with important *Arabidopsis WRKY* genes that helped us identify the genes that are associated with flowering. *ClWRKY36* is clustered with *Arabidopsis AtWRKY2* and *AtWRKY34* (**Figure 2**), both of which play an important role in pollen development through interaction with the *VQ20* protein (Lei et al., 2017). Similarly, *ClWRKY45* clustered with *AtWRKY71* (**Figure 2**), which play a key role in the regulation of flowering, directly and indirectly, by influencing other flowering-associated genes (Yu et al., 2016, 2018).

Gene Structure of *WRKY* Members and Protein Motif Analysis

For the determination of intron-exon structure similarity, Gene Structure Display Server (GSDS)[6] database has been used. Variation among *ClWRKY* members was observed in terms of a number of introns. The minimal number of introns in a gene was one, which occurred in seven members including *ClWRKY27, ClWRKY58, ClWRKY59, ClWRKY76, ClWRKY86, ClWRKY92,* and *ClWRKY125* while *ClWRKY90* had 9 introns, which is the maximal in the whole family. The overall average number of introns in the *ClWRKY* family was three (**Figure 3**). The number of introns in group I ranged from 1 to 5, group IIa varied from 3 to 9, group IIb from 2 to 6, group IIc from 1 to 2, group IId from 1 to 4, and group IIe from 1 to 4. Group III varied from 1 to 6 (**Supplementary Table 2**). The minimal number of exons was two, which appeared in *ClWRKY27, ClWRKY58, ClWRKY59, ClWRKY76, ClWRKY86, ClWRKY92,*

FIGURE 1 | A screenshot of the MEGA X version 10.2.6, showing variation in the conserved domain (WRKYGQK) of *ClWRKY* proteins, and the variation of the conserved domain is encircled in different colors.

and *ClWRKY125,* while the maximal number of exons was 10, which occurred in *ClWRKY87* and *ClWRKY90* (**Supplementary Table 2**). The overall average number of exons in the *ClWRKY* family was four (**Figure 3**). The exon numbers varied within the groups; group I ranged from 2 to 6, group IIa from 4 to 10, group IIb from 3 to 7, group IIc from 2 to 3, group IId from 2 to 5, group IIe from 2 to 4, and group III from 2 to 7, respectively. The average number of exons within the groups is as follows: group I has 4, group IIa has 6, group IIb has 5, group IIc has 3, group IId has 3, group IIe has 3, and group III has 3, respectively (**Supplementary Table 2**). Protein motif analyses were performed on the *ClWRKY* gene family. The first two motifs represented by green and yellow colors are *WRKY* protein motifs (**Figure 4**). At least one or two *WRKY* protein motifs have been identified among all the gene sequences. This suggests that *WRKY* is a conserved domain.

CpG Island Analysis of the Promoter Region of *ClWRKY* Gene Family

CpG island is the region of DNA that is rich in CpG sites and is often located near the promoters of genes. Ten members were found to have CpG islands; among them, seven members contained CpG in their promoter region, two in the cross-promoter and gene region, and one member was found to have it in the gene body. All the ten members had one CpG island. *ClWRKY115* had the shortest island with 438 bp, while *ClWRKY83* had the longest island with a length of 1,065 bp (**Table 1**). The genes with the CG-rich content are the obvious means of DNA methylation and a prominent source of epigenetic improvement.

Identification of DNA Methylation

Total genomic DNA of *C. lavandulifolium* was extracted from the root, stem, leaf, little buds, median buds, and first flowers

(flowers at just opening time). These tissues were taken at different growth stages of the plant, which include the young stage, adult stage, and flowering stage. The methylation and unmethylation primers were designed for the ten different genes using the "Methyl Primer Express version 1.0" software. These genes were selected due to the presence of CpG islands. The MSP was carried out for the following ten genes: *ClWRKY9, ClWRKY31, ClWRKY33, ClWRKY41, ClWRKY83, ClWRKY97, ClWRKY100, ClWRKY115, ClWRKY129*, and *ClWRKY130*. The methylation status detected by MSP was analyzed through gel electrophoresis. The MSP results showed the presence of methylation in *ClWRKY31, ClWRKY100*, and *ClWRKY129* at different stages in different tissues. These genes showed significant methylation status in all tissue samples at all growth stages of the plant (**Figures 5A–C**). However, the methylation status detection through bisulfite sequencing can give us more clear results compared with the MSP method.

Cis-Acting Elements in the Promoter Region of *ClWRKY*

The *cis*-acting elements in the promoter region are really important for gene expression (Rombauts et al., 1999). The 2,000 bp sequence upstream of the genes was submitted to the PlantCARE software and searched for *cis*-acting elements. A range of *cis*-acting elements was identified in the promoter region of the Cl*WRKY* family, which were highly diverse.

A total of 110 *cis*-acting elements were found in the promoter region of *ClWRKY* members. Most of these elements have a diverse role in the plant life cycle. According to our objectives, we screened out the elements that can play a role in plant growth and development and particularly in flowering induction. Therefore, we selected 15 *cis*-acting elements on the basis of their possible role in flowering-related traits and the overall development of
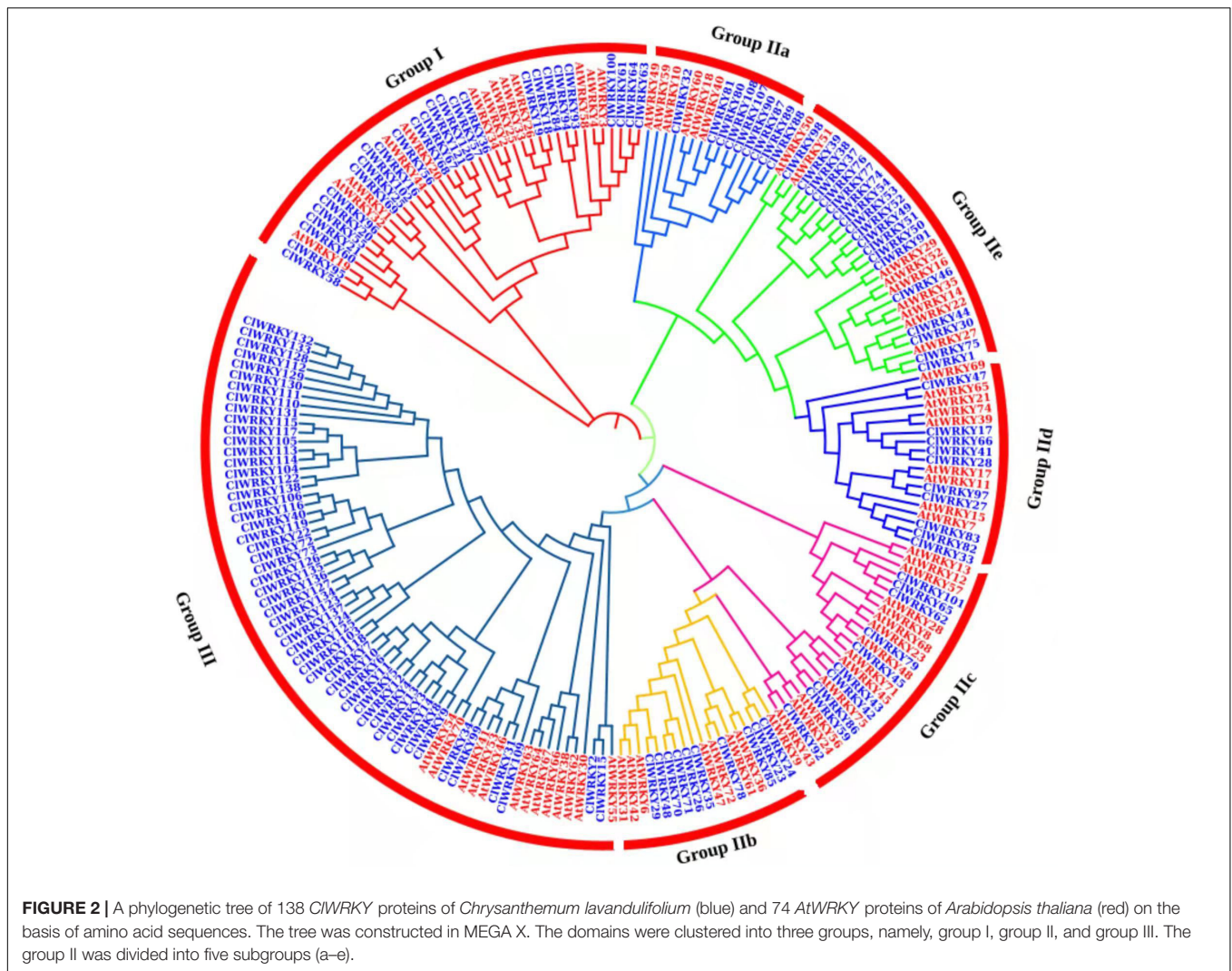
FIGURE 2 | A phylogenetic tree of 138 *ClWRKY* proteins of *Chrysanthemum lavandulifolium* (blue) and 74 *AtWRKY* proteins of *Arabidopsis thaliana* (red) on the basis of amino acid sequences. The tree was constructed in MEGA X. The domains were clustered into three groups, namely, group I, group II, and group III. The group II was divided into five subgroups (a–e).

the plant. The detailed features of the 15 *cis*-acting elements are provided in **Supplementary Table 4**.
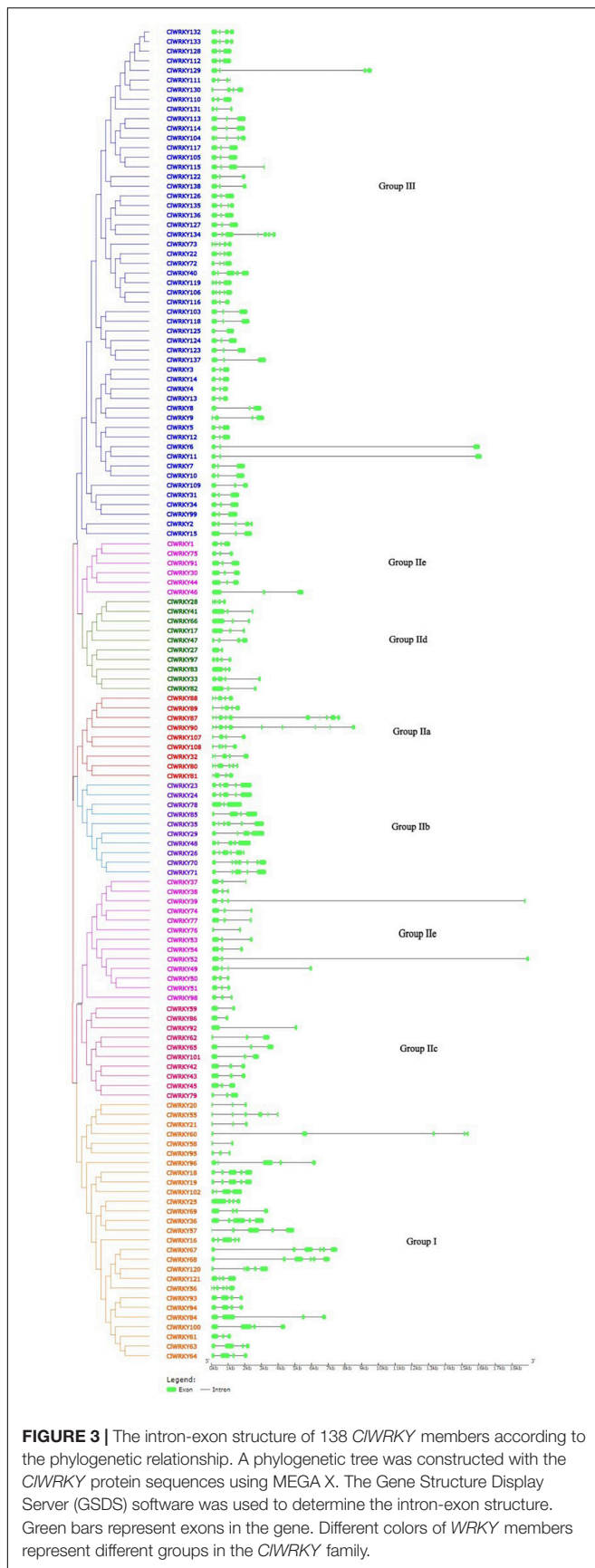
Most of the genes have important *cis*-acting elements, such as auxin responsiveness (AuR) and gibberellin responsive elements (GRE), associated with flowering-related traits (**Figure 6**). W-box was most widely available among the *ClWRKY* family. *ClWRKY45* and *ClWRKY36*, the genes that resulted in high expression at the capitulum stage, had some important elements involved in endosperm expression, elements involved in auxin responsiveness, and ethylene-responsive elements (AuR, EE, and ERE), which are supposed to play a role in flower development. Methyl jasmonate (MeJAR), which plays important role in flowering induction, was present in most of the *ClWRKY* members, especially in group III members, which indicates that *WRKY* members may play a role in the overall development of a plant.

The *de novo* motif analysis in MEME identified 15 motifs (**Supplementary Figure 2**). Tomtom tool comparison showed homology with some important motifs, such as motifs similar to MEME12 and MEME14, which are involved in seed germination, pollen tube growth, and WRKY binding sites (**Supplementary**

Table 5). The enriched motifs identified in MEME are mostly different from the motifs found in the PlantCare software. The simple enrichment analysis (SEA) in MEME identified 95 known enriched motifs. Among them, 49 motifs encoded ethylene, which is essential for plant growth and development. Other important motifs included are WRKY binding sites, TEOSINTE BRANCHED, cycloidea and PCF (TCP) binding sites, and so on (**Supplementary Table 6**).

qRT-PCR Analyses

Different *ClWRKY* genes having special characters were selected on the basis of previous analysis. Total RNA was extracted from different tissues (root, stem, leaves, and flowers) *of C. lavandulifolium* at different stages (young, adult, and flowering). The expression pattern of 19 *WRKY* genes was observed in all tissues at different stages of the plant's life span (**Figure 7**). Among them, 10 genes were selected due to the presence of CG-rich regions, and nine genes were selected as they showed close association with important *Arabidopsis WRKY* genes that are associated with floral traits. Most of the genes exhibited higher expression at the flowering stage in the capitulum
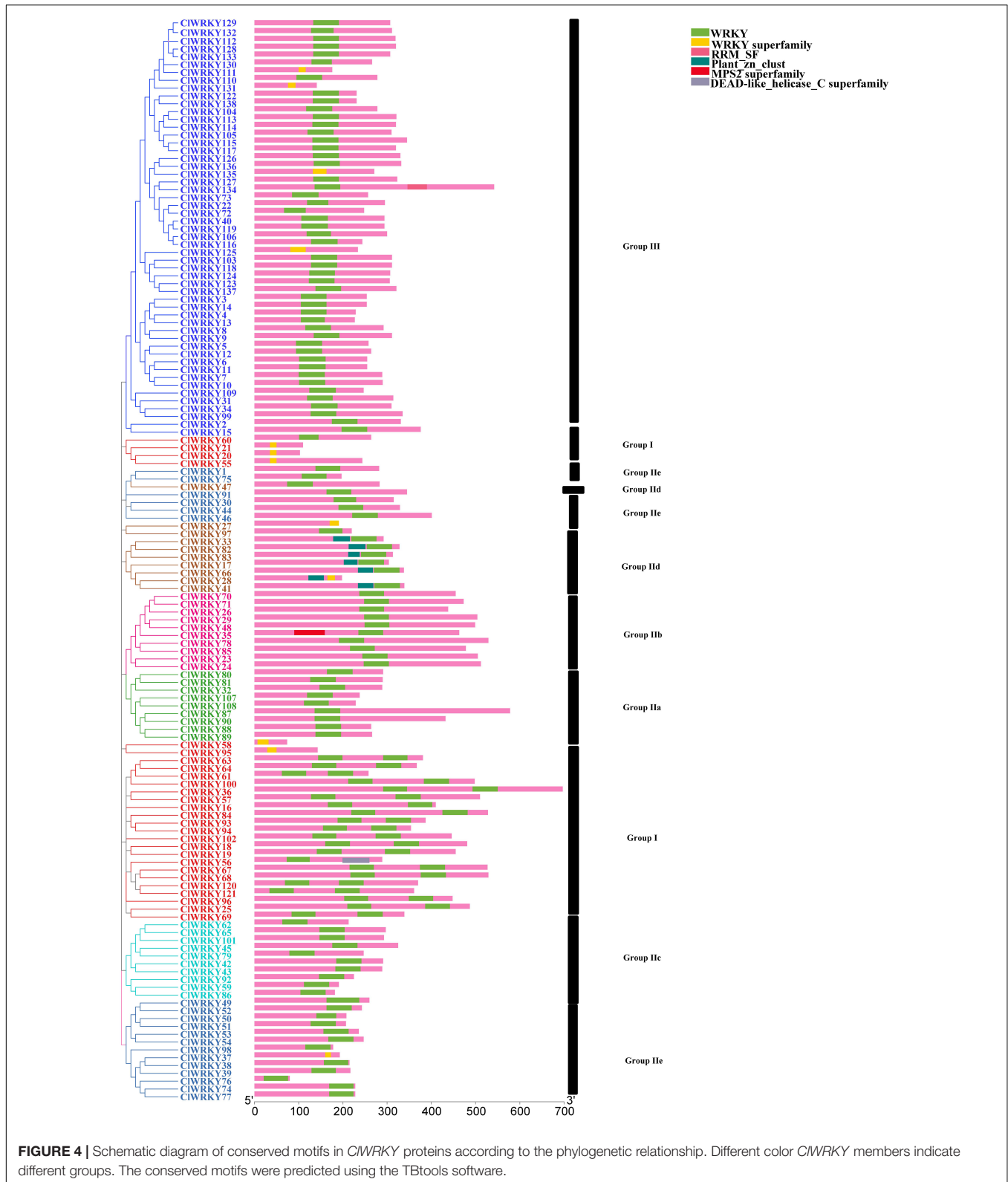
FIGURE 3 | The intron-exon structure of 138 *ClWRKY* members according to the phylogenetic relationship. A phylogenetic tree was constructed with the *ClWRKY* protein sequences using MEGA X. The Gene Structure Display Server (GSDS) software was used to determine the intron-exon structure. Green bars represent exons in the gene. Different colors of *WRKY* members represent different groups in the *ClWRKY* family.

tissues. The highest expression in the capitulum tissues at the flowering stage was exhibited by *ClWRKY36*, *ClWRKY45*, *ClWRKY57*, *ClWRKY59*, and *ClWRKY82* (**Figure 7**). The expression of these genes was higher in the flowering stage, especially in the first flowering (FF) stage and median bud stage, which suggests their role in flowering regulation. These genes were selected for qRT-PCR as they clustered with flowering-related At*WRKY* genes (**Figure 2**). On the basis of previous analysis, *ClWRKY36* clustered with *ClWRKY71*, so it might have a basic role in the control of flowering time and plant development, as *WRKY71* homolog was found to promote flowering in woodland strawberries (Lei et al., 2020). *ClWRKY31*, *ClWRKY33*, *ClWRKY41*, *ClWRKY97*, *ClWRKY100*, *ClWRKY129*, and *ClWRKY130*, which were selected on the basis of having CpG rich regions (**Supplementary Table 1**), showed higher expression in capitulum tissues (**Figure 7**). In addition, *ClWRKY31*, *ClWRKY100*, and *ClWRKY129* were detected with DNA methylation (**Figure 5**), which is linked with flowering regulation and is one of the basic sources of epigenetics. Interestingly, *ClWRKY83* remains stable across all the tissues and stages as it is constantly expressed in all samples of *C. lavandulifolium*. It might play a role in adaptability. Moreover, *ClWRKY83* showed close association with important *AtWRKY7* of *Arabidopsis*. The other *WRKY* member's expressions showed less or non-significant changes at different stages in all tissues.

We also investigated *ClLFY* (leafy), as it not only promotes flowering time but also influences other genes that regulate flowering-related traits (Yang et al., 2016). The expression of this gene was high in the median bud and adult leaf stages, but low in stem and root (**Figure 7**). Besides, *ClLFY* may promote flowering time in *C. lavandulifolium* by making association with *ClWRKY36* and *ClWRKY45*.

DISCUSSION

WRKY TFs are one of the largest transcription families, playing an important role in plant development and resistance against different biotic and abiotic stresses. *WRKY* TFs are spread broadly across higher plants (Li et al., 2014). *WRKY* family investigation is limited to *C. morifolium* with 15 genes (Song A. et al., 2014). Being a model plant for chrysanthemums (Dong-ru et al., 2019), the characterization of the *WRKY* gene family in *C. lavandulifolium* is of great importance. For the first time, we identified 138 *WRKY* members in *C. lavandulifolium*. Out of 138 members, 118 were properly conserved in the "WRKYGQK" domain. Substitution of glutamine occurs instead of lysine in thirteen *WRKY* heptapeptide domains. This WRKYGKK sequence is reported to be a major variant in many studies (Song H. et al., 2014; Song et al., 2016a,b). *ClWRKY12* (WRKYGHK) "Q" replaced by "H" *ClWYKY20* and 21 (WRKYGQN) "K" replaced by "N," *ClWRKY27* (WKKYGEQK) "K" replaced by "Q," *ClWRKY97* (WKKYGEK) "Q" replaced by "E," and *ClWRKY131* (WRKNGQK) "Y" replaced by "N" as their respective domains were also reported in carrot (Nan and Gao, 2019). The variation in the heptapeptide domain of *WRKY* genes

FIGURE 4 | Schematic diagram of conserved motifs in *ClWRKY* proteins according to the phylogenetic relationship. Different color *ClWRKY* members indicate different groups. The conserved motifs were predicted using the TBtools software.
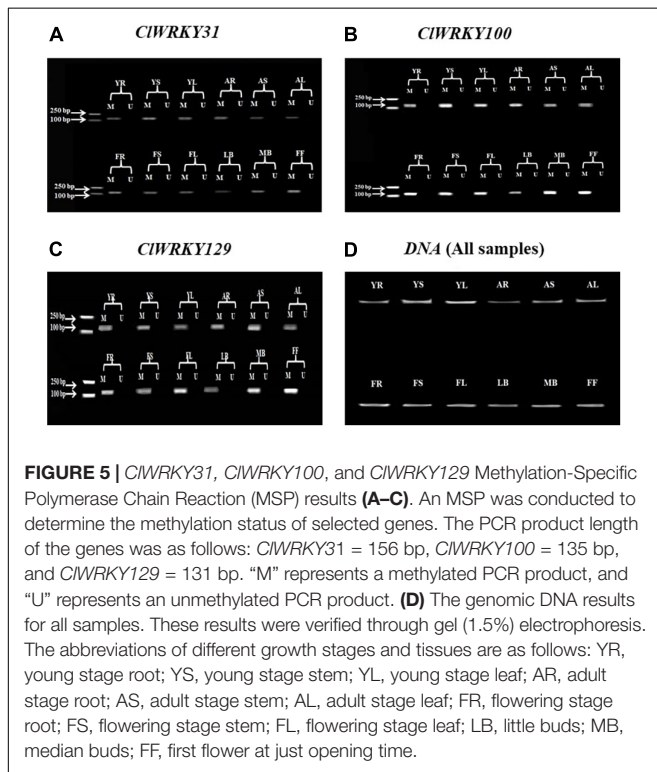
has been largely demonstrated (Yamasaki et al., 2005; Brand et al., 2013; Rinerson et al., 2015; Yang et al., 2020); however, variations in aa sequence were different from our findings. The variation

in the WRKYGQK conserved domain mainly occurs from Q to K aa. The same kind of substitution was observed in the *WRKY* gene family of *Camelina sativa* (Song et al., 2020).

TABLE 1 | Details of each of the ten genes with a CpG island.

NO	WRKY Gene	Island #	Start	End	Length	Region
1	*ClWRKY9*	1	−1239	−593	647	Promoter
2	*ClWRKY31*	1	−1049	−251	799	Promoter
3	*ClWRKY33*	1	−4	566	571	Promoter + Gene Body
4	*ClWRKY41*	1	−1296	−688	609	Promoter
5	*ClWRKY83*	1	−2000	−935	1,065	Promoter
6	*ClWRKY97*	1	−80	471	552	Promoter + Gene Body
7	*ClWRKY100*	1	−2000	−1429	571	Promoter
8	*ClWRKY115*	1	−2000	−1562	438	Promoter
9	*ClWRKY129*	1	6494	6950	457	Gene Body
10	*ClWRKY130*	1	−878	−251	628	Promoter

The 138 ClWRKY genes were analyzed for CG-rich regions using the "Methyl Primer Express version 1.0" software.

FIGURE 5 | *ClWRKY31, ClWRKY100*, and *ClWRKY129* Methylation-Specific Polymerase Chain Reaction (MSP) results **(A–C)**. An MSP was conducted to determine the methylation status of selected genes. The PCR product length of the genes was as follows: *ClWRKY31* = 156 bp, *ClWRKY100* = 135 bp, and *ClWRKY129* = 131 bp. "M" represents a methylated PCR product, and "U" represents an unmethylated PCR product. **(D)** The genomic DNA results for all samples. These results were verified through gel (1.5%) electrophoresis. The abbreviations of different growth stages and tissues are as follows: YR, young stage root; YS, young stage stem; YL, young stage leaf; AR, adult stage root; AS, adult stage stem; AL, adult stage leaf; FR, flowering stage root; FS, flowering stage stem; FL, flowering stage leaf; LB, little buds; MB, median buds; FF, first flower at just opening time.

The *ClWRKY* gene family is composed of 138 genes, which is bigger than the *AtWRKY* gene family (Rushton et al., 2010). The *ClWRKY* family was classified into three groups. The classification of *ClWRKY* members was according to the classification of the *WRKY* family in *Arabidopsis*. Most of the *ClWRKY* members clustered in the corresponding groups. However, some of the *ClWRKY* members with one *WRKY* domain clustered in group I, which included, *ClWRKY58, 95, 60, 56, 20, 21,* and *ClWRKY55*. Among them, *ClWRKY20, 21,* and *55* possessed a unique pattern "WNSIVV" which was conserved in these three members. Clustering of one domain *WRKY* of factors in group I is consistent with the study of the *WRKY* TF family in *Linum usitatissimum* (Yuan et al., 2021). The same results are reported in *Cicer arietinum* (Kumar et al., 2016).

Some *WRKY* members with one conserved domain and clustered into group I have been reported in peanut (*Arachis hypogaea* L.) (Zhao et al., 2020).

The intron-exon structure seems to be diverse, starting from 1 to 9 introns. Diversification in intron-exon structure may play an important role in the evolution of genes (Xu et al., 2012). In this study, we have identified different intron constitutions within gene groups. Group I has four introns on average, group IIa has 5, group IIb has 4, and all other groups have an average of three introns. This suggests that the latter groups originated from group I and earlier subgroups of group II. The same trend of intron presence in different groups of *WRKY* members in eggplant was reported (Yang et al., 2020). A total of six motifs were detected; among them, two motifs were considered to be *WRKY* domains. The conserved domain "WRKYGQK" was almost included in all of the members; however, some of the *WRKY* members showed a slight variation as "WRKYGKK" domain as suggested by Liu et al. (2021) and Qu et al. (2021).

The *cis*-acting elements are involved in plant development and flowering induction. There are several elements, such as auxin, gibberellins, and jasmonic acid, which affect flowering-related traits. Jasmonates, cytokinins, and auxin control second bud growth in chrysanthemum (Sun et al., 2021). Auxin controls plant growth and development (Singla et al., 2006). The petal is the most important factor influencing the chrysanthemum's ornamental value. Auxin signaling is associated with petal growth in chrysanthemums (Wang J. et al., 2017). AuR is found in most *ClWRKY* members and may play a role in plant development and flowering induction. GRE was abundant in *ClWRKY*, an important hormone associated with flowering induction in chrysanthemum (Dong et al., 2017). CmBBX24 regulates flowering in chrysanthemum in conjunction with gibberellin synthesis (Zhu et al., 2020). In addition, the key recognition site for the *WRKY* gene, the W-box containing the TGAC sequence, was observed in most *ClWRKY* members. The W-box contributes under abiotic stress conditions (Dhatterwal et al., 2019) by influencing the early senescence of rice flag leaves (Liu et al., 2016). The presence of flower-associated *cis*-acting elements in most genes indicates the potential of *ClWRKY* to play a role in flowering control in *C. lavandulifolium*.

DNA methylation has an impact on growth and development, with CG content, or CpG, being the most abundant source of DNA methylation (Dong-ru et al., 2019). Various methods such as methylation-sensitive amplification polymorphism and MSP have been applied to methylated genes (Herman et al., 1996; Coronel et al., 2018). In this study, we have identified ten genes with strong CpG content in the promoter region (**Table 1**), which may cause epigenetic changes in *C. lavandulifolium* (Chip and Dresselhaus, 2019). Three genes having methylated sites, i.e., *ClWRKY31, ClWRKY100,* and *ClWRKY129*, were identified on the basis of MSP results. These genes can regulate gene expression and may play a role in flowering-related traits, as they are expressed significantly in capitulum tissues at the flowering stage. *CmMET1* can affect methylation and regulate candidate genes associated with flowering in *C. morifolium* (Kang et al., 2021). DNA methylation changes in the promoter and gene body regions can influence gene expression and function of the protein
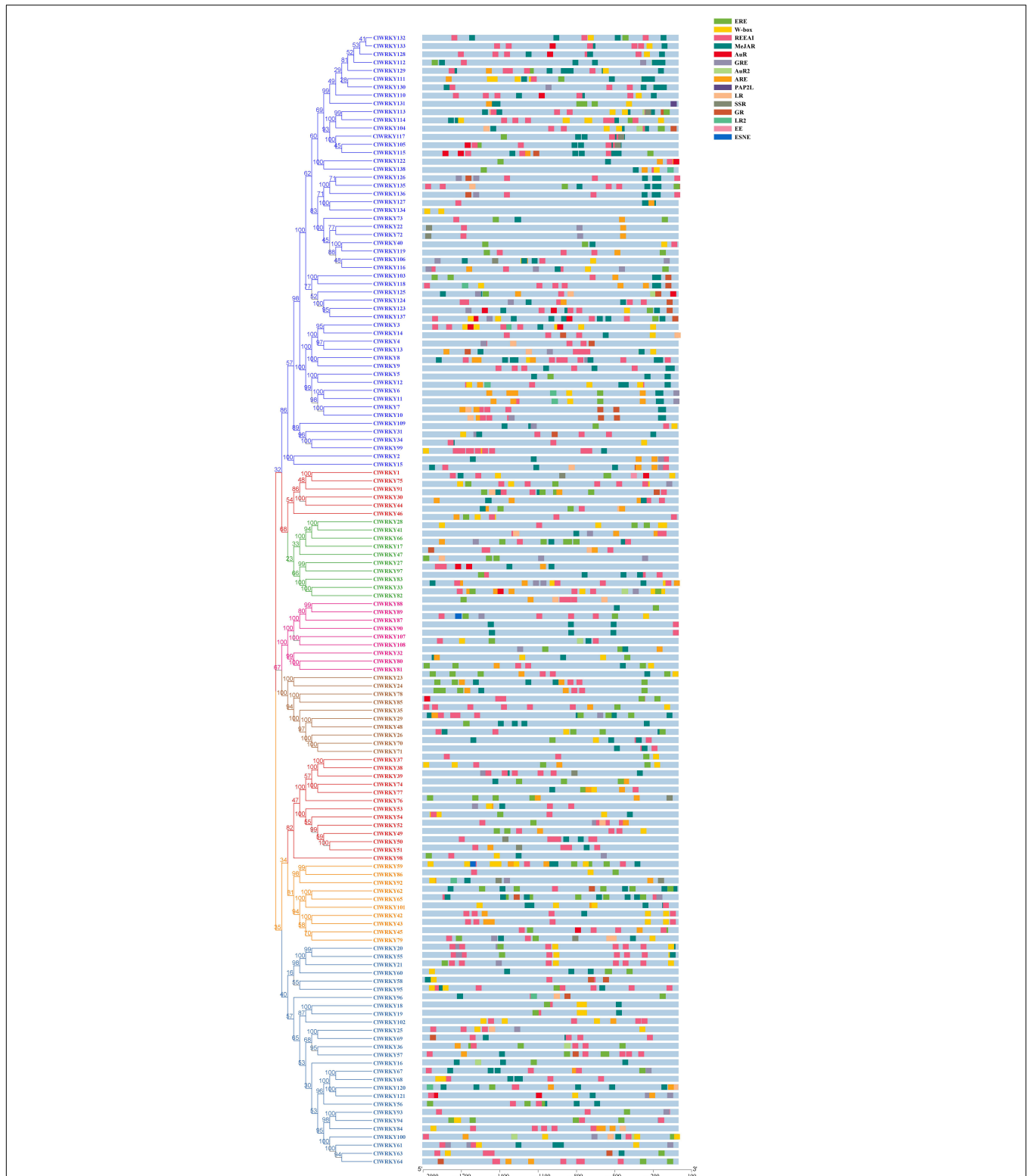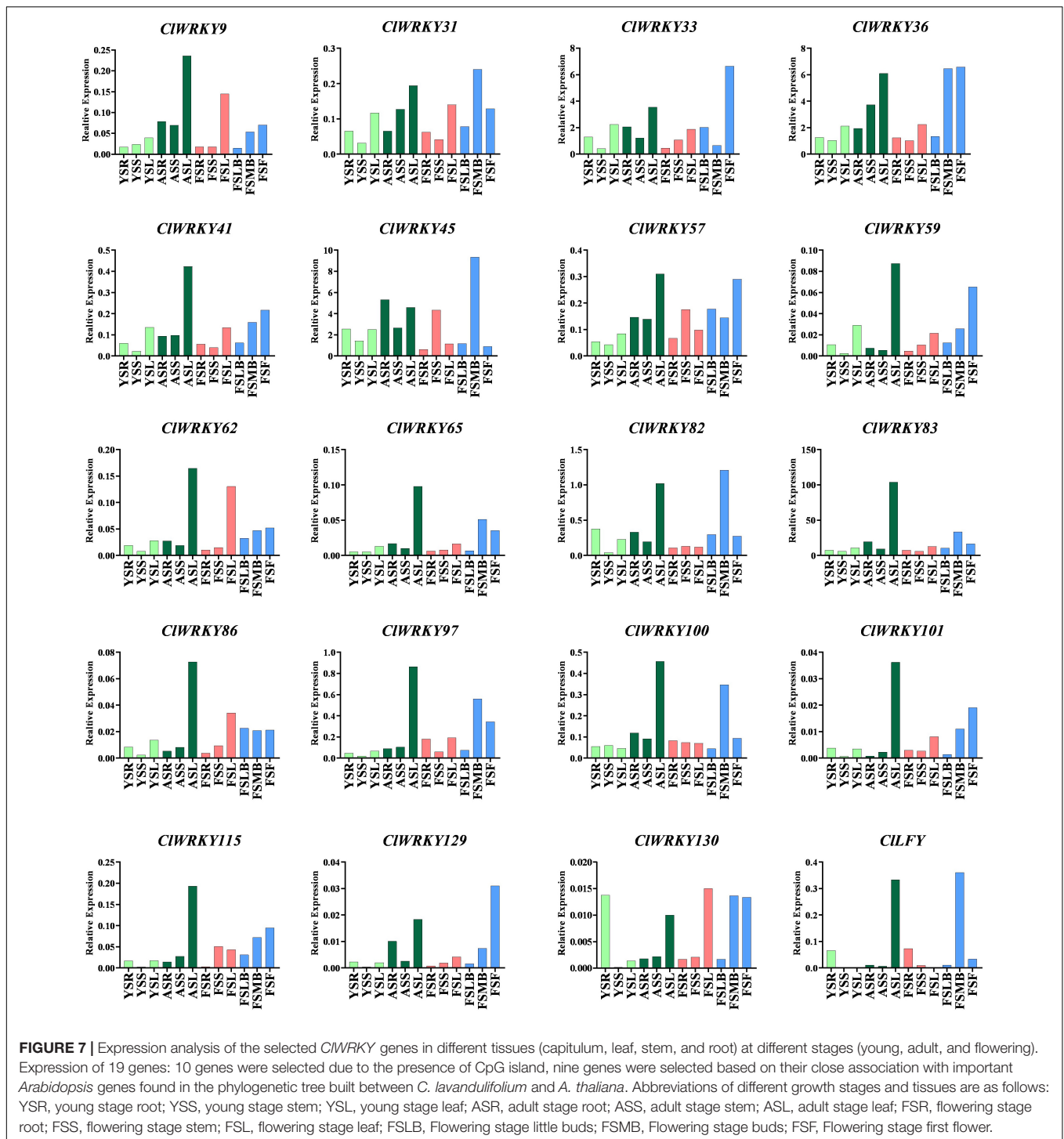
FIGURE 6 | *Cis*-acting element analysis in the promoter region of the *ClWRKY* family. The PlantCARE software was used to search the *cis*-acting elements. Abbreviation of important *cis*-acting elements are as follows: EE, endosperm expression; ARE, auxin-responsive elements; GR, gibberellin responsiveness; LR, light responsiveness; REEAI, regulatory element essential for anaerobic induction; AuR, elements involved in auxin responsiveness; SSR, seed-specific regulation; MeJAR, methyl jasmonate responsiveness; ERE, ethylene-responsive elements; GRE, gibberellin responsive elements; ESNE, endosperm specific negative expression; LR2, elements involved in light response; PAP2L, plant-AP2 such as AuR2, part of auxin responsiveness; and W-box, recognition site of *WRKY* TFs.

FIGURE 7 | Expression analysis of the selected ClWRKY genes in different tissues (capitulum, leaf, stem, and root) at different stages (young, adult, and flowering). Expression of 19 genes: 10 genes were selected due to the presence of CpG island, nine genes were selected based on their close association with important Arabidopsis genes found in the phylogenetic tree built between C. lavandulifolium and A. thaliana. Abbreviations of different growth stages and tissues are as follows: YSR, young stage root; YSS, young stage stem; YSL, young stage leaf; ASR, adult stage root; ASS, adult stage stem; ASL, adult stage leaf; FSR, flowering stage root; FSS, flowering stage stem; FSL, flowering stage leaf; FSLB, Flowering stage little buds; FSMB, Flowering stage buds; FSF, Flowering stage first flower.

(Miguel and Marum, 2011), the morphology of floral characters, photoperiod-related sensitivity, and fruit ripening (Sun et al., 2014; Xiao et al., 2020). We studied only four stages to detect methylation, and it will be better to explore the methylation status in more stages in the future.

This study focuses on the expression of ClWRKY members to find their role in plant development, especially their influence

over flower induction. The expression analysis revealed variations of different genes in different organs. ClWRKY45 showed significant expression at the flowering stage in the capitulum tissue, which may be related to some morphological characters and floral organ development. In the same fashion, ClWRKY45 clustered with Arabidopsis AtWRKY71, a prominent gene that controls flowering characteristics (Yu et al., 2016, 2018),

providing strong evidence for our results. Moreover, *ClWRKY36* and *ClWRKY57* are significantly expressed in capitulum at the flowering stages and have close homology with *Arabidopsis AtWRKY2* and *AtWRKY34*, indicating the role of *ClWRKY36* and *ClWRKY57* in flowering, as *Arabidopsis* homologs are the key regulators of pollen development (Lei et al., 2017). *ClWRKY59* is expressed significantly in the first flower tissue and can positively regulate flowering, as *ClWRKY59* homolog, *AtWRKY75*, promoted flowering in *Arabidopsis* (Zhang et al., 2018). *ClWRKY82* exhibited higher expression in the median bud stage, which indicates its potential to play a role in flowering regulation. *AtWRKY7*, a *ClWRKY82* homolog, regulates plant growth (Chen et al., 2019). The qRT-PCR results indicate that *ClWRKY83* is a stable gene as it expresses across all the tissues at different stages of plant life and may play a role in adaptability. The higher expression of three genes in capitulum tissues, including *ClWRKY31, ClWRKY100,* and *ClWRKY129*, which are methylated as well, can play an important role in flowering-related traits. These results suggest that *ClWRKY33, ClWRKY36,* and *ClWRKY45,* along with *ClWRKY31, ClWRKY100,* and *ClWRKY129,* can play a crucial role in the genetic and epigenetic improvement of the chrysanthemum. *ClWRKY45, ClWRKY36,* and *ClWRK129,* in particular, have the potential to alter flowering-related traits. The overall investigation of the *ClWRKY* domain is providing theoretical insight into *C. lavandulifolium* transcriptional activation and inactivation mechanisms for the improvement of plant development.

CONCLUSION

This study aimed to identify the *WRKY* gene family in *C. lavandulifolium* and explore the potential of *WRKY* genes for genetic improvement. A total of 138 *WRKY* genes were analyzed in *C. lavandulifolium*. All the data results are unique to explore the underlying mechanism of the WRKY TF in *C. lavandulifolium* plant improvement. The gene structure analysis suggested the vast variation among *WRKY* members. The *WRKY* domain remains conserved across all the members of this family. Ten *WRKY* genes possessed CG-rich content in the promoter region where three genes exhibit methylation status.

This research can provide a basis to study the role of *WRKY* genes in the improvement of chrysanthemum plants, especially in flowering traits.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

WZ designed the research project, coordinated, and supervised the study. MA performed all the experiments and wrote the manuscript. MA and KD did most of the experimental work and analyzed the data. WuY, AP, and WaY performed specific experiments during the research. All authors read and approved the final manuscript.

FUNDING

ACKNOWLEDGMENTS

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.861193/full#supplementary-material

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