Genetics and Molecular Research

In silico identification and characterization of the WRKY gene superfamily in pepper (*Capsicum annuum* L.)

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Genet. Mol. Res. 15 (3): gmr.15038675
Received March 30, 2016
Accepted July 14, 2016
Published September 23, 2016
DOI http://dx.doi.org/10.4238/gmr.15038675

ABSTRACT. The WRKY family is one of the most important transcription factor families in plants, involved in the regulation of a broad range of biological roles. The recent releases of whole-genome sequences of pepper (*Capsicum annuum* L.) allow us to perform a genome-wide identification and characterization of the WRKY family. In this study, 61 CaWRKY proteins were identified in the pepper genome. Based on protein structural and phylogenetic analyses, these proteins were classified into four main groups (I, II, III, and NG), and Group II was further divided into five subgroups (IIa to IIe). Chromosome mapping analysis indicated that *CaWRKY* genes are distributed across all 12 chromosomes, although the location of four

CaWRKYs (*CaWRKY58-CaWRKY61*) could not be identified. Two pairs of *CaWRKYs* located on chromosome 01 appear to be tandem duplications. Furthermore, the phylogenetic tree showed a close evolutionary relationship of WRKYs in three species from Solanaceae. In conclusion, this comprehensive analysis of CaWRKYs will provide rich resources for further functional studies in pepper.

Key words: WRKY; Transcription factors; Phylogenetic analysis; Pepper

INTRODUCTION

WRKY is one of the most important transcription factors in higher plants. As a sequence-specific DNA-binding transcription factor, the WRKY protein contains at least one WRKY domain, which is responsible for the special *cis*-element (w-box: TTGACC/T) binding activity. All WRKY domains contain a highly conserved peptide (WRKYGQK) at the N terminus, which is followed by a zinc-finger structure (Cx4-5Cx22-23HxH or Cx7Cx23HxC; Rushton et al., 2010). The WRKY domain is assembled into a four-stranded β-sheet and a zinc-binding pocket for w-box binding and protein interaction activity. This could help regulate corresponding physiological processes by activating/repressing transcription of target genes (Yamasaki et al., 2005). It is well known that WRKY originated in lower eukaryotes gradually evolved into a large superfamily in higher plants (Rushton et al., 2010). Only one group of WRKY proteins (known as Group I) has been identified both in higher plants and in lower eukaryotes such as the nonphotosynthetic slime mold, *Dictyostelium discoideum*, and the green alga, *Chlamydomonas reinhardtii*. Thus, members of this group are believed to be most similar to the ancestral protein (Yamasaki et al., 2005; Rushton et al., 2010).

Based on the number of WRKY domains and the corresponding zinc-finger structure, WRKY proteins are classified mainly into three groups (I, II, and III; Huang et al., 2012). Group I contains two zinc-finger motifs (CX4CX22-23HXH), Group II contains one zinc-finger motif (CX4CX22-23HxH), and Group III contains one zinc-finger motif (CX7CX23-24HXC or CX4CX23HXC). Group II proteins can be further divided into five subgroups (IIa, IIb, IIc, IId, and IIe; Huang et al., 2012), and phylogenetic analyses have indicated an additional possible level of classification as IIa+b, IIc, and IId+e (Zhang and Wang, 2005). Recently, Huang et al. (2012) found that among 81 tomato SlWRKYs, 15 were classified into Group I, 52 into Group II, and 11 into Group III. The remaining three SlWRKYs were placed in a new group labeled as none group (NG).

Since the first identification of plant WRKY proteins in sweet potato (Ishiguro and Nakamura, 1994; Rushton et al., 1996), many WRKYs from various plant species have been identified and characterized (Liu et al., 2014; Cai et al., 2015; Sun et al., 2015). Studies of WRKY transcription factors are no longer limited to model plants such as *Arabidopsis* and *Oryza sativa* (rice; Eulgem and Somssich, 2007; Mao et al., 2007). In members of Solanaceae, such as potato, eggplant, and tomato, WRKYs have been identified and characterized across the genome (Huang et al., 2012; Huang and Liu, 2013; Yang et al., 2015). Recently, *LeWRKY* was cloned in tomato and was induced by jasmonic acid and *Botrytis cinerea*, but not by salicylic acid (Lu et al., 2015).

In pepper (*Capsicum annuum* L.), the only proteins that have been reported to be involved in the regulation of various stress responses are several CaWRKYs and CaHsfA2 (Huh et al., 2012; Dang et al., 2014; Guo et al., 2014; Cai et al., 2015). Prior to this study, pepper CaWRKY had not been comprehensively analyzed at the whole-genome level. Recently, whole-genome sequencing for pepper has provided an opportunity for comprehensive identification and analysis of CaWRKY transcription factors (Qin et al., 2014). In the current study, we identified a total of 61 CaWRKYs based on the Pepper Genome Database (PGD; http://peppersequence.genomics.cn; Qin et al., 2014). Further bioinformatic analyses included comparisons of conserved domains, assessment of gene structure and classification, mapping onto chromosomes, and phylogenetic inference. Our results will provide resources for future studies on the functions of *CaWRKY* genes.

MATERIAL AND METHODS

Identification and intron-exon configuration of CaWRKYs in pepper

The annotated protein sequences of *Capsicum annuum* L. 'Zunla-1' were downloaded from the PGD (Release 2.0, http://peppersequence.genomics.cn; Qin et al., 2014). A BLASTp was performed using the HMM profile (Pfam: PF03106) of the conserved WRKY domain (http://pfam.xfam.org/). Default parameters were employed and the e-value was set at $1e^{-5}$. The full set of non-redundant putative CaWRKYs was then further checked for the presence of WRKY domains using Pfam 27.0 (Punta et al., 2012). Genomic DNA sequences, including the encoding sequences of CaWRKYs were also downloaded from the PGD. The intron-exon structure was visualized using GSDS 2.0 (http://gsds.cbi.pku.edu.cn).

Multiple-sequence alignment and chromosome mapping of CaWRKYs

To reveal the level of sequence conservation of these CaWRKY proteins, the sequences of the specific WRKY domain(s) of each CaWRKY were detected using SMART7.0 (http://smart.embl-heidelberg.de/; Letunic et al., 2012). Preliminary sequence manipulations were performed using DNAMAN 6.0. Multiple-sequence alignment of CaWRKY domains was conducted using BioEdit 7.0.

Chromosome mapping of each *CaWRKY* was performed with MapDrawV2.1 based on gene information provided in the PGD. Tandem duplications of *CaWRKY* genes were identified using the method of Yang et al. (2008) and Huang et al. (2012). Genes were identified as tandem duplicates if they were found within 100 kb of each other, using Smith-Waterman alignment (e-values $\leq 1 \times 10^{-5}$).

Phylogenetic analysis of CaWRKYs

Amino acid sequences of all CaWRKYs were aligned using ClustalX version 1.83 with default settings (http://www.clustal.org/; Thompson et al., 1997). A phylogenetic analysis of the WRKY conserved domains was conducted in MEGA version 5.05 (Tamura et al., 2011). Relative branch support was evaluated using bootstraps (1000 replicates). Branch lengths were calculated by pairwise comparison of genetic distances, and missing data were treated by pairwise deletions of gaps.

RESULTS

Identification of CaWRKY proteins from the pepper genome

A total of 61 CaWRKYs in *C. annuum* L. 'Zunla-1' were identified and chosen for analysis. These proteins were named CaWRKY01 to CaWRKY61 (Table 1). Among the 61, CaWRKY33 is the longest protein (869 aa), CaWRKY58 is the shortest (137aa), and the average length is 358 bp. Detailed information, including accession number, WRKYGQK peptide, zinc-finger structure, WRKY domain number, and group classification are listed in Table 1 and **Table S1**.

Table 1. WRKY gene family in pepper.

Gene symbol	Gene locus	WRKY domain Conserved heptapeptide	Zinc-finger type	Domain number	Group
CaWRKY01	Capana01g000165	WRKYGQK	Deficiency	1	NG
CaWRKY02	Capana01g000167	WRKYGQK	C2H2	1	IIe
CaWRKY03	Capana01g002803	WRKYGQK	C2H2	1	IIc
CaWRKY04	Capana01g003441	WRKYGQK	C2H2	1	IIc
CaWRKY05	Capana01g004471	WRKYGQK	C2HC	1	III
CaWRKY06	Capana01g004472	WRKYGQK	C2HC	1	III
CaWRKY07	Capana02g000212	WRKYGQK	C2H2	1	IIe
CaWRKY08	Capana02g000680	WRKYGQK	C2H2	1	IId
CaWRKY09	Capana02g000918	WRKYGQK	C2H2	1	IIb
CaWRKY10	Capana02g001642	WRKYGQK	C2H2	1	IIe
CaWRKY11	Capana02g002230	WRKYGQK	C2H2	1	IIb
CaWRKY12	Capana02g003053	WRKYGQK	Deficiency	1	NG
CaWRKY13	Capana02g003339	WRKYGQK/ WRKYGQK	C2H2	1	I
CaWRKY14	Capana02g003661	WRKYGQK	C2H2	2	IIc
CaWRKY15	Capana03g000473	WRKYGQK	C2H2	1	IIa
CaWRKY16	Capana03g001099	WRKYGQK	C2H2	1	IIb
CaWRKY17	Capana03g001962	**WRKYGMK**	C2H2	1	NG
CaWRKY18	Capana03g002072	WRKYGQK	C2H2	1	III
CaWRKY19	Capana03g002134	WRKYGQK	C2H2	1	NG
CaWRKY20	Capana03g002635	WRKYGQK	C2HC	1	III
CaWRKY21	Capana03g003085	WRKYGQK/WRKYGQK	C2H2	2	I
CaWRKY22	Capana03g003279	WRKYGQK	C2H2	1	IId
CaWRKY23	Capana04g000568	WRKYGQK	C2H2	1	IId
CaWRKY24	Capana04g001820	WRKYGQK/WRKYGQK	C2H2	2	I
CaWRKY25	Capana05g002502	WRKYGQK/WRKYGQK	C2H2	2	I
CaWRKY26	Capana06g001008	WRKYGQK	C2H2	1	IIb
CaWRKY27	Capana06g001110	WRKYGQK	C2H2	1	IIa
CaWRKY28	Capana06g001506	WRKYGQK/WRKYGQK	C2H2	2	I
CaWRKY29	Capana06g002128	WRKYGQK	C2HC	1	III
CaWRKY30	Capana06g003072	WRKYGQK	C2H2	1	IId
CaWRKY31	Capana07g000181	WRKYGQK/WRKYGQK	C2H2	2	I
CaWRKY32	Capana07g000528	WRKYGQK	C2H2	1	III
CaWRKY33	Capana07g001256	WRKYGQK/WRKYGQK	C2H2	2	I
CaWRKY34	Capana07g001387	WRKYGQK	C2H2	1	IIb
CaWRKY35	Capana07g001809	WRKYGQK	C2H2	1	IIe
CaWRKY36	Capana07g001968	WRKYGQK	C2H2	1	IIc
CaWRKY37	Capana07g002350	WRKYGQK/WRKYGQK	C2H2	2	I
CaWRKY38	Capana07g002454	WRKYGQK/WRKYGQK	C2H2	2	I
CaWRKY39	Capana08g000429	**WRKYGKK**	C2H2	1	IIc
CaWRKY40	Capana08g000683	WRKYGQK	C2H2	1	IIa
CaWRKY41	Capana08g001012	WRKYGQK	C2H2	1	IIe
CaWRKY42	Capana08g001044	WRKYGQK	C2HC	1	III
CaWRKY43	Capana08g001961	WRKYGQK	C2H2	1	IIb
CaWRKY44	Capana09g000676	WRKYGQK	C2H2	1	IIc
CaWRKY45	Capana09g001251	WRKYGQK/WRKYGQK	C2H2	2	I
CaWRKY46	Capana09g001790	WRKYGQK	C2HC	1	NG
CaWRKY47	Capana10g000205	**WRKYGQN**/no conserved stretch	C2H2	2	I
CaWRKY48	Capana10g000754	WRKYGQK	C2H2	1	IIe
CaWRKY49	Capana10g001220	WRKYGQK	C2HC	1	III
CaWRKY50	Capana10g001548	WRKYGQK	C2HC	1	III
CaWRKY51	Capana10g001791	WRKYGQK/**WRKYGHK**	C2H2	2	I
CaWRKY52	Capana10g001805	WRKYGQK	C2H2	1	NG
CaWRKY53	Capana11g001882	WRKYGQK/WRKYGQK	C2H2	2	I
CaWRKY54	Capana11g001905	WRKYGQK	C2H2	1	IIc
CaWRKY55	Capana12g001134	WRKYGQK	C2H2	1	IIa
CaWRKY56	Capana12g001826	**WRKYGKK**	C2H2	1	IIc
CaWRKY57	Capana12g001851	WRKYGQK	C2H2	1	IIc
CaWRKY58	Capana00g000429	KKKGEK	C2H2	1	IIc
CaWRKY59	Capana00g001033	WRKYGQK	C2H2	1	IIc
CaWRKY60	Capana00g003083	WRKYGQK	C2H2	1	IId
CaWRKY61	Capana00g004112	**WRKYGKK**	C2H2	1	IIc

NG means none group. CaWRKY01, CaWRKY12, CaWRKY17, CaWRKY19, CaWRKY46, and CaWRKY52 were not assigned to any group. The variants of conserved WRKYGQK peptide are shown in bold and no conserved WRKYGQK stretch exists in the C-terminal of CaWRKY47.

Classification and structural analysis of *CaWRKY* genes

As the most prominent structural feature of WRKY transcription factors, a total of 74 WRKY domains were identified in the 61 CaWRKY proteins. When there were two domains in the same WRKY protein, we designated them as the N-terminal WRKY domain (I-NTWD) and the C-terminal WRKY domain (I-CTWD).

CaWRKY01 and CaWRKY12 encoded truncated proteins, and they were excluded from the alignment of CaWRKY domains in ClustalX. The results classified the CaWRKYs into four groups (I, II, III, and NG). Thirteen CaWRKYs were classified into Group I, and they contained two WRKY domains with the C2H2 type of zinc finger structure (CX4CX22-23HX1H). Group II comprised 33 CaWRKYs that each contained only one WRKY domain with the C2H2 type of zinc-finger (CX4-5CX23HX1H). These were further divided into five subgroups: IIa (4 proteins), IIb (6), IIc (12), IId (5), and IIe (6). Group III contained nine CaWRKY members that also had one WRKY domain, but the zinc-finger (C2HC: CX7CX23-24HX1H/CX4CX23HX1H) was different in the other two groups. The remaining four CaWRKYs (CaWRKY17, CaWRKY19, CaWRKY46, and CaWRKY52) did not seem to fit into any of the groups above because of their distinctive WRKY domain structure and were classified in NG. The detailed amino acid sequences of the 74 CaWRKY domains are in **Table S2**.

Although the WRKYGQK peptide is known to be highly conserved, sequence variation was still found in six of these CaWRKYs (Table 1). In addition to the most common variant (WRKYGKK), three other variants (WRKYGMK, WRKYGQN, and WRKYGHK) were also identified in our study. Strikingly, a new variant (KKKGEK) was observed for the CaWRKY58 protein and a WRKY domain deletion event had occurred in the C-terminal WRKY domain of CaWRKY47. In addition, the expected variant, C-X33, was identified in the zinc-finger structure of CaWRKY32 (Table 1).

Most CaWRKY members have 2-3 introns, except for those in Group I and Group IIb, in which most members seem to have at least 4 introns (Figure 1). CaWRKY33 in Group I has the most introns (12), while several CaWRKYs have only one intron (CaWRKY14 and 58 in Group IIc, CaWRKY06 in Group III; Figure 1).

Multiple-sequence alignment of the WRKY gene superfamily

As shown in Figure 2, most of the WRKYGQK variants were members of Group IIc, including WRKYGKKs in CaWRKY39, CaWRKY56, and CaWRKY61, and KKKGEK in CaWRKY58. The other variants were in Group I and NG. By analyzing the WRKY domain sequence of CaWRKY members in the NG, we found that some of the more unusual variations were observed in the WRKY domains of CaWRKY17, CaWRKY19, and CaWRKY46, compared to members of Groups I, II, and III. For example, the amino acid residue immediately after the first Cys (C) of the zinc-finger in CaWRKY17 and CaWRKY19 is Asn (N), instead of T, D, or S. For CaWRKY46, both the amino acids adjacent to the second His (H) of the zinc-finger are Leu (L) (Figure 2).

Chromosomal distribution and duplication of *CaWRKY* genes

The genomic locations of 57 of the 61 identified *CaWRKY* genes across the twelve pepper chromosomes. The remaining four genes (*CaWRKY58* to *CaWRKY61*) could not be

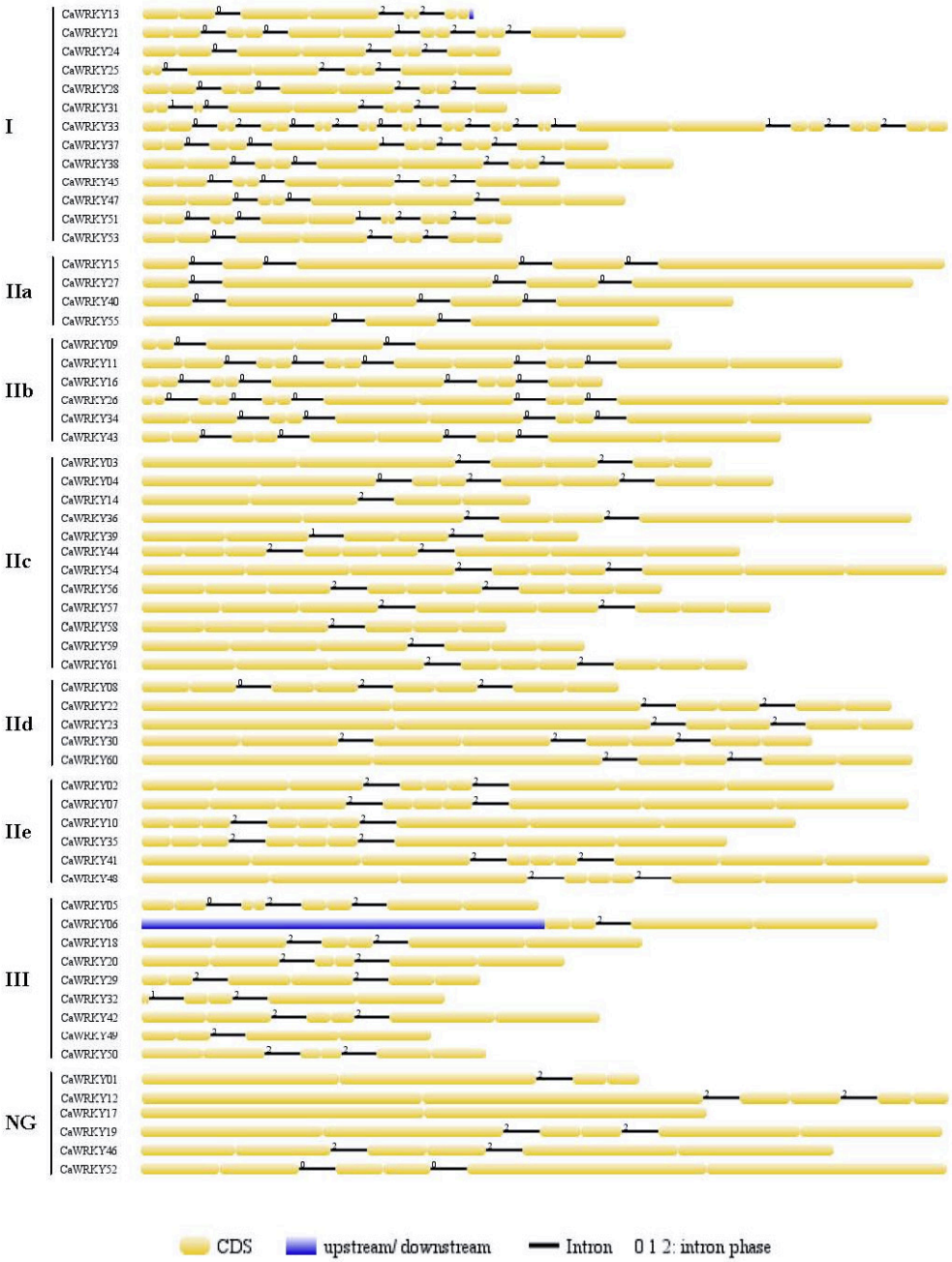

Figure 1. Intron-extron structure of CaWRKY genes in pepper. The upstream/downstream, extron and intron are shown with blue rectangle, yellow cylinder and black line, respectively. Intron phase numbers (0, 1, 2) were labeled on the top of each *CaWRKY* gene sketch map.

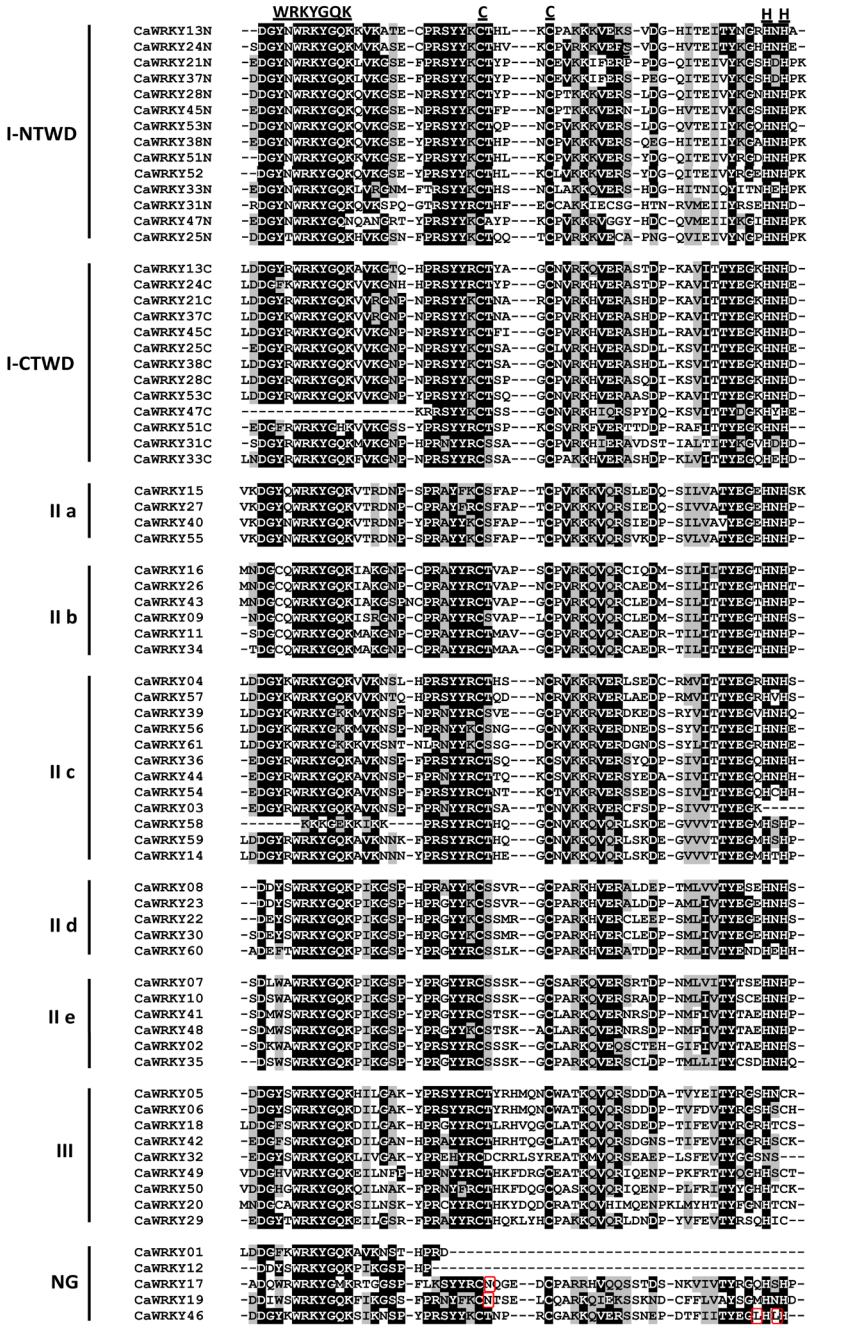

Figure 2. Multiple-sequence alignment of conserved WRKY domains of different groups in pepper. CaWRKY domains that belong to the same group are clustered together. The conserved amino acids are emphasized with black background. The conserved heptapeptide (WRKYGQK) and zinc-finger key structure [cysteine (C), histidine (H)] are labeled on top. The amino acid variations of NG group of members are labeled with red blocks.

located on any chromosome and were assigned to chromosome 00. Although the sequenced size of chromosome 02 (164.0 M) only accounts for 5% of the assembled pepper genome (3.36 G), it includes 8 of 61 *CaWRKY*s (~13%). In contrast, chromosome 05 (217.27 M) only contains one, *CaWRKY25*. Overall, more *CaWRKY* genes are found on chromosomes 01, 02, 03, 06, 07, 08, and 10 than on chromosomes 04, 05, 09, 11, and 12 (Figure 3).

Two pairs of tandem duplications of *CaWRKY* genes were found. A pair of homologous genes was considered a tandem duplication if the genes were within 100 kb of each other. As shown in Figure 3, these pairs of genes (*CaWRKY01*/*CaWRKY02*, *CaWRKY05*/*CaWRKY06*) were both located on chromosome 01.

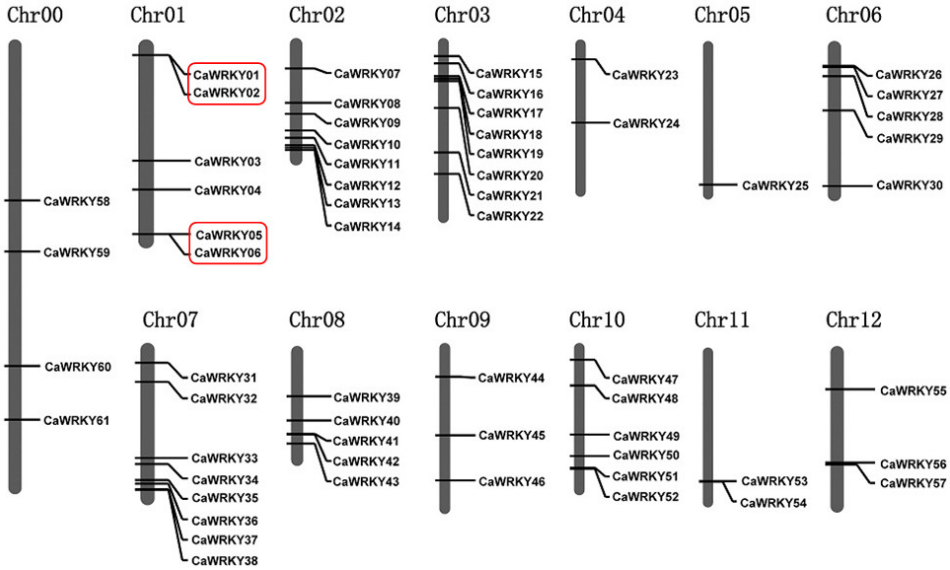

Figure 3. Chromosome distribution of CaWRKY genes. Tandemly duplication genes are labeled with red rectangles.

Phylogenetic analysis of the WRKY gene family in pepper, tomato, and potato

We inferred unrooted phylogenetic relationships among the CaWRKY proteins in pepper, using an alignment of amino acid sequences of 72 WRKY domains (including N-terminal and C-terminal domains of Group I members; Figure 4). Eight at WRKYs from *Arabidopsis* were also selected for phylogenetic analysis. The relationships of CaWRKYs in the tree confirm the groupings noted above (Figure 4 and Table 1). Three main clusters were observed, cluster 1 (Group I, IIa+b, and IIc), cluster 2 (Group IId and IIe), and cluster 3 (Group III; Figure 4). The most strongly supported clusters and subclusters are I+IIc and IIa+b (in cluster 1) and cluster 2 (IId+e). Interestingly, independent distinct gene expansion events occurred in cluster 2.

To further illuminate the evolutionary relationships among WRKY genes in Solanaceae, we ran a separate phylogenetic analysis including amino acid sequences of 264 WRKY domains (including N-terminal and C-terminal domains of members of Group I) from pepper, tomato, and potato (Figure 5). Incomplete WRKY domains (CaWRKY01 and CaWRKY12 in pepper, and StWRKY21 and StWRKY39C in potato) were excluded from

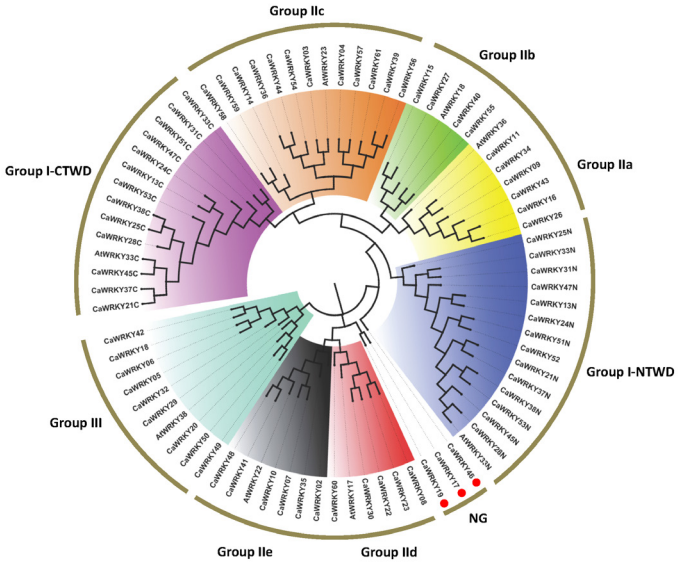

Figure 4. Phylogenetic tree of CaWRKY proteins in pepper using the neighbor joining method by MEGA 5.0. The WRKY domains of each group are clustered together, and each clade was labeled with different colors. The three CaWRKYs in no-color clade (CaWRKY17, CaWRKY19 and CaWRKY46) were defined as none group (NG) and emphasized with red spots.

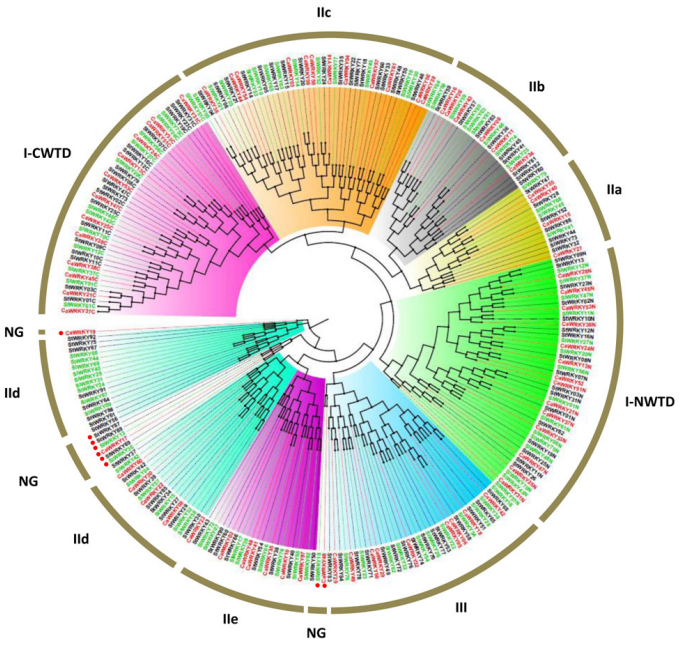

Figure 5. Phylogenetic tree of WRKY proteins among pepper, tomato and potato using the neighbor joining method by MEGA 5.0. Each WRKY group is labeled with different colors. None group (NG) WRKYs are emphasized with red spots.

the phylogenetic analysis. The amino acid sequences of all 268 WRKY domains are available in **Table S2**. The resulting topology of this tree is completely consistent with the CaWRKY phylogenetic tree (Figure 4), but reveals a more complex set of relationships (Figure 5). The interspersed distribution of WRKY domains from the three species (pepper, tomato, and potato) in all of the groups suggests that the expansion of WRKY genes occurred in an ancestor of these three species. In addition, WRKY gene expansions were observed for nine members (CaWRKY17, CaWRKY19, CaWRKY46, StWRKY69, StWRKY87, StWRKY89, SlWRKY13, SlWRKY18, and SlWRKY36; Figure 5), suggesting the evolutionary uniqueness of these WRKY members in the long-term evolution of this gene family.

DISCUSSION

The WRKY transcription factor family has been noted in many plant species for its regulatory roles in a wide range of biological processes. Seven WRKY protein-encoding genes from pepper had been reported to play critical roles in biological processes, including pathogen resistance and high-temperature tolerance (Park et al., 2006; Lim et al., 2011; Wang and He, 2011; Huh et al., 2012; Wang et al., 2013; Dang et al., 2013, 2014; Cai et al., 2015). Based on the recently released pepper genome sequence of *C. annuum* 'Zunla-1', we identified the entire family of 61 CaWRKY proteins in pepper. These CaWRKYs are classified into Group I, Group II (IIa, IIb, IIc, IId, and IIe), Group III, and NG, which possess 21.3, 54.1% (6.6, 9.8, 19.7, 8.2, and 9.8%), 14.8, and 9.8% of all CaWRKYs, respectively (Table 1 and **Table S2**).

Gene duplication is an important factor in the evolution of gene families. In our study, about 7% (4 of 61) *CaWRKY* genes were shown to result from tandem duplication (Figure 3). The different numbers of introns of *CaWRKY* genes in different groups suggest that some of the loss/gain events occurred prior to the speciation of *Capsicum* (Figure 1). A phylogenetic tree of WRKYs from three species in Solanaceae (pepper, tomato, and potato) confirmed that members of Group II can be reorganized into three clusters, including Group IIa+b, IIc, and IId+e (Figures 4, 5). A unique gene expansion within Group IId+e had been identified in tomato (Huang et al., 2012) and potato (Huang and Liu, 2013). In the present study, nine WRKYs (three CaWRKYs, three SlWRKYs, and three StWRKYs) were shown to form a separate subclade in GroupIId+e, demonstrating that a unique WRKY gene expansion has occurred in Solanaceae (Figures 4 and 5). We propose that these WRKYs play specific roles in Solanaceae plants.

In this study, genome-wide identification and analysis of the *CaWRKY* gene superfamily in pepper were performed using bioinformatic methods. The results showed at least 61 members of the family across the genome. In addition, studies of exon-intron structure, multiple-sequence alignment, chromosome mapping, and phylogenetic analyses of these 61 CaWRKYs were also conducted. The results provide important insights for future studies of the structure, evolution, and biological functions of this critical transcription factor family in pepper.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by the Zhejiang Provincial Natural Science Foundation of China

(#Q15C150010), the National Natural Science Foundation of China (#31301774, #31272156, and #3150110311), the Young Talent Cultivation Project of Zhejiang Academy of Agricultural Sciences (#2015R23R08E07, #2015R23R08E09), the Public Agricultural Technology Research in Zhejiang (#2016C32101, #2015C32049), and the Technological System of Ordinary Vegetable Industry (#CARS-25-G-16).

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Supplementary material

Table S1. CaWRKY gene family.

Table S2. WRKY domain of the WRKY gene superfamily in Solanaceae plants.