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# Genome Identification and Characterization of *WRKY* Transcription Factor Gene Family in Mandarin (*Citrus reticulata*)

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**Abstract:** *WRKY* proteins are an important group of transcription factors (TFs) gene family and were identified primarily in plants. *WRKY* TFs play vital roles in modulating gene expression when plants face detrimental effects due to the environment. In the current study, we focused on using the mandarin citrus (*Citrus reticulata*) genome to understand the impact of the *WRKY* gene family on the extraction of alleles mining in mandarins. The mining of the *C. reticulata* genome identified 46 *CrWRKY* genes that were classified into three main groups (G1, G2, and G3) further with five subclasses (IIa, IIb, IIc, Iid, and IIe) in the G2 group, and all were presented on 29 scaffolds representing numerous segmental duplications of 100% events established. Multiple sequence analysis predicted the presence of the "WRKYGQK" domain and metal-chelating zinc-finger motif $C_2H_2$ in 45 genes, while the "WRKYGQK" domain was replaced with "WRKYGKK" only in *CrWRKY20*. The comparative relationship of *CrWRKY* with other plant species using dual synteny analysis revealed that the divergence between *C. reticulata* and *C. grandis* occurred after the evolutionary divergence of *C. clementine*, *C. sinensis*, *C. medica*, and *C. ichangensis*. The possible functions of the *CrWRKY* genes in mitigating environmental effects were predicted using cis-regulatory elements analysis and in silico RNAseq analysis, for the development of plants. These results provide a robust platform and absence of knowledge for the functional identification from key genes of *CrWRKY* genes in the mandarin for the possible use to improve key desirable agronomic and consumer-driven fruit quality traits in mandarins and related species.

**Keywords:** *Citrus reticulata*; expression pattern; genome; *WRKY* transcription factor gene family



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## 1. Introduction

The plant, a sessile organism, faces many environmental stresses and evolves various mechanisms to mitigate these environmental fluctuations [1]. Gene expression regulation using various transcription factors plays a significant role in response to ever-changing internal and external stimuli [2]. *WRKY* was identified as a transcription factor gene family in plants and is vital to regulate different pathways against many abiotic and biotic stresses [3]. The first gene, *SPF1* containing the *WRKY* domain, was identified in 1994 from sweet potatoes [4]. The *WRKY* name is derived from the four conserved amino acids present in the *WRKY* domain. *WRKY* domain consists of 60 highly conserved amino

acids, with a conserved heptapeptide stretch WRKYGQK at its N-terminus, followed by a CX4–5CX22–23HXH or CX7Cx23HXC zinc-finger (ZF) motif in the C-terminal [5].

DNA binding motifs in the zinc-finger domain of *WRKY* transcription factors exhibit a conserved structure [6]. Protein-DNA interaction is eliminated by substituting conserved cysteines and histidine residues that form the characteristic zinc-finger structure when binding to a zinc atom [7]. *WRKY* gene could be classified into three subgroups based on the number of *WRKY* domains and type of ZF motif. Group I has two *WRKY* domains and one ZF structure [8]. Group II has one *WRKY* domain and a C2H2 (Cx4-5Cx22-23HxH) type of ZF structure. Based on variants in the ZF motif, Group II can also be further divided into five subgroups, including II-a, II-b, II-c, II-d, and II-e. Proteins from Group III have only one *WRKY* domain but with a $C_2HC$ motif [9–11]. Previous studies have reported the role of *WRKY* TFs in biochemical and physiological processes, growth, development, and biotic and abiotic stress response [12]. *WRKY* protein directly binds to its associated cis-acting element, the W box (TTGACC/T), and regulates the cold tolerance in plant responses to abiotic stress. When plants are exposed to cold, their gene expressions are changed [13]. Many plant species have already been identified and characterized with the *WRKY* gene family (Table 1) due to their complicated functions in plant growth and development (both Angiosperms and Gymnosperms) [14].

**Table 1.** Numbers of *WRKY* genes in various plants.

| Plant Species | No. of WRKY Genes | References |
|---|---|---|
| Wheat (*Triticumaestivum* L.) | 171 | [15,16] |
| Grapes (*Vitisvinifera*) | 80 | [17] |
| Japonica (*Oryza sativa* subsp. *Japonica*) Indian Wild Rice (*Oryza nivara*) | 89<br>97 | [18] |
| Arabidopsis (*A. thaliana*) | 72 | [19] |
| Corn (*Zea maize*) | 140 | [20] |
| Cucumber (*Cucumissativus*) | 61 | [21] |
| Pineapple (*Ananascomosus*) | 54 | [22] |
| Barely (*Hordeumvulgare*) | 86 | [23] |
| Licorice (*Glycyrrhizaglabra*) | 87 | [24] |
| Papaya (*Carica papaya*) | 66 | |
| Sorghum (*Sorghum bicolor*) | 68 | [25] |
| Poplar (*Populus* spp.) | 104 | |
| Moss (*Physcomitrella patens*) | 38 | [25] |

Grape-vine plant *VvWRKY1* transgenic tobacco (*Nicotianatabacum*) plants were susceptible to a fungus variety. Still, the exhibited-ectopia expression of the grape-vine *VvWRKY2* increased the resistance of the necro-trophic fungi *Pythium*, *B. cinerea*, and *Alternariatenuis* resistance. *CaWRKY1* of chili pepper (*Capsicum annuum*), acts as a defense negative-regulator, viral-induction genes silencing of *CaWRKY1* gene reduced *Xanthomonas* growth, showing that overexpression of the gene increased hypersensitivity cell death to tobacco mosaic virus and *P. syringae*. Mildew Locus A (MLA) provides isolate-specific resistance to barley powdery mildew (*Blumeriagraminis*). MLA is physically engaged with *HvWRKY1* and *HvWRKY2*; it triggered the pathogen-associated molecular pattern molecules (PAMP) with their two repressors for basal defense, which work as a primary defense in the nucleus and disrupt WRKY repressor functions [25,26].

The genus *Citrus* includes more than 162 species belonging to the order Geraniales, family Rutaceae, and subfamily Aurantoideae. Mandarin (*Citrus reticulata*) is a primitive species and the most important citrus fruit crop worldwide. Recent studies indicate that farmers face several problems due to biotic and abiotic stresses such as diseases, drought, cold, and soil salinity resulting in a decrease in mandarin production. Traditional breeding methods have been used successfully over the years to improve *Citrus*; however, these methods are limited by slow growth, incompatibility, polyembryony, and parthenocarpy [27]. *WRKY* genes have been reported from *C. sinensis* (51), *C. unshiu* (1), and *C. clementina* (48).

However, due to their essential role in the early response to pathogens and abiotic stresses, several *WRKY* genes were intensively studied in *C. reticulata*. The citrus *WRKY* family is potentially involved in several plant developmental processes and abiotic and biotic stress responses. Thus, members of this family could be suitable candidates for different citrus breeding and improvement programs [28].

This study used tactical bioinformatics tools to identify and characterize genes associated with the *WRKY*TFs in the *C. reticulata* genome. A deterministic strategy was used to identify member of the *WRKY* gene class in *C. reticulata*. *WRKY* genes were classified with their intron/exon distribution pattern, chromosome distribution, presence of conserved motifs, comparative phylogenetic analysis, cis-regulatory elements, enrichment analysis, transcriptome analysis, and following the expression inhibitory miRNA. Further, the genome-wide association studies of *WRKY* genes in *C.reticulata* provided a reference and convenience for practical analysis and cloning of key targeted genes.

## 2. Materials and Methods

### *2.1. Database Search, Classification, and Retrieval of Sequences*

WRKY domain sequence [13], obtained from the Pfam database (PF03106) (http://pfam.xfam.org/family/PF03106 accessed on 17 July 2022), was used to identify and download WRKY TFs gene members in *C. reticulata* genome from the Citrus Genome Database (CGD) (https://www.citrusgenomedb.org/ accessed on 18 July 2022) [29] using the BLAST-P (Protein-Basic Local Alignment Search Technique) algorithm. The physical and chemical properties such as protein length (AA residues), molecular weight (MW), and theoretical isoelectric point (pI) were calculated using the online program ExPASy (https://web.expasy.org/protparam/ accessed on 20 July 2022) [30] while sub-cellular localization was found using WOLF PSORT (https://wolfpsort.hgc.jp/ accessed on 22 July 2022). The information about gene IDs, chromosomal positions, and genome sequences was retrieved from the Citrus Genome Database. The order of physical position was used to rename these *WRKY* encoding genes.

### *2.2. Analyzing the Structure of Genes*

The coding sequence (CDS) and genomic sequence of *CrWRKY* genes were downloaded from Citrus Genome Database (CGD) (https://www.citrusgenomedb.org/citrus downloads/Citrus reticulata/C.reticulataHzau v1 accessed on 28 July 2022). Gene Structure Display Server (GSDS v2.0) software was used to visualize gene structure using these sequences (http://gsds.cbi.pku.edu.cn/ accessed on 31 July 2022) [31]. A phylogenetic tree was added to understand the gene association better.

### *2.3. Phylogenetic Analysis*

To explore the evolutionary relationships among the *WRKY* genes, a neighbor-joining method-based phylogenetic tree was constructed using the molecular evolutionary genetic analysis (MEGA11) package [32]. For this purpose, the MUSCLE program performed the multiple sequence alignment of identified *WRKY* amino acid sequences with default parameters. The unrooted tree was generated using the neighbor-joining strategy, which summarizes the evolutionary distances between 97 members of the *WRKY* gene family. The tree was visualized and presented in circular form, and the closely related sequences were grouped in different clads.

### *2.4. Cis-Elements and Conserved Motifs Analysis*

The promoter sequence of 1000 bp (base pair) upstream to the start codon was retrieved from the Citrus Genome Database to analyze *cis*-regulatory elements (CREs). Promoter sequences of *CrWRKY* genes were used to identify CREs from PlantCare Database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [33].

The Multiple EM for Motif Elicitation (MEME) tools (http://meme.nbcr.net/meme/) [34] were used to understand the motif pattern and structures with the maximum 10 numbers of

motif sets and default value sets or other variables to analyze concluded peptide sequence of *CrWRKY* genes.

### *2.5. Analysis of Synteny and Duplication*

Using the default configuration, duplicated genes event was analyzed through MCScanX (Multiple Collinearity Scan toolkit) [35]. Syntenic and dual syntenic maps were created using TBtools [36] to find the synteny link between the paralogous genes of *C. reticulata* and orthologous genes of *CrWRKY* in *Arabidopsis*, *C. sinensis*, *C. Clementina*, *C. grandis*, *C. medica*, and *C. ichangensis.*

TBtools software [36] was used to calculate Ka/Ks substitution values from the aligned protein sequences of *CrWRKY* genes. These values were used to calculate the time of divergence (T) and the molecular evolution rate of every gene pair. The formula T = Ks/2R was used to calculate the divergence time equation, where R of *C. reticulata* is $1.7 \times 10^{-8}$. Gene IDs and their length were used to map the scaffold by TBTools software [36].

### *2.6. Expression Analysis of CrWRKY in Different Organs*

To investigate the expression patterns of all *CrWRKY* genes tissues, we obtained previously generated RNA-seq (GEO accession no. GSE94810, GSE67560, and GSE74384) and EST (NCBI) data for Mandarin citrus (*C. reticulata*) plant tissues including nucellarembryony, ovule, fruit, flower (before and after anthesis), roots, and leaves. The experiment (GSE74384) was conducted at Huazhong Agricultural University (Wuhan, Hubei Province, China) to identify the candidate gene related to nucellarembryony initiation (NEI) and polyembryony (PE) formation from mature Mandarin citrus (*C. reticulata*) trees grown under natural conditions. The monoembryonic clementine (CM, *C.clementina*) and the polyembryonic Ponkan (PK, *C. reticulata* Blanco) ovules were collected for roots, leaves, flower and fruit from three biological replications (trees) at anthesis after anthesis/50% flowering (DAF) (day 0 DAF) and subsequently at 3, 7, 14, 21, and 28 days after flowering (DAF). Samples were taken right before and after the appearance of Nucellar Embryo Initials (NEI) cells were used to create messenger RNA-seq libraries. PK/CM at pre-NEI and NEI stages with biological replicates. The expression pattern of all 46 *CrWRKY* genes was retrieved from this dataset. The experiment (GEO accession no. GSE94810) focused on understanding the wild-type (WT) of *C. reticulata* cv. Suavissima. Fruits were collected from the field of Wenzhou (Zhejiang Province, P.R. China; [37]). The fruit color is one of the most important appearance quality traits. The flavedo of the *C. reticulata* cv. Suavissima wild and its mutant were collected at 21 DAF of the wild-type and mutant-type and 30 DAF of the wild- and mutant-type after storage for RNA extraction, which had slight phenotypic differences [37]. Reads per kilobases per million mapped reads (RPKM) values from RNA-seq data were log2 transformed for expression profiling. Expression patterns with hierarchical clustering were displayed in Heatmap Illustrator in TBtools software [36].

### *2.7. Comprehensive Analysis of microRNAs*

psRNA Target using CDS sequences of *CrWRKY* genes and its default parameters was used to determine the micro-RNA (miRNA) sequences associated with *CrWRKY* genes in *C. reticulata* [38]. Previous in vivo and in vitro research was used to determine the potential function of the discovered miRNA.

## 3. Results

### *3.1. Identification of WRKY TFs in Mandarin (C. reticulata)*

Initial analysis identified 87 genes that encode the WRKY domain in *C. reticulata* genome proteins. The proteins encoded by the identical gene isoforms and proteins containing a truncated WRKY DNA binding domain were excluded from the analysis. A total of 46 non-redundant *CrWRKY* genes were identified and used for further analysis. These non-redundant *WRKY* protein sequences from *C. reticulata* included the highly conserved WRKYGQK motif in all sequences except *CrWRKY20*, which contain WRKYGKK.

Fourteen of sixty-one AA in the *WRKY* domain were 100% conserved in all *CrWRKY* peptide sequences in *C. reticulata* (Figure 1).

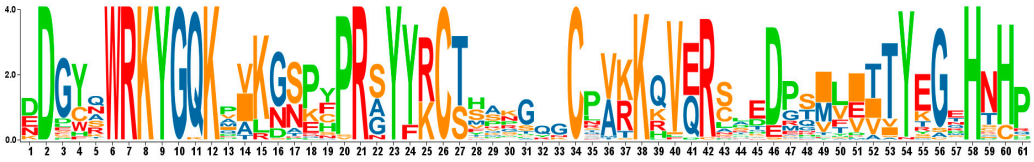

**Figure 1.** The sequence logos were based on alignments of *Citrus reticulata WRKY* domains. *WRKY* domain was highly conserved across all *WRKY* proteins in *C. reticulata* while the zinc-finger C2-H2 domain was highly conserved in *WRKY*.

The sizes of the proteins encoded by *CrWRKY* genes ranged from 159 to 602 AA. The molecular weight ranged from 18.23697 to 65.19112 kD, with *CrWRKY20* being the smallest and *CrWRKY30* being the most extended protein (ExPASy; https://web.expasy.org/protparam/) (Table 1).

### *3.2. Gene Structure, Conserved Motif, and Domain*

The number and structure of introns and exons play a crucial role in determining evolutionary relationships among different genes [39]. Exon–intron structures of *CrWRKY* genes were comprehensively demonstrated phylog (Figure 2) enetically, revealing the gene distribution pattern and their number. The intron numbers varied from one to five in *CrWRKY* genes (Figure S2). Three genes contain one intron (6.5%), twenty-three *CrWRKY* genes retained two introns (50%), seven genes owned three introns (15.21%), nine genes had four introns (19.56%) and four genes had five introns (8.69%). All of the *CrWRKY* genes in subfamily G3, 2D, and 2E possessed two introns, while the several introns in the *CrWRKY* gene in subfamily G1 varied from two to five. Group 2a *CrWRKY* genes introns ranged from three to four and 2b from three to five. Group 2C also contained variable numbers of introns ranging from one to three (Table 1, Figure S2).

All 10 motifs identified in the mandarin *WRKY* proteins were studied using the MEME program in TBtools [36] (Figure 3). *WRKY* domain was consistent in all the *CrWRKY* proteins. It was also determined that *CrWRKY* proteins from the same group have similar motifs, indicating the involvement of these conserved motifs in group-specific activities among the whole gene family. The presence of two *WRKY* domains in Group 1 (G1) further supports the phylogenetic distribution of the gene family. G1 contains two WRKY domains, collectively made up of 5-6 motifs, but *CrWRKY2* showed ambiguity by having 4 motifs and an additional domain (Plant-znclust) at the C-terminus region. Group 2A showed a single *WRKY* domain and most of them having 4 motifs but *CrWRKY19* displayed an additional motif (motif 10) in its structure. In Group 2B, *CrWRKY27* and *CrWRKY34* also contained bZIP and Phage_GPO superfamily domains in their N-terminus regions. Except for *CrWRKY23*, all the members of Group 2B had 7 motifs. In the case of Group 2C, all the members exhibited complete homogeneity in the number of motifs and domains, except for *CrWRKY8*, which had an additional motif (motif 6) at N-terminus. However, *CrWRKY26* contained motif 4 instead of motif 6, which was unusual for the whole of Group 2D (Figure 3). Furthermore, the only member from the whole group lacked the Plant-znclust domain. Other than motifs 1, 2, and 6, *CrWRKY32* also had motif 9. *CrWRKY39* was the only member from the whole gene family with no *WRKY* domain and had the Herpes-BLLF1 superfamily in its place. While coming towards the motifs, this group displayed conservation of motifs 1 and 2 among its members. All the members of Group 3 had the same number of motifs and conserved domain structure. This comparable distribution of conserved motifs between the *WRKY* proteins in each group corresponds to the similarity in the functioning of these genes.

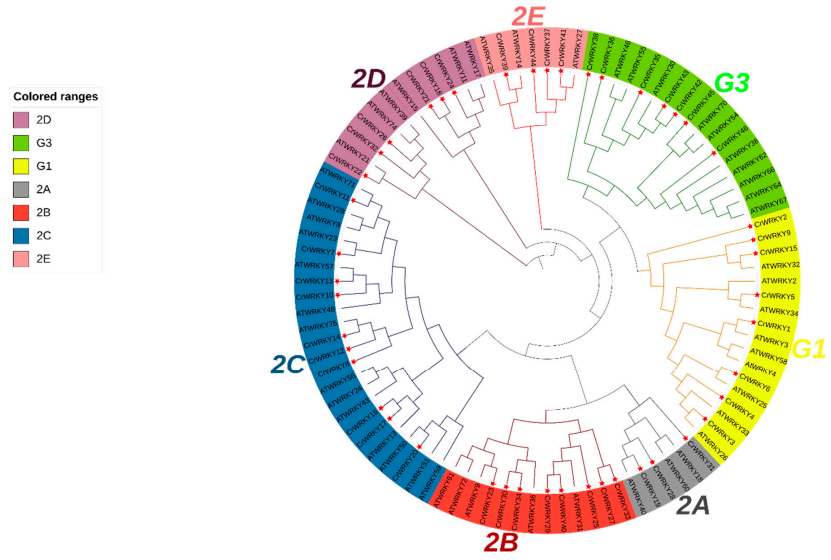


**Figure 2.** Phylogenetic relationship between the *WRKY* genes of *C. reticulata* and *A. thaliana*. The genes of *C. reticulata* were marked with red stars. *CrWRKY* genes were categorized into seven subgroups. G1 is represented by the yellow color, 2A is shown as the grey color, red represents 2B, blue shows 2C, the 2D group of *CrWRKYs* is denoted in purple color, pink represents the 2E group, and G3 is represented by green color. The evolutionary history was inferred using the NJ method with 1000 bootstraps. This analysis involved 95 *WRKY* genes.

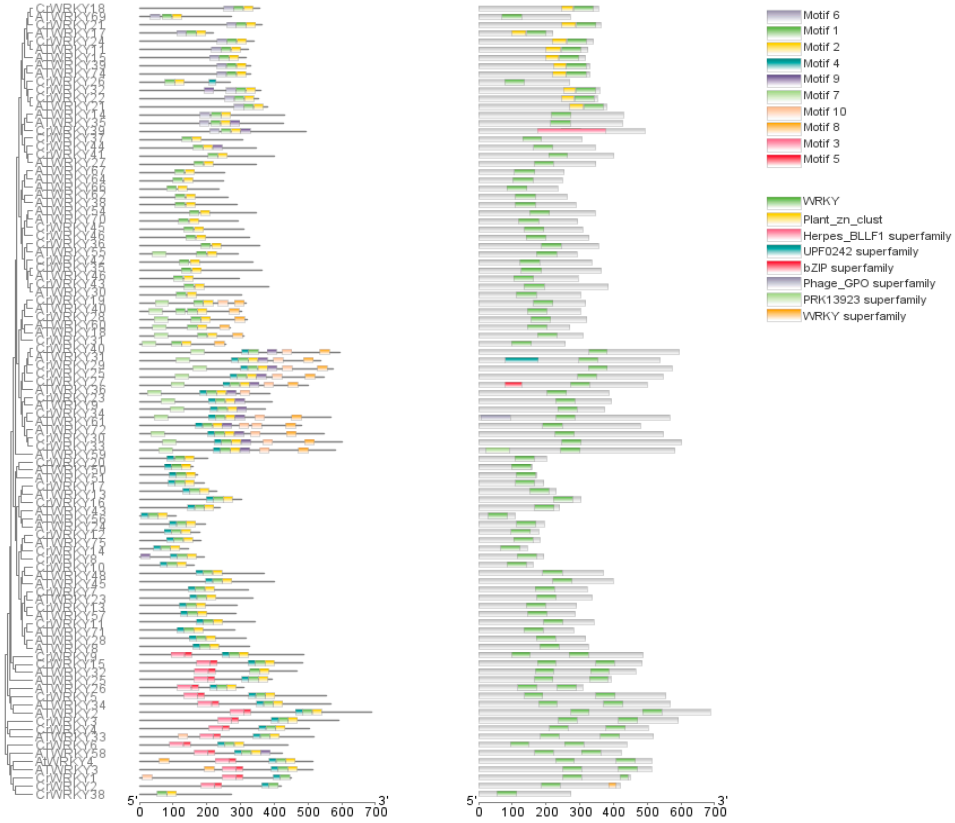


**Figure 3.** The distribution of 10 motifs on 46 *CrWRKY* proteins of Mandarin citrus using MEME version 4.9.0 and interlinking it with a phylogenetic tree to better understand their association. The bars represent motifs with different color codes for different types of motifs.

### *3.3. Comparative Phylogenetic Relationship of C. reticulata WRKY Gene Family with Arabidopsis*

The evolutionary relationship between *A. thaliana* and *C. reticulata* was determined by generating a phylogenetic tree using the neighbor-joining (NJ) method (Figure 2). Results depicted that these total 96 *WRKY* proteins can be categorized into three main groups G1, G2, and G3, based on the domains and motifs present in these peptide sequences. More precisely, this grouping was based on the number of *WRKY* domains and the nature of the zinc finger in the peptide sequences. Members of G1 contain two WRKY domains each, while the members of other groups contain only a single WRKY domain in their sequence. The zinc finger of Group 3 was of C2HC-type while Group 1 and 2 had C2H2-type of zinc fingers. Group 2 was further classified into 2A, 2B, 2C, 2D, and 2E, depending on their initial amino acid sequences [40].

Group G1 contained 17 proteins, of which 8 belonged to *C. reticulata* and 9 were from *Arabidopsis*. G2 had 62 members, divided into 5 subgroups. 2A had 6 members (3 from each organism); 2B contained 13 proteins, 8 belonging to *C. reticulata*; 2C was the most prominent group with 25 members, out of which 10 were from *C. reticulata*; 2D had 13 members (6 from *C. reticulata*). Four out of seven proteins in Group 2E belonged to citrus, whereas G3 had 17 proteins, out of which 7 were from *C. reticulata* (Figures 2 and S1a–g).

### *3.4. Gene Location and Duplication*

Orthologous Gene Pairs between *C. reticulata* and Other Plant Species

Orthologous gene pairs provide information about the evolutionary relationship between different plant species. To understand the evolution of the *CrWRKY* gene, we analyzed the syntenic relationships between *C. reticulata* and other citrus species in the *Rutacaeae* family. To better understand the evolution and relationship of *CrWRKY* genes in monocot and dicot plants, a dual syntenic analysis was also performed between *C. reticulata* and *Arabidopsis*, *C. reticulata*, and *O.sativa*. Collinearity analysis revealed that 39 collinear orthologous *WRKY* gene pairs were identified in the Mandarin citrus/*Arabidopsis* and 14 among Mandarin citrus/rice pairs, respectively (Figure 4). The number of orthologous events of *CrWRKY-AtWRKY* was higher than of *CrWRKY-OsWRKY*, indicating that the divergence between citrus and *Arabidopsis* occurred after the divergence of rice and the common ancestor of dicotyledons. The high level of syntenic conservation between the citrus and *Arabidopsis* indicated that *WRKY* TFs in citrus might share similar structures and functions to those of orthologs in *Arabidopsis*.

Syntenic analysis was also performed on the whole genome of *C. reticulata* to discover any *CrWRKY* duplication event within the *C. reticulata* genome. Gene location on the scaffold predicted that *CrWRKY* genes belonging to the same subgroup were present on the different scaffolds and linked, suggesting that these genes might have emerged from segmental duplication (Figure 4; Table S2). The maximum number of *CrWRKY* genes (six) were present on S7, followed by S21, which contains four genes (Figure S4). Results showed that 100% of segmental duplication events possibly lead to *CrWRKY* genes and that the evolution of *CrWRKY*s may have been driven, at least in part, by segmental duplication events.

Seventy-three syntenic collinear lines observed between *C. reticulata* and *C. grandis* were higher than all other species of citrus. Collinear *WRKY* genes were identified in *C. reticulata*/*C. clementine* (70), *C. reticulata*/*C. sinensis* (69), *C. reticulata*/*C. medica* (60) and *C. reticulata*/*C. ichangensis* (36). It indicated the divergence between *C. reticulata* and *C. grandis* occurred after the divergence of *C. clementine*, *C. sinensis*, *C. medica*, and *C. ichangensis* (Figures 4 and 5).

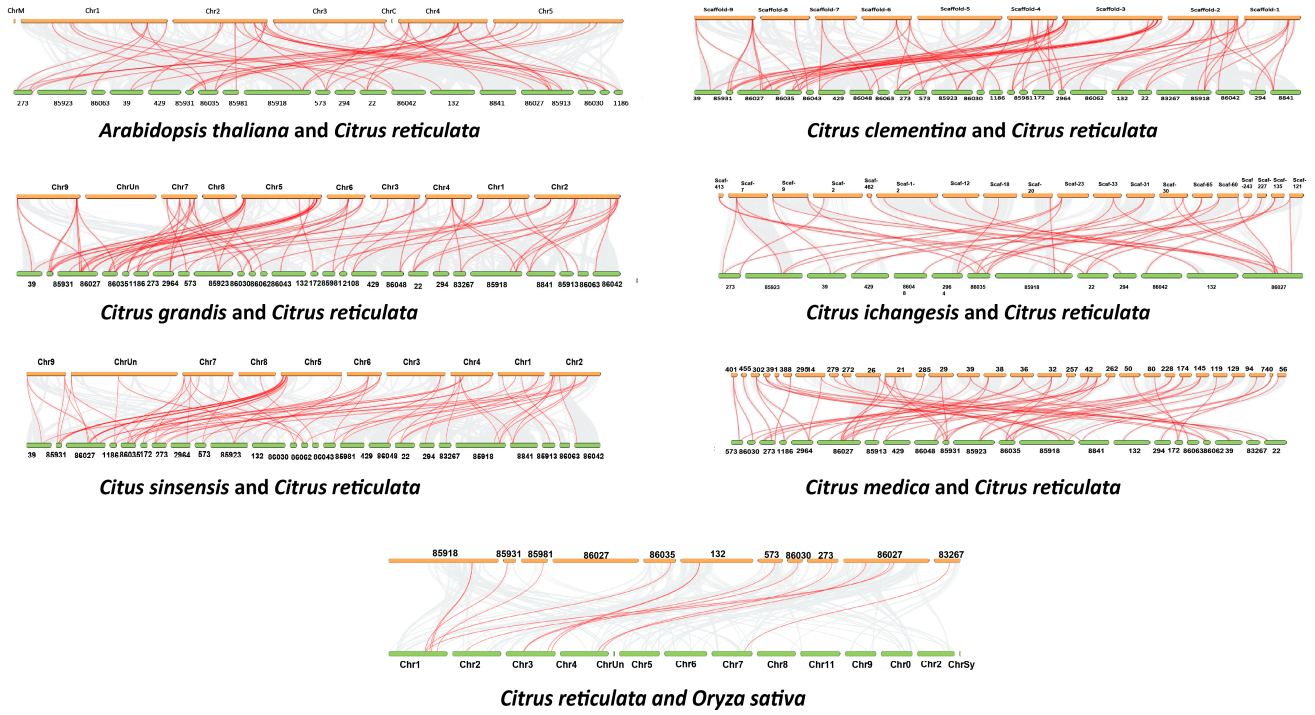


**Figure 4.** Dual synteny analysis of Dicot: *C. reticulata-Arabidopsis*, *C. reticulata-C. clementina*, *C. reticulate-C. grandis*, *C. reticulate-C. ichangensis*, *C. reticulate-C. sinensis*, and *C. reticulata-C. medica*. Orange bars represent chromosomes and scaffolds of Arabidopsis, *C. clemetina*, *C. grandis*, *C. ichangensis*, *C. sinensis*, and *C. medica*, respectively, while the green bars represent *C. reticulata*. Red lines show the duplicated genes in the respective genomes. Monocot: the orange bars represent scaffolds of *C. reticulata*, and the green bars represent chromosomes of *Oryza sativa*. Red lines show the duplicated genes in the respective genomes.

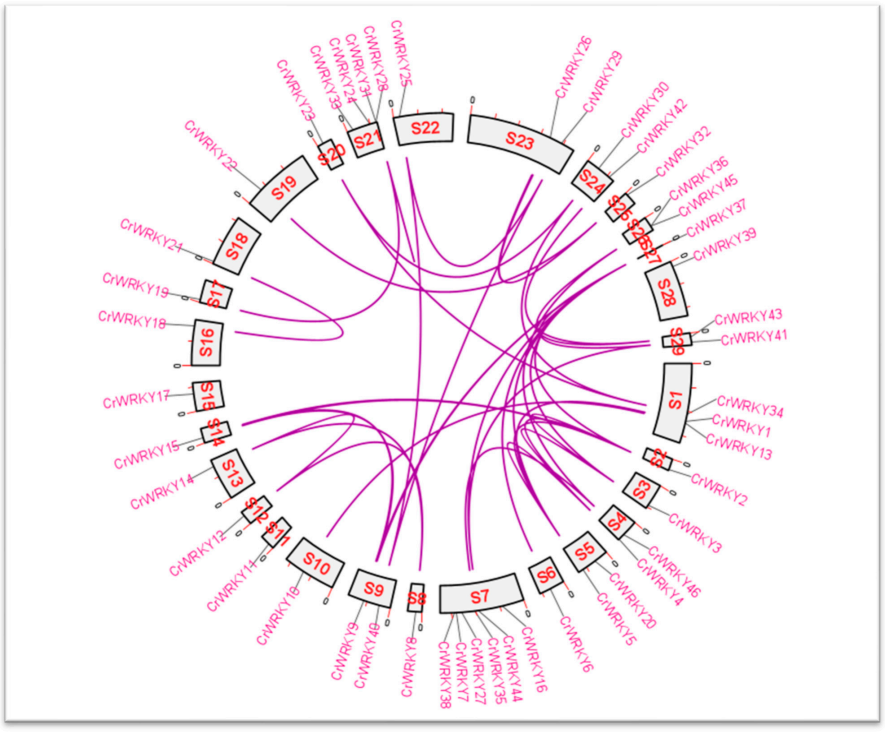

**Figure 5.** Genome-wide synteny analysis of *WRKY* genes showed 100% segmental duplication dominance. Joining lines (purple color) were showing the duplicated *CrWRKY* genes in the genome.

The time of duplication of *CrWRKY* genes was assessed by the KaKs calculator in tbtool software [36] (Figure 6). Ka shows the number of nonsynonymous substitutions per nonsynonymous site while Ks show the number of synonymous substitutions per synonymous site and the ratio between these two was calculated by Ka/Ks. The estimated ratio between different gene pairs *(CrWRKY15_CrWRKY2, CrWRKY4_CrWRKY2,CrWRKY9_CrWRKY38, CrWRKY41_CrWRKY44,CrWRKY3_CrWRKY2,CrWRKY4_CrWRKY3,CrWRKY36_CrWRKY43, CrWRKY14_CrWRKY12,CrWRKY12_CrWRKY8,CrWRKY14_CrWRKY8,CrWRKY13_CrWRKY10, CrWRKY37_CrWRKY44,CrWRKY9_CrWRKY15,CrWRKY2_CrWRKY38,CrWRKY21_CrWRKY18, CrWRKY26_CrWRKY32,CrWRKY23_CrWRKY34,CrWRKY28_CrWRKY19,CrWRKY31_ CrWRKY28,CrWRKY40_CrWRKY29,CrWRKY25_CrWRKY29,CrWRKY34_CrWRKY30,CrWRKY23_ CrWRKY30,CrWRKY41_CrWRKY37,CrWRKY22_CrWRKY32,CrWRKY14_CrWRKY12, CrWRKY6_CrWRKY38,CrWRKY3_CrWRKY38,CrWRKY43_CrWRKY42,CrWRKY45_CrWRKY46 and* CrWRKY35_CrWRKY46) were less than 1; while *CrWRKY5_ CrWRKY2, CrWRKY4_ CrWRKY5, CrWRKY1_ CrWRKY5* and *CrWRKY5_ CrWRKY38* held more than 1 ratio. The highest estimated date of duplication was 352.1051365 (MYA) in *CrWRKY31_CrWRKY28* and the lowest estimated date of duplication was 53.91711764 (MYA) in *CrWRKY15_CrWRKY2* (Table S3).

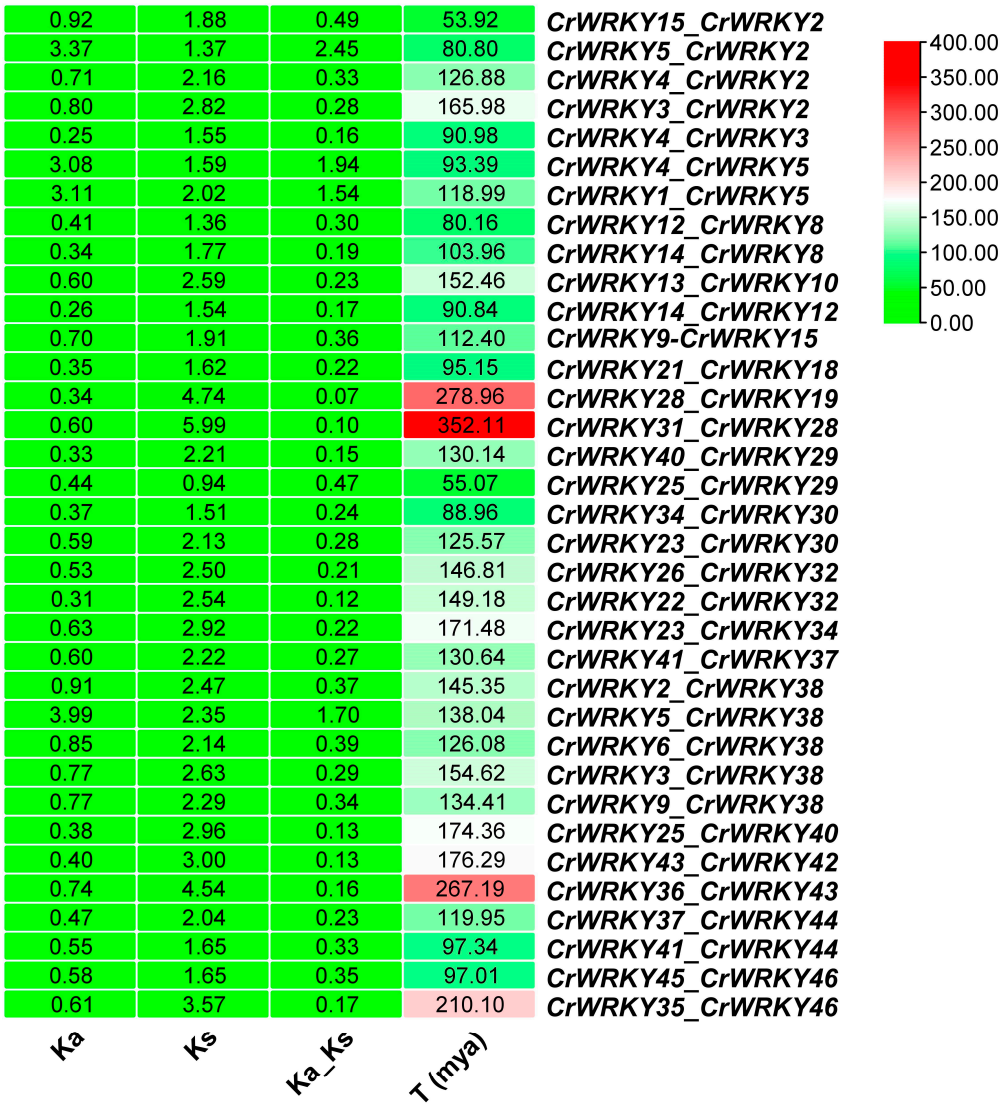


**Figure 6.** Time of gene duplication were estimated for different paralogous pairs of *CrWRKY* genes based on Ks and Ka values. The analyses was performed using TBtools (Software). Ka/Ks represents the ratio of nonsynonymous (Ka) versus synonymous (Ks) mutations.

### 3.5. Cis-Regulatory Elements Analysis

Cis-regulatory elements (CREs) are DNA binding motifs present at the promoter region that regulate the transcription of a gene [41]. In silico analysis can be performed to evaluate the putative function of various genes using CREs analysis [42]. Various CREs were detected in the promoter regions of *CrWRKY* genes (Figure 7). Phytohormone-responsive elements, such as MeJARE (MeJA-responsive element), ABRE (abscisic acid-responsive element), SARE (salicylic acid-responsive element), ethylene-responsive elements (ERE) and Gibbereline response elements GARE were detected in the promoter regions, suggesting that multiple phytohormones might regulate the expression of *CrWRKY* genes. Additionally, some stress-related *CREs*, such as DSRE (defense and drought stress-responsive element), LTRE (low-temperature-responsive element), and HSRE (heat stress-responsive element), were also found in the *CrWRKY* gene promoter regions (Table S1); these results indicated that *CrWRKY* genes might be closely related to the responses to multiple abiotic and biotic stresses. Moreover, two plant hormone-related elements (ABRE and MeJARE) and a stress-responsive element (HSRE) were frequently detected in the putative promoters of *CrWRKY* genes. Notably, each *CrWRKY* promotor contained multiple copies of LRE (light-responsive element), suggesting that *CrWRKY* genes were an important component of light response in *C. reticulata*. Similarly, *CrWRKY5*, *CrWRKY6*, *CrWRKY9*, and *CrWRKY22* contained multiple LRE suggesting their role in light responsiveness. *CrWRKY6*, *CrWRKY9*, and *CrWRKY16* had multiple ABAR CREs that showed their role in abscisic acid responsiveness. MeJA element showed their regulation of methyl jasmonate responsiveness in *CrWRKY9*, *CrWRKY18*, *CrWRKY35*, and *CrWRKY42*. Defense and drought resistance signaling specific CREs were observed in *CrWRKY2*, *CrWRKY6*, and *CrWRKY9*.

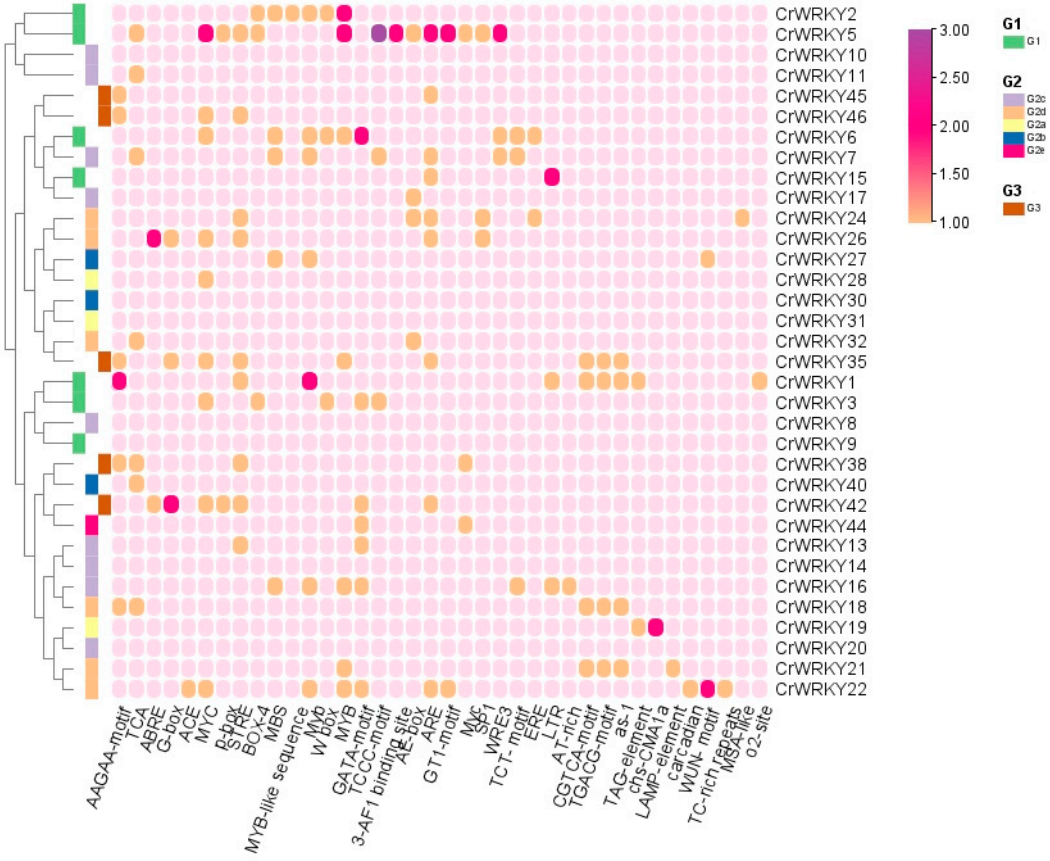


**Figure 7.** *Cis*-regulatory elements in putative *CrWRKY* gene promoters were associated with different plant stresses, hormone responses, growth, and developmental processes. G1, G2, and G3 showed the groups of *WRKY* genes.

### *3.6. Expression Analysis of the CrWRKY Gene in Different Organs*

To investigate the expression of *CrWRKY* genes, computational Expressed Sequence Tag (EST) analysis was accomplished for all 46 *CrWRKY* genes using NCBI nucleotide blast and interpreted using a heatmap with a hierarchical cluster in TBtools [36]. Results revealed that most of the *CrWRKY* genes expressed in leaves compared to the fruit. *CrWRKY7*, *CrWRKY22*, *CrWRKY28*, and *CrWRKY38* genes showed expression during fruit development in contrast to the expression of other genes in the leaf (Figure 8).

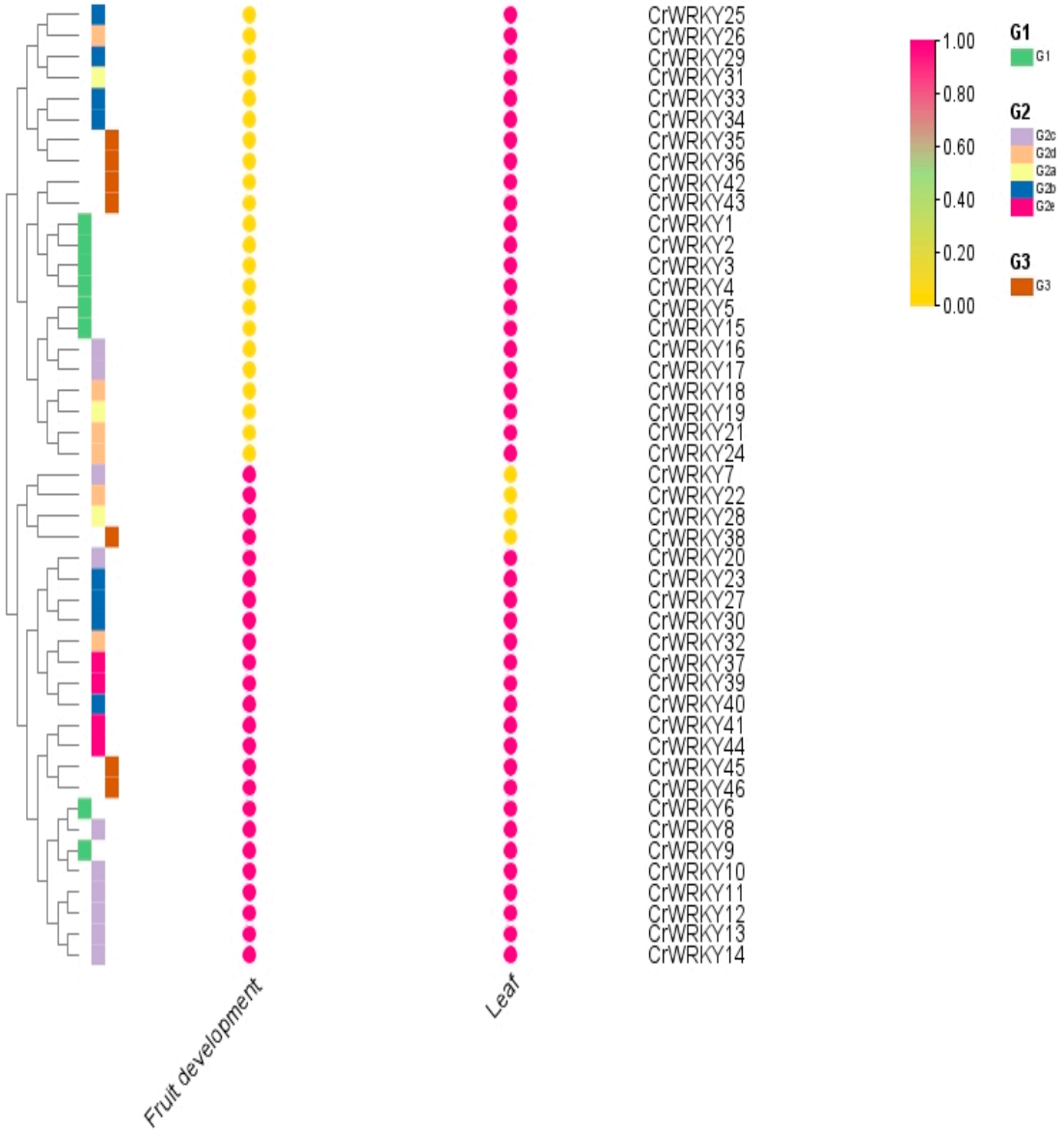


**Figure 8.** Heat map showing the expression profile of the *CrWRKY* genes in different organs of *C. reticulata.* G1, G2, and G3 show the groups of *WRKY* genes.

### *3.7. Expression Analysis of CrWRKY Genes during Nucellarembryony Initiation*

Next-generation RNA sequencing analysis is an important tool to find and compare the expression of genes under different organ development and stress responses. An experimental NGS RNA-seq (GSE74384) data were downloaded from the NCBI GEO database. In the current study, transcriptome profiling was employed to compare differentially expressed genes (DEGs) of ovules at and before the appearance of nucellarembryony initial cells. Results demonstrated that *CrWRKY35* and *CrWRKY43* expression were significantly down-regulated during NEI in monoembryonic cultivars CM and *C. clementina*.

*CrWRKY8* was down-regulated considerably in polyembryonicPonkan (PK, *C. reticulata* Blanco) during the beginning of the new embryo initiation (NEI) compared to pre-NEI. LEC1, L1L, FUS3, ABI3, and ABI5 were involved in the somatic embryogenesis of citrus in vitro but differentially not expressed in polyembryonic and monoembryonic ovules. The AP2/ERF and WRKY domains were up-regulated in polyembryonic cultivars compared to monoembryonic cultivars [43] (Figure 9).

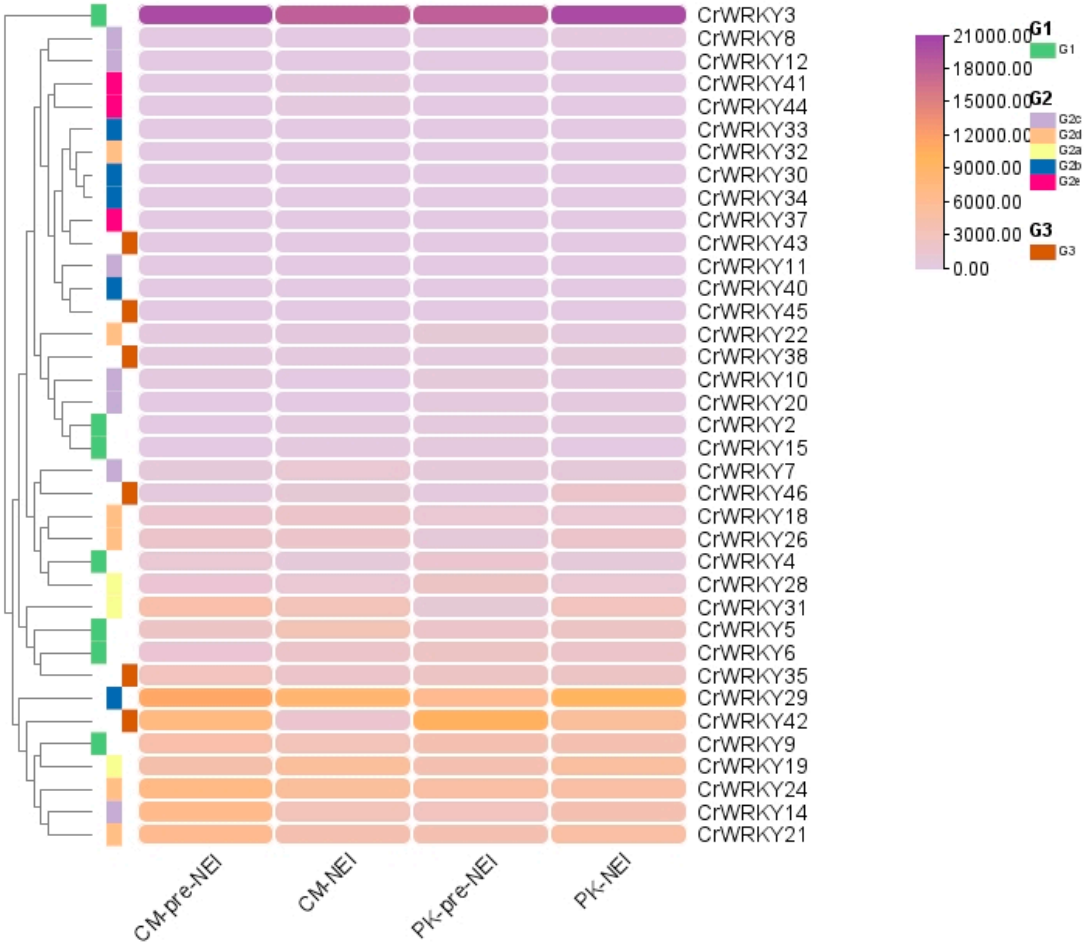


**Figure 9.** Heat map showing the expression profile of the *CrWRKY* genes in monoembryonic clementina (CM) and polyembryonic ponkon (PK) citrus cultivars at pre-nucellar embryony initial cells (pre-NEI) and nucellar embryony initial cells (NEI) stages. G1, G2, and G3 indicateed the groups of *WRKY* genes.

### *3.8. Fruit Color Expressed in Wild- and Mutant-Type of Stay-Green Citrus*

The experiment (GEO accession no. GSE94810) data were planned to illuminate the potential regulation of citrus fruits' color formation process and compare the different gene expression patterns between a spontaneous stay-green mutant and its wild-type. Read per Kilo-bases per million Mapping reads (RPKM) values from RNA-seq data were log2 transformed for expressions profiling [36].

The color expression of the flower and profiles of wild and mutant stay-green *CrWRKY* are represented in the form of a heat map. Results demonstrated that *CrWRKY7*, *CrWRKY11*, *CrWRKY21*, *CrWRKY24*, *CrWRKY29*, *CrWRKY30*, *CrWRKY31*, *CrWRKY35*, *CrWRKY40*, *CrWRKY41*, *CrWRKY45*, and *CrWRKY46* were highly significant and showed down-regulated expression in wild-type. *CrWRKY7*, *CrWRKY21*, *CrWRKY30*, *CrWRKY32*, *CrWRKY36*, *CrWRKY40*, *CrWRKY43*, and *CrWRKY44* were highly significant in mutant-type. NAC TFs play a significant role in the ripening process of climacteric and non-climacteric fruit [37] (Figure 10).

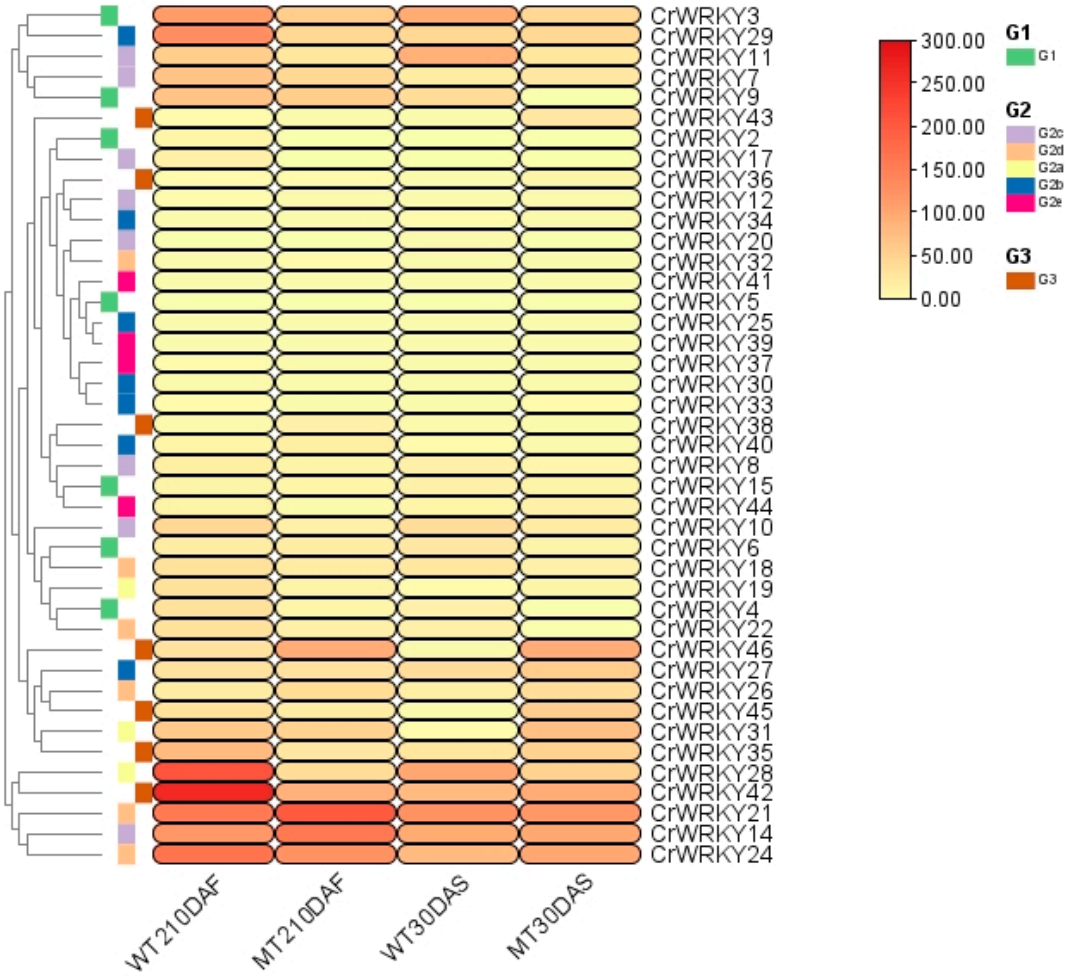


**Figure 10.** Heat map showing the expression profile of the *CrWRKY* genes in wild-type and mutant-type. The wild-type and mutant-type with 21 days after 50% flowering (DAF), and wild-type and mutant-type with 30 DAF after storage for RNA extraction and analysis.

### *3.9. CrWRKY Gene Expression Profiles in HBL Infection*

The experiment (GEO accession no.GSE67560) data were downloaded from NCBI Geo to illuminate the potential regulation of *Citrus Huanglongbing* (HLB) disease detected in the leaves and roots of citrus. HLB is a worldwide uncontrollable and worldwide citrus disease, Gram-negative Candidatus Liberibacterphloem-inhabiting α-Proteobacteria causes this Ca. *Liberibacterasiaticus* (CLas) is a pathogenic species of this class, which is transmitted by grafting with HLB-infected bud woods and by a phloem-feeding psyllid, *Diaphorinacitri. P*hosphorus-starvation-induced miR399 significantly induced phosphorus levels to alleviate symptoms of HLB in the infected tree [44]. In roots, CLas-responsive genes were exploited for HLB resistance to HBL in breeding. Some *WRKYs* involved in SA- and JA-dependent defense pathways were up-regulated and targeted in CLas-infected roots [45].

The expression patterns of 44 out of 46 *CrWRKY* genes were identified through root transcriptome analysis. Among the 46 *CrWRKY* genes, 26 *CrWRKY* DEGs were significantly up-regulated. However, the remaining 20 genes were down-regulated in HLB-infected roots. CrWRKY28 exhibited the highest transcription level in HBL as compared to the control. *CrWRKY10,46,45,24,29,3,26,35,42,4,18,44,13,20,14*) showed slightly higher expression than the control but less than *CrWRKY28.* The expression analysis of several genes (*CrWRKY9/21/32/7/5/22/8*) showed slightly higher expression in the control. In addition, the expression of some *CrWRKYs* (*CrWRKY31/12/36*) slightly decreased in the control (Figure 11). These results indicated that these genes might play roles in many aspects of citrus root development for improving yield and fruit growth.

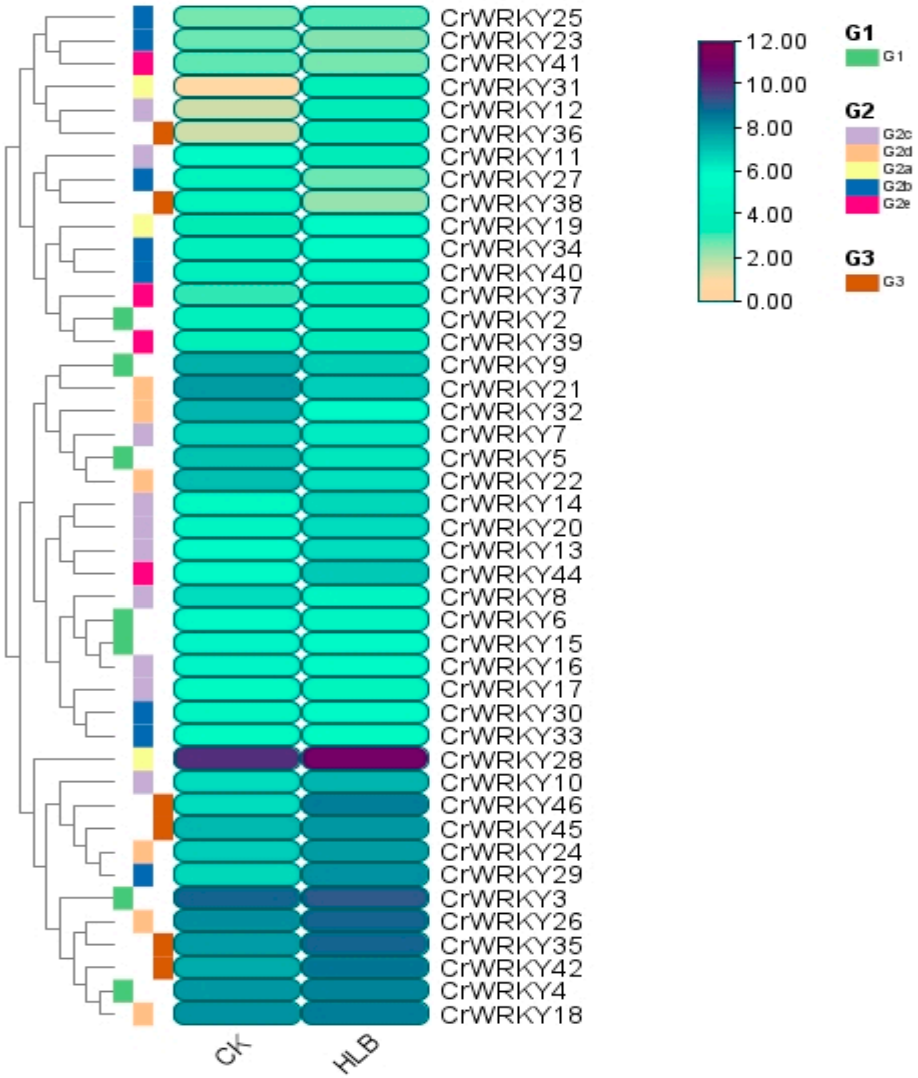


**Figure 11.** Heat map showing the expression profile of the *CrWRKY* genes in HLB (*Citrus Huanglongbing*) and control.

### *3.10. miRNA Targets*

Sequences of mature miRNA targeting *CrWRKY* genes were retrieved from psRNA. using an online web tool (https://plantgrn.noble.org/psRNATarget/analysis). A total of 55 miRNA sequences targeting 22 *CrWRKY* genes (*CrWRKY3*, *CrWRKY4*, *CrWRKY5*, *CrWRKY6*, *CrWRKY7*, *CrWRKY9*, *CrWRKY16*, *CrWRKY20*, *CrWRKY21*, *CrWRKY22*, *CrWRKY24*, *CrWRKY25*, *CrWRKY27*, *CrWRKY30*, *CrWRKY32*, *CrWRKY33*, *CrWRKY36*, *CrWRKY39*, *CrWRKY40*, *CrWRKY42*, *CrWRKY43*, and *CrWRKY45*) were detected. The observed expectation value ranged from 3 to 5 and the minimum length of the miRNA was between 21 bp and a maximum of 22 bp. miRNA sequences targeting *CrWRKY1*, *CrWRKY2*, *CrWRKY8*, *CrWRKY10*, *CrWRKY11*, *CrWRKY12*, *CrWRKY13*, *CrWRKY14*, *CrWRKY15*, *CrWRKY17*, *CrWRKY18*, *CrWRKY19*, *CrWRKY23*, *CrWRKY26*, *CrWRKY28*, *CrWRKY29*, *CrWRKY31*, *CrWRKY34*, *CrWRKY35*, *CrWRKY37*, *CrWRKY38*, *CrWRKY41*, *CrWRKY44*, and *CrWRKY46* were not found (Table S4). Putative functions of the identified miRNA sequences were interpreted using data from previously performed experiments (Table S4).

## 4. Discussion

*C. reticulata* plays a significant part in the worldwide economy [46]. Conventional methods coupled with molecular approaches and tools employed through bioinformatic approaches can improve the fruit quality and production traits of *C. reticulata*. *WRKY*

protein family is one of the major TFs in citrus and has also been identified in many other organisms. Here, we consistently detected 46 *WRKY* genes from the WGS of *C. reticulata*. Sequence alignment and phylogenetic analysis divided *WRKY* genes into three main groups (G-I, G-II, G-III). This classification depends on the WD motif and the conserved ZF motif. There were 8 and 7 members of *CrWRKY* in G-I and G-III, while G-II was the largest group containing 31 members (Figure 2). Furthermore, in *A. thaliana* and *Populas* [47], G-I houses the maximum gene members of *WRKY*; while G-III contains the largest number of *WRKYs* in rice [19]. Most of the *WRKY* genes in *C. reticulata* are present in G-II, suggesting that the evolutionary history of G-II may experience further duplication of genes. Currently, *WRKY* genes identified in the genome of lower *bryophytes,* unicellular protoplast, slime mold *myxomycetes*, *ferns,* and *Chlamydomonas monocytogenes* that relate to G-I, indicating that G-I is most likely the novel form of protein expression and they developed 1.5 billion years ago in a eukaryotic cell, before thallophytes [48]. A diverse alignment study of *CrWRKY* domain structure revealed two main types of conserved introns in *CrWRKY* proteins (Table S5): V-type and R-type introns [49]. The R-type intron was found only in subgroups I, II-c, II-d, II-e, and III. Therefore subgroups II-a and II-b have V-type introns, which they discovered as an additional type of introns, which were positioned at the fourth AA-residue (K) after the second C-residue metal-chelating zinc-finger motif [19]. It is unclear whether the *WRKY* function is affected by adding homologous introns in distinct locus. The constant sequence of the *WRKY* gene is the heptapeptide sequence WRKYGQK. Furthermore, with respect to the domain feature and the phylogenic tree (Figures 1 and 2), it showed three interesting observations: (i) all Group I members have two *WRKY* domains and two zinc-finger structures; (ii) subgroup II-c has several mutations, indicating that perhaps the selective force and evolutionary patterns of *CrWRKY* genes are different among categories, such as *CrWRKY20* possess lack of ZF structure the six amino acid residues (WRKYGQ), mutations were observed in the Q position which is replaced by K; (iii) *CrWRKY47* belong to none of the group because of lack of its domain and ZF structure. All these results implied the *CrWRKY* genes may have experienced loss of the *WRKY* domain loss during the evolution.

The Group III WRKY gene accounts for 20% of the family members in higher plants, but it does not exist in some lower plants, such as bryophytes. Studies have shown that almost all Group III *WRKY*s in *Arabidopsis* are related to the response to biotic stress response [40], which indicates that Group III *WRKYs* are relatively the latest in the evolutionary history of terrestrial plants. However, in *CrWRKY*, all of G-I have deficient expression levels in fruit development even those not expressed in fruit development (Figure 8). Two of them (*CrWRKY6* and *CrWRKY9)* are considered pseudogenes that are expressed both in fruit and leaf development, implying that G-III members may have little to do with the fruit formation only expressed in *CrWRKY38, 45*and *46* genes, which is inconsistent with the Arabidopsis.

Tissue expression analysis showed that members of G-II expressed highly in both tissue's development. Members of G-IIe expressed in both developments without apparent different expression levels. However, other subgroups of G-II showed different expressions in fruit and leaf development (Figure 8). Combined with their expression in the various organs (nucleus, chloroplast, cytoplasm, cytoplasm nucleus, vacuoles, plasmids, extra nucleus, peroxisomes, golgi plasmids, mitochondria, and cytoskeleton), all groups showed high expression in the nucleus, but *CrWRKY16* of G- IIc was lacking expression (Figure S3).

Regardless of the fact that *C. reticulata* has 46 *CrWRKY* genes, members of G-I (*CrWRKY1* is the ortholog of *AtWRKY3*; *CrWRKY3*, *CrWRKY4* are homologues of *AtWRKY26* and *AtWRKY33*; *CrWRKY6* is the homologue of *AtWRKY25*; *CrWRKY5* is the homology of *AtWRKY34*; *CrWRKY9*, *CrWRKY2* and *CrWRKY15* are the homology of *AtWRKY32*); members of G-III (*CrWRKY38*, *CrWRKY42*, *CrWRKY43*, *CrWRKY35* and *CrWRKY36* are homology of *AtWRKY30*; *CrWRKY45* is the homology of *AtWRKY70*; *CrWRKY46* is the homology of *AtWRKY38*; *CrWRKY39* is the homology of *AtWRKY14*; members of G-IIe (*CrWRKY44*, *CrWRKY37* and *CrWRKY41* are the homology of *AtWRKY27*); mem-

bers of G-IId (*CrWRKY18*, *CrWRKY21* and *CrWRKY24* are homology *AtWRKY17* and *AtWRKY11*; *CrWRKY22* is homology of *AtWRKY21*; *CrWRKY32* and *CrWRKY26* are homology of *AtWRKY39* and *AtWRKY74*); members of G-IIc (*CrWRKY11* is homology of *AtWRKY71*; *CrWRKY7* is homology of *AtWRKY23*; *CrWRKY10* and *CrWRKY13* are homology of *AtWRKY57*; *CrWRKY8*, *CrWRKY12* and *CrWRKY14* are the homology of *AtWRKY75*; *CrWRKY16* and *CrWRKY17* are homology of *AtWRKY13*; *CrWRKY20* is the homology of *AtWRKY50*); members of G-IIa (*CrWRKY19* and *CrWRKY28* are homology of *AtWRKY40*; *CrWRKY31* is the homology of *AtWRKY18* and *AtWRKY60*); members of G-IIb (*CrWRKY29*, *CrWRKY40*, *CrWRKY25*, *CrWRKY27* and *CrWRKY33* are homology of *AtWRKY31*; *CrWRKY30* and *CrWRKY34* are the homology of *AtWRKY36*; *CrWRKY23* is the homology of *AtWRKY9*) (Figure 2).

Through the structural analysis of *CrWRKY* genes, it is found that *CrWRKY* genes have different numbers of exons/introns as in their ortholog *A.thaliana* (as predicted by phylogeny analysis) (Figure S2). Furthermore, the motif analysis indicated that all the *CrWRKY* and *AtWRKY* have the same motif structures except *CrWRKY1, CrWRKY8, CrWRKY26, CrWRKY32, CrWRKY19, CrWRKY40, CrWRKY38, AtWRKY36, AtWRKY55* and *AtWRKY40* of different clades have variant motif structures.

RNA-seq analysis helps to identify the significant *PmWRKYs* in the cold tolerance of *Prunusmume* [50]. Some *PmWRKY* genes changed their expression during chilling stress in the *P. mume* cultivar 'Zhusha'. According to [50], *PmWRKY6* and 11 were identified as up-regulated or down-regulated over twofold after experiencing cold acclimation in the winter. Although some conditions were controlled during winter, *PmWRKYs* were reported to evaluate their cold response through artificially low-temperature conditions. Cold temperature signaling pathways are classified into ABA-dependent or ABA-independent pathways; both modulate the functional gene expression, such as *KIN1*, *COR47*, and *RD29A*, that increase the cold resistance in plants. WRKY TrFs were reported as a significant role in the ABA signal transduction pathway [51]. According to the authors, 17 genes respond to ABA treatment. Especially after this treatment, PmWRKY18 was expressed significantly up-regulated, while other genes were not significantly different compared to its control. So, they concluded that *PmWRKY18* of G-II may involve in ABA-dependent cold signaling pathway. *PmWRKY18*is homology of *AtWRKY18. AtWRKY18* belongs to G-IIa, and the other genes of *A. thaliana* in G-IIaare*AtWRKY40*, and *AtWRKY60* [50].

According to our research, the homologies of *AtWRKY18, AtWRKY40*, and *AtWRKY60* in G-IIa of *C. reticulata* are *CrWRKY19*, *CrWRKY28,* and *CrWRKY31. AtWRKY18, AtWRKY40*, and *AtWRKY60* served as positive and negative regulators in ABA-dependent pathways for inhibiting seed and root development (Table S6). Moreover, to further know about the gene structure and their function, CRE has an essential function in binding the TrFs by their respective target site and helps to regulate gene expression. *CrWRKY* genes have CRE that are related to LRE, LTRE, and different hormone responsiveness to play a significant role in plant growth and development (Table S1 and Figure 7). So, these genes may be involved in photosynthesis and ABA responses. However, further research is required to find out the exact function of these CRE. Members of G-I (*CrWRKY1*, *CrWRKY2*, *CrWRKY5*, *CrWRKY6*, and *CrWRKY9*); G-IIb (*CrWRKY27*); G-IIc (*CrWRKY7*, *CrWRKY16*); G-IIc (*CrWRKY18*, *CrWRKY21*, *CrWRKY22*, *CrWRKY24*); and G-III (*CrWRKY35*, *CrWRKY38*, *CrWRKY45*, and *CrWRKY46*) play a significant role in ABAR and LTRE that are directly or indirectly involved in cold stress.

Evolutionary changes play a vital role in the process of gene duplication. Duplicated genes on the same scaffolds are tandem duplication. However, duplication of the genes on different scaffolds is a segmented mode of duplication [26]. All the *CrWRKYs* show segmental duplication events, while tandem duplication events were lacking(Figure 5). The lack of tandem duplication in *CrWRKY* genes might be possible for the minimum number of genes. Segmental duplication was a major driver of WRKYs expansion during the citrus evolutionary process. Twenty-nine scaffolds of *C. reticulata* genes were identified, possibly all scaffolds resulting from segmented duplication due to their presence on different scaf-

folds. We have deliberated the Ka/Ks ratio of an expected duplicated gene in *C. reticulata* by using the R-value as r = 1.7 × $10^{-8}$ and calculated the expected time of divergence or duplication in the homologs (Table S3 and Figure 6). To further find out the homologs of *C. reticulata* in other crops, dual synteny blocks of *C. reticulata* were constructed with one monocot (*O. sativa*) and six different dicot crops (i.e., *A. thaliana*, *C. sinensis*, *C. grandis*, *C. ichangensis*, *C. clementina*, and *C. medica*), separately to determine the connection between them. Dual syntenic block of *C. reticulata* and *O. sativa* genome predicted 14 duplicated genes that contain 6 scaffolds on chr 1, chr 2, and 7 having a single number of the scaffold, 4 scaffolds present on chr 3 and 2 on chr 4 (Figure 4). Dual syntenic block of *C. reticulata* and *A. thaliana* genome predicted 39 duplicated scaffolds that contain nine scaffolds in chr 1, 13 scaffolds on chr 2, 4 scaffolds on chr 3, 15 scaffolds in chr 4 and 7 on chr 5 had duplicated scaffolds in *A. thaliana* as it was shown by joining threads between respective chromosomes and scaffolds (Figure 4). Dual syntenic block predicted 69 duplicated genes between *C. reticulata* and *C. sinensis* genome, while 73, 36, 70, and 60 duplicated genes of *C. reticulate* with *C. grandis*, *C. ichangensis*, *C. clementina,* and *C. medica*, respectively (Figure 4).

MicroRNAs (miRNAs) are important regulatory entities of plants. They regulate almost all biological processes of plants, such as plant development and growth, during abiotic and biotic stress [38,52]. They are extremely conserved and are very specific in function. We found miRNA targeting *CrWRKY* genes from psRNA Target (online web tool). A total of 55 miRNAs from 26 different miRNA families targeting *CrWRKY22* genes were detected (Table S4). Cre-MIR530a, Cre-MIR530b, and Cre-MIR530c target *CrWRKY25* and *CrWRKY16* which are used as blocking miR for the improvement of yield [53]; Cre-MIR3954a target *CrWRKY4* that trigger phasiRNAs which affect flowering time in plants [54]; Cre-MIR170e, Cre-MIR170f and Cre-MIR170j target *CrWRKY39* and *CrWRKY42* their functions are still unknown; Cre-MIRN5527 target *CrWRKY33*; Cre-MIR156a, Cre-MIR156c, Cre-MIR156e, and Cre-MIR156f target *CrWRKY21, CrWRKY42*, and *CrWRKY32* act to modulate lateral development of roots, leaf, and branching morphology [55], Cre-MIR159a, Cre-MIR159c, Cre-MIR159e, Cre-MIR159f, Cre-MIR159g, and Cre-MIR159h, target*CrWRKY32* and *CrWRKY5* are involved in miR-159-GAMYB pathway which functioned in vegetative tissues [56]; Cre-MIR166a target *CrWRKY24* that is involved in up-regulation upon concomitant depression and salinity stress [57],Cre-MIR168a targeted *CrWRKY7* which play important role in modulation of small RNA regulation by Argonaute1 [58]; Cre-MIR172a and Cre-MIR172b target *CrWRKY36* and *CrWRKY45* which are involved in triggering gene repression [59]; Cre-MIR477e target *CrWRKY16* that trigger PAL (phenylalanine ammonia-lyase) to enhance susceptibility [60], Cre-MIR164a target*CrWRKY32* act as negatively regulator in drought resistance [61], Cre-MIR2275a and Cre-MIR2275b target *CrWRKY43* that led to abundant 24 nucleotide phasi-RNAs in different plants [62]; Cre-MIR391a target *CrWRKY3* act as regulatory factor in root development and growth [63], Cre-MIR3951a target *CrWRKY6* and *CrWRKY39* used to encode MYB transcriptional factor [64]; Cre-MIR395a, Cre-MIR395b, Cre-MIR395c, Cre-MIR395d and Cre-MIR395e target *CrWRKY47* respond to regulate sulphate assimilation [65]; Cre-MIR396a target *CrWRKY4* it act against fungal pathogens [66]; Cre-MIR5522, Cre-MIR5675a target *CrWRKY43*, *CrWRKY40* and *CrWRKY9* have not any discovered functions; Cre-MIR5523 target *CrWRKY20* and *CrWRKY30* are functioned as negative regulation in drought stress [67]; Cre-MIR5524, Cre-MIR5525, Cre-MIR5526 and Cre-MIR5541 target *CrWRKY20* and *CrWRKY30* which have still unidentified functions; Cre-MIR5527 target*CrWRKY16* unidentified functions; Cre-MIRN834a target *CrWRKY32* have unknown functions; Cre-MIRN845a target *CrWRKY27* is used to trigger Transposon derived small RNAs [68]; Cre-MIRN876a and Cre-MIRN876b target *CrWRKY6*; and *CrWRKY22* acts as an inhibitor [69].

## 5. Conclusions

In summary, systematic genome-wide analyses of the *WRKY* gene in Mandarin citrus identified forty-six WRKY genes in *C. reticulata.* These genes were categorized into seven subgroups by their functional and structural properties (Table S5). It is concluded that these

genes have diverse functional networks but mainly in the leaf and flower developmental processes. miRNA data on possibly targeted *CrWRKY* genes during root, leaf, flowering, and fruit development provided a base for further studies in *C. reticulata* to increase yield and help cope with stress, which badly affects the plant. The detailed computational inspection of *CrWRKY* proteins revealed in the current study could be used for cloning and selection at the molecular level, portraying gene expression and studying their interactions with different transcription factors.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13061182/s1, Figure S1a–g: The sequence logos and its motif analysis are based on alignments of *Citrus reticulata WRKY* domains. *WRKY* domain is highly conserved across all *WRKY* proteins in *C. reticulata* while the Zinc-finger C2-H2 domain is highly conserved in *WRKY.* Groups are also mentioned which show *the WRKY* domain in all groups and the structure of the Zinc-finger motif. Black bars representing the start and end point of *WRKY* domain & C2H2/ C2HC. Single alphabet on the respective number representing the highly conserved nature of that respective amino acid in s all *CrWRKY* genes sequence; Figure S2: Phylogenetic relationships and gene structures of the *CrWRKY* genes. (A) The phylogenetic tree was constructed based on the full-length sequences of *CrWRKY* genes. (B) Intron-Exon structures of the *CrWRKY* genes. Yellow boxes indicate exons; and black lines indicate introns. Figure S3: Heat Map representing Sub-cellular localization of all 46 *CrWRKY* genes. Skin colour represents the absence of the respective gene in that cell region; yellow mustard represents the minimum and maximum value of the gene in that region. Figure S4: Distribution of *CrWRKY* genes on citrus scaffolds, it is predicting the possible gene duplication on the different chromosome and show segmented duplication. Table S1: Cis-regulatory elements in putative CrWRKY promoters were associated with different plant developmental processes. Table S2: Scaffold Renaming. Table S3: Ka/Ks ratio duplicated gene pairs in Citrus reticulata. Table S4: miRNA targets prediction of *CrWRKY* genes. The miRNA data was downloaded from psRNATarget (Online Web tool). Table S5: R-type and V-type are differentiated by red (R) and purple (V) in the sequence alignment of *CrWRKY* genes. Table S6: *A. thaliana* with its role in plants.

**Author Contributions:** Conceptualization, M.S., Q.A., M.A.S. and F.A.; Methodology, M.S., S.S., M.A.S. and F.A.; Software, N.M., M.S., U.H. and M.A.S.; Validation, M.S., M.A.S. and A.A.; Formal analysis, M.S. and F.A.; Investigation, N.M., M.S., M.F. and F.A.; Resources, N.M., M.S. and Q.A.; Data curation, N.M. and M.S.; Writing—original draft, N.M. and F.A.; Writing—review & editing, M.F., Q.A., U.H., A.A. and F.A.; Supervision, M.S.; Project administration, M.S.; Funding acquisition, M.S. All authors have read and agreed to the published version of the manuscript.

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