



Identification of the WRKY Gene Family in *Camellia oleifera* and Expression Analysis under Cold Stress

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Abstract

The WRKY transcription factor family plays critical regulatory roles in plant stress responses, but the response of its members to cold stress remains unclear in *Camellia oleifera*. To identify the WRKY gene family members in *C. oleifera* and characterize their response to cold stress, WRKY transcription factors were screened from a third-generation full-length transcriptome using hidden Markov model search, BLAST homology alignment and conserved domain verification, and their sequence characteristics were revealed through phylogenetic analysis, conserved motif prediction and physicochemical property analysis. Expression patterns under cold stress (−10 °C) were further analyzed in two *C. oleifera* materials (wild, LT; cultivated, GW1) using second-generation transcriptome data. A total of 22 CoWRKY transcription factors were identified and classified into groups I, II and III, with group II being predominant (13 members). All members were hydrophilic proteins containing conserved WRKY domains. Expression profiling revealed stage-specific up-regulation of most members under cold stress, with stronger induction in LT than in GW1. Notably, Co-WRKY-IIb-2 maintained a high expression level throughout the treatment, making it a key candidate gene involved in the cold stress response. This study provides important genetic resources and a theoretical basis for molecular breeding for cold tolerance in *C. oleifera*.

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Keywords: *Camellia oleifera*, WRKY transcription factor, gene family identification, cold stress, expression analysis

1. Introduction

Camellia oleifera, an evergreen tree of the genus *Camellia* in the family Theaceae, is an important woody oil crop endemic to China, mainly distributed in the Yangtze River Basin and the regions to its south. The seed oil of *C. oleifera* is rich in unsaturated fatty acids, particularly oleic acid, and is known as the “oriental olive oil”, occupying an important position in China's woody edible oil industry^[1-2]. As a typical subtropical evergreen species, *C. oleifera* is sensitive to environmental conditions, and cold stress is one of the major environmental factors limiting the expansion of its cultivation area and the stability of its yield^[3]. Low temperature reduces photosynthetic efficiency in leaves, disrupts the stability of the cell membrane system, and induces the accumulation of reactive oxygen species, leading to growth inhibition and, in severe cases, frost damage or even plant death^[4]. Therefore, mining genes associated with cold tolerance and dissecting the molecular mechanisms underlying the cold response are of great significance for breeding for cold tolerance in *C. oleifera*.

The WRKY gene family is one of the important transcription factor families in plants and is widely involved in the regulation of plant growth and development, signal transduction and stress responses^[5-7]. WRKY proteins contain a highly conserved WRKY domain that recognizes the W-box cis-acting element in the promoters of target genes, thereby regulating the expression of downstream genes; they also possess a characteristic zinc-finger motif, which maintains the spatial structure of the protein and strengthens its DNA-binding capacity^[8]. Based on the number of WRKY domains and the type of zinc-finger motif, the WRKY family can be divided into three groups (I, II and III), and group II can be further subdivided into five subgroups (IIa–

Ile)^[9]. And members of different subfamilies differ in structure and function, whereas members within the same subfamily share higher sequence conservation and similar regulatory functions^[10]. To date, 72 WRKY members have been identified in *Arabidopsis thaliana*^[11] and approximately 100 in rice, indicating that this family has undergone substantial expansion and functional diversification during plant evolution^[12, 13].

Under cold stress, plants rapidly activate complex signal transduction networks to enhance stress tolerance, in which WRKY transcription factors play important regulatory roles. Studies have shown that WRKY genes can be induced by low temperature and enhance cold tolerance by regulating the expression of genes involved in the CBF signaling pathway, the ABA signaling pathway and the reactive oxygen species scavenging system^[14, 15]. WRKY proteins can also improve plant tolerance to cold stress by modulating the content of osmotic adjustment substances, the activity of protective enzymes and the stability of the antioxidant system^[16]. Different WRKY members respond to low temperature with distinct temporal patterns and functional divisions: some participate in cold signal perception at the early stage, whereas others function in the maintenance of cold tolerance at later stages. *C. oleifera* is a cold-sensitive species, and the functions of its WRKY genes under cold stress remain to be elucidated^[17].

Although some WRKY family members have been preliminarily identified in *C. oleifera*, systematic analyses of their expression under cold stress based on full-length transcriptome data and materials with contrasting cold tolerance are still lacking^[18-20]. In this study, members of the WRKY gene family in *C. oleifera* were systematically identified based on third-generation full-length transcriptome data, and analyzed their phylogenetic classification, physicochemical properties and conserved motifs. Furthermore, using time-course second-generation transcriptome data under cold stress (−10 °C), the expression patterns of WRKY genes were compared between the wild material LT and the cultivated material GW1, and key candidate genes responsive to cold stress were identified. This work provides a theoretical basis and genetic resources for molecular breeding for cold tolerance in *C. oleifera*.

2. Materials and Methods

2.1. Plant Materials and Data Sources

The plant materials were the cultivated *C. oleifera* cultivar “Ganwu 1” (GW1, low cold tolerance) and wild *C. oleifera* collected from a high-altitude (approximately 900 m) site on Mount Lu (LT, high cold tolerance). Leaves of both materials were collected for RNA extraction and sequenced on the PacBio Sequel II platform; after data filtering and assembly, unigene sequences and their predicted protein sequences were obtained and used for WRKY gene family identification. Cold stress treatment was conducted in an artificial climate chamber at −10 °C, and leaves of both materials were sampled at 0, 1, 3, 6, 16 and 32 h. Total RNA was extracted and sequenced on the Illumina NovaSeq 6000 platform. Gene expression was quantified against the third-generation full-length transcriptome, and the expression level of each gene was measured as FPKM (fragments per kilobase of exon per million mapped fragments). All transcriptome sequencing data are sourced from China National Center for Bioinformatics (project accession number PRJCA076102). *Arabidopsis thaliana* WRKY protein sequences were

obtained from the TAIR database^[21].

2.2. Identification of WRKY Gene Family Members

The hidden Markov model (HMM) profile corresponding to the conserved WRKY domain (Pfam accession PF03106) was downloaded from the Pfam database, and the whole-proteome sequences of *C. oleifera* were searched using the Simple HMM Search function of TBtools (v2.154) with an E-value threshold of 1×10^{-5} ; both sequence-based and domain-based scores were considered for candidate screening^[22, 23]. In parallel, the WRKY protein sequences of *Arabidopsis* were used as queries in a BLASTP search against the *C. oleifera* proteome, with an E-value of 1×10^{-5} and a maximum of five best hits retained for each query^[24]. The intersection of the candidate sequences obtained by the two methods was taken, and redundant entries were removed. Each candidate protein was then subjected to conserved domain verification using the NCBI-CDD and SMART databases, and sequences with incomplete or abnormal WRKY domains were discarded^[25, 26]. The final set of *C. oleifera* WRKY gene family members was thus determined.

2.3. Phylogenetic Analysis and Gene Nomenclature

The identified CoWRKY protein sequences were combined with *Arabidopsis* WRKY protein sequences and aligned using the MUSCLE algorithm implemented in MEGA software with default parameters^[27, 28]. A neighbor-joining (NJ) phylogenetic tree was constructed using the p-distance model with 1,000 bootstrap replicates, and missing data were treated by partial deletion. The tree was visualized using the iTOL online tool^[29]. According to the *Arabidopsis* WRKY classification scheme (group I, subgroups IIa–IIe, and group III), each member was assigned to a group or subgroup, and all members were uniformly named in the format “Co-WRKY + subgroup designation + number”^[9].

2.4. Analysis of Physicochemical Properties and Conserved Motifs

The physicochemical properties of each CoWRKY protein, including the number of amino acids, molecular weight, theoretical isoelectric point (pI), instability index, aliphatic index and grand average of hydropathicity (GRAVY), were calculated using ExPASy ProtParam via TBtools^[30]. Conserved motifs were predicted using the online MEME tool, with the number of motifs set to 10 and other parameters kept at default values^[31].

2.5. Expression Analysis Under Cold Stress

The expression levels (FPKM) of the 22 CoWRKY genes at the six treatment time points were extracted from the transcriptome FPKM expression matrix. A heat map was generated using the HeatMap function of TBtools with row-wise normalization, and the expression differences between the two materials at different treatment stages were compared to screen candidate genes responsive to cold stress.

3. Results

3.1. Identification of WRKY Gene Family Members in *C. oleifera*

Through three successive screening steps, i.e., HMM search, BLAST and conserved domain verification, a total of 22 highly reliable WRKY gene family members were finally identified from the third-generation full-length transcriptome of *C. oleifera*, and were uniformly named according to the

results of phylogenetic classification (Table 1).

Table 1: Members and nomenclature of the WRKY gene family in *Camellia oleifera*

Gene name	Gene ID	Protein ID
<i>Co-WRKY-I-1</i>	PB.22366.1	PB.22366.1 m.59038
<i>Co-WRKY-I-2</i>	PB.18609.1	PB.18609.1 m.47218
<i>Co-WRKY-I-3</i>	PB.23674.1	PB.23674.1 m.63198
<i>Co-WRKY-I-4</i>	PB.22808.1	PB.22808.1 m.60454
<i>Co-WRKY-IIb-1</i>	PB.25785.1	PB.25785.1 m.69857
<i>Co-WRKY-IIb-2</i>	PB.21923.1	PB.21923.1 m.57630
<i>Co-WRKY-IIc-1</i>	PB.34188.1	PB.34188.1 m.93700
<i>Co-WRKY-IIc-2</i>	PB.33576.1	PB.33576.1 m.91932
<i>Co-WRKY-IIc-3</i>	PB.23997.1	PB.23997.1 m.64323
<i>Co-WRKY-IIc-4</i>	PB.30258.1	PB.30258.1 m.83068
<i>Co-WRKY-IIc-5</i>	PB.30877.1	PB.30877.1 m.84763
<i>Co-WRKY-IId-1</i>	PB.31410.1	PB.31410.1 m.86138
<i>Co-WRKY-IId-2</i>	PB.30153.1	PB.30153.1 m.82788
<i>Co-WRKY-IId-3</i>	PB.30726.1	PB.30726.1 m.84340
<i>Co-WRKY-IId-4</i>	PB.26167.1	PB.26167.1 m.71032
<i>Co-WRKY-IId-5</i>	PB.26377.1	PB.26377.1 m.71654
<i>Co-WRKY-IIe-1</i>	PB.25673.1	PB.25673.1 m.69515
<i>Co-WRKY-III-1</i>	PB.32429.1	PB.32429.1 m.88840
<i>Co-WRKY-III-2</i>	PB.20111.1	PB.20111.1 m.51857
<i>Co-WRKY-III-3</i>	PB.24721.1	PB.24721.1 m.66607
<i>Co-WRKY-III-4</i>	PB.25679.1	PB.25679.1 m.69529
<i>Co-WRKY-III-5</i>	PB.30991.1	PB.30991.1 m.85052

3.2. Phylogenetic Analysis and Gene Classification

A joint phylogenetic tree was constructed from the 22 CoWRKY proteins and the *Arabidopsis* WRKY proteins (Fig. 1). Based on the *Arabidopsis* classification criteria, combined with the topology of the phylogenetic tree and the conserved domain characteristics of each protein, the 22 CoWRKY members were classified into three groups (I, II and III): four members in group I (*Co-WRKY-I-1* to *Co-WRKY-I-4*), each containing two WRKY domains; five members in group III (*Co-WRKY-III-1* to *Co-WRKY-III-5*),

which possess a C2HC-type zinc-finger motif; and 13 members in group II, accounting for 59.1% of the family, which were further divided into four subgroups, IIb (2), IIc (5), IId (5) and IIe (1), while no member of subgroup IIa was detected. Members of subgroup IIc were most densely clustered on the phylogenetic tree, suggesting that this subgroup may play an important regulatory role in the growth, development and environmental stress responses of *C. oleifera*.

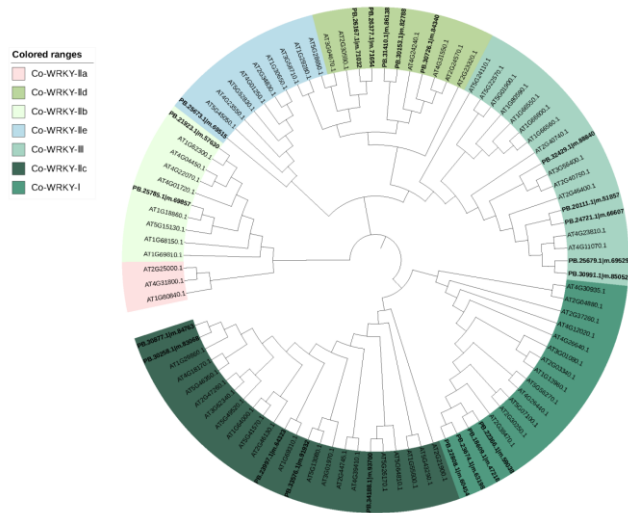


Fig 1: Phylogenetic tree of WRKY proteins from *Camellia oleifera* and *Arabidopsis thaliana*

3.3. Physicochemical Properties of Cowrky Proteins

The physicochemical properties of the 22 CoWRKY proteins were analyzed (Table 2). The numbers of amino acid residues ranged from 176 to 613, and the molecular weights ranged from 18.97 to 68.01 kDa, indicating considerable variation in protein composition among family members. The theoretical pI values ranged from 4.86 to 9.84, with 13 acidic and 9 basic

proteins, suggesting that different members may have distinct DNA-binding capacities and regulatory modes under different microenvironments. Instability index analysis showed that 20 members had an instability index greater than 40 and were thus unstable proteins; only *Co-WRKY-IId-3* (33.42) and *Co-WRKY-IIc-2* (32.52) were stable. Overall, this is consistent with the characteristic of transcription factors

being transiently synthesized and rapidly degraded, which enables plants to respond promptly to external stimuli. The aliphatic index ranged from 40.15 to 80.46, indicating differences in thermostability among members. The GRAVY values of all CoWRKY proteins were negative (−1.031 to

−0.286), indicating that all of them are hydrophilic proteins, consistent with the basic feature of transcription factors that are localized in the nucleus and perform transcriptional regulation.

Table 2: Physicochemical properties of CoWRKY proteins in *Camellia oleifera*

Gene name	Number of amino acid	Molecular weight (kDa)	Theoretical pI	Instability index	Aliphatic index	Grand average of hydropathicity
<i>Co-WRKY-I-1</i>	613	68.01	8.49	60.51	45.2	−0.923
<i>Co-WRKY-I-2</i>	469	52.62	8.54	53.98	40.15	−1.031
<i>Co-WRKY-I-3</i>	597	66.32	6.95	65.11	54.41	−0.852
<i>Co-WRKY-I-4</i>	560	61.74	8.12	68.64	49.82	−0.892
<i>Co-WRKY-IIb-1</i>	544	59.08	6.53	43.56	60.07	−0.691
<i>Co-WRKY-IIb-2</i>	594	64.44	6.23	53.01	60.05	−0.654
<i>Co-WRKY-IIc-1</i>	194	21.68	6.27	50.41	42.32	−1.023
<i>Co-WRKY-IIc-2</i>	210	23.78	9.45	32.52	68.67	−0.495
<i>Co-WRKY-IIc-3</i>	294	32.11	5.94	57.55	49.4	−0.875
<i>Co-WRKY-IIc-4</i>	357	39.66	6.97	61.81	61.99	−0.599
<i>Co-WRKY-IIc-5</i>	337	37.22	6.27	61.28	44.78	−0.823
<i>Co-WRKY-IId-1</i>	349	38.17	9.29	50.55	80.46	−0.286
<i>Co-WRKY-IId-2</i>	346	38.25	9.65	51.87	73.27	−0.607
<i>Co-WRKY-IId-3</i>	176	18.97	9.84	33.42	61.48	−0.576
<i>Co-WRKY-IId-4</i>	323	36.18	9.83	49.85	63.1	−0.794
<i>Co-WRKY-IId-5</i>	345	38.95	9.75	46.6	64.14	−0.856
<i>Co-WRKY-IIe-1</i>	421	46.56	5.42	64.27	64.56	−0.848
<i>Co-WRKY-III-1</i>	327	37.1	6.59	53.79	57.89	−0.830
<i>Co-WRKY-III-2</i>	355	40.51	4.86	49.76	61.83	−0.693
<i>Co-WRKY-III-3</i>	363	39.8	5.22	56.43	53.53	−0.805
<i>Co-WRKY-III-4</i>	361	40.63	5.08	55.35	55.1	−0.756
<i>Co-WRKY-III-5</i>	341	38.62	5.46	49.05	58.01	−0.838

3.4. Conserved Domain and Motif Analysis

Conserved domain analysis showed that all CoWRKY proteins contain a typical WRKY conserved domain, and its core heptapeptide sequence WRKYGQK was highly conserved in most members, with only slight variations in a few members; this region constitutes the functional basis for DNA binding of WRKY proteins. A total of 10 conserved motifs were predicted by MEME analysis (Figs. 2 and 3). The types and arrangement of motifs differed among members, but members within the same phylogenetic clade shared a highly consistent motif composition, indicating functional conservation among members of the same clade. The motif composition was highly consistent with the phylogenetic classification, further confirming the reliability of the classification.

3.5 Expression Patterns of CoWRKY Genes Under Cold Stress

A heat map of the expression levels of the 22 CoWRKY genes was generated based on transcriptome FPKM data at 0, 1, 3, 6, 16 and 32 h of treatment at −10 °C (Fig. 4). The results showed that the responses of different CoWRKY genes to cold stress differed markedly over time: the expression of

most genes changed little during the early stage (0–6 h), whereas the expression of most genes increased markedly during the middle and late stages (16–32 h), showing a distinct stage-specific response. According to their expression trends, the genes could be roughly divided into three types: continuously induced, early-responsive and repressed. Members up-regulated rapidly at the early stage may be involved in the perception of cold signals, whereas those continuously up-regulated at later stages may be associated with the establishment and maintenance of cold tolerance. Comparison of the two materials revealed that the expression levels and induction amplitudes of multiple CoWRKY genes in the wild material LT were markedly higher than those in the cultivated material GW1, indicating a more sensitive cold response in LT. Among them, *Co-WRKY-IIb-2*, *Co-WRKY-IId-3*, *Co-WRKY-IIc-2* and *Co-WRKY-I-4* maintained high transcript levels throughout the treatment period and are the main candidate genes for the cold stress response in *C. oleifera*. Combined with the phylogenetic analysis, these highly expressed candidate genes mostly belong to group II, suggesting that group II WRKY genes may represent a key functional branch in the cold-tolerance regulation of *C. oleifera*.

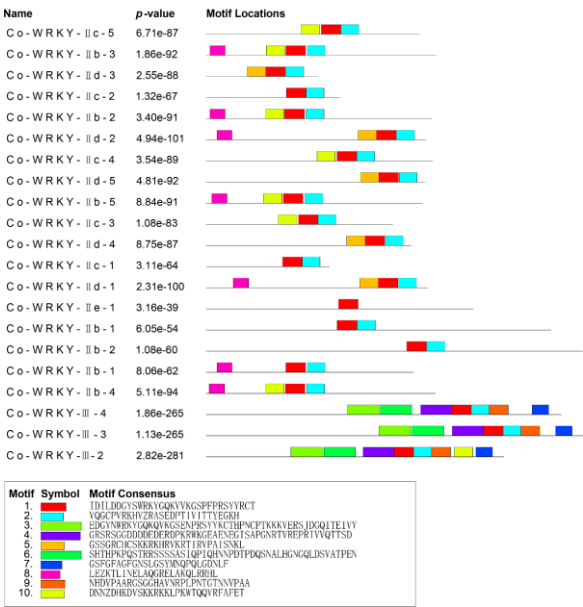


Fig 2: Conserved motif distribution of CoWRKY proteins in *Camellia oleifera*

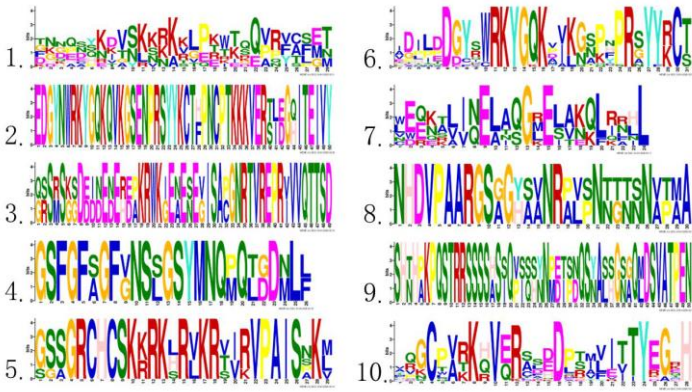


Fig 3: Sequence logos of the conserved motifs of CoWRKY proteins in *Camellia oleifera*

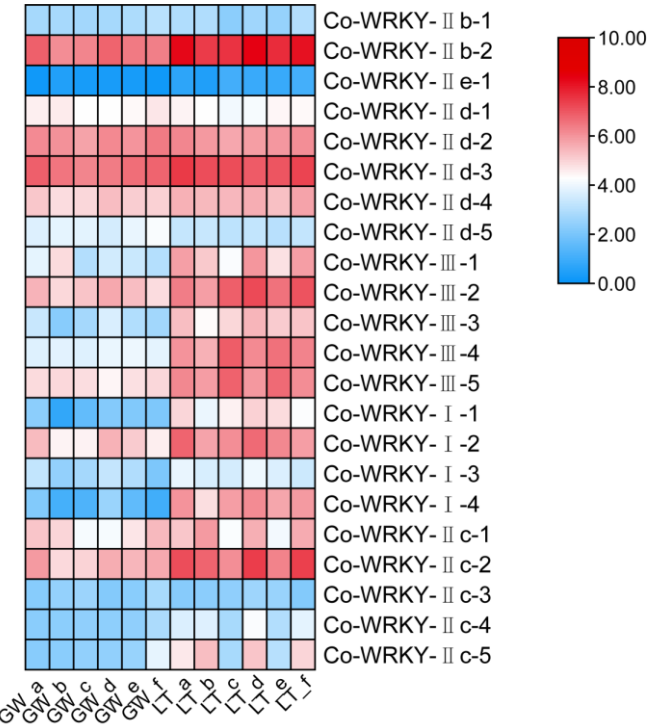


Fig 4: Expression profiles of CoWRKY genes under cold stress (a–f correspond to 0, 1, 3, 6, 16 and 32 h, respectively)

4. Discussion

In this study, 22 CoWRKY members were identified from the third-generation full-length transcriptome of *C. oleifera*, which is fewer than the numbers reported in *Arabidopsis* (72 members) and rice (approximately 100 members), and also fewer than the 89 members identified from the *C. oleifera* whole-genome data [11, 12, 18, 19]. This discrepancy is mainly attributable to the difference in data types: genomic data can theoretically cover all family members, whereas the leaf full-length transcriptome used here can only capture genes actually expressed in a specific tissue under specific conditions, so tissue-specific or very lowly expressed members may have been missed. Studies on the close relative tea plant (*Camellia sinensis*) have likewise shown that genome-wide identification yields a more complete WRKY family than transcriptome-based approaches [32]. Notably, group II members accounted for the highest proportion (59.1%) in our identification, consistent with the predominance of group II in the *C. oleifera* genome-wide studies, indicating that group II is likely the most functionally diversified branch of the plant WRKY family [18, 19]. Segmental duplication has been recognized as an important driver of WRKY family expansion in plants, and the evolutionary pattern of the WRKY family in *C. oleifera* appears to be consistent with that in other plants [33].

In terms of physicochemical properties, all CoWRKY proteins were hydrophilic, consistent with their characteristics as nuclear-localized transcription factors that bind DNA. Most members had an instability index greater than 40 and were thus unstable proteins; WRKY transcription factors are generally subject to stringent multi-level regulation at the transcriptional, translational and protein-turnover levels, and such a “short-lived” nature allows them to be rapidly synthesized and eliminated upon cold or other stress stimuli, which is conducive to the precise regulation of signal transduction [34]. The pI values of the members ranged from 4.86 to 9.84, with both acidic and basic proteins present; different members have different optimal pH environments, which may affect their binding to W-box cis-acting elements. Time-course expression analysis under cold stress showed that the response of CoWRKY genes was distinctly stage-specific: changes were small at the early stage (0–6 h), whereas extensive up-regulation occurred at the middle and late stages (16–32 h). This pattern is similar to the expression behavior of WRKY genes in response to abiotic stresses in wheat and maize, suggesting that plants may first perceive cold signals and initiate a primary response, and then activate more downstream genes to acquire cold tolerance [35–37]. The expression levels of multiple CoWRKY genes in the wild material LT were higher than those in the cultivated material GW1; in particular, *Co-WRKY-IIb-2*, *Co-WRKY-IIc-3*, *Co-WRKY-IIc-2* and *Co-WRKY-I-4* maintained high expression throughout the treatment period, and most of them belong to group II. These results suggest that group II WRKY genes constitute the main functional type in the regulation of cold tolerance in *C. oleifera*. This inference is consistent with the high cold tolerance of LT, which likely results from its long-term adaptation to the low-temperature environment at high altitude on Mount Lu. [17].

Notably, this study was based on transcriptome data and may not have covered all tissue-specific or lowly expressed WRKY members, so the family size is likely underestimated. In addition, the expression patterns of the candidate genes have not yet been validated by quantitative real-time PCR,

and their biological functions remain to be confirmed by genetic transformation and other experiments. Future work will improve the family identification by incorporating whole-genome data and will systematically verify the functions of key candidate genes such as *Co-WRKY-IIb-2*.

5. Conclusion

In this study, 22 WRKY gene family members were identified from the third-generation full-length transcriptome of *C. oleifera* and classified into three groups (I, II and III), among which group II accounted for the highest proportion (59.1%) and subgroup IIc was the most densely clustered. All CoWRKY proteins are hydrophilic, most are unstable proteins, and their theoretical pI values range from 4.86 to 9.84. Members within the same phylogenetic clade share the same conserved motif composition, validating the reliability of the classification. Under cold stress (−10 °C), the expression of CoWRKY genes showed a clear stage-specific pattern, with a much greater up-regulation amplitude in the middle and late stages (16–32 h) than in the early stage (0–6 h). Multiple members in the wild material LT showed higher induction levels than in the cultivated material GW1, indicating a more sensitive cold response in LT. *Co-WRKY-IIb-2*, *Co-WRKY-IIc-3*, *Co-WRKY-IIc-2* and *Co-WRKY-I-4* were continuously highly expressed throughout the treatment, among which *Co-WRKY-IIb-2* was the most prominent and represents a key candidate gene for the cold stress response. These results provide important genetic resources and a theoretical basis for dissecting the molecular mechanism of the cold response and for breeding for cold tolerance in *C. oleifera*.

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