Characterization and Divergence Analysis of Duplicated *R2R3-MYB* Genes in Watermelon

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ABSTRACT. Watermelon (*Citrullus lanatus*) is an economically important cucurbit (Cucurbitaceae) crop cultivated globally for its nutritional benefits. Fruit development in watermelon is characterized by fast fruit expansion attributed to unique biological processes. *Myeloblastosis* (*MYB*) family genes play important roles in plant growth and development, especially *R2R3-MYB*-type genes. However, the evolution of *R2R3-MYB* family genes in the watermelon genome and whether they participate in the regulation of watermelon fruit development remain unknown. To address these questions, duplication modes of *R2R3-MYB* family genes were identified and their expression profiles were investigated during watermelon fruit development. A total of 48 duplicated gene pairs were identified among the 89 *R2R3-MYBs* in watermelon. Segmental and transposed duplication events play major roles in the *R2R3-MYB* family gene expansion process. The ratio of nonsynonymous mutation and synonymous mutation analysis indicated that all the duplicated *R2R3-MYBs* experienced negative selection. Gene structures and cis-element compositions in promoter sequences exhibited abundant divergences between the *R2R3-MYB* duplicated genes. Transcriptome analyses of seed, rind, and flesh during fruit development showed that only two duplicated gene pairs had significantly similar expression patterns, whereas divergent expression profiles were found between the remaining duplicated gene pairs. Tissue-specific and development stage-specific divergent expression patterns demonstrated that neo-functionalization occurred between watermelon *R2R3-MYB* duplicated genes. The current study provides valuable information for further functional analyses of *R2R3-MYBs* in watermelon.

Transcription factors (TFs) are extensively involved in the regulation of plant growth and development. *MYB* is an important TF gene family with the largest members (Dubos et al., 2010). Its N-terminal region has a highly conserved DNA binding domain, which is called MYB domain. A *MYB* gene usually contains one to four incomplete MYB domain repeats (R) of ≈ 52 amino acid residues. Based on the number of MYB repeats, the *MYB* family gene can be divided into four types, namely, *4R-MYB*-type with four repeats, *3R-MYB*-type with three repeats, *R2R3-MYB*-type with two repeats, and *MYB*-related-type with a single repeat or partial *MYB*-related repeats (Jia et al., 2004). *R2R3-MYB* is dominant and has the largest members in most plants (Rosinski and Atchley, 1998), and it has a wide range of functions, including the regulation of plant growth and development, primary and secondary metabolism, and response to biotic and abiotic stresses (Stracke et al., 2001).

Gene duplication has an important role in the expansion of the gene family and functional diversity. Large-scale gene duplication and single-gene duplication are two types of gene duplication models that widely exist in eukaryotic genomes (Wang et al., 2013b). Large-scale gene duplication includes whole-genome duplication (WGD) and segmental duplication (Salman-Minkov et al., 2016). Single-gene duplication is also

prevalent in plant genomes and consists of three duplication models, including transposed duplication, proximal duplication, and tandem duplication (Freeling, 2009). Transposed duplication generates a duplicated gene on a new chromosome position via replicative transposition (Panchy et al., 2016). Tandem duplication results in a gene pair produced by an unequal cross between alleles (Hahn, 2009). The proximal gene pairs are two duplicated genes separated by a small number of genes (Wang et al., 2012). The WGD in watermelon (*Citrullus lanatus*) has seven major triplications dating back to between 76 and 130 million years ago and generated 302 paralogous relationships covering 29% of the genome (Guo et al., 2013). The segmental duplication event reportedly has a major role in the expansion of the *MYB* gene family in pineapple (*Ananas comosus*) (Liu et al., 2017).

After duplication, mutations increasingly accumulate in the regulatory and coding regions of duplicated genes during the course of evolution (Zhu et al., 2014). Different regulatory regions may lead to changes in expression levels, and different coding regions may result in new functions (Long and Thornton, 2001). The function-differentiated duplicated genes may undergo positive or negative selection. The ratio of nonsynonymous mutation (K_a) to synonymous mutation (K_s) is used to estimate the selection pressure of duplicated genes (Zhang et al., 2006). Many evolutionary models have been proposed to elucidate the gene status after duplication, including the short- and long-term retention or loss function (Qiao et al., 2018). Furthermore, three functional models are used to describe the

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functions of the retained duplicated genes, including gene dosage, sub-functionalization, and neo-functionalization (Flagel and Wendel, 2009). Gene dosage is one mechanism that affects the functions by changing the genes number of copies after duplication, and recent research has suggested that the presence of gene dosage may be beneficial to gene duplication (Panchy et al., 2016). The sub-functionalization model suggests that each duplicated gene retains its original ancestral function during the natural mutational process (Freeling et al., 2015). Sub-functionalization was found between the duplicated *MYB* genes *ScAN2* and *ScAN1* in potato (*Solanum commersonii*) in the response to cold stress (D'Amelia et al., 2018). The phenomenon that one duplicated gene keeps the ancestral function while the other gene acquires a new function is called neo-functionalization (Duarte et al., 2006). Neo-functionalization has been reported for the B-class *MADS-box* duplicated genes of *PFGLO1* and *PFDEF* in *Physalis floridana* (Zhang et al., 2015).

Watermelon is an economically important cucurbit crop widely grown for its large, edible fruit. The watermelon planted area and total yield are ranked among the world's top 10 for fresh fruits (Food and Agriculture Organization of the United Nations, 2018). Because a whole genome sequence of the crop is available, watermelon has become a crop widely researched by plant breeders and plant physiologists. A total of 162 *MYB* genes have been identified in the watermelon genome, and expression patterns of 25 *MYB* genes were measured in watermelon seedlings under abiotic stresses (Xu et al., 2018). This study provides a fundamental understanding of the possible roles of *MYB* family genes in watermelon stress tolerance. Fruit development is one of the most important biological processes of watermelon; it determines the economic value of watermelon production. *MYB* genes have been reported to widely participate in the regulation of fruit development (Machemer et al., 2011). However, the roles *R2R3-MYB* genes in regulating watermelon fruit development and whether the functions of duplicated *R2R3-MYB* genes are different during fruit development are still unknown.

In this study, duplicated *R2R3-MYB* gene pairs were identified in the watermelon genome. Their expression profiles in rind, flesh, and seeds were determined during fruit development. Expression patterns of duplicated genes were associated with their Ka/Ks values, gene structures, and cis-element compositions. This study aimed to identify the *R2R3-MYB* genes involved in watermelon fruit development and clarify the function divergences of duplicated *R2R3-MYB* genes.

Materials and Methods

IDENTIFICATION OF R2R3-MYB GENES. The genome of watermelon cultivar 97103 (v1) was used as the reference genome (Guo et al., 2013). All protein sequences and coding sequences were downloaded from the Cucurbit Genomics Database (Zheng et al., 2019). The hidden Markov model (HMM) profile of the MYB DNA-binding domain (PF00249) was downloaded from the Pfam database (El-Gebali et al., 2019). HMMER (Finn et al., 2011) was applied to identify the *MYB* family members with a cutoff of $E \leq 0.01$. Protein domains of *R2R3-MYB* were validated by SMART (Letunic and Bork, 2018). Gene structures were analyzed using the Gene Structure Display Server (Hu et al., 2015). The MEME online tool (version 5.1.0; National Institutes of Health, Bethesda,

MD) was used to investigate conserved domains. Weblogo (Crooks et al., 2004) was used to draw the sequence logos. Protein modeling was predicted using SWISS-MODEL (Waterhouse et al., 2018).

IDENTIFICATION OF DUPLICATED GENES AND CIS-ELEMENT ANALYSIS. MCScanX-transposed was used to identify and classify gene duplication modes (Wang et al., 2013a). Circos (Krzywinski et al., 2009) was used to display the synteny relationships. KaKs_Calculator1.2 (Zhang et al., 2006) was applied to estimate the Ks and Ka values. Bedtools (Quinlan and Hall, 2010) was used to obtain the 1-kb upstream sequence for each gene. Cis-elements were predicted using PlantCARE (Lescot et al., 2002). The cis-elements index value of the duplicated genes were calculated using the following equation: $[1 - (\text{common cis-elements} / \text{all cis-elements})]$. This is used to measure the dissimilarity of cis-elements between two duplicated genes. The value of the cis-element index ranged from 0 to 1, and the larger value indicated greater divergence.

TRANSCRIPTOME DATA ANALYSIS. The transcriptome data of watermelon seeds sampled at 25, 31, 37, and 49 d after pollination (DAP) under BioProject ID PRJNA319011 (University of Nebraska, Lincoln, NE) and transcriptome data of watermelon fruit flesh and rind sampled at 10, 18, 26, and 34 DAP under BioProject ID SRP012849 (Boyce Thompson Institute, Ithaca, NY) were downloaded from the National Center for Biotechnology Information Sequence Read Archive (Bethesda, MD) database. All the annotated gene sequences of the watermelon cultivar 97103 reference genome were used as the reference transcriptome. Kallisto (Bray et al., 2016) was used to calculate the transcript per million (TPM) value for each gene. RStudio (version 1.0.136; RStudio, Boston, MA) was used to conduct the data analysis and draw the plots.

Results

IDENTIFICATION OF WATERMELON R2R3-MYB GENES. The HMM profile of MYB was used to search for watermelon genome-wide annotated proteins; 164 *MYB* genes were identified. These *MYB* genes were further validated and classified by searching both the Pfam and SMART databases, resulting in 89 *R2R3*-type genes, 5 *R3*-type genes, and 70 *MYB*-related-type genes. No *R4*-type genes were found in the watermelon genome. The *R2R3*-type genes were selected for further analysis and named *CIR2R3-MYB*. Information regarding these genes is listed in Supplemental Table 1. Sequence logo was used to exhibit the characteristics of *CIR2R3-MYB* conserved motifs (Fig. 1). Results revealed 53 amino acid residues in the R2 repeat and 71 amino acid residues in the R3 repeat. Highly conserved Trp residues were found on positions 6, 26, and 46 of the R2 repeat and 25, 45, 64 on the R3 repeat, respectively. The three-dimensional protein structure models showed that each of the two domains build an HTH structure, which is the main characterization of *R2R3-MYB* family genes. Amino acids such as Glu-10, Gly-22, Arg-37, Lys-40, Ser-41, Lys-45, Cys-42, Leu-44, Asn-48, and Leu-50 in the R2 repeat and Glu-10, Ala-29, Thr-56, Asp-57, Asn-58, Lys-61, and Asn-62 in the R3 repeat were also conserved among watermelon *R2R3-MYB* genes.

DUPLICATION EVENTS OF CIR2R3-MYB GENES. A total of 48 *CIR2R3-MYB* duplicated gene pairs were identified, including 27 segmental duplicated pairs, 19 transposed duplicated pairs, 1 proximal duplicated pair, and 1 tandem duplicated pair (Supplemental Table 2). Duplicated gene pairs and their

genome locations are displayed in the Circos plot (Fig. 2). The duplicated genes were distributed on all 11 watermelon chromosomes and tended to cluster at the two ends of each chromosome. Chromosome 5 harbored the largest number of

Fig. 1. Domains of *CIR2R3-MYB* family genes and protein three-dimensional (3D) structure models of R2 and R3 MYB repeats. (A) R2 domain. (B) R3 domain. The bit score indicates the information content for each position in the sequence and the purple asterisks indicate the conserved tryptophan residues (Trp, W). (C) R2 repeats 3D structure model. (D) R3 repeats 3D structure models.

duplicated genes. Specially, mutually segmental duplicated events were found among the triple genes of *Cla013979-Cla020663-Cla022601*, *Cla005982-Cla007586-Cla021520*, *Cla010332-Cla019115-Cla018610*, and *Cla007715-Cla019451-Cla001341*.

GENE STRUCTURE DIVERGENCES OF DUPLICATED *CLR2R3-MYB* GENES. To compare gene structures of duplicated genes, DNA and mRNA sequences of each gene were aligned to analyze the exon-intron composition (Fig. 3). Exons ranged from one to four in the duplicated genes. The duplicated gene pair *Cla017337-Cla015165* had the highest number of exons, whereas duplicated gene pairs *Cla020702-Cla022542*, *Cla020633-Cla022601*, *Cla004362-Cla013979*, *Cla013979-Cla020633*, and *Cla013979-Cla022601* harbored only one exon in each gene. A total of 36 duplicated gene pairs comprised similar exon-intron compositions. The remaining 12 duplication gene pairs had different gene structures, including three segmental duplicated gene pairs and nine transposed duplicated gene pairs. The different gene structures probably lead to functional divergences between the duplicated genes.

CIS-ELEMENTS DIVERGENCES OF DUPLICATED *CLR2R3-MYB* GENES. To compare the cis-regulatory element composition in the promoter region between the duplicated gene pair, a 1-kb upstream sequence of each gene was selected and the putative

cis-regulatory elements were identified (Supplemental Table 3). To quantify the dissimilarity of cis-element composition between duplicated genes, the cis-element index was calculated for each gene pair. The density distribution of the cis-element index for segmental and transposed duplicated genes is shown in Fig. 4A. The cis-element index of segmental and transposed duplicated genes ranged from 0.55 to 0.85 and 0.54 to 0.8, respectively, demonstrating that wide divergences occurred in the promoter regions of duplicated gene pairs.

The cis-element types were summarized. The top 30 cis-element types are shown in Fig. 4B. Function annotations of these cis-elements were related to hormone regulations, cellular development, and stress response. For hormone regulation, the functions of cis-elements were mainly involved in responses related to salicylic acid, auxin, and abscisic acid metabolism. Cellular development mainly included meristem-specific activation, endosperm expression, palisade mesophyll cells, leaf morphology development, shoot-specific expression, and cell-cycle regulation. The remaining cis-elements were related to stress physiology, such as responses to light, low-temperature, heat, drought, dehydration, anaerobic induction, wound, and anoxic-

Fig. 3. Gene structures of duplicated *R2R3-MYB* gene pairs. Green boxes represent the coding sequence (CDS) regions. Black lines show the intron regions. Yellow indicates transposed duplicated gene pairs. Purple indicates segmental duplicated gene pairs. The proximal and tandem duplicated gene pairs each have a pair of duplicated genes.

specific inducibility. The cis-element sequences CAAT-box and TATA-box were found in all *CIR2R3-MYB* genes. The light response of cis-elements Box-I, Box-4, and GAG-motif occurred in the promoter regions of most duplicated *CIR2R3-MYB*s. A total of 59 duplicated *CIR2R3-MYB* genes had the ARE cis-acting regulatory element, which is essential for anaerobic induction. Skn-1 motif was found in 62 duplicated

genes, suggesting these genes are putatively involved in the regulation of seed development. The 5UTRPy-rich stretch and G-box were also identified in more than 50 *CIR2R3-MYB* promoter sequences, suggesting that these genes probably have high expression levels. TC-rich repeats, which are involved in defense and the stress response, were also found in the promoter regions of 54 *CIR2R3-MYB* genes.

Fig. 4. The cis-element summary and density distribution of the cis-element index. (A) The cis-elements index density of duplicated gene pairs. (B) The cis-element summary. Annotations of cis-elements are listed in Supplemental Table 3.

KA AND KS SUBSTITUTION RATES OF DUPLICATED CIR2R3-MYB GENES. Ka, Ks, and their ratios were calculated. The density distribution of Ks values is presented in Fig. 5A. The Ks values ranged from 0.42 to 3.95. The smallest Ks value was observed in the tandem duplicated gene pair *Cla014140-Cla014141*. A peak appeared at ≈ 2.8 in the density distribution plot, indicating that the duplication events occurred at one major stage and varied with the time of evolutionary process.

The Ka/Ks value is an index estimating the selection pressure during the gene evolution process. The Ka/Ks values of the *R2R3-MYB* duplicated gene pairs ranged from 0.06 to 0.32, with an average of 0.15 (Fig. 5B). All the Ka/Ks values were less than 1.00, suggesting that *CIR2R3-MYB* duplicated genes had undergone purifying selection during the evolution process. Gene pairs with different duplication models tended to have different Ka/Ks value distributions. The segmental duplicated gene pairs had relatively low Ka/Ks values, whereas the transposed duplicated gene pairs displayed relatively high Ka/Ks values. A maximum Ka/Ks value of 0.32 occurred in the tandem duplicated gene pair *Cla014140-Cla014141*, suggesting that the two genes might experience more relaxed purifying selection.

EXPRESSION DIVERGENCES OF DUPLICATED CIR2R3-MYB GENES. RNA-seq data derived from the flesh and rinds of watermelon fruit at 10, 18, 26, and 34 DAP and from seeds at 25, 31, 37, and 49 DAP were used to determine the expression profiles of *CIR2R3-MYB* family genes. A total of 34 genes were not expressed during watermelon fruit development. The remaining 55 genes were involved in the regulation of fruit development and maturity. Among them, 10 genes including *Cla006761*, *Cla021243*, *Cla011548*, *Cla013099*, *Cla007114*, *Cla020633*, *Cla013668*, *Cla022601*, *Cla010413*, and *Cla013979* were constitutively expressed in all tissues and at all developmental stages. However, some *CIR2R3-MYB* genes exhibited tissue-specific expression patterns. Twelve *CIR2R3-MYB* genes including *Cla007815*, *Cla007846*, *Cla015834*, *Cla013461*, *Cla018610*, *Cla021474*, *Cla018967*, *Cla010415*,

Cla017337, *Cla017267*, *Cla017012*, and *Cla016980* were only expressed in seeds. *Cla021520*, *Cla019115*, and *Cla017797* were specifically expressed in flesh, whereas *Cla005982*, *Cla008278*, *Cla019115*, and *Cla021520* were specifically expressed in the rinds.

To compare expression patterns of the duplicated genes, pairwise transcript abundances were illustrated in the heatmap (Fig. 6). A total of eight duplicated gene pairs were not expressed during watermelon fruit development, including one tandem duplicated gene pair, one proximal duplicated gene pair, two transposed duplicated pairs, and four segmental duplicated gene pairs. Three segmental duplicated gene pairs of *Cla013979-Cla022601*, *Cla020633-Cla022601*, and *Cla013979-Cla020633* were expressed in all tissues and at all stages of watermelon fruit development. To further quantify the expression similarity of duplicated gene pairs, Pearson's correlation coefficient was calculated for each expressed duplicated gene pair during watermelon fruit development. The significant correlation coefficients were marked aside the corresponding gene pairs ($P < 0.05$). A transposed duplicated gene pair of *Cla004362-Cla013979* and a segmental duplicated gene pair of *Cla020702-Cla022542* exhibited significantly similar expression patterns, demonstrating that the duplicated gene pairs had putatively similar functions during watermelon fruit development. Although the expression patterns were significantly correlated, significantly different transcript abundances were also observed between the two duplicated gene pairs. The remaining 38 duplicated gene pairs exhibited divergent expression patterns, suggesting different regulatory roles during fruit development.

Tissue-specific divergent expression patterns were observed between duplicated gene pairs. Five transposed duplicated gene pairs exhibited tissue-specific divergent expression patterns. *Cla019999* was expressed in rind and flesh, but the expression of its duplicated gene *Cla009903* was not detected in the two tissues. *Cla009263* was expressed in all tissues, but *Cla000767* was not expressed in the fruit. Similarly, *Cla015834* was

Fig. 5. Density distribution of synonymous mutation (Ks) and boxplot of nonsynonymous mutation (Ka)/Ks values: (A) density distribution of Ks for the *CIR2R3-MYB* duplicated gene pairs and (B) Ka/Ks value distribution of *CIR2R3-MYB* duplicated gene pairs.

Fig. 6. Expression profiles of duplicated *CIR2R3-MYB* gene pairs in seed, fruit flesh, and rind during watermelon fruit development. The color scale at the left of each dendrogram represents log2 expression values, with black indicating high levels and white indicating low levels of transcript abundance. r = correlation coefficient expression of two duplicated genes.

specifically expressed in the seed, but its duplicated gene *Cla016689* was not expressed in the fruit. *Cla019223* was expressed in rind and flesh, but its duplicated gene *Cla001702* was not expressed in the fruit. *Cla013668* was expressed in all tissues and at all stages, but expression of its duplicated gene *Cla005321* was not detected in the fruit. Tissue-specific divergent expression patterns were also found in the four segmental duplicated gene pairs. *Cla019115* was only expressed in the rind, but its duplicated gene *Cla010332* was not expressed in the fruit. *Cla018501* was expressed in all tissues, whereas its duplicated gene *Cla018967* was only expressed in the seed. *Cla018610* was only expressed in the seed, but its duplicated gene *Cla019115* was only expressed in the rind. *Cla018610* was only expressed in the seed, but its duplicated gene *Cla010332* was not expressed in the fruit.

Development stage-specific divergent expression patterns were also observed between duplicated gene pairs. Regarding transposed duplicated gene pairs, *Cla018657* was not expressed in fruit, but its duplicated gene *Cla017496* was expressed in rinds and flesh at all stages, as well as in the seed at 10 DAP. *Cla012519* was expressed in all tissues and at all stages, but its duplicated gene *Cla007846* was only expressed in the seed at 25 and 31 DAP. *Cla007815* was only expressed in seed at 25 DAP, whereas its duplicated gene *Cla010193* was not expressed in fruit. Similar results were also observed in segmental duplicated gene pairs. For instance, *Cla020702* was expressed in all tissues, but its duplicated gene *Cla022542* was only expressed in the rinds at 10 and 18 DAP. Similarly, *Cla017337* was only expressed in the seed at 25 and 49 DAP, but its duplicated gene *Cla015165* was expressed in all tissues. *Cla019199* was expressed in all tissues but not in the seed at 25 DAP, whereas its duplicated gene *Cla010415* was only expressed in the seed at 25 DAP. *Cla003441* was expressed in the rind and flesh at 10 DAP. However, *Cla019223* was only expressed in the rind and flesh at 18, 26, and 34 DAP.

Discussion

R2R3-MYB transcription factors are widely involved in the regulations of plant morphogenesis, growth, metabolism, developmental processes, and response to biotic or abiotic stresses. However, duplication types of watermelon *R2R3-MYB* and their possible roles regulating watermelon fruit development have been ignored. In this study, the duplicated *CIR2R3-MYB* genes were identified, and their expression profiles were investigated during fruit development to understand their potential roles during watermelon fruit development.

A total of 89 *R2R3-MYB* genes were identified in the watermelon genome; this number was similar to that of previous reports (Xu et al., 2018) but larger than that reported for cucumber (*Cucumis sativus*) (Ren et al., 2009) and smaller than that identified in *Arabidopsis thaliana* (Stracke et al., 2001). All *CIR2R3-MYBs* have two incomplete R. Every R forms an HTH structure, which is consistent with *R2R3-MYB* family characteristics identified in the present study. The first Trp residue in the R3 repeat was replaced by some hydrophobic amino acids like Phe or Ile. Similar results were also observed in *A. thaliana* (Stracke et al., 2001) and cucumber (Li et al., 2012). However, some other residues, such as Glu, Gly, Lys, Ser, Cys, Leu, Thr, Asp, and Asn, were specifically conserved in watermelon *R2R3-MYB* family genes.

A total of 48 *CIR2R3-MYB* duplicated gene pairs were identified in this study. Segmental duplication accounted for

56.25% of the total duplication events, demonstrating that it has a major role in *CIR2R3-MYB* gene expansion. Similar results were also reported for pineapple (Liu et al., 2017). Results of the present study suggested that genes produced by segmental duplications were retained during evolution (Cannon et al., 2004). In addition, mutually segmental duplicated events were found among the triple genes of *Cla013979-Cla020663-Cla022601*, *Cla005982-Cla007586-Cla021520*, *Cla010332-Cla019115-Cla018610*, and *Cla007715-Cla019451-Cla001341*, which probably occurred along with the triplicate evolution of the watermelon genome (Guo et al., 2013). One proximal gene pair and one tandem gene pair were detected in *CIR2R3-MYBs*. Low frequencies of tandem and proximity duplicated gene pairs in the *R2R3-MYB* family were also found in *A. thaliana* (Cannon et al., 2004). Transposed duplication accounted for 41.7% of the total duplication events, which also had an important role in the expansion of *CIR2R3-MYB* genes. Similar results were also reported for family genes related to sorbitol metabolism pathways (Qiao et al., 2018).

Different cis-regulatory elements in the promoter sequences of duplicated genes may produce different expression patterns. In this study, most duplicated gene pairs had divergent regulatory elements. There were four transposed duplicated gene pairs with a cis-element index more than 0.8, suggesting that their expressions can be regulated by several different trans-acting factors (Qiao et al., 2018). Several studies have shown that the duplicated genes inherited the transcription regulatory models from their ancestors, but that their cis-regulatory regions were divergent after evolution (Haberer et al., 2004). The highly divergent cis-regulatory elements between *CIR2R3-MYB* gene pairs potentially lead to sub-functionalization or neo-functionalization between the duplicated genes.

Expression patterns of *CIR2R3-MYB* duplicated genes were measured in flesh, rind, and seed during watermelon fruit development. Only two duplicated gene pairs, *Cla004362-Cla013979* and *Cla020702-Cla022542*, had similar expression patterns, whereas less nonsynonymous mutations, similar gene structures, and cis-element compositions were observed. This suggested that function redundancy or sub-functionalization probably occurred between the two genes. Similar results were also observed between *GmPIN* duplicated genes in soybean (*Glycine max*) (Liu and Wei, 2017). The other 38 duplicated gene pairs exhibited tissue-specific or development stage-specific divergent expression patterns during watermelon fruit development. Different gene structures and cis-element compositions, as well as relatively high nonsynonymous mutations, were observed between these duplicated pairs, suggesting that neo-functionalization probably occurred after duplication. Similar results were also reported between the segmental duplication of *MYB* homologous genes in maize (*Zea mays*) (Zhang et al., 2000). Neo-functionalization potentially leads to the importance of *CIR2R3-MYB* duplicated genes in the regulation of watermelon fruit development.

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Supplemental Table 1. Characteristics of watermelon *R2R3-MYB* genes.

Gene no.	Strand	Chromosome	Start	End	Amino acids (no.)	CDS ^z	PI ^z	MW ^z	Subcellular localization
<i>Cla000320</i>	–	Chr0	5441327	5443057	257	774	8.89	29414.86	Nuclear
<i>Cla011345</i>	+	Chr1	1490069	1490918	258	777	5.55	28832.15	Nuclear
<i>Cla011417</i>	+	Chr1	2156319	2158502	319	960	5.94	35875.21	Nuclear
<i>Cla011548</i>	–	Chr1	3667889	3669976	267	804	5.55	30525.15	Nuclear
<i>Cla003566</i>	+	Chr1	4923858	4925042	234	705	6.53	26565.03	Nuclear
<i>Cla003441</i>	–	Chr1	13111643	13111643	369	1110	5.86	41407.99	Nuclear
<i>Cla009156</i>	–	Chr1	23463077	23464793	292	879	5.04	32898.89	Nuclear
<i>Cla013979</i>	+	Chr1	27060335	27061240	301	906	9.21	32265.26	Nuclear
<i>Cla014058</i>	–	Chr1	27774930	27776082	317	954	6.62	35714.91	Nuclear
<i>Cla014140</i>	–	Chr1	28564024	28565238	345	1038	6.22	38505.43	Nuclear
<i>Cla014141</i>	–	Chr1	28574505	28575956	340	1023	6.7	38471.62	Nuclear
<i>Cla001341</i>	–	Chr1	31189128	31190559	384	155	5.53	43708.15	Nuclear
<i>Cla009903</i>	+	Chr1	33941450	33942325	291	76	6.3	33343.10	Nuclear
<i>Cla007715</i>	+	Chr2	571745	573152	255	68	8.4	29455.92	Mitochondrial
<i>Cla007719</i>	–	Chr2	613115	614296	335	1008	5.77	37434.8	Nuclear
<i>Cla007815</i>	+	Chr2	1567270	1568681	212	639	10.47	23950.87	Extracellular
<i>Cla007846</i>	–	Chr2	1878624	1880236	223	672	8.46	25463.5	Nuclear
<i>Cla015834</i>	+	Chr2	4004234	4005597	251	756	9.11	28075.99	Mitochondrial
<i>Cla006761</i>	+	Chr2	8804391	8805317	308	927	8.24	32944.9	Nuclear
<i>Cla000767</i>	–	Chr2	20007833	20008749	239	720	7.1	27899.14	Extracellular
<i>Cla020285</i>	–	Chr2	21232550	21233415	254	765	8.59	28806.89	Nuclear
<i>Cla019999</i>	–	Chr2	25025952	25027283	309	930	8.42	35124.34	Nuclear
<i>Cla013461</i>	+	Chr2	29168969	29172276	336	1011	5.34	38321.35	Nuclear
<i>Cla013280</i>	+	Chr2	30696468	30697466	264	795	6.16	30249.56	Nuclear
<i>Cla008710</i>	–	Chr2	31694415	31695536	288	867	8.59	33052.3	Nuclear
<i>Cla008544</i>	–	Chr2	33245766	33247436	328	987	5.99	36264.56	Nuclear
<i>Cla008278</i>	+	Chr3	1909428	1909428	266	801	5.61	30033.83	Nuclear
<i>Cla008279</i>	–	Chr3	1920922	1922199	242	729	6.11	28069.61	Cytoplasmic
<i>Cla005093</i>	–	Chr3	3060617	3062192	316	951	8.52	36045.4	Cytoplasmic
<i>Cla019451</i>	–	Chr3	5375019	5376260	338	1017	5.7	38048.66	Nuclear
<i>Cla005135</i>	+	Chr3	28818868	28820147	355	1068	6.22	40355.74	Nuclear
<i>Cla018501</i>	–	Chr4	22684515	22685586	291	876	6.14	32803.44	Nuclear
<i>Cla018610</i>	–	Chr4	23722295	23723553	317	954	6.37	35474.55	Nuclear
<i>Cla018657</i>	+	Chr4	24039901	24040706	229	690	7.94	26494.39	Nuclear
<i>Cla021243</i>	–	Chr5	1532336	1534468	304	915	6.03	34460.56	Nuclear
<i>Cla021474</i>	–	Chr5	3477830	3478897	246	741	8.89	28929.53	Nuclear
<i>Cla021520</i>	–	Chr5	3810766	3811720	263	792	6.22	30481.24	Cytoplasmic
<i>Cla021776</i>	–	Chr5	6104841	6107931	305	918	5.48	34873.03	Nuclear
<i>Cla021810</i>	–	Chr5	6470072	6471183	310	933	5.52	33926.55	Extracellular
<i>Cla006186</i>	+	Chr5	8241841	8242392	125	378	9.84	14628.88	Nuclear
<i>Cla013099</i>	+	Chr5	10845617	10846297	226	681	8.13	25583.8	Nuclear
<i>Cla007114</i>	–	Chr5	15916504	15917655	383	1152	9.33	43351.52	Nuclear
<i>Cla004039</i>	–	Chr5	16992217	16993691	265	798	8.11	30566.8	Nuclear
<i>Cla020901</i>	–	Chr5	25765521	25766551	275	828	8.61	30904.84	Cytoplasmic
<i>Cla020702</i>	–	Chr5	27538516	27539187	223	672	8.84	24808.61	Nuclear
<i>Cla020633</i>	+	Chr5	28144577	28145407	276	831	8.6	30369.25	Nuclear
<i>Cla010193</i>	+	Chr5	31333372	31335482	382	1149	6.17	42662.97	Nuclear
<i>Cla006720</i>	+	Chr6	3703046	3704814	309	930	6.4	34989.25	Nuclear
<i>Cla009263</i>	+	Chr6	5124399	5130931	286	861	6.2	32793.22	Nuclear
<i>Cla007586</i>	–	Chr6	7993305	7994841	286	861	5.79	32581.28	Nuclear
<i>Cla007626</i>	+	Chr6	8519859	8521167	271	816	9.19	32043.22	Nuclear
<i>Cla018803</i>	–	Chr6	22295406	22296925	335	1008	5.93	38060.39	Nuclear
<i>Cla018967</i>	–	Chr6	23938722	23940516	269	810	8.25	30541.06	Nuclear
<i>Cla019115</i>	–	Chr6	25199695	25200299	164	495	9.93	19103.97	Mitochondrial
<i>Cla019144</i>	+	Chr6	25444109	25445516	373	1122	6.2	42267.44	Nuclear
<i>Cla019199</i>	+	Chr6	25934308	25936822	470	1413	6.08	54517.78	Extracellular
<i>Cla019223</i>	+	Chr6	26181330	26182648	362	1089	6.38	41240.78	Nuclear
<i>Cla005982</i>	–	Chr7	1933675	1936069	255	768	5.85	28778.24	Cytoplasmic

Continued next page

Supplemental Table 1. Continued.

Gene no.	Strand	Chromosome	Start	End	Amino acids (no.)	CDS ^z	PI ^z	MW ^z	Subcellular localization
<i>Cla004362</i>	–	Chr7	5095273	5096094	273	822	7.02	30128.52	Nuclear
<i>Cla012581</i>	+	Chr7	24209665	24210419	173	522	8.87	20092.68	Nuclear
<i>Cla012590</i>	–	Chr7	24306363	24307941	352	1059	6.29	38832.77	Nuclear
<i>Cla010722</i>	+	Chr7	29157052	29158336	315	948	5.4	36057.45	Nuclear
<i>Cla012519</i>	+	Chr8	20064	21159	279	840	8.75	30980.1	Nuclear
<i>Cla013871</i>	–	Chr8	15614824	15616294	295	888	9.42	34267.85	Nuclear
<i>Cla013668</i>	+	Chr8	17810586	17814452	554	1665	5.29	61217.91	Nuclear
<i>Cla022542</i>	–	Chr8	24477181	24477870	229	690	9.46	25648.75	Nuclear
<i>Cla022601</i>	+	Chr8	24979792	24980664	290	873	9.12	317271	Nuclear
<i>Cla022660</i>	+	Chr8	25499664	25501221	209	630	8.69	23660.77	Mitochondrial
<i>Cla015165</i>	–	Chr9	3809851	3811275	277	834	5.82	31978.94	Cytoplasmic
<i>Cla016229</i>	+	Chr9	11171832	11171832	305	918	6.08	34245.44	Nuclear
<i>Cla001702</i>	+	Chr9	15834488	15837110	260	783	8.69	29165.39	Nuclear
<i>Cla013009</i>	+	Chr9	22873188	22874895	306	921	6.04	34638.03	Nuclear
<i>Cla010332</i>	–	Chr9	30413537	30414657	311	936	7.94	34889.17	Nuclear
<i>Cla010413</i>	–	Chr9	31388005	31389474	201	606	5.44	23274	Nuclear
<i>Cla010415</i>	+	Chr9	31403568	31406870	419	1260	6.31	47797.02	Cytoplasmic
<i>Cla004450</i>	–	Chr10	4615148	4616284	321	966	5.95	35527.88	Nuclear
<i>Cla005321</i>	+	Chr10	5821997	5824222	523	1572	7.04	56840.06	Nuclear
<i>Cla017337</i>	–	Chr10	17127134	17128365	223	672	6.11	25572.81	Extracellular
<i>Cla017267</i>	–	Chr10	18051587	18053674	317	954	5.36	36267.87	Nuclear
<i>Cla017100</i>	+	Chr10	20002954	20007003	182	549	6.65	21182.84	Nuclear
<i>Cla017012</i>	+	Chr10	20930755	20932103	312	939	6.23	34947.11	Nuclear
<i>Cla016980</i>	–	Chr10	21382991	21384272	230	693	9.45	26154.38	Nuclear
<i>Cla017463</i>	–	Chr10	22830473	22831888	291	876	8.19	32239.23	Nuclear
<i>Cla017496</i>	+	Chr10	23259634	23261049	346	1041	7.90	38611.66	Nuclear
<i>Cla017787</i>	–	Chr10	26143507	26144679	232	699	6.56	27850.19	Nuclear
<i>Cla017797</i>	+	Chr10	26225140	26227302	306	921	6.39	34132.94	Nuclear
<i>Cla022852</i>	–	Chr11	14690954	14694226	294	885	6.46	32590.96	Nuclear
<i>Cla016504</i>	–	Chr11	22030736	22031902	208	627	6.15	24074.28	Mitochondrial
<i>Cla016689</i>	+	Chr11	23821985	23822997	232	699	6.1	26856.72	Nuclear

^zCDS = coding sequence, PI = theoretical isoelectric point, MW = molecular weight.

Supplemental Table 2. Duplication information of *R2R3-MYB* gene pairs in watermelon.

Duplicate gene pairs	E-value	Mode	Ka ^z	Ks ^z	Ka/Ks	Cis-elements index
<i>Cla001341-Cla007715</i>	1E-49	Segmental	0.57	2.65	0.22	0.70
<i>Cla001341-Cla019451</i>	5E-98	Segmental	0.27	2.23	0.12	0.56
<i>Cla003441-Cla019223</i>	2E-88	Segmental	0.33	3.59	0.09	0.75
<i>Cla007586-Cla005982</i>	1E-73	Segmental	0.41	3.13	0.13	0.78
<i>Cla007715-Cla019451</i>	2E-50	Segmental	0.52	2.94	0.18	0.71
<i>Cla013280-Cla008279</i>	2E-76	Segmental	0.30	2.22	0.13	0.76
<i>Cla013979-Cla020633</i>	2E-77	Segmental	0.29	3.61	0.08	0.73
<i>Cla013979-Cla022601</i>	4E-87	Segmental	0.34	3.10	0.11	0.63
<i>Cla015834-Cla020285</i>	1E-116	Segmental	0.22	3.09	0.07	0.60
<i>Cla017012-Cla010332</i>	2E-84	Segmental	0.33	2.54	0.13	0.76
<i>Cla017100-Cla010413</i>	8E-77	Segmental	0.24	2.11	0.12	0.71
<i>Cla017337-Cla015165</i>	2E-71	Segmental	0.29	3.74	0.08	0.63
<i>Cla017797-Cla005093</i>	2E-68	Segmental	0.48	3.25	0.15	0.69
<i>Cla017797-Cla008710</i>	4E-72	Segmental	0.48	3.17	0.15	0.63
<i>Cla018501-Cla018967</i>	2E-80	Segmental	0.33	1.79	0.19	0.79
<i>Cla018610-Cla010332</i>	2E-98	Segmental	0.10	1.87	0.06	0.76
<i>Cla018610-Cla019115</i>	2E-90	Segmental	0.09	1.49	0.06	0.66
<i>Cla019115-Cla010332</i>	8E-86	Segmental	0.15	2.05	0.07	0.70
<i>Cla019199-Cla010415</i>	2E-62	Segmental	0.60	2.69	0.22	0.54
<i>Cla019999-Cla005135</i>	1E-89	Segmental	0.31	3.86	0.08	0.68
<i>Cla020633-Cla022601</i>	1E-103	Segmental	0.27	3.71	0.07	0.79
<i>Cla020702-Cla022542</i>	8E-95	Segmental	0.19	2.48	0.08	0.59
<i>Cla021474-Cla007626</i>	1E-75	Segmental	0.35	1.97	0.18	0.73
<i>Cla021520-Cla005982</i>	1E-70	Segmental	0.48	2.96	0.16	0.67
<i>Cla021520-Cla007586</i>	2E-69	Segmental	0.45	2.83	0.16	0.74
<i>Cla021776-Cla009263</i>	2E-65	Segmental	0.42	3.10	0.14	0.69
<i>Cla022852-Cla018803</i>	5E-82	Segmental	0.30	1.56	0.20	0.79
<i>Cla000320-Cla017496</i>	1E-63	Transposed	0.49	2.74	0.18	0.81
<i>Cla000767-Cla009263</i>	3E-49	Transposed	0.68	2.65	0.26	0.74
<i>Cla004039-Cla003566</i>	5E-67	Transposed	0.49	3.19	0.15	0.72
<i>Cla004362-Cla013979</i>	7E-46	Transposed	0.51	2.74	0.19	0.85
<i>Cla006186-Cla019223</i>	5E-47	Transposed	0.32	3.95	0.08	0.74
<i>Cla007815-Cla010193</i>	7E-53	Transposed	0.45	3.02	0.15	0.67
<i>Cla009156-Cla011548</i>	1E-80	Transposed	0.39	3.56	0.11	0.76
<i>Cla009903-Cla019999</i>	3E-54	Transposed	0.50	2.86	0.18	0.68
<i>Cla010722-Cla006720</i>	2E-64	Transposed	0.47	2.22	0.21	0.67
<i>Cla012519-Cla007846</i>	3E-82	Transposed	0.34	2.93	0.12	0.55
<i>Cla012581-Cla007846</i>	1E-48	Transposed	0.50	2.96	0.17	0.75
<i>Cla013009-Cla014140</i>	1E-75	Transposed	0.46	2.69	0.17	0.71
<i>Cla013668-Cla005321</i>	5E-63	Transposed	0.62	2.33	0.27	0.73
<i>Cla013871-Cla017496</i>	3E-58	Transposed	0.43	3.32	0.13	0.68
<i>Cla016229-Cla019451</i>	2E-74	Transposed	0.47	3.23	0.14	0.85
<i>Cla016689-Cla015834</i>	2E-43	Transposed	0.57	2.94	0.19	0.74
<i>Cla018657-Cla017496</i>	2E-57	Transposed	0.46	2.92	0.16	0.73
<i>Cla022660-Cla018803</i>	8E-60	Transposed	0.42	2.48	0.17	0.84
<i>Cla001702-Cla019223</i>	4E-48	Transposed	0.60	2.85	0.21	0.76
<i>Cla007715-Cla007719</i>	1E-48	Proximal	0.56	2.76	0.20	0.71
<i>Cla014140-Cla014141</i>	7E-151	Tandem	0.13	0.41	0.32	0.74

^zKa = synonymous mutation, Ks = nonsynonymous mutation.

Supplemental Table 3. Cis-regulatory elements annotations.

Cis-elements	Annotation	Function
AuxRE	Hormone	Part of an auxin-responsive element
TCA-element	Hormone	Cis-acting element involved in salicylic acid responsiveness
ERE	Hormone	Ethylene-responsive element
motif IIb	Hormone	Abscisic acid responsive element
ABRE	Hormone	Cis-acting element involved in the abscisic acid responsiveness
CGTCA-motif	Hormone	Cis-acting regulatory element involved in the MeJA-responsiveness
TGACG-motif	Hormone	Cis-acting regulatory element involved in the MeJA-responsiveness
GARE-motif	Hormone	Gibberellin-responsive element
P-box	Hormone	Gibberellin-responsive element
TATC-box	Hormone	Cis-acting element involved in gibberellin-responsiveness
TGA-element	Hormone	Auxin-responsive element
TGA-box	Hormone	Part of an auxin-responsive element
SARE	Hormone	Cis-acting element involved in salicylic acid responsiveness
AuxRR-core	Hormone	Cis-acting regulatory element involved in auxin responsiveness
5UTR Py-rich stretch	Cellular development	Cis-acting element conferring high transcription levels
CCGTCC-box	Cellular development	Cis-acting regulatory element related to meristem-specific activation
CAT-box	Cellular development	Cis-acting regulatory element related to meristem expression
GCN4_motif	Cellular development	Cis-regulatory element involved in endosperm expression
Skn-1_motif	Cellular development	Cis-acting regulatory element required for endosperm expression
HD-Zip 1	Cellular development	Element involved in differentiation of the palisade mesophyll cells
HD-Zip 2	Cellular development	Element involved in the control of leaf morphology development
as-2-box	Cellular development	Involved in shoot-specific expression and light responsiveness
MSA-like	Cellular development	Cis-acting element involved in cell-cycle regulation
MBSI	Cellular development	MYB binding site involved in flavonoid biosynthetic gene regulation
MBSII	Cellular development	MYB binding site involved in flavonoid biosynthetic gene regulation
AACA_motif	Cellular development	Involved in endosperm-specific negative expression
MRE	Stress	MYB binding site involved in light responsiveness
HSE	Stress	Cis-acting element involved in heat stress responsiveness
LTR	Stress	Cis-acting element involved in low-temperature responsiveness
TC-rich repeats	Stress	Cis-acting element involved in defense and stress responsiveness
MBS	Stress	MYB binding site involved in drought-inducibility
ARE	Stress	Cis-acting regulatory element essential for the anaerobic induction
GC-motif	Stress	Enhancer-like element involved in anoxic specific inducibility
WUN-motif	Stress	Wound-responsive element
DRE	Stress	Cis-acting element involved in dehydration, low temperature, and salt stresses
Box-W1	Stress	Fungal elicitor responsive element
ELI-box3	Stress	Elicitor-responsive element
AT-rich sequence	Stress	Element for maximal elicitor-mediated activation (2 copies)
Sp1	Stress	Light-responsive element
MNF1	Stress	Light-responsive element
GT1-motif	Stress	Light-responsive element
Box I	Stress	Light-responsive element
TCCC-motif	Stress	Part of a light-responsive element
TCT-motif	Stress	Part of a light-responsive element
GATA-motif	Stress	Part of a light-responsive element
GAG-motif	Stress	Part of a light-responsive element
GA-motif	Stress	Part of a light-responsive element
AE-box	Stress	Part of a module for light response
Box II	Stress	Part of a light-responsive element
CATT-motif	Stress	Part of a light-responsive element
chs-CMA1a	Stress	Part of a light-responsive element
Gap-box	Stress	Part of a light-responsive element
I-box	Stress	Part of a light-responsive element
G-Box	Stress	Cis-acting regulatory element involved in light responsiveness
ACE	Stress	Cis-acting element involved in light responsiveness
ATC-motif	Stress	Part of a conserved DNA module involved in light responsiveness
ATCT-motif	Stress	Part of a conserved DNA module involved in light responsiveness
Box 4	Stress	Part of a conserved DNA module involved in light responsiveness

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Supplemental Table 3. Continued.

Cis-elements	Annotation	Function
3-AF1 binding site	Stress	Light-responsive element
AAAC-motif	Stress	Light-responsive element
4cl-CMA2b	Stress	Light-responsive element
Pc-CMA2a	Stress	Part of a light-responsive element
AT1-motif	Stress	Part of a light-responsive module
L-box	Stress	Part of a light-responsive element
rbcS-CMA7a	Stress	Part of a light-responsive element
LAMP-element	Stress	Part of a light-responsive element
LS7	Stress	Part of a light-responsive element
ATCC-motif	Stress	Part of a conserved DNA module involved in light responsiveness
H-box	Stress	Cis-acting regulatory element involved in light responsiveness
Box III	Stress	Protein binding site (involved in light responsiveness)
EIRE	Stress	Elicitor-responsive element
chs-Unit 1 m1	Stress	Part of a light-responsive element
chs-CMA2a	Stress	Part of a light-responsive element
ACA-motif	Stress	Part of gapA in (gapA-CMA1) involved with light responsiveness