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Functional Characterization and in Silico Analysis of Phytoene Synthase Family Genes Responsible for Carotenoid Biosynthesis in Watermelon (*Citrullus lanatus* L.)

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Abstract: Carotenoids are the main pigments in watermelon (*Citrullus lanatus* L.) fruit and contribute to its aesthetic and nutritional value. Phytoene synthase (PSY) is reported to be the first rate-limiting enzyme in carotenogenesis and controls the carotenoid flux. This study aimed to identify *PSY* genes responsible for carotenoid biosynthesis in the red-fleshed watermelon cultivar LSW-177. The *PSY* gene members *CIPSY1*, *CIPSY2* and *CIPSY3* were characterized and their catalytic activities were displayed in the heterologous complementation assay. The transcript levels of *CIPSY* genes at the different developmental stages of LSW-177 fruit and the promoter sequence of *CIPSY1* were also analyzed. Transcription factors involved in regulating the *CIPSY1* expression were scanned with previous RNA-seq data of the different stages during fruit ripening. Results showed that the PSY proteins from watermelon LSW-177 contained the conserved PSY domains and exhibited the ability to condense GGPP into phytoene in *E. coli*. *CIPSY1* is the dominant carotenogenic gene during fruit ripening; and can be induced by light and hormones. Furthermore, *Cla013914* and *Cla007950* that, respectively encode the transcription factors WD40-like protein and bZIP, likely upregulate *CIPSY1* during fruit ripening. In conclusion, *CIPSY1* play a dominant role in carotenoid biosynthesis during watermelon fruit ripening and is regulated by complex light and hormone-responsive networks.

Keywords: watermelon; carotenoid; phytoene synthase; promoter; transcription factor

1. Introduction

The watermelon is an important fruit crop with cultivars that differ in flesh pigmentation due to varying composition and levels of carotenoids [1]. The major carotenoid in the red-fleshed cultivars is lycopene, whereas β -carotene is the predominant carotenoid in cultivars with yellow flesh [1]. Carotenoids are a large group of natural and lipid-soluble pigments present in photosynthetic organisms some non-photosynthetic organisms, including plants, algae, cyanobacteria and some bacteria [2]. More than 1100 natural carotenoids had been identified so far, of which most are C_{40} compounds (<http://carotenoiddb.jp>) [3]. Carotenoids capture light during photosynthesis and also protect plants from photodamage [4,5]. They are also precursors of vitamin A [6], phytohormones [7,8]

and various flavor and aroma-inducing compounds [9]. In addition, carotenoids and their metabolites can reduce the risk of cancers and improve nutritional quality of horticultural crops [10,11].

The biosynthetic pathways of carotenoids have been studied in many species, including *Arabidopsis* [12], tomato [13] and citrus fruits [14], etc. Phytoene synthase (PSY), the first rate-limited enzyme of the pathway, condenses GGPP into phytoene and controls the carotenoid flux [15,16]. The number of *PSY* gene families varies across species due to duplication events. While *Arabidopsis* contains only one copy of the *PSY* gene [15], two *PSY* genes have been reported in carrot [17] and three paralogs have been identified in tomato [18–20], cassava [21] and members of the grass family (such as rice [22], maize, [23], etc.). Furthermore, six *PSY* gene family members have been described in *Malus × domestica* and four genes have been amplified [24]. A new *MdPSY5* has reported with highly expression in leaves and during fruit ripening of *M. domestica* cv. Fuji' [25]. It was also reported that six members of the *PSY* gene family were expressed and encoded functional proteins in *Brassica napus* L. [26]. The presence of multiple *PSY* genes is indicative of distinct localization (photosynthetic versus non-photosynthetic tissues), as well as developmental stage and environmental response-specific functions [27]. In melon for example, *CmPSY2* is the predominant transcript in roots, whereas *CmPSY1* is abundant in all other tissues [28]. In addition, tomato *SIPSY1* promotes carotenoid accumulation in the fruit while *SIPSY2* mainly functions in the green tissues and *SIPSY3* in roots [18–20]. Similarly, in maize, *ZmPSY1* has a dominant role in endosperm carotenogenesis and *ZmPSY2* is upregulated by light and associated with photosynthesis [29].

Carotenoid biosynthesis is regulated by phytohormones and environmental factors [30]. The light-responsive bHLH transcription factor PIF1 represses the *Arabidopsis* *PSY* gene by binding directly to a G-box motif in its *PSY1* promoter [31]. Chromatin immunoprecipitation assays indicated that tomato PIF1a specifically binds to the PBE box of the *PSY1* promoter to repress its expression and eventually inhibits carotenoid biosynthesis [32]. Ethylene-responsive factors such as ERFs and RAP2.2 on the other hand transcriptionally activate *PSY* and increase carotenoid accumulation in *Arabidopsis* leaves [33]. Recent studies have identified MADS-box transcription factors such as TAGL1 [34], RIN [35] and FUL11 [36] in tomato, which markedly increase carotenoid synthesis and accumulation during fruit ripening by directly or indirectly activating *SIPSY1*. Other MADS-box proteins like *SIMADS1* [37] and *SIFYEL* [38] are known to repress *SIPSY1*, while *SICMB1* shows a positive regulatory effect during fruit ripening [39]. The NAC family of TFs also participates in carotenogenesis and affects the transcription of *PSY* gene. *SINAC4* transcriptionally activates *SIPSY1* [40] while *SINAC1* has the opposite effect [41]. In citrus fruits, *CsMADS6* can bind to the promoters of *CsPSY* and *CsPDS* and increase their expression levels [14]. RAP2.2 and *CubHLH1* are carotenoid metabolism-related TFs that have been isolated from *Satsuma mandarin* [33,42]. In addition, the abundance and stability of the *PSY* proteins are also regulated post-transcriptionally. The ORANGE (Or) protein enhances *PSY* protein stability and increases the enzymatically active form in the plastids of *Arabidopsis* [43] and sweet potato [44]. On the other hand, the Clp protease physically interacts with *PSY* protein to enhance its degradation [45]. Both regulators maintain the homeostasis of plastid *PSY* protein to assure optimal abundance for carotenoid biosynthesis.

Previous studies show that the *CILCYB* gene controls the flesh color of watermelon [46,47], and the abundance of *CILCYB* correlates negatively with lycopene accumulation [48]. However, given the role of *PSY* in carotenoid flux, we systematically characterized the functions and comparative gene expression patterns of the duplicated *PSY* genes in watermelon. We also analyzed the promoter sequences and *cis*-regulatory structures of *CIPSY1* and identified putative transcription factors involved in fruit ripening.

2. Materials and Methods

2.1. Plant Material and Lycopene Measurement

The lycopene-rich red fleshed watermelon (*Citrullus lanatus* L.) cultivars LSW-177 and W1-1 were grown in the greenhouse of Northeast Agricultural University (44°04' N, 125°42' E), Haerbin, China. The plants were placed under field management using standard horticultural procedures [49]. Samples were scooped from the center of three intact watermelon fruits at five different ripening stages (10, 18, 26, 34 and 42 DAP or days after pollination) and stored at −80 °C until further analysis. The lycopene content was estimated in 3 g flesh per sample as previously described [48]. Three technical replicates were tested per sample.

2.2. Isolation of PSY cDNA Sequences and Corresponding Genomic Sequences

Total RNA was extracted using the RNeasy pure plant kit (Qiagen Biotech, Beijing, China) according to the manufacturer's instructions and 1 µg RNA was reverse transcribed to first-strand cDNA using the RevertAidTM-MuLV Kit (MBI, Vilnius, Lithuania) and an oligo dT primer. The open reading frames (ORFs) of watermelon PSY (*CIPSY*) gene were amplified using primers (Table S1) synthesized according to the sequence available in the Cucurbit genomics database (CuGenDB) (<http://cucurbitgenomics.org/>). The amplified PCR products were cloned into the pMD19-T Easy vector (TaKaRa, Kyoto, Japan) and transformed into *Escherichia coli* strain DH5α. The genomic DNA of watermelon samples was extracted using the PlantGen DNA Kit (CWBiotech, Beijing, China) and the promoter and coding regions of *CIPSY1* gene were amplified and sequenced.

2.3. Protein Sequence Analysis

The signal peptide and transmembrane regions of *CIPSY* proteins were predicted using CBS prediction servers and the putative conserved domains were analyzed with the help of NCBI conserved domains. The resulting amino acid sequences were aligned using Clustal X v.2.0 [50] and a phylogenetic tree was constructed using MEGA 7.0 by neighbor-joining method and 1000 bootstrap replications [51]. The genomic and coding sequences of *CIPSYs* from watermelon and other species were compared using TBtools JRE1.6 [52]. The conserved motifs for all PSY protein sequences were detected by multiple expectation maximization for motif elicitation (MEME v.5.1.1) online tools (<http://meme-suite.org/tools/meme>).

2.4. Real-Time PCR Analysis

Primers specific for *CIPSYs* exons were designed using the Primer 5.0 software to avoid genomic amplification (Table S1). First, strand cDNA was synthesized using random primers and RT-PCR was performed using SYBR green master mix (TOYOBO, Osaka, Japan) in the iQ5 Gradient Real Time PCR system (Bio-Rad, Berkeley, CA, USA) following the manufacturers' protocols. The reaction conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Relative gene expression was calculated using comparative C_T method [53], with the watermelon *CIYLS8* gene as the internal control [54]. Three technical replicates were tested for each sample.

2.5. Plasmids and Functional Complementation

To test whether the watermelon PSY genes encode functional enzymes, heterologous complementation was performed for the carotenoid biosynthetic genes. The pACCRT-E plasmid carrying the bacterial *crtE* gene that encodes geranylgeranyldiphosphate synthase (GGPS) was transformed into *E. coli* [55]. Fragments of *CIPSY* genes coding sequences were amplified and cloned into the pET28a vector (Novagen, Germany) to generate pETPSY1, pETPSY2 and pETPSY3 constructs. The bacteria carrying pACCRT-E plasmid was co-transformed with the respective *CIPSY*

constructs or empty pET28a vector (negative control); (*E. coli* harboring the pACCRT-EB vector with phytoene-producing *crtE* and *crtB* genes was used as the positive control. The suitably transformed *E. coli* cells were cultured at 37 °C in Luria–Bertani (LB) liquid medium containing 100 µg mL^{−1} ampicillin and 35 µg mL^{−1} chloramphenicol till OD₆₀₀ reached 0.6–0.8. After inducing protein expression with 0.05 mM isopropyl-β-D-thiogalactopyranoside (IPTG), the cells were cultured at 28 °C for 2 days in the dark without shaking. The cells were harvested by centrifuging at 5000× *g* for 15 min at 4 °C and washed twice with sterile distilled water. The pellets were resuspended in 5 mL hexane/acetone (1:1) solution containing 1% butylated hydroxytoluene (BHT) and sonicated twice on ice. Samples were centrifuged at 4000× *g* to remove the lysed cells, and the supernatant was dried under nitrogen. The dried extracts were resuspended in acetone for high-performance liquid chromatography (HPLC) as previously described [56].

2.6. In Silico Analysis of CIPSY1 Promoter Elements and Related Transcription Factors in Watermelon

The *CIPSY1* promoter sequence was amplified from the genomic DNA of both cultivars using specific primers (Table S1). The nucleotide start site of the *CIPSY1* gene was determined using transcription start site (TSS) program of the Softberry software and the *cis*-regulatory elements were predicted with plantCARE [57] and PLACE [58,59]. The RNA-seq data of watermelon at different ripening stages are available in the NCBI SRA database under the numbers SRX2037189 for cultivar LSW-177 [49] and SUB4001164 for cultivar W1-1 [60]. The expression levels of different transcription factors and *CIPSY1* were analyzed at the different stages of fruit ripening.

3. Results

3.1. Characterization of Watermelon PSY Gene and Protein Sequences

Three watermelon *PSY* genes were searched in the CuGenDB and BLAST annotation indicated that each mapped to different chromosomes (1, 7 and 2). To determine their role in carotenogenesis, we amplified the *CIPSY1*, *CIPSY2* and *CIPSY3* coding sequences from the LSW-177 cultivar. The full-length 1266 bp *CIPSY1* sequence encoded a 421 amino acid protein with a predicted isoelectric point (*pI*) of 6.86. The putative protein encoded by *CIPSY2* consisted of 355 amino acids and a *pI* of 8.46, while the deduced *CIPSY3* protein had 388 amino acids and a *pI* of 8.87. Multiple alignment of the predicted proteins indicated 74.5% homology between *CIPSY1* and *CIPSY2* and 67.6% between *CIPSY3* and *CIPSY2*. The variable sequences were concentrated in the N-terminal regions and predicted as transit peptides. Both *CIPSY2* and *CIPSY3* contained a shorter N-terminal sequence compared to *CIPSY1* (Figure 1). According to the NCBI conserved domains database, watermelon *PSY*s have six motifs in common (Figure 1 and Figure S1), including the substrate binding pocket, Mg²⁺ binding site, active site lid residues, catalytic residues and two aspartate-rich sites (DXXXD). The catalytic domains are mainly concentrated between 144–193 aa and 299–348 aa (Figure 1, yellow and pink background). A transmembrane region was also predicted in all three proteins (Figure 1, and Figure S2).

Comparison of the genomic and cDNA sequences revealed that the genomic sequence of *CIPSY3* (3180 bp) was longer than that of both *CIPSY1* (2886 bp) and *CIPSY2* (1902 bp). Furthermore, the coding sequence of *CIPSY1* consisted of 6 exons split by 5 introns similar to that of *AtPSY* and *SIPSYs*, compared to 5 exons in both *CIPSY2* and *CIPSY3* (Figure 2). Analysis of the conserved motifs with MEME identified the same regions as described above. Phylogenetic analysis of *CIPSY* proteins with that of *PSY* proteins from other plants showed that the central region was conserved across the species, whereas the N-terminal sequences were diverse on account of the variations in plastid transit peptides. Proteins from watermelon are more closely related to the ones from melon and grouped, respectively. *CIPSY1*, *CIPSY2* and *CIPSY3* were classified into two distinct clades, indicating their different functionality (Figure 2).

Figure 1. Alignment of watermelon and *Arabidopsis* phytoene synthase (PSY) proteins. Multiple sequences were aligned using the ClustalX software v.2.0 [50]. The predicted chloroplast cleavage site by chloroP is underlined with red color (<http://www.cbs.dtu.dk/services/ChloroP/>). Black boxes and arrows indicate the putative active site DXXXD. Catalytic domains are highlighted with yellow and pink and the potential transmembrane region with green (<http://www.cbs.dtu.dk/services/TMHMM/>).

Figure 2. Gene structures of watermelon and other plant PSYs and the distribution of conserved motifs. Left panel shows the phylogenetic tree. The tree was constructed using MEGA7 with a bootstrap of 1000 by the neighbor-joining method [51]. The motif distribution is shown in the middle, wherein rectangles of different colors represent conserved motifs. Color coding is the same as in Figure 1. Exon–intron distribution is shown in the right panel, wherein the black line and blue boxes represent introns and exons, respectively. The database accession numbers are: *AtPSY1* (AAA32836), *SIPSY1* (CAA42969), *SIPSY2* (ABU40771), *SIPSY3* (Soly01g005940), *MdPSY1* (KT189149), *MdPSY2* (KT189150), *MdPSY3* (KT189151), *MdPSY4* (KT189152), *CmPSY1* (MELO3C025102.2.1), *CmPSY2* (MELO3C025102.2.1), *CmPSY2* (MELO3C016185.2.1).

3.2. Functional Complementation

A heterologous complementation assay was performed to evaluate whether watermelon *PSY* genes encode functional enzymes. Briefly, the coding sequence of *CIPSY1*, *CIPSY2* and *CIPSY3* genes were each introduced into GGPP-expressing *E. coli* that harbored the pACCRT-E plasmid. phytoene-producing bacteria that carried the pACCRT-EB plasmid was used as the positive control.

Introduction of the *CIPSY1*, *CIPSY2* and *CIPSY3* construct in the pACCRT-E cells resulted in a peak with retention time and spectral qualities similar to that of phytoene (Figure 3). These results indicated all the three *PSY* genes from watermelon encoded functional proteins and were able to catalyze the conversion of GGPP to phytoene in bacteria.

Figure 3. Functional complementation of watermelon *PSY* proteins in bacteria. *E. coli* cells harboring the pACCRT-EB vector that contains the *crtE* and *crtB* genes encoding for phytoene was the positive control. *E. coli* cells harboring the pACCRT-E vector that contains the *crtE* gene encoding for GGPP were transformed with pET28a (empty vector), *CIPSY1*, *CIPSY2* and *CIPSY3* constructs. The peak of phytoene (indicated by an arrow) was observed in cells with *CIPSY1*, *CIPSY2* and *CIPSY3* constructs.

3.3. Carotenoid Synthesis and Accumulation in Watermelon Fruit

To further determine the role of *CIPSY* proteins in watermelon carotenogenesis during the ripening period, we measured the lycopene content in the LSW-177 fruit at the different stages. The flesh color of LSW-177 gradually deepened from white to red by 26 DAP due to accumulation of lycopene, which peaked at 42 DAP and corresponded to the deepest red color (Figure 4A). We next detected the transcript levels of *CIPSY1*, *CIPSY2* and *CIPSY3* in the fruit flesh at different stages of ripening. *CIPSY3* did not alter significantly during ripening, indicating that it does not regulate watermelon fruit maturation. While both *CIPSY1* and *CIPSY2* had similar transcript profiles till 18 DAP, the *CIPSY1* levels were 3.3-fold higher than that of *CIPSY2* at 26 DAP and peaked at 42 DAP, which was consistent with the pattern of lycopene accumulation (Figure 4B). Based on these results and previous reports detailing lack of correlation between the *CIPSY2* levels and lycopene content in watermelon of different flesh color [61], we hypothesize that *CIPSY1* has a dominant role in the carotenoid biosynthetic pathway during watermelon fruit ripening.

Figure 4. Lycopene content and *CIPSY* gene expression levels in watermelon fruits at different development stages. **(A)** Lycopene concentration in watermelon fruits harvested at different time points (days after pollination); **(B)** relative expression of *CIPSY* genes at the same time points. Error bars indicate standard errors from three biologic replicates. Different letters indicate significant differences in expression patterns for *CIPSY* genes ($p < 0.05$).

3.4. Cis-Regulatory Structures of *CIPSY1* Promoter

The regulatory motifs of most plant genes lie within 2000 bp upstream of the start codon [62,63]. To identify the *cis*-regulatory elements of *CIPSY1* and compare the motifs in different watermelon cultivars, we amplified their *CIPSY1* genomic sequences 2000 bp upstream of the ATG codon. The reference sequence of red-flesh watermelon 97103 was obtained from Cucurbit genomics database (<http://cucurbitgenomics.org/>). The predicted TSS was located at about 500 bp upstream from ATG. Putative *cis*-regulatory elements in the *CIPSY1* gene promoter were searched using plantCARE and PLACE programs. Most of the motifs were light-related, including Box 4, G-Box, GT1-motif, chs-CMA1a, AE-box, TCT-motif and Sp1 (Figure 5 and Table S2). Furthermore, hormone-related elements such as ABA-responsive motif ABRE and MeJA-responsive TGACG-motif were also identified in the *CIPSY1* gene promoter in all three cultivars. The presence of the ARE motif in the *CIPSY1* gene of the three cultivars indicated that is likely induced by anaerobic conditions. Interestingly, the promoter sequence of *CIPSY1* from W1-1 and 97103 harbored one G-box motif compared to two in LSW-177. The ATCTA motif, which is recognized by the transcription factor RAP2.2, was only found in the *CIPSY1* promoter in LSW-177 and not in W1-1 and 97103.

Figure 5. Promoter and 5' untranslated regions of *CIPSY1* gene from red-flesh watermelon LSW-177, W1-1 and 97103 cultivars. Putative *cis*-regulatory motifs were identified with plantCARE [57] and PLACE [58,59]. Motifs associated with light and hormonal response are highlighted in different colors. Motif positions are relative and not drawn to scale. Red arrows indicate differences between cultivars.

3.5. WD40-Like and bZIP Regulate CIPSY1 Gene Expression during Watermelon Fruit Ripening

We previously annotated and classified genes that are differentially expressed during watermelon fruit ripening [49]. To further identify transcription factors (TF) that potentially bind to the *CIPSY1* promoter and regulate its expression during ripening, we analyzed the RNA-seq data from the different stages of the LSW-177 (10, 18, 26, 34 and 42 DAP) and W1-1 (WA, WB, WC, WD and WE) cultivars. Both transcriptomic and subsequent qPCR analysis indicated that *CIPSY1* transcript levels increased rapidly in the first three stages, suggesting that the upstream TFs likely exert their effects in the initial stages of fruit maturation. We then screened for the differentially expressed TFs in these stages based on p -value <0.05, q value <0.05 and fold change >1. Twenty-two TFs were upregulated in the LSW-177 cultivar, including AP2, bHLH, bZIP, MYB, WD40-like and WRKY (Figure 6A and Table S3). In the W1-1 fruits, 50 TFs were upregulated (Figure 6B and Table S4). The *Cla013914* and *Cla007950* genes, respectively encoding the WD40-like and bZIP TFs were upregulated in both cultivars, indicating that they likely increase *CIPSY1* expression levels during fruit ripening.

Figure 6. Expression patterns of upregulated transcription factors (TFs) and *CIPSY1* gene (Cla009122) at different developmental stages of the LSW-177 and W1-1 cultivars. (A) Expression patterns of 22 upregulated TFs from 10 DAP to 42 DAP in LSW-177; (B) expression patterns of 50 upregulated TFs from 8 DAP to 40 DAP in W1-1. Red and blue boxes indicate differential increase ratio. Red arrows indicate similarity between the cultivars.

4. Discussion

The content and composition of carotenoids in the fleshy part of watermelon are highly important traits for breeding new varieties since they contribute to the appearance and nutritional value [64,65]. Many transcriptome and QTL genetic linkage experiments had been performed to reveal the genes

responsible for carotenoid biosynthesis in watermelon fruit ripening [46,49,65,66]. Phytoene synthase (PSY) has been confirmed to catalyze the first committed step of carotenogenesis and therefore controls the carotenoid flux through the pathway in many species [67,68]. The aim of this study was to analyze the functionality of *CIPSY* proteins in order to determine which one was responsible for the carotenoid biosynthesis in the watermelon fruit. In this study, we cloned and identified three PSY-encoding genes from the red-flesh LSW-177 cultivar and designated them as *CIPSY1*, *CIPSY2* and *CIPSY3*.

The PSY isozymes showed extensive homology in their amino acid sequences with the exception of the N-terminal region. The high diversity in the potential transit peptide at the N-terminal of *CIPSY*s possibly affect their localization in photosynthetic versus non-photosynthetic tissues, which is also previously observed in *Brassica napus* and apple PSY proteins [24–26]. In silico analysis further showed the presence of the active aspartate-rich motifs DELVD and DVGED, squalene/PSY signature one and two in each *CIPSY* protein at the same positions as in *AtPSY* [15], indicating that these motifs are highly conserved across different species and may origin from the same ancestor [24,69,70]. In addition, the watermelon PSY proteins were phylogenetically closer to that of other species than among themselves. For instance, *CIPSY1* is organized into six exons similar to that of its homolog in melon (*CmPSY1*), while the five exon–four intron structure of *CIPSY2* and *CIPSY3* is comparable to that of *MdPSY3/4* and *MdPSY1/2* in apples [24]. Furthermore, the structure of *CIPSY2* is more divergent with *CmPSY2* gene. Taken together, the nonconserved intron/exon structure suggest duplication events and functional diversity.

Complementation assay confirmed that all *CIPSY* proteins can condense GGPP into phytoene in bacteria. However, they exhibited distinctive expression profiles at the different stages of fruit ripening. The *CIPSY1* transcripts showed a rapid surge in the initial stages of ripening in LSW-177 cultivars, which corresponded to maximum lycopene accumulation, and both showed a subsequent decline in the fully ripened fruit. Guo et al. [66] reported a similar pattern of *CIPSY1* expression in the red-flesh 97103 watermelon cultivar. In contrast, its paralog *CmPSY1* is predominantly expressed in the ripe melon fruit [28]. Wang et al. [61] analyzed *CIPSY2* transcripts in six watermelon cultivars with distinct fruit pigmentation and found that the expression profile of *CIPSY2* was not consistent with lycopene accumulation. The red-fleshed MSW28 cultivar had the highest lycopene content, while *CIPSY2* displayed lower levels. Finally, *CIPSY3* expression levels did not alter significantly across the different development stages. Taken together, the *CIPSY1* gene plays the dominant role in carotenogenesis during watermelon fruit ripening.

Various light- and hormone-responsive elements were identified in the *CIPSY1* gene promoter regions from different watermelon cultivars, indicating complex regulatory mechanisms. Chs-CMA1a, Box 4, GT1-motif, AE-box and Sp1 motifs were the common light-responsive elements across LSW-177, W1-1 and 97103 cultivars. In *Arabidopsis*, PIF1 represses *AtPSY* transcription by directly binding to a G-box element in its promoter [31]. *MabZIP4* directly binds to the *BanAAT* promoter in banana, while *MabZIP5* binds to the promoters of *MaMT1*, *MaACY1*, *MaAGT1* and *BanAAT* via the G-box motif, implicating diverse functional roles of *MabZIP*s during fruit ripening [71]. Fruit growth and ripening are also controlled by multiple phytohormones. For instance, ABA regulates fruit ripening in the watermelon 97103, W1-1 and Pi186490 cultivar [60,72]. We identified ABA and MeJA-responsive elements in the *CIPSY1* promoter of LSW-177, W1-1 and 97103 cultivars, suggesting that multiple factors involved in hormone synthesis and signaling regulate *CIPSY1* expression during fruit ripening. The ABA-responsive element (ABRE) ACGTG is the major *cis*-element for ABA-responsive gene expression and TFs containing basic-domain leucine zipper (bZIP) bind to the ABRE sequence in *Arabidopsis* [73,74]. Furthermore, the MeJA-responsive motif TGACG is a binding site for bZIP that is involved in the MeJA signal transduction pathway [75]. We hypothesize that bZIP regulates *CIPSY1* expression via these motifs during watermelon fruit ripening. In addition, an RAP2.2 motif that is recognized by the ethylene response factors (ERF) RAP2.2 were only detected in LSW-177, indicating the distinct regulatory mechanisms between the different cultivars.

Further screening of the transcriptome data of LSW-177 and W1-1 revealed the TFs Cla013914 and Cla007950 as potential upstream activators of *CIPSY1*. *Cla013914* encodes an unknown protein containing the WD40/YVTN repeat-like-containing domain (IPR015943). The WD40 TF typically contains 4–16 tandem repeat WD motifs that form a scaffold to regulate the assembly and binding of multiple protein complexes involved in plant growth and development [76,77]. A total of 54 putative WD40 genes were differentially expressed between the red-fleshed and white-fleshed peach fruits at five developmental stages [78]. In blueberries, high *MYB-bHLH-WD40* expression correlated positively with anthocyanin accumulation and color development [79]. Similar expression patterns have also been detected in the *Lycium* fruit [80]. *Cla007950* encodes a protein of the bZIP family that shows high homology with *AtbZIP42*. As one of the largest TF families in plants, the bZIP family controls multiple biologic processes, including development, plant growth, fruit ripening and stress responses. Based on these findings, it is highly likely that Cla013914 and Cla007950 activate *CIPSY1* in watermelon fruits and promote carotenoid biosynthesis.

5. Conclusions

This work presents the structural and functional characterization of phytoene synthase proteins in red-fleshed watermelon LSW-177. Taken together, higher expression levels of *CIPSY1* and the ability to generate phytoene in a heterologous system, suggest that *CIPSY1* play a dominant role in the carotenoid biosynthesis of watermelon during fruit ripening. This provides a target for watermelon fruit-color breeding. We also analyzed the promoter sequences of *CIPSY1* and selected *Cla013914* and *Cla007950* as the candidate genes that may positively regulate *CIPSY1* expression in watermelon during fruit ripening. This study also provide important cues to unveil the underlying mechanisms of carotenoid biosynthesis in watermelon fruit.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4395/10/8/1077/s1>, Table S1: PCR primers used in the research; Table S2: Putative *cis*-regulatory elements related to specific function present in *CIPSY1* promoter from red-fleshed watermelon cultivars LSW-177, W1-1 and 97103; Table S3: Excel file containing the upregulated transcription factors in LSW-177 at five developmental stages; Table S4: Excel file containing the upregulated transcription factors in W1-1 at five developmental stages; Figure S1: The motifs of watermelon PSY1 protein analyzed using NCBI conserved domains; Figure S2: The transmembrane sequence of watermelon *CIPSY1* protein as predicted with CBS prediction servers.

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