Comprehensive Analysis of TIFY Transcription Factors and Their Expression Profiles under Jasmonic Acid and Abiotic Stresses in Watermelon

Youxin Yang,¹ Golam Jalal Ahammed,² Chunpeng Wan,¹ Haoju Liu,³ Rongrong Chen,³ and Yong Zhou³,⁴

¹Jiangxi Key Laboratory for Postharvest Technology and Nondestructive Testing of Fruits & Vegetables, Collaborative Innovation Center of Postharvest Key Technology and Quality Safety of Fruits and Vegetables, College of Agronomy, Jiangxi Agricultural University, Nanchang 330045, China
²College of Forestry, Henan University of Science and Technology, Luoyang 471023, China
³College of Bioscience and Bioengineering, Jiangxi Agricultural University, Nanchang 330045, China
⁴Key Laboratory of Crop Physiology, Ecology, and Genetic Breeding, Ministry of Education, Jiangxi Agricultural University, Nanchang 330045, China

Correspondence should be addressed to Rongrong Chen; r_r_chen@163.com and Yong Zhou; yzhoujxau@163.com

Received 18 April 2019; Accepted 26 August 2019; Published 1 October 2019

Academic Editor: Wilfred van IJcken

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The TIFY gene family is plant-specific and encodes proteins involved in the regulation of multiple biological processes. Here, we identified 15 TIFY genes in the watermelon genome, which were divided into four subfamilies (eight JAZs, four ZMLs, two TIFYs, and one PPD) in the phylogenetic tree. The ClTIFY genes were unevenly located on eight chromosomes, and three segmental duplication events and one tandem duplication event were identified, suggesting that gene duplication plays a vital role in the expansion of the TIFY gene family in watermelon. Further analysis of the protein architectures, conserved domains, and gene structures provided additional clues for understanding the putative functions of the TIFY family members. Analysis of qRT-PCR and RNA-seq data revealed that the detected ClTIFY genes had preferential expression in specific tissues. qRT-PCR analysis revealed that nine selected TIFY genes were responsive to jasmonic acid (JA) and abiotic stresses including salt and drought. JA activated eight genes and suppressed one gene, among which ClJAZ1 and ClJAZ7 were the most significantly induced. Salt and drought stress activated nearly all the detected genes to different degrees. These results lay a foundation for further functional characterization of TIFY family genes in Citrullus lanatus.

1. Introduction

TIFY transcription factors are plant-specific transcriptional regulators characterized by the presence of a highly conserved motif (TIF(F/Y)XG) in the TIFY domain (previously called ZIM domain) with a length of approximately 36 amino acids (aa) [1, 2]. According to their domain architectures, the TIFY proteins can be divided into four subfamilies, including TIFY, ZIM-like (ZML), jasmonate ZIM-domain (JAZ), and PEAPOD (PPD). Except for the TIFY subfamily, which only harbors the TIFY domain, members of other three subfamilies possess some additional domains [3]. For example, the ZML subfamily proteins possess a GATA zinc-finger DNA-binding domain and a CCT domain (CONSTANS, CO-like, and TOC1) [4, 5]. The JAZ subfamily proteins have a conserved jasmonic acid- (JA-) associated domain (Jas, also known as CCT-2 motif) with a SLX₂FX₂KRX₂RX₂PY (X represents any amino acid) consensus sequence, while the PPD subfamily proteins contain a typical PPD domain in the N-terminus and a truncated Jas motif lacking the conserved P and Y residues [6–8].

To date, a number of TIFY genes have been functionally characterized in Arabidopsis [9–12], rice [13–15], wheat [16, 17], tomato [18], cotton [19], and other plants [20–22].
AtTIFY1/AtZIM is the first identified TIFY gene in plants, and overexpression of AtTIFY1/AtZIM promotes the elongation of the petiole and hypocotyl, which is independent of gibberellin and brassinosteroids [4, 23]. AtZML1/AtTIFY2b and AtZML2/AtTIFY2a are important components of the CRYPTOCHROME1-(cry1-) mediated response to excess light [24]. In tomato, SlJAZ2 was described as an important regulator of the transition from vegetative growth to reproductive growth [18]. In addition, many JAZ genes were found to play key roles in JA signal transduction and participate in the regulation of various developmental processes and responses to biotic and abiotic stresses in plants. For example, OsTIFY3/OsJAZ1 can regulate floral development and root elongation by interacting with OsMYC2 and OsCOI1b in rice, and substitution or deletion of the core segments of OsJAZ1 can affect the specificity and sensitivity of JA signaling during flower and root development [13, 25]. In addition, OsJAZ1 was identified as a negative regulator of rice drought resistance, partially by regulating the JA and ABA signaling pathways [14]. Another rice JAZ member, OsJAZ8, can confer resistance to rice bacterial blight by regulating JA-responsive volatile compounds [26]. Additionally, rice plants expressing OsJAZ8 and OsJAZ8AC (C-terminal truncated) under the control of the salt-inducible ZOS3-11 promoter exhibited higher tolerance to salt stress in early stages [27].

Recently, genome-wide surveys of the TIFY gene family have been conducted in various plant species such as Brachypodium distachyon [28], maize [29], moso bamboo (Phyllostachys edulis) [30], pigeon pea ( Cajanus cajan) [31], Brassica rapa [5], tomato (Solanum lycopersicum) [32], Gossypium species [33], wheat (Triticum aestivum) [16], poplar (Populus trichocarpa) [6, 34], and pear ( Pyrus pyrifolia) [35]. However, no genome-wide information of this gene family is available in watermelon, an important agricultural crop susceptible to various biotic and abiotic stresses during growth and development [36].

In this study, we predicted and classified the TIFY family genes of watermelon and analyzed their distribution patterns, phylogenetic relationships, and protein and gene structures. In addition, we examined the expression profiles of several CITIFY genes in various tissues during fruit development of watermelon as well as under diverse abiotic stresses. Our findings lay a solid foundation for further understanding the role of TIFY genes in the growth and development of watermelon.

2. Materials and Methods

2.1. Genome-Wide Identification of TIFY Genes in Watermelon. To identify the TIFY family members in watermelon, the hidden Markov model (HMM) profiles of the TIFY domain (PF06200), Jas domain (PF09425), and CCT domain (PF06203) were obtained from the Pfam database (http://pfam.sanger.ac.uk/), and these domains were used as queries to search the watermelon genome database (http://cucurbitgenomics.org/organism/1) using HMMER 3.0 software (http://hmmer.org/) with an E value cutoff of 1e-3. Arabidopsis and rice TIFY protein sequences were also used as queries to obtain the watermelon TIFYs by BlastP through searching the watermelon genome database with a cutoff E value of 1e-3. According to previous reports [2, 37], Arabidopsis and rice TIFY protein sequences were downloaded from the Arabidopsis genome database at TAIR (The Arabidopsis Information Resource, https://www.arabidopsis.org/) and from the rice genome database at RGAP (Rice Genome Annotation Project, http://rice.plantbiology.msu.edu/), respectively. The nonredundant sequences were subsequently confirmed with the Pfam database and the SMART database (http://smart.embl-heidelberg.de/).

2.2. Protein Properties, Sequence Analyses, and Phylogenetic Tree Construction. The biochemical features including isoelectric point (pl) and molecular weight (MW) of watermelon TIFY proteins were calculated with the ProtParam tool (http://web.expasy.org/protparam/). Conserved motifs were identified using the MEME tool (http://meme-suite.org/tools/meme) with the maximum number of motifs set as 10. The exon-intron structures of watermelon TIFY genes were displayed by the GSDS tool (http://gsds.cbi.pku.edu.cn) based on the alignment of coding region sequences (CDS) with the corresponding genomic sequences. For phylogenetic tree construction, TIFY protein sequences from watermelon, tomato, rice, and Arabidopsis were aligned by Clustal Omega with default parameters. Then, the alignments of protein sequences were used to construct the phylogenetic tree by MEGA 7.0 using the Neighbor-Joining (N) method, with parameters of 1,000 bootstrap replicates and pairwise deletion.

2.3. Chromosomal Location and Duplication Analysis. The genetic positions of watermelon TIFY genes on chromosomes were obtained from the watermelon genome database, and the MapChart software was used to present the chromosomal positions and relative distance of CITIFY genes on the basis of their ascending order of physical position (bp). Gene duplications were conducted using multiple collinear scanning toolkit (MCScanX) software with the default parameters as previously reported [38].

2.4. In Silico Expression Analysis of CITIFY Genes. The inbred line 97103 strand-specific RNA-seq of both the flesh and rind at four pivotal stages of fruit development (10 days after pollination, 10 DAP; 18 DAP; 26 DAP; and 34 DAP) was analyzed [39]. Fragments per kilobase of exon model per million mapped (FPKM) values were log2-transformed and heat maps with hierarchical clustering were plotted using the OmicShare tools (http://www.omicshare.com/tools).

2.5. Plant Materials and Treatments. Watermelon (Citrullus lanatus L. Xinong 8) plants were cultured in a growth room (25°C/19°C, 12 h light/12 h dark, 200 μmol μm−2 s−1). At the four-leaf stage, watermelon seedlings grown in hydroponics with Hoagland’s solution were treated with JA and abiotic stresses. For JA treatment, the leaves were sprayed with 100 μM methyl jasmonate (MeJA) solution. For abiotic stress treatments, the plants were exposed to 200 mM NaCl solution for 24 h (salt stress), or under 20% PEG-6000 (ω/ν) for 24 h (drought stress) under the same photoperiod and light
Table 1: CITIFY family genes identified in watermelon.

<table>
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<th>Gene name</th>
<th>Gene ID</th>
<th>Map position (bp)</th>
<th>CDS length</th>
<th>Protein length (aa)</th>
<th>MW (kDa)</th>
<th>pI</th>
<th>TIFY motif</th>
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<td>Cla009781</td>
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<td>13.05</td>
<td>10.13</td>
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3.1. Identification and Characterization of TIFY Family Members in Watermelon.

A total of 15 CITIFY family genes were identified in the watermelon genome, which were further examined using SMART and PFAM to confirm that their coding protein sequences contain the TIFY, Jas, ZIM, and PPD domains. The results revealed that these 15 CITIFY genes consisted of eight ClJAZs, four ClIZMLs, two ClTIFYs, and one ClPPD. These genes were named according to their chromosomal positions and the domains that they contained as described in other plant species [6, 34, 35]. The amino acid sequences of TIFY family members varied from 118 aa (ClJAZ1) to 450 aa (ClTIFY2) in length, from 13.05 kDa (ClJAZ1) to 47.43 kDa (ClTIFY2) in theoretical MW, and from 4.73 (CIZML1) to 10.56 (ClJAZ5) in pI values (Table 1).
3.3. Protein Architecture and Conserved Domain Analysis of TIFYs in Watermelon. The protein sequence analysis showed that all the watermelon TIFY proteins contained the TIFY domain (PF06200), with the conservation at the amino acid level of TIFYXG, except for ClZMLs, whose TIFY domain sequences were TLS(F/Y)XG (Table 1; Figure S1). In addition, thirteen of these 15 watermelon TIFY proteins (except for ClTIFY1 and ClTIFY2) possessed the Jas domain (PF09425). The conserved sequences of ClJAZs and ClZMLs were SLXRF(L/F)(E/Q)KRKXRX5PY and SLXRFR(E/Q)KRKXRX,Y, while CIPPD contained a truncated Jas motif lacking the conserved P and Y residues (Figure S1). Moreover, the GATA zinc-finger (ZnF_GATA, PF00320) domain was represented by CX2CX20CX2C in all the four ClZML proteins (Figure S1), which was also described in Arabidopsis [23] and pigeon pea [31].

To further explore the diversity of the conserved domains of watermelon TIFY proteins, the MEME online tool was employed, and 10 conserved motifs were identified and named as motifs 1–10. Among them, motifs 1 and 5 or motifs 1 and 10 composed the TIFY domain, and motif 2 was annotated as the Jas domain (Figure 2). Nearly all watermelon

Figure 1: Phylogenetic relationships of TIFY family proteins of watermelon, tomato, rice, and Arabidopsis. The phylogenetic tree was created by MEGA 7.0 using the NJ method with 1000 bootstrap replicates.
TIFY proteins contained motifs 1 and 2, with the exception of ClTIFY1 and ClTIFY2, both of which lacked motif 2 but harbored an additional motif 6. In addition, motif 3 was annotated as the ZnF_GATA domain, motifs 2 and 4 made up the CCT domain, and both domains were only present in the ClZML proteins. In addition, both ClJAZ1 and ClJAZ2 contained motif 9 at their N-terminus, which was associated with a classical EAR motif (LXLXL) (Figure 2; Table S2).

Moreover, some watermelon TIFY proteins had unique motifs. For example, motif 8 was observed in ClJAZ7, ClJAZ8, and ClPPD1 but was absent in other watermelon TIFY proteins (Figure 2).

3.4. Gene Structure Analysis of ClTIFY Genes. To understand the structural components of the ClTIFY genes, the gene structures of the ClTIFY genes were analyzed by alignment.
with the CDS sequences and corresponding genomic DNA sequences. The results revealed that the CIFY genes contained 1–9 introns, and the number of introns in each subfamily varied. For example, in the JAZ subfamily, CIJAZ4 and CIJAZ6 had six introns; CIJAZ3, CIJAZ5, and CIJAZ8 harbored four introns; CIJAZ2 and CIJAZ7 contained two introns; while CIJAZ1 possessed only one intron (Figure 3). It can be observed that all ZML subfamily genes have longer genomic sequences than the CIFY genes of other subfamilies, and the intron number of ClZMLs was 4–9. However, some members in the same subfamily shared similar intron numbers but with different intron lengths, such as CIJAZ4 and CIJAZ6, CIJAZ3 and CIJAZ5, and CIFY1 and CIFY2 (Figure 3).

3.5. Chromosome Distribution and Duplication Analysis of CIFY Genes. The 15 CIFY genes were distributed among eight chromosomes, with chromosomes 8 and 9 containing the most genes (three genes for each), followed by chromosomes 3, 6, and 7 (two genes for each), while chromosomes 1, 4, and 5 contained the fewest CIFY genes (one gene for each) (Figure 4). The segmental and tandem duplication events among the CIFY genes were further determined. As a result, three paralogous gene pairs (CIJAZ1/CIJAZ2, CIJAZ3/CIJAZ5, and CIFY1/CIFY2) were identified.
ClJAZ3/ClJAZ8, and ClJAZ7/ClJAZ8) were found to be related to segmental duplication events, while only one tandem duplication event was identified on chromosome 8 (ClZML1/ClZML2).

3.6. Tissue-Specific Expression Profiles of the CITIFY Genes.
To gain insights into the expression profiles of the CITIFY genes in various tissues, qRT-PCR analysis was carried out to examine the expression of nine selected CITIFY genes in leaves, roots, fruits, flowers, and stem apices. The qRT-PCR data revealed the preferential expression of these CITIFY genes (Figure 5). Among them, ClJAZ1, ClJAZ4, and ClJAZ7 were highly and preferentially expressed in flowers, leaves, and fruits, respectively. In addition, ClJAZ3, ClZML1, and ClZML2 had higher expression in fruits than in other tissues. Interestingly, two TIFY subfamily members, CITIFY1 and CITIFY2, were highly expressed in roots and stem apices (Figure 5). Additionally, CIPP1 expression was also high in stem apices, but relatively lower in fruits, flowers, and other tissues. The results showed that the CITIFY genes had overlapping but spatially varying expression, indicating that they may play important roles in specific tissues.

3.7. Characterization of the Expression of CITIFYs during Watermelon Fruit Development.
The expression profiles of CITIFYs during fruit development in watermelon were analyzed according to the transcriptome data from a previous study [39]. The results showed that four CITIFY genes were differentially expressed during watermelon flesh and rind development (Figure 6). During flesh development, ClJAZ4 showed an observable accumulation of transcripts. During the development of rind, ClJAZ4 and ClJAZ8 exhibited specifically higher expression at some time points, while ClJAZ5 showed lower transcripts at all stages of rind development (Figure 6). These findings indicated that these genes might play a role in the fruit development of watermelon.

To gain more insights into the roles of CITIFY genes in response to hormone and various stresses, the expression of nine selected CITIFY genes was
analyzed by qRT-PCR under various stress treatments including salt, drought, and JA. The results suggested that most of the genes exhibited obvious variations in expression under these treatments. Under drought stress, the expression of all detected CITIFY genes was significantly upregulated (Figure 7). Among them, CIJA77 and CIJA71 showed 166- and 31-fold increases at specific time points after drought treatment, respectively, while the other seven CITIFY genes exhibited 2- to 5-fold increases in expression compared with the control (Figure 7).
It was observed that nearly all the detected *ClTIFY* genes were highly induced by salt treatment, with the exception of *ClJAZ4*, which seemed to be insensitive to salt treatment (Figure 8). Notably, the transcripts of *ClJAZ1*, *ClJAZ3*, and *ClJAZ7* displayed more dramatic increases at 1 h than at other time points after treatment (Figure 8), implying that they play vital roles in the response of watermelon to salt stress in earlier periods.

Upon JA treatment, eight out of the nine selected *ClTIFY* genes exhibited significantly upregulated expression, except for *ClPPD1*, whose expression was significantly reduced at all time points (Figure 9). Among the upregulated *ClTIFY* genes, *ClJAZ1* and *ClJAZ7* were the most significantly induced ones, exhibiting 533- and 77-fold changes, respectively.

### 4. Discussion

In the present study, we systematically identified 15 *TIFY* family genes from the watermelon genome, including eight *ClJAZs*, four *ClZMLs*, two *ClTIFYs*, and one *ClPPD*. Since only one *PPD* gene was present in the watermelon genome, the number of *TIFY* genes in watermelon was smaller than that in other plant species, whose numbers of *TIFY* genes range from 18 to 54, such as *Arabidopsis* (18) [37], rice (20) [2], *Brachypodium distachyon* (21) [28], *pear* (21) [35], poplar (24) [6, 34], maize (30) [29], *Brassica rapa* (36) [5], *wheat* (49) [16], *Gossypium hirsutum* (50), and *G. barbadense* (54) [33]. This difference might be attributed to gene duplication events, including large segmental duplications and small-scale tandem duplications, which have been proven to play a key role in the expansion and function diversification of genes in *TIFY* gene family [2, 28, 41]. In this study, only three segmental duplications and one tandem duplication were observed (Figure 4), and the number is much smaller than that in other plants, indicating that the identified *TIFY* genes are indispensable for the growth and development of watermelon.

Phylogenetic analysis showed that watermelon *TIFY* proteins can be divided into four subfamilies (JAZ, ZML, TIFY, and PPD), and the JAZ subfamily can be further classified into five groups (Figure 1). It was observed that some JAZ proteins were clustered in monocot- or eudicot-specific patterns, suggesting that these JAZ proteins might have predated the divergence between monocotyledonous and dicotyledonous plants (Figure 1). Multiple sequence alignments showed...
that the Jas motif of ClJAZs is strikingly similar to the special consensus sequence of SLX2FX2RX2RX5PY in other plants [3, 5], which has been reported to participate in the formation of the JA-Ile-COI1-JAZ complex [42]. However, the sequence of conserved Jas domains from ClZML proteins was SLXRFR(E/Q)KRKXRX7Y, which is slightly different from the characteristic sequence (Figure S1), suggesting that ClJAZs and ClZMLs might play different roles in regulating jasmonate responses in watermelon. We further detected 10 conserved motifs by MEME, and some conserved motifs were unique to specific subfamilies in watermelon TIFY proteins (Figure 2), which may contribute to the function diversification of TIFY proteins. Considering that the exon/intron organization can provide additional clues for understanding the evolutionary relationships among gene families [43], the gene structures of ClTIFY genes were determined in this study. Similar to the case in other plants [1, 31], ClZML genes have longer structures and a larger number of introns than other genes (Figure 3). Besides, ClZML genes exhibited a relatively similar pattern of exon/intron organization but had different intron numbers, indicating that gain or loss of introns occurred during the evolution of ClZML genes, which may lead to the functional divergence [43, 44]. Moreover, the JAZ subfamily genes exhibited the highest variability in exon/intron organization, while ClJAZ4 and ClJAZ6 had the same number of introns and similar lengths of exons (Figure 3), suggesting that these two genes are highly conserved during evolution and may have similar functions.

The signal molecule JA plays vital roles in plant growth, development, and responses to environmental stresses. Previous studies have revealed that the TIFY genes play vital roles in various biological processes of plants, such as petiole and hypocotyl elongation [4], lamina size and curvature [9, 11], flower development [13, 25, 45], and seed germination [46]. TIFYs may regulate plant development through the JA signaling pathway. For example, some JAZ proteins can interact with the WD-Repeat/bHLH/MYB complexes to repress JA-mediated trichome initiation and anthocyanin accumulation in Arabidopsis [47]. In Arabidopsis, JAZ4 and JAZ8 competitively interact with WRKY57 to mediate JA-induced leaf senescence [48]. In this study, all the selected ClTIFY genes were regulated by JA and exhibited preferential expression in specific tissues (Figures 5 and 9), suggesting that these genes play specific roles in regulating the normal development of plants probably through the JA signaling pathway. It is noteworthy that a majority of ClTIFY

![Graphs showing expression levels of different ClTIFY genes](image-url)

**Figure 9:** Differential expression detected for nine ClTIFY genes in response to JA treatment at 0 h, 1 h, 3 h, 9 h, and 24 h. Bars represent mean value ± SD of three independent experiments. Columns with different letters are significantly different (P < 0.05).
genes were highly expressed in flowers and fruits (Figure 5), and four CITIFY genes might play crucial roles in watermelon fruit development (Figure 6), suggesting that CITIFY genes may function in the development of the flower and fruit of watermelon. It should be noted that abiotic stress can increase the JA content, and TIFY genes were also shown to play vital roles in response to abiotic stresses through the JA signaling pathway. For example, overexpression of apple MdJAZ2 in Arabidopsis decreased JA sensitivity and increased the tolerance to salt and drought stresses during seedling development [49]. Moss PnJAZ1 acts as a repressor to mediate the crosstalk between JA and ABA signaling pathways, and thus increases tolerance to salt stress [21]. In this study, the vast majority of the detected CITIFYs exhibited differential accumulations under salt and drought stresses. Similar results were also reported in other plants, such as apple [41] and B. rapa [5]. In addition, nearly all the detected CITIFYs were upregulated by JA treatment, with the exception of CIPPD1, whose expression was downregulated (Figure 9). It is worth noting that CfJAZ1 and CfJAZ7 showed the most remarkable increases in expression under JA and drought treatments (Figures 7 and 9), indicating that they may play essential roles in regulating the response to drought stress by the JA-mediated signaling pathway. CfJAZ1 was clustered together with AtJAZ7 in the JAZ V group (Figure 1), and overexpression of AtJAZ7 was found to confer drought tolerance in Arabidopsis [50]. Under salt treatment, CfJAZ7 exhibited the highest expression among the detected CITIFYs (Figure 8), demonstrating that it may play a major role in salt stress response. Similarly, overexpression of OsTIFY11a/OsJAZ9 in rice resulted in significantly enhanced tolerance to salt and dehydration stresses, and suppression of OsJAZ9 resulted in reduced salt tolerance through the regulation of JA signaling [2, 15]. Further studies of these CITIFY genes are needed to unravel their regulatory roles in the development of watermelon and in abiotic stress response through the JA signaling pathway.

5. Conclusions

In this study, a total of 15 TIFY genes were identified in the watermelon genome, including eight CIJAZs, four CIZMLs, two CITIFYs, and one CIPPD. The analysis of qRT-PCR and RNA-seq data revealed that some TIFY genes play organ-specific roles. Expression patterns of the nine selected TIFY genes in response to JA and abiotic stress indicated that they may be involved in abiotic stress response by the JA signaling pathway. Our findings lay a foundation for a further functional characterization of the TIFY family genes in watermelon and a clarification of how TIFY genes can be utilized for the improvement of watermelon via biotechnological strategies.

Data Availability

The original data of the TIFY family genes are available from the watermelon genome database (http://cucurbitgenomics.org/organism/1).

Conflicts of Interest

The authors declare that they have no conflict of interest.

Authors’ Contributions

Youxin Yang and Yong Zhou conceived and designed the experiments. Youxin Yang, Rongrong Chen, and Yong Zhou performed the experiments. Youxin Yang, Golam Jalal Ahammed, Chunpeng Wan, Haoju Liu, Rongrong Chen, and Yong Zhou analyzed data. Youxin Yang, Rongrong Chen, and Yong Zhou wrote the manuscript. All authors read and approved the manuscript.

Acknowledgments

This work was jointly supported by the National Natural Science Foundation of China (31560572), the Natural Science Foundation of Jiangxi Province of China (20171BAB214030), and the Natural Science Foundation of Jiangxi Educational Committee (GJJ160393 and GJJ180172).

Supplementary Materials

Supplementary 1. Supplementary Fig. S1 Alignment of the amino acid sequences of watermelon TIFY proteins.

Supplementary 2. Supplementary Table S1 Primer sequences used in qRT-PCR.

Supplementary 3. Supplementary Table S2 Sequences and lengths of CITIFY motifs.

References


