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Genome-Wide Identification of *WRKY* Genes and Their Response to Cold Stress in *Coffea canephora*

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Abstract: *WRKY* transcription factors are known to play roles in diverse stress responses in plants. Low temperatures limit the geographic distribution of *Coffea canephora* Pierre ex A.Froehner. The *WRKYs* of *C. canephora* are still not well characterized, and the response of *C. canephora WRKYs* (*CcWRKYs*) under cold stress is still largely unknown. We identified 49 *CcWRKYs* from the *C. canephora* genome to gain insight into these mechanisms. These CcWRKYs were divided into three groups that were based on the conserved WRKY domains and zinc-finger structure. Gene expression analysis demonstrated that 14 *CcWRKYs* were induced during the cold acclimation stage, 17 *CcWRKYs* were preferentially upregulated by 4 °C treatment, and 12 *CcWRKYs* were downregulated by cold stress. Subsequently, we carried out a genome-wide analysis to predict 14,513 potential CcWRKY target genes in *C. canephora*. These isolated genes were involved in multiple biological processes, and most of them could be grouped by the response to stimulus. Among the putative CcWRKY target genes, 235 genes were categorized into response to the cold process, including carbohydrate metabolic, lipid metabolic, and photosynthesis process-related genes. Furthermore, the qRT-PCR and correlation analysis indicated that CcWRKY might control their putative targets that respond to cold stress. These results provide a basis for understanding the molecular mechanism for *CcWRKY*-mediated cold responses.

Keywords: WRKY transcription factor; *Coffea canephora*; cold stress; coffee; target genes

1. Introduction

WRKY transcription factors (TFs) were first reported in sweet potato as DNA-binding proteins [1]. WRKY TFs not only regulate plant growth and development, but they also play a key role in biotic and abiotic stress responses [2]. The WRKY domain characterizes the WRKY proteins, which contains approximately 60 amino acids with a highly conserved WRKYGQK heptapeptide [2–5]. The WRKY proteins also have a zinc-finger motif at the C-terminus [3,4]. Based on the number of WRKY domains and the pattern of zinc finger motifs, the WRKY proteins can be divided into three groups I, II, and III. Proteins from group I include two WRKY domains and a C_2H_2 ($CX_{4\text{-}5}CX_{22\text{-}23}HXH$) zinc finger motif, while proteins from groups II and III contain a single WRKY domain and a C_2H_2 or C_2HC ($CX_7CX_{23}HXC$) zinc finger motif, respectively [3,4]. Furthermore, group II WRKY proteins can be subdivided into five subgroups (IIa-IIe) [3–5].

WRKY proteins usually bind to the W-box [(C/T)TGAC(T/C)] *cis*-element in the promoter of their target genes to regulate the downstream genes that are involved in all types of biological functions,

including diverse abiotic and biotic stress responses and different plant development processes [3–5]. Owing to their functions, WRKY proteins have been systematically analyzed in numerous plants at the genomic level in Arabidopsis (*Arabidopsis thaliana* (L.) Heynh.), field mustard (*Brassica rapa* L.), tomato (*Solanum lycopersicum* L.), black cottonwood (*Populus trichocarpa* Torr. & A.Gray ex. Hook.), papaya (*Carica papaya* L.), grape (*Vitis vinifera* L.), peach (*Prunus persica* (L.) Batsch 1801 not Stokes 1812 nor (L.) Siebold & Zucc. 1845), wild strawberry (*Fragaria vesca* L.), soybean (*Glycine max* (L.) Merr.), cucumber (*Cucumis sativus* L.), rice (*Oryza sativa* L.), stem-orchid (*Dendrobium officinale* Kimura et Migo), and the thorny shrub *Caragana intermedia* Kuang et H.C.Fu [3,6–16]. Among them, many WRKY proteins have been cloned and shown to be associated with abiotic stress responses, such as cold [17,18], salt [19], and drought [20]. For example, cold stress and ABA treatment upregulated *CsWRKY46*, and the overexpression of *CsWRKY46* increases the freezing and chilling tolerance in cucumber [20]. *OsWRKY71* is highly upregulated in response to cold stress, and the overexpression of *OsWRKY71* can increase the survival rate under cold treatment in rice [21].

WRKY proteins that are involved in plant biotic responses have also been broadly characterized. For instance, the overexpression of *OsWRKY76* in rice results in decrease in the blast disease resistance [22]. Overexpression of *OsWRKY45-2* can enhance the resistance to bacterial blight [23]. Overexpression of *VvWRKY1* in tobacco shows enhanced resistance to fungal pathogens [24]. The role of WRKY proteins involves different plant development processes, such as trichome morphogenesis, flowering, seed development, root development, dormancy, and germination; additionally, senescence has been widely reported [25–31].

Coffea canephora, which belongs to the Rubiaceae family, is one of the key commercial crops in subtropical and tropical developing countries, and it accounts for approximately 30% of the world coffee bean production [32,33]. Commonly known as robusta coffee, *C. canephora* is a species that is considered as one of the parents of *Coffea arabica*, which represents 70% of the coffee bean production in the world [34,35]. For cup quality, *C. canephora* is famous for its dark color and intense flavor and bitterness when compared with *C. arabica* [36]. For agronomic traits, *C. canephora* can provide a higher high-temperature, drought, and coffee-rust tolerant plant as compared to *C. arabica* [33,36,37]. However, like most subtropical and tropical plants, *C. canephora* is sensitive to low temperatures [33]. Low temperature is one of the main factors limiting the geographic distribution of *C. canephora*. Developing cold-tolerant *C. canephora* plants and understanding its cold response mechanisms are exploited in genotype screening and breeding programs of the coffee industry.

The aim of the current research was to identify the *WRKY* genes in the *C. canephora* genome, to classify their expression patterns, and to reveal the putative CcWRKY targets and their regulatory biological processes under cold stress. Our results might provide insight regarding the molecular significance of *CcWRKYs* under cold stress.

2. Materials and Methods

2.1. Plant Materials and Cold Treatment

One-year-old seedlings of *C. canephora* were provided by the Germplasm Repository of Coffee (Coffea spp.), RuiLi City, Ministry of Agriculture (RuiLi, Yunnan, China). The plants were grown at a constant temperature of 24 °C under a long-day cycle (16 h light/8 h cark) in a greenhouse before cold treatment. Cold treatments were carried out as described by a previous report [38], with some modifications. The plants were transferred into a growth chamber and submitted to 24 °C/20 °C (day/night) for seven days, followed by seven days at 13 °C/8 °C (day/night) to express cold acclimation ability, and followed by three days at 4 °C/4 °C (day/night). During the cold treatment, the following parameters were fixed: photoperiod (16 h light/8 h dark), humidity (60%), and luminosity (600–650 μmol m^{-2} s^{-1}). At the end of each treatment time point, five plants were taken out and the two top pairs of recent mature leaves were sampled, frozen immediately in liquid nitrogen, and then stored at −80 °C for further analysis. Three independent biological replicates were obtained and analyzed.

2.2. WRKY Gene Family Identification

The entire genome sequence of *C. canephora* was downloaded from the Coffee Genome Hub (http://coffee-genome.org/) [34]. All of the protein sequences were used as queries to search against the profile Hidden Markov Model (HMM) by HMMER v3.1b2 software (http://hmmer.org/) with Pfam HMM library Pfam 31.0, and the WRKY DNA-binding domain (PF03106, E < 0.1) was isolated [39]. A similar method was applied for the isolation of the *AtWRKY* gene family from TAIR10 (www.arabidopsis.org).

2.3. Chromosomal Location of CcWRKYs

All of the putative *CcWRKY* genes were mapped to *C. canephora* chromosomes based on information available at the Coffee Genome Hub (http://coffee-genome.org/) with Tools of 'Advanced Search'. Figures were modified with Inkscape (version 0.92, Inkscape's Contributors, https://inkscape.org/).

2.4. Sequence Alignment and Phylogenetic Analysis

Multiple alignments of the protein sequences of *C. canephora* and *Arabidopsis* were performed while using ClustalX2 software with default settings [40]. MEGA6 software using a Poisson model constructed the phylogenetic trees, with the following options: pairwise deletion, homogeneous pattern, and 1000-replicate bootstrap [41].

2.5. Exon-Intron Structure Analysis of CcWRKY Ggenes

The exon-intron structures of the *CcWRKY* genes were determined according to the alignments of their full genomic DNA sequences and their respective coding sequences (http://coffee-genome.org). The gene structure diagrams were obtained from the online program GSDS (Gene Structure Display Server, http://gsds.cbi.pku.edu.cn) [42].

2.6. Conserved Motifs Search and GO Enrichment

To identify the conserved motifs of CcWRKY proteins, the full-length protein sequences of *CcWRKY* genes were submitted to MEME (Multiple Em for Motif Elicitation, http://meme-suite.org/tools/meme) with the following criteria: 0 or 1 per sequence for number of repetitions; maximum number of motifs, 100. The motifs with *E* value ≤ 0.1 were selected for further analysis [43]. GO enrichment was carried out using agriGO (http://bioinfo.cau.edu.cn/agriGO/index.php) [44].

2.7. Identification and Analysis of Potential CcWRKY Target Genes

The potential binding site of the WRKY transcription factor, W-box element (C/TTGACT/C), was scanned in the 1,000 bp upstream regions from all 25,572 putative genes of *C. canephora* (http://coffee-genome.org/) while using PlantPAN 2.0 (http://plantpan2.itps.ncku.edu.tw/). As previously reported, genes with ≥ 3 putative WRKY binding sites were considered as potential CcWRKY target genes [15]. The GO information for CcWRKY target genes was downloaded from PLAZA (version 4.0, https://bioinformatics.psb.ugent.be/plaza/) and then submitted to WEGO 2.0 (http://wego.genomics.org.cn/) for functional classification [45,46].

2.8. qRT-PCR Analysis

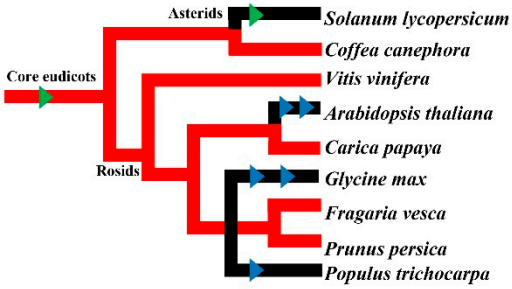
The samples from *C. canephora* were stored at −80 °C until use. RNA was isolated using the RNAiso Plus Reagent (TAKARA BIO INC., Shiga, Japan), according to the manufacturer's protocols. The first-strand cDNA was synthesized using the PrimeScript™ RT reagent Kit with gDNA Eraser (TAKARA BIO INC., Shiga, Japan). The concentration of cDNA was determined and then diluted to 12.5 ng/µl. PCR was performed using the QuantStudio™ 7 Flex Real Time PCR System (Applied Biosystems®, Foster City, CA, USA). The reactions were prepared in a total volume of 20 µl containing 2 µl cDNA, 10 µl of SYBR® *Premix Ex Taq*™ II (Tli RNaseH Plus) (TAKARA BIO INC., Shiga, Japan), 1.0 µl of each primer at 10 µM, and 6 µl distilled water. PCR was performed with the following

program: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, and 56 °C for 30 s. All of the primer sequences that were used in this study are listed in Table S1. The PCR data were analyzed based on the $2^{-\Delta\Delta C}{}_{T}$ method [47], and re-represented by a heatmap, as described by previous research [9] using MeV software [48] with the average mean values of three independent biological replicates. All qRT-PCR was carried out using a sample from the three biological repeats with triplicate technique repeats for each sample.

3. Results

3.1. Isolation of the WRKY Genes in C. canephora

Based on the HMM (Hidden Markov Model) search, 49 protein sequences were identified as members of the WRKY family in *C. canephora*. These proteins were named from *CcWRKY01* to *CcWRKY49*, according to their order on the *C. canephora* chromosomes. Table S2 lists the locus ID, CDS (coding sequence) length, protein length, and genome location of the genes. With the average sequence length of 1132 bp, the CDS sizes of *CcWRKY* genes varied from 312 bp (*CcWRKY28*) to 2985 bp (*CcWRKY26*). The genomic sequence of the *CcWRKY* genes ranged from 827 bp to 10,852 bp (Table S2). For comparative genomic analyses, we collected the WRKY protein coding genes in other plants from previous publications. Finally, 81, 59, 72, 52, 188, 62, 58, and 104 *WRKY* genes were collected from *S. lycopersicum*, *V. vinifera*, *A. thaliana*, *C. papaya*, *G. max*, *F. vesca*, *P. persica*, and *P. trichocarpa*, respectively [8–16,49]. The number of *CcWRKY* genes was similar to *V. vinifera*, *C. papaya*, *F. vesca*, and *P. persica*; but less than *S. lycopersicum*, *A. thaliana*, *G. max*, and *P. trichocarpa* (Figure 1).



	Group								Total
	I	IIa	IIb	IIc	IId	IIe	III	UG/IV	
Solanum lycopersicum	15	5	8	16	6	17	11	3	81
Coffea canephora	10	3	6	15	6	4	5		49
Vitis vinifera	12	3	8	16	6	6	6	2	59
Arabidopsis thaliana	12	3	8	19	9	8	13		72
Carica papaya	9	3	6	13	8	3	8	2	52
Glycine max	32	14	33	42	21	20	26		188
Fragaria vesca	10	3	8	13	6	7	15		62
Prunus persica	10	3	8	15	7	7	8		58
Populus trichocarpa	50	5	9	13	13	4	10		104

Figure 1. Evolutionary relationships of *WRKY* genes in *C. canephora* and other species. UG, ungrouped, indicates the subfamily members from a distinct group in a combined phylogenetic tree. Green arrowheads indicate all genome triplication events. The blue arrowheads show all genome duplication events. The red lines trace lineages of five species that have not undergone further polyploidization. The number of WRKY genes in *Solanum lycopersicum*, *Vitis vinifera*, *Glycine max*, *Fragaria vesca*, *Prunus persica*, and *Populus trichocarpa* were collected from previous studies [8–16,49]. The phylogeny and genome duplication history of core eudicots was also cited from a previous study [34].

Based on the previously study of phylogeny, whole-genome duplication, and chromosomal structure [34], forty-six of the *CcWRKYs* could be mapped to one of eleven chromosomes of *C. canephora*. *CcWRKY47–49* (*Cc00_g06830*, *Cc00_g13890*, *Cc00_g21560*) was putatively located on 'Chromosome Unknown'. *CcWRKYs* were not evenly distributed across the chromosomes of the *C. canephora* genome. The most abundant distribution was located on Chromosome 2 (ten *CcWRKYs*, 20.4%), and the least abundant was located on Chromosome 3 (one *CcWRKYs*, 6.1%) (Figure 2). Most of the *CcWRKYs* (38 genes) that are located on the ancestral blocks correspond to the seven core eudicot chromosomes (Figure 2).

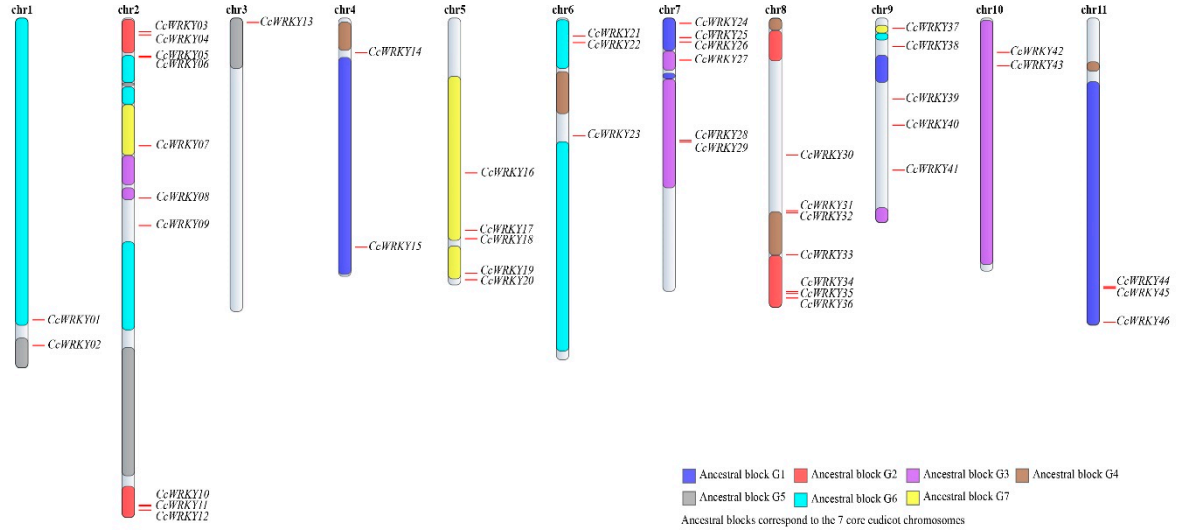


Figure 2. Chromosomal location of 49 *CcWRYKs*. *CcWRKY47–49* (*Cc00_g06830*, *Cc00_g13890*, *Cc00_g21560*) were putatively located on 'Chromosome Unknown'. The chromosomal structure and gene locations were obtained from the Coffee Genome Hub (http://coffee-genome.org/) [33].

3.2. Phylogenetic Analysis and Motif Identification of the CcWRKY Family

To analyze the evolution and relationships among the *CcWRKYs*, we carried out a phylogenetic analysis of the *CcWRKYs* and *A. thaliana WRKY* (*AtWRKY*) genes. The *Arabidopsis* genome (TAIR10) also has been analyzed by HMM search, as described above for *C. canephora*. Finally, 72 *AtWRKY* genes were isolated. An unrooted phylogenetic tree was constructed for 49 *CcWRKYs* and 72 *AtWRKYs* while using the NJ (neighbor-joining) method in MEGA6 (Figure 3). According to the results of the phylogenetic analyses using the predicted WRKY domains and zinc finger structures, the 49 *CcWRKY* genes were divided into three main groups, group I, II and III (Figure 3, Table S2). Table S2 shows the number of WRKY domains and the type of zinc finger motifs. Ten of the CcWRKY proteins contained two complete WRKY domains and a C_2H_2-type ($CX_{4\text{-}5}CX_{22\text{-}23}HXH$) zinc finger motif, and these proteins were classified into group I. The 34 CcWRKY proteins in group II were categorized into five subgroups that were based on the classifications of WRKY genes in Arabidopsis [3]. Additionally, three, six, fifteen, six, and four CcWRKY proteins were found in subgroups II a, II b, II c, II d, and II e, respectively. Subgroup II c showed higher divergence than the other subgroups. There were five CcWRKY proteins that constituted group III, which contained one WRKY domain and a C_2HC-type ($CX_7CX_{23}HXC$) zinc finger motif. WRKY proteins were characterized by the WRKY domain, which included a conserved WRKYGQK heptapeptide [3]. In *C. canephora*, one variant in the signature WRKY domain, WRKYGKK, was identified in three CcWRKYs: CcWRKY28, CcWRKY33, and CcWRKY43 (Table S2).

The conserved motifs and sequences imply important roles in the gene functions. A total of ten motifs (*E* value ≤ 0.1) were identified from the 49 *CcWRKY* proteins (Table 1). Motifs 1 and 2 were highly conserved in all of the CcWRKY proteins, and motif 1 contained the C-terminal WRKY domain, while motif 2 included the zinc finger motifs. Motifs 3 and 4 were found in all the group I CcWRKY proteins, and motif 3 was composed of the N-terminal WRKY domain in group I. In addition to these four highly conserved motifs, motifs 6, 7, and 10 were specific to group II a and II b; motifs 8 and 9 were only found in group II a and I, respectively.

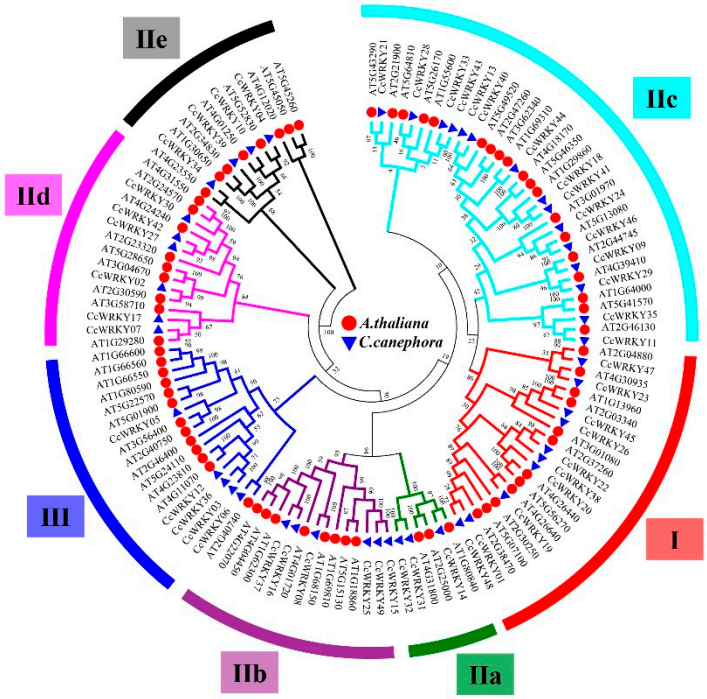


Figure 3. Phylogenetic tree of CcWRKY proteins. The multiple protein sequence alignment was performed using ClustalX2. The NJ (neighbor-joining) tree was constructed in MEGA6 while using a Poisson model with the following options: pairwise deletion, homogeneous pattern, and 1000-replicate bootstrap. Different clades (or subclades) are indicated by the colors of the branch lines, respectively. The red cycles indicate the gene ID from Arabidopsis, and the blue triangle indicates the gene from *C. canephora*.

Table 1. Conserved motifs identified in the CcWRKY proteins. The underlined letters indicate the conserved WRKY domains and zinc finger motifs.

Motif	*E* Value	Sites	Width	Best Possible Match	Groups
1	9.2×10^{-1082}	49	31	EVDILDDGYRWRKYGQKVVKGNPNPRSYYKC	I, II a, II b, II c, II d, II e, III
2	1.2×10^{-850}	49	29	GCPVRKQVQRCLEDMSILITTYEGTHNHP	I, II a, II b, II c, II d, II e, III
3	8.2×10^{-242}	10	40	AEDGYNWRKYGQKQVKGSEYPRSYYKCTHPNCPVKKKVER	I
4	1.1×10^{-74}	10	24	LDGQITEIVYKGNHNHPKPQSTRR	I
5	5.8×10^{-65}	37	15	QKKTRKPRFAFQTRS	I, II a, II b, II c, II d, III
6	3.3×10^{-59}	8	39	MGEVMEENQKLRMHLDRVMKEYRALQMQFHDMVQQEPNK	II a, II b
7	2.4×10^{-53}	9	32	SFADTLSAATAAITADPNFTAALAAAISSIIG	II a, II b
8	1.7×10^{-31}	6	29	VAATAMASTTSAAASMLMSGSTTSTSGLL	II b
9	5.3×10^{-21}	6	33	LTIPPGLSPTSFLESPVLLSNIKAEPSPTTGTF	I
10	2.7×10^{-11}	7	19	ISASAPFPTVTLDLTQNPN	II a, II b

3.3. Exon–Intron Organization of CcWRKY Genes

Gene structure analysis revealed that the *CcWRKY* genes consisted of, on average, four exons. Nineteen *CcWRKY* genes (38.8%) contained three exons (Table S2 and Figure S1). The remaining genes contained two exons (six genes), four exons (eleven genes), five exons (five genes), six exons (six genes), seven exons (one gene, *CcWRKY37*), or 12 exons (one gene, *CcWRKY26*). The diversity of the exon number may be related to the functional diversity of *CcWRKYs*. In this analysis, we noted that *CcWRKYs* in the same group usually contained a similar number of exons. In group I, the number of exons ranged from four to twelve, while the genes that were involved in group II contained two to seven exons, and the number of exons in group III ranged from three to four. The exon pattern

similarity in the same group might be the result of gene duplication events. These results were similar to the observations that were obtained for *Vitis vinifera* [9,50].

3.4. CcWRKYs Expression in Response to Cold Stress

To obtain additional information regarding the responses of the *CcWRKYs* in cold stress, we carried out qRT-PCR using the two top pairs of recent mature leaves of one-year old *C. canephora* seedlings that had been subject to cold treatments. Twelve genes were downregulated by cold treatments (Figure 4A, 4B). Fourteen genes were induced by the cold acclimation process (13 °C) and sustained the up-regulation at 4 °C, which indicated that these genes might be involved in cold acclimation in *C. canephora* (Figure 4C). Seventeen genes were preferentially upregulated by 4 °C cold treatment (Figure 4D). Two *WRKY* genes showed no significantly changed (below a two-fold change) expression level under cold treatment, indicating that they might not be involved in cold responses (Figure 4E). Four genes, *CcWRKY06*, *CcWRKY21*, *CcWRKY25*, and *CcWRKY38*, were excluded from cold treatments due to their high Ct value (≥35 cycles) in the templates of normal and cold-treated leaves. From Figure 4C and 4D, a number of *CcWRKY* genes might be considered as candidate transcription factors for further study in relation to cold responsiveness: *CcWRKY01*, *CcWRKY10*, *CcWRKY13*, *CcWRKY14*, *CcWRKY15*, *CcWRKY24*, *CcWRKY30*, *CcWRKY35*, *CcWRKY37*, *CcWRKY40*, *CcWRKY41*, *CcWRKY48*, and so on.

3.5. CcWRKYs Response to Cold Stress by Mediating Multiple Biological Processes

To analyze the CcWRKY-mediated regulatory processes, we examined the potential promoter regions of all the annotated genes in *C. canephora*. A total of 25,574 putative gene promoters from *C. canephora* were screened. Finally, 14,513 genes were found have at least three W-box elements in their putative promoter regions (Table S3). To obtain more information regarding these potential CcWRKY target genes, the GO information was downloaded from PLAZA 4.0 and then analyzed by WEGO software [45,46]. Of the 14,513 genes, 11,527 genes exhibited GO annotation and they were categorized into 58 functional groups under three major categories: cellular components, molecular functions, and biological process (Figure 5). Metabolic process (6079, 52.7%), cellular process (5596, 48.5%), and response to stimulus (2865, 24.9%) were the most highly represented GO terms in the category of biological process. Cell (5503, 47.7%), cell part (5500, 47.7%), organelle (4135, 35.9%), and membrane (2850, 24.7%) were the most abundant cellular component ontologies. In the molecular function category, binding (7106, 61.6%) and catalytic activity (5313, 46.1%) were the most abundant terms. Furthermore, we also found that 39 *CcWRKY* genes were included in the putative CcWRKY genes, which indicated that the CcWRKY genes regulated themselves (Table S3).

To reveal the regulatory network of the *CcWRKY* genes under cold stress, all of the potential CcWRKY target genes that were involved in the biological process of response to cold (GO:0009409) were isolated, and 235 genes were obtained (Table S4). Functional classification showed that the categories of membrane (58 genes), cold acclimation (21 genes), lipid metabolic process (21 genes), carbohydrate metabolic process (20 genes), hormone-mediated signaling pathway (15 genes), response to sucrose stimulus (13 genes), and photosynthesis process (12 genes) were highlighted (Table S4). Furthermore, 31 GO items were significantly (false discovery rate, FDR < 0.05) represented, including hexose catabolic process, cellular carbohydrate metabolic process, glucose metabolic process, monosaccharide catabolic process, calcium-dependent phospholipid binding, UDP-glucosyltransferase activity, fatty acid biosynthetic process, and others (Table 2).

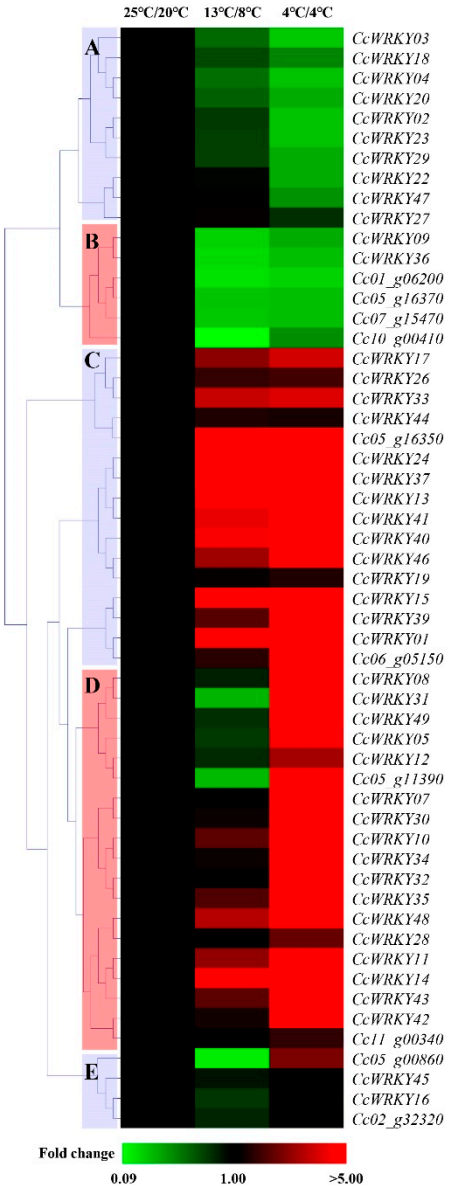


Figure 4. qRT-PCR analysis of expression patterns of *CcWRKYs* and their ten putative targeted genes under cold stress. **A**, genes were downregulated by 4 °C/4 °C cold treatments. **B**, genes downregulated by 13 °C/8 °C and 4 °C/4 °C cold treatments. **C**, genes were upregulated by 13 °C/8 °C and 4 °C/4 °C cold treatments. **D**, genes were upregulated by 4 °C/4 °C cold treatments. **E**, genes with no significantly changed expression under cold treatment.

Table 2. GO enrichment of the 235 putative CcWRKY target genes involved in the biological processes of response to cold (GO: 0009409).

GO Term	Ontology	Description	No. [1]	FDR [2]
GO:0019320	P	Hexose catabolic process	9	5.9×10^{-5}
GO:0044262	P	Cellular carbohydrate metabolic process	17	5.9×10^{-5}
GO:0006006	P	Glucose metabolic process	10	5.9×10^{-5}
GO:0006007	P	Glucose catabolic process	9	5.9×10^{-5}
GO:0046365	P	Monosaccharide catabolic process	9	5.9×10^{-5}
GO:0006091	P	Generation of precursor metabolites and energy	11	5.9×10^{-5}

Table 2. *Cont.*

GO Term	Ontology	Description	No. [1]	FDR [2]
GO:0006096	P	Glycolysis	8	5.9×10^{-5}
GO:0005975	P	Carbohydrate metabolic process	28	5.9×10^{-5}
GO:0005544	F	Calcium-dependent phospholipid binding	6	6.0×10^{-5}
GO:0044275	P	Cellular carbohydrate catabolic process	9	1.1×10^{-4}
GO:0046164	P	Alcohol catabolic process	9	1.1×10^{-4}
GO:0016052	P	Carbohydrate catabolic process	10	1.5×10^{-4}
GO:0019318	P	Hexose metabolic process	10	1.5×10^{-4}
GO:0005996	P	Monosaccharide metabolic process	10	2.3×10^{-4}
GO:0006066	P	Alcohol metabolic process	11	4.0×10^{-4}
GO:0005509	F	Calcium ion binding	12	8.3×10^{-4}
GO:0005543	F	Phospholipid binding	6	9.9×10^{-4}
GO:0044282	P	Small-molecule catabolic process	9	1.8×10^{-3}
GO:0006414	P	Translational elongation	5	2.9×10^{-3}
GO:0044265	P	Cellular macromolecule catabolic process	10	3.2×10^{-3}
GO:0009057	P	Macromolecule catabolic process	11	5.2×10^{-3}
GO:0044248	P	Cellular catabolic process	11	9.3×10^{-3}
GO:0044281	P	Small-molecule metabolic process	23	1.6×10^{-2}
GO:0009058	P	Biosynthetic process	43	1.6×10^{-2}
GO:0035251	F	UDP-glucosyltransferase activity	5	1.8×10^{-2}
GO:0008289	F	Lipid binding	6	1.8×10^{-2}
GO:0009056	P	Catabolic process	12	2.2×10^{-2}
GO:0046527	F	Glucosyltransferase activity	5	2.5×10^{-2}
GO:0044237	P	Cellular metabolic process	77	2.9×10^{-2}
GO:0006633	P	Fatty acid biosynthetic process	5	3.6×10^{-2}
GO:0050794	P	Regulation of cellular process	23	4.3×10^{-2}

[1] Number of genes in our study included in the 235 putative CcWRKY target genes. [2] FDR, false discovery rate.

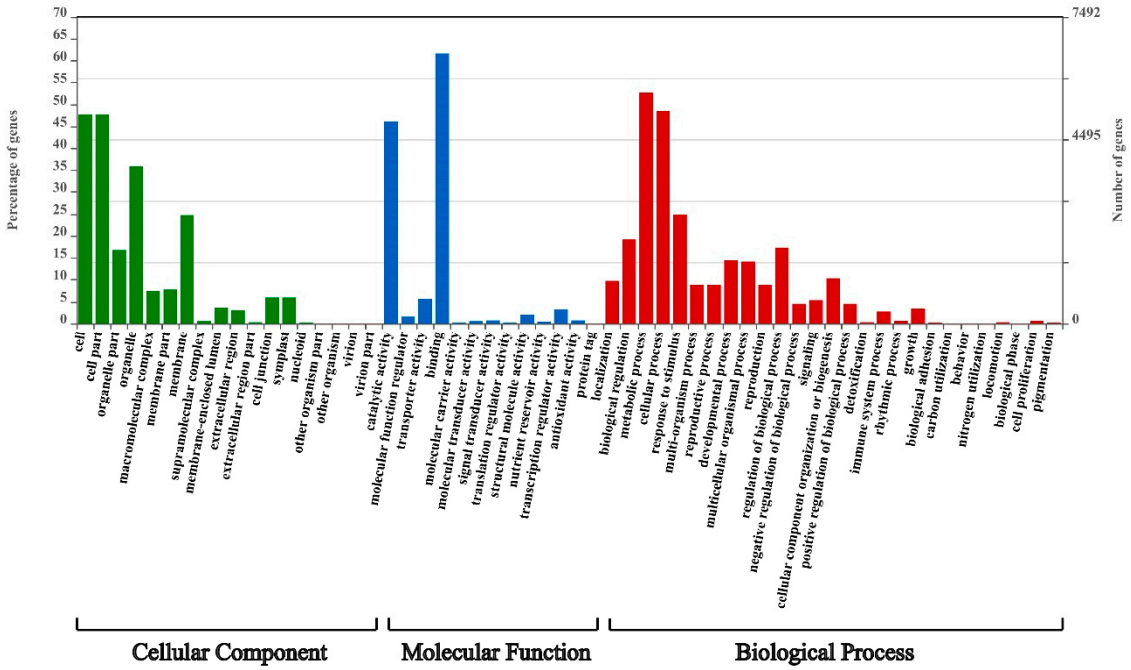


Figure 5. Gene ontology (GO) analysis of CcWRKY target genes. Categories pertaining to cellular components (green color), molecular functions (blue color), and biological processes (red color) were defined by GO classification.

3.6. Confirmations of the Expression Patterns of the Putative CcWRKY Target Genes under Cold Stress

Ten randomly selected putative CcWRKY target genes that involved in the biological process of cellular carbohydrate metabolic were analyzed by qRT-PCR to determine their responses to cold stress to which *CcWRKYs* responded (Table S5). As shown in Figure 4, three genes (*Cc05_g16350*, *Cc06_g05150*, and *Cc05_g11390*) were induced by cold treatment, four genes (*Cc01_g06200*, *Cc05_g16370*, *Cc07_g15470*, and *Cc10_g00410*) were downregulated by cold treatment, two genes (*Cc11_g00340* and *Cc02_g32320*) were almost no changed, and one gene (*Cc05_g00860*) was slightly only increased by 4 °C treatment. Furthermore, we calculated the Pearson correlation coefficients (PCC) between the CcWRKY genes and the ten selected genes based on the Ct value. With PCC above 0.9, one to four *CcWRKYs* were found to be co-expressed with the target genes, except *Cc05_g11390* (Figure 4 and Table S5). These analyses indicated that the *CcWRKYs* directly regulated the putative target of response to cold stress.

4. Discussion

4.1. WRKY Genes in Coffee (Coffea spp.)

Owing to the important roles of WRKY transcription factors in plant kingdoms, genome-wide analyses of the *WRKY* gene family have been performed in *A. thaliana*, *B. rapa*, *S. lycopersicum*, *P. trichocarpa*, *C. papaya*, *V. vinifera*, peach (*P. persica*), *F. vesca*, soybean (*G. max*), *C. sativus*, *O. sativa*, *D. officinale*, and *C. intermedia* [3,6–16,18,49]. In coffee plants, 22 *WRKY* genes have been isolated based on the EST database [51]; however, only four *CcWRKY* genes were involved in their study. In the current study, we first provided a systematical analysis of *CcWRKY* genes and then identified 49 *CcWRKYs* based on the information regarding the *C. canephora* genome sequence (Table S1). A comparison of the number of *CcWRKYs* in *C. canephora* with other sequenced plants [8–16,49], the number of *CcWRKY* genes was similar to *V. vinifera*, *C. papaya*, *F. vesca*, and *P. persica*; but, less than *S. lycopersicum*, *A. thaliana*, *G. max*, and *P. trichocarpa* (Figure 1), which might be due to the whole-genome polyploidization event of *S. lycopersicum*, *A. thaliana*, *G. max*, and *P. trichocarpa*, because of the γ triplication at the origin of the core eudicots [34,50].

4.2. Phylogenetic Analysis, Conserved Motifs, and Structure of the CcWRKYs

In this study, we identified 49 CcWRKYs and divided into three major groups (Table S2, Figure 3), based on the motif features of the zinc finger and the number of WRKY domains. Ten CcWRKYs with two WRKY domains belonged to group I. 34 CcWRKYs with one WRKY domain and the zinc finger structure of CX4-5CX22-23HXH were assigned to group II and accounted for the largest proportion of 69.5%, and 5 CcWRKYs contained a WRKY domain and C2HC-type ($CX_7CX_{23}HXC$) zinc finger motif and were classified into group III. Our findings are consistent with those of *S. lycopersicum*, *V. vinifera*, *A. thaliana*, *C. papaya*, *G. max*, *F. vesca*, *P. persica*, and *Caragana intermedia* [8–10,12–16], which contained the largest numbers of group II WRKY genes and showed no WRKY domain loss.

The variation in the number of group III *WRKY* genes was considered to be one of the causes of diversity of *WRKY* gene family size [52]. In *C. canephora*, five group III *CcWRKY* genes were fewer than *S. lycopersicum*, *A. thaliana*, *G. max*, and similar to *V. vinifera* and *C. papaya* (Figure 1). The smaller number of group III *WRKY* genes in *C. canephora*, *V. vinifera* and *C. papaya* might due to the different pattern of duplication events.

The highly conserved WRKYGQK heptapeptide characterizes the WRKY proteins [3]. However, this protein sequence can be replaced by some variants, such as WRKYGQR, WRKYGKK, WRKYGYA, and WRKYGYK [4]. In our analysis, the WRKYGKK variant was observed in CcWRKY28, CcWRKY33, and CcWRKY43 proteins (Table S2). This result is consistent with recent reports regarding other species, such as *V. vinifera* (VvWRKY8, VvWRKY13, VvWRKY14, and VvWRKY24), radish (*Raphanus sativus* L.) (RsWRKY79, 105, 109, 113, 120, and 121) and *C. intermedia* (CiWRKY41–1, 2, 50 and 51) [9,16,53].

Using MEME, we analyzed the conserved motifs of the CcWRKYs, and ten conserved motifs were identified (Table 1). Motifs 1 and 2 were consistent with the C-terminal WRKY domain and zinc finger motifs separately and were located in all the CcWRKYs. Motifs 3 and 4 were unique to group I, whereas motifs 6, 7, and 10 were specific to group IIa and IIb. The similar motif composition in the same groups or sub-groups indicated their conserved protein architecture.

The exon-intron structural diversification also provided some important clues regarding the evolution of gene families that is caused by the fusions and rearrangement of different chromosome parts [52,54]. In the current study, genes that are involved in the same group usually contain a similar structures, such as four to six exons that were found in group I, except *CcWRKY26* (12 exons), all *CcWRKYs* in group IIe that contained three exons, except *CcWRKY39* (four exons), and all of the *CcWRKYs* in group III that have three exons, in addition to *CcWRKY36* (four exons) (Table S2 and Figure S1). Given all of that, the conserved protein architecture, motif compositions, and similar gene structures strongly supported the phylogenetic analysis results.

4.3. Identification of CcWRKYs and Their Target's Responses to Cold Stress in C. canephora

WRKY genes participate in the cold response, and they have been reported in several plants, such as *V. vinifera* and *D. officinale* [17,18]. To analyze the detailed response of *CcWRKY* genes to cold stress, the *C. canephora* plants were submitted to cold treatments, and the expression patterns were analyzed using qRT-PCR. Based on the results of analysis, *CcWRKYs* can be divided into five groups: cold acclimation-induced genes (14 genes, Figure 4C), 4 °C preferentially induced genes (17 genes, Figure 4D), downregulated by 4 °C/4 °C cold treatments (10 genes, Figure 4A), and downregulated by cold treatments (two genes, Figure 4B), and unchanged genes (two genes, Figure 4E). Furthermore, the GO items of these 14,513 genes were grouped into 58 functional groups. Based on the GO analysis, the processes of metabolic process, cellular process, and response to stimulus were highly represented (Figure 5).

To obtain additional information regarding the regulatory network under cold stress in *C. canephora*, the putative target genes of CcWRKYs that are involved in the GO process of response to cold stress (GO:0009409) were isolated, and 235 genes were obtained (Table S4). Functional classification and GO enrichment analysis revealed that the 235 genes can be grouped into multiple biological processes, such as carbohydrate metabolic, fatty acid biosynthetic, hormone-mediated signaling, photosynthesis, and others (Table 2 and Table S4), indicating that the CcWRKY-mediated cold-responsive regulatory network may cover these processes. Out of 10 putative *CcWRKY* target genes that were randomly selected, only five genes were upregulated upon cold treatment, while four genes were downregulated (Figure 4), indicating that some CcWRKY transcription factors might act as negative regulators. Particularly, three putative target genes would be potent candidate target genes for cold stress-related maker development in *C. canephora*. The expression of three genes was matched to corresponding WRKY protein: Cc05_g16350/CcWRKY44, Cco5_g11390/CcWRKY12, and CC06_g05150/CcWRKY01 (Figure 4). In addition, several putative target genes from Table S5 would be also good candidate for further study and application in breeding: CcSFR2 (Cc03_g00500), CcDREB1D/CBF4 (Cc09_g09540), CcCOR413 (Cc07_g05360 and Cc02_g18110), and CcCOR314 (Cc09_g09540). (Figure 4). Taken together, our analysis extends the knowledge regarding how WRKYs regulate downstream genes in response to cold stress.

5. Conclusions

In summary, 49 *CcWRKYs* were identified from the *C. canephora* genome. These CcWRKYs were divided into three groups that were based on the conserved WRKY domains and zinc finger structure. Ten conserved motifs were identified from the 49 CcWRKY proteins, and motif 1 contained the C-terminal WRKY domain. In addition to the highly conserved WRKYGQK motif, one variant motif (WRKYGKK) of the WRKY domain was isolated. The qRT-PCR results demonstrated that 14 *CcWRKYs* could be induced during the cold acclimation stage, 17 *CcWRKYs* were preferentially

upregulated by 4°C treatment, and 12 *CcWRKYs* were downregulated by cold stress. There were 14,513 potential CcWRKY target genes that were isolated from the *C. canephora* genome, and most of them were grouped into response to stimulus process. In addition, 235 genes of the potential targets were categorized in response to cold process, and these genes can also be grouped into carbohydrate metabolic, lipid metabolic, and photosynthesis processes. The qRT-PCR results and co-expression analysis showed that the CcWRKYs might directly control the targets responding to cold stress. Our results not only provide clues for future analyses of the mechanisms that are used by *CcWRKYs* to mediate cold responses, but also extend our knowledge regarding the WRKY family in plants.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/10/4/335/s1. Figure S1: Gene structures of the *CcWRKYs*, blue boxes represent CDS regions, and solid lines indicate intron regions. Table S1: List of primers used in this study. Table S2: Characteristic features of *WRKY* Transcription factor gene family identified in *Coffea Canephora*, the red color letters indicate the variant WRKY domain. Table S3: Potential WRKY target genes in *Coffea canephora*, genes with at least three W-box elements in their putative promoters were consider as the potential CcWRKY target genes. Table S4: Potential CcWRKY target genes involved in response to cold stress process. Table S5: Information for 10 selected target genes of CcWRKYs in *Coffea canephora* for qRT-PCR verification, the co-expressed *CcWRKYs* were the CcWRKY genes that had Pearson correlation coefficients (PCC) ≥ 9.0 with the putative targets based on the Ct value. "—" indicates no correlational CcWRKY genes.

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