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Research Paper

Allelic diversity of phytoene synthase gene influences the transcription level in citrus fruit among a citrus F_1 hybrid population

Aiko Sugiyama[1)], Yoshinori Ikoma[2)], Hiroshi Fujii[2)], Tomoko Endo[2)], Hirohisa Nesumi[2)], Takehiko Shimada*[2)] and Mitsuo Omura[3)]

[1)] *The United Graduate School of Agriculture Science, Gifu University*, Gifu 501-1193, Japan
[2)] *National Agriculture and Food Research Organization Institute of Fruit Tree and Tea Science*, Shimizu, Shizuoka 424-0292, Japan
[3)] *Faculty of Agriculture, Shizuoka University*, Suruga, Shizuoka 422-8529, Japan

Phytoene synthase (PSY) is one of the key regulatory enzyme on the biosynthesis and accumulation of carotenoid in citrus fruits. The transcriptional diversity of *PSY* is mainly attributed to the structural variation in promoter region among *PSY* alleles. In aim to clarify how this transcriptional diversity is regulated among them, *PSY* alleles responsible for carotenoid biosynthesis in the fruits are characterized and their promoter sequences were compared. Based on gene structure and expression pattern of *PSY* homologues on the clementine mandarin genome sequence, *PSY* alleles responsible for carotenoid biosynthesis are derived from a single locus in the scaffold 6. AG mapping population possessed four *PSY* alleles derived from parent lines of A255 and G434, and their F_1 individuals with *PSY-g2* allele tended to have low transcription level. From sequence comparison of their promoter regions, the *cis*-motif alternation from MYBPZM to RAV1AAT might be a candidate to influence the transcription level. Among the ancestral pedigree varieties of AG mapping population, the transcription level of *PSY* correlated with genotypes of MYBPZM and RAV1AAT motifs in the promoter region of *PSY* alleles, so that homozygous genotype of MYBPZM showed higher transcription level while heterozygous genotype of MYBPZM and RAV1AAT showed lower transcription level.

Key Words: citrus, carotenoid, allele, phytoene synthase, *cis*-regulatory motif.

Introduction

Most photosynthetic organisms produce carotenoids, which are essential for both plant and animal lives (Wong *et al.* 2004). Carotenoids are involved in various functions in plants, including phyto-hormone precursor action (Schwartz *et al.* 2003) and environmental adaptation through the modulation of the photosynthetic apparatus (Demmig-Adams and Adams 2002). Citrus fruit contains significant amounts of various carotenoids and more than 100 different kinds of carotenoids have been isolated in *Citrus* (Gross 1987). To date, many studies have described the isolation and characterization of the physiological and molecular features of carotenoid biosynthesis during fruit development and ripening in citrus (Kato *et al.* 2004, Rodrigo *et al.* 2004). The composition and content varied of carotenoids among citrus varieties rather than species, and the carotenoid diversity in cultivated citrus is highly influenced by genetic factors (Fanciullino *et al.* 2006). As a typical example, satsuma mandarin (*Citrus unshiu* Marc.) mainly accumulates β-cryptoxanthin (B-Cry) in the flavedo and juice sacs in mature fruit (Goodner *et al.* 2001, Ikoma *et al.* 2001), while sweet orange (*Citrus sinensis* Osbeck) fruit accumulates violaxanthins, predominantly 9-cis-violaxanthin (Lee and Castle 2001, Molnár and Szabolcs 1980). The carotenoid accumulation that occurs during citrus fruit ripening is highly regulated by the coordinated expression of carotenoid biosynthetic genes, and the differences in the variance of transcripts encoding biosynthetic enzymes is significantly associated with the carotenoid composition and content among varieties (Kato *et al.* 2004). In addition, several transcription factors, such as *RAP2.2* (Welsch *et al.* 2007) and *CubHLH1* (Endo *et al.* 2016), related to carotenoid metabolism were isolated and characterized in *Arabidopsis thaliana* and satsuma mandarin. However, the genetic information about how carotenoid composition and contents would extend the variation among citrus varieties at a molecular level has been quite limited.

In the Japanese citrus breeding program, the enrichment of carotenoids, especially B-Cry with health-promoting properties (Sugiura *et al.* 2011), is an important object

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*Corresponding author (e-mail: tshimada@affrc.go.jp)

with the aim of expanding citrus fruit consumption. To advance the molecular breeding for the enrichment of B-Cry, Sugiyama *et al.* (2011) examined quantitative trait locus (QTL) mapping for each carotenoid's content level using the F_1 hybrid population (AG population) between breeding lines 'Okitsu 46 gou' (A255) and 'Kankitsu Chukanbohon Nou 5 gou' (G434) with wide variation in carotenoid content and composition. A major QTL for B-Cry was detected on the Gn0005 locus in linkage group 6 of the G434-map and had a LOD value of 3.4. Various QTLs for the other carotenoid contents were also detected but most of them had low LOD values. This result was reasonable considering that carotenoid composition and content were affected by transcriptional amounts among various carotenoid biosynthetic genes. Recently, Shimada *et al.* (2014) constructed a citrus framework genetic map anchored by 708 gene-based markers using the same AG population. In this AGI map, major carotenoid biosynthetic genes were mapped as follows: *phytoene synthase* (*PSY*), on linkage group (LG)-4, *phytoene desaturase* (*PDS*) on LG-3, *ζ-carotene desaturase* (ZDS) on LG-9, *β-ring hydroxylase* (*HYb*) on LG-3, *zeaxanthin epoxidase* (*ZEP*) on LG-2, and *9-cis-epoxycarotenoid dioxygenase* (*NCED*) on LG-06. The other carotenoid biosynthetic genes could not be mapped owing to the low polymorphic features of genomic sequence and so on. Among the mapped loci of carotenoid biosynthetic genes, an eQTL contributing to *ZEP* expression level was located on *ZEP* locus of the genetic map for AG population (Sugiyama *et al.* 2014) and pairs of allelic *ZEP* gene with different sequences on *ZEP* locus were observed. In mandarin and orange, there are at least four *ZEP* alleles and the amounts and accumulation patterns of their transcripts were different during fruit development (Sugiyama *et al.* 2010). Therefore, the allelic variation among carotenoid biosynthetic genes would likely to extend wide variation in the carotenoid contents and composition among citrus varieties. In addition to *ZEP* expression, the eQTL analysis of *PSY* also suggested that the major regulation site for *PSY* expression might be tightly associated with *PSY* locus (Gn0009 locus) assigned by *PsyG-CT* marker (Sugiyama *et al.* 2014). PSY is a rate-limiting key enzyme in the carotenoid biosynthetic pathway and the flux of carotenoid pathway is generally controlled by multiple *PSYs* in plants. It is reported that *Arabidopsis* genome possesses one *PSY* gene while tomato and rice has two and three *PSYs* with different roles in plant development and tissues-specific expression or seasonal different expression (Bartley and Scolnik 1993, Ruiz-Sola *et al.* 2012, Welsch *et al.* 2008). In citrus, many cDNAs for *PSY* have been isolated and functionally characterized during fruit development (Kato *et al.* 2004, Maheswary *et al.* 2006, Rodrigo *et al.* 2004), however, little is known about how many loci or copies citrus *PSYs* comprise in the genome and how transcriptional variation of *PSY* is regulated to extend among citrus varieties.

In this study, we investigated the allelic diversity on genomic sequence and gene expression patterns of *PSY* homologues in clementine mandarin (*C. clementine* hort ex. Tanaka) genome, and inquired how transcriptional variation of *PSYs* in eQTL analysis was occurred in the AG population. The *PSY*s in the AG population comprised four alleles (*PSY-a1* and *PSY-a2* from A255, *PSY-g1* and *PSY-g2* from G434) which were derived from the parent lines, and F_1 individuals with *PSY-g2* tended to show low transcription level in the fruits. The sequence analysis was carried out to compare the promoter structures of four alleles and possible *cis*-regulatory elements to influence the transcription level were discussed.

Materials and Methods

Plant materials and preparation of DNA and RNA

All plants used in the experiments were cultivated in the research field of the National Agriculture and Food Research Organization Institute of Fruit Tree and Tea Science, Citrus Research Center, Okitsu, Shizuoka, Japan. Forty-eight F_1 individuals bearing the fruits, which were obtained from crossing A255 and G434, were used for genetic analyses. The female parent of A255 was derived from 'Sweet spring' ['Ueda unshiu' (*C. unshiu* Marc.) × 'Hassaku' (*C. hassaku* hort ex. Tanaka)] × 'Trovita' orange, and the male parent of G434 was derived from 'Lee' [clementine mandarin × 'Orlando' tangelo ('Duncan' grapefruit (*C. paradise* Macf.) × 'Dancy' tangerin (*C. tangerina*) × 'Mukaku kishu' (*C. kinokuni* hort. Tanaka). Genomic DNA was extracted from fresh and fully expanded leaves of F_1 individuals and their parent varieties according to the method of Dellaporta *et al.* (1983). Total RNA was extracted from juice sacs on the middle of November (more than three fruits per individual) from the AG population using the method described by Ikoma *et al.* (1996).

In addition, various tissues were collected from clementine mandarin and immediately frozen by liquid nitrogen for and mRNA expression analysis, as follows: flower, leaf, stem, young whole fruit at DAF30, and juice sacs and peels at DAF60, DAF120 and DAF180. Total RNAs were also extracted from these samples.

Sequence comparison of PSYs among clementine mandarin, A255 and G434

Blastx search was carried out using a query nucleotide sequence of *CitPSY* (AF22021) against the clementine mandarin genome in the public databases (http://phytozome.jgi.doe.gov/pz/portal.html). Four nucleotide sequences show high homology against *CitPSY* and their nucleotide sequences were downloaded. Their deduced amino acid sequences were compared with those of *PSY* alleles detected in A255 and G434 and those of citrus *PSYs* published in the public DNA database. The alignment analysis and phylogenetic analysis were carried out using the computer program Genetyx-Win Ver.11.0.3 (Software Development, Tokyo, Japan). Phylogenetic tree was constructed under the unweighted pair group maximum average method based on the amino acid sequences.

Reverse transcriptase (RT)-PCR for various tissues of clementine mandarin

The cDNA was synthesized from 1 μg of total RNA by QuantiTect® Reverse Transcription (Qiagen, Hilden, Germany) using a poly-dT primer. Gene-specific primer sets for clementine mandarin *PSY* homologues were designed to prohibit the cross amplification between them. Primer sequences were described as follows: Ciclev10011841m.g: forward primer (5′-ATGTCTGTTGCATTGCTATGG-3′) and reverse primer 5′-GTCGATTCCAGATGAGCAAG-3′), Ciclev10018150m.g: forward primer (5′-TGGTCATC CCTGTTAATATATAGAGAG-3′) and reverse primer 5′-TGATCAGTAGGGACAAATCAAACTACC-3′), Ciclev10015582m.g: forward primer (5′-ATCACCTTGTT GTGCTGAATCTTTGAAGAGAG-3′) and reverse primer 5′-ATCGAGTTTTTCTCTCTGGGGATGTTTATGTTC-3′). The primer sets for Ciclev10018272m.g were not designed owing to the incomplete gene structure. Elongation factor 1 alpha (*EF1-α*) is used as an endogenous control gene and the primer sequences are refereed to our previous report (Endo *et al.* 2006).

RT-PCR was conducted by the condition of 30 PCR cycles. Each cycle was composed of 94°C for 30 sec., 56°C for 1 min. and 72°C for 1 min. The reaction mixture consisted of 100 mM Tris-HCl (pH 8.0), 50 mM of KCl, 1.5 mM of $MgCl_2$, 0.2 mM each of dNTPs, 5 mM of each primer, 5 ng of cDNA and 1 U of *AmpliTaq* in a 20 μl reaction volume. PCR reaction mixtures were electrophoresed on 1.5% agarose gel at the 100 V. Gel was stained in the EtBr solution and the amplified fragments were detected on the UV trans-illuminator.

Association analysis of PSY *genotype and expression in F_1 individuals of the AG population*

To determine the genotypes in F_1 individuals, TaqMan allele-discriminating PCR was carried out using the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 7300 (Applied Biosystems). TaqMan allele-specific primer/probe sets for *PSY* were utilized referring to the information of Gn0009 SNP markers encoding *PSY* (Shimada *et al.* 2014).

The total *PSY* expression level was evaluated using a TaqMan MGB probe with a primer/probe set reported by Kato *et al.* (2007). Total RNA (0.2 μg), through on-column DNase digestion by an RNeasy MiniKit (Qiagen, Hilden, Germany), was used to synthesize cDNA with random hexamers at 37°C for 60 min using TaqMan reverse transcription reagents (Applied Biosystems). The *PSY* expression level was estimated using quantitative reverse transcription PCR (qRT-PCR). The TaqMan Ribosomal RNA Control Reagent VIC probe (Applied Biosystems) was used as an endogenous control. The ABI PRISM 7300 Sequence Detection System Software (Applied Biosystems) analyzed the gene expression levels. The qRT-PCR score was normalized by the expression of 18 S ribosomal RNA and were relatively adjusted based on the score of A255, which had an estimated value of 1.0. Each reaction of contained 900 nM primers, 250 nM TaqMan MGB probe, and 2.5 μL of template cDNA or DNA. The thermal cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s.

Sequence analysis for the promoter regions of four PSY *alleles*

The 1.2 kbps of fragments for four *PSY* alleles were obtained by PCR amplification using *AmpliTaq* Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) protocols. The primers were designed referred to the public databases for the draft genome sequences of clementine mandarin and sweet orange (http://phytozome.jgi.doe.gov/pz/portal.html) as following; sense primer: 5′-TTTCCCAC TTTGCACAGCGTCAGTC-3′ in the promoter region, antisense primer: 5′-AAAAGGATTGATGAAAGAAGGCA-3′ in the primary exon. The amplified PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), transformed into *Escherichia coli* strain XL-1 Blue, and sequenced using a BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems) with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). These nucleotide sequences were registered in DDBJ (*PSY-a1*: LC169453, *PSY-a2*: LC169454, *PSY-g1*: LC169455, *PSY-g2*: LC169456). Genetyx-Win ver. 11.0.3 (Software Development) were used for the alignment analysis. The 1.2 kbps of promoter sequences were applied to a homology search using a plant *cis*-acting regulatory DNA element (PLACE, http://www.dna.affrc.go.jp/htdocs/PLACE/) database.

The genotype of *cis*-regulatory motif (MYBPBZ or RAV1AAT) located around –330 bps in the promoter region of *PSY* alleles was investigated for ten parental varieties and two parent lines of AG mapping population as following; ‘Duncan’ grapefruit, ‘Dancy’ tangerin, clementine mandarin. ‘Mukaku kishiu’, Hassaku, ‘Ueda unshiu’, ‘Trovita’ orange, ‘Orlando’, ‘Lee’, ‘Sweet spring’, A255 and G434. The following primer set was used to amplify the promoter region around 330 bps of upstream region of *PSY* alleles for sequence analysis (sense primer: 5′-AGTGGCCATTGTTA CAGTTC-3′, antisense primer: 5′-CCAGAGAAAATGGT GAGTGG-3′). The transcription level of *PSY* was also investigated using juice sac tissues on the middle of November by qRT-PCR. These analyses were carried out under above described methods.

Results

Gene structures of PSY *homologues on the clementine mandarin genome*

In the clementine mandarin genome sequence, there are four homologues with high homology with *CitPSY* as follows: Ciclev10018150m.g, Ciclev10018272m.g, Ciclev10015582m.g and Ciclev10011841m.g. Ciclev10011841m.g is annotated as chloroplastic phytoene

synthase and is located from 21,390,477 bps to 21,396,087 bps in the scaffold 6, which was equivalent to *Gn0009* locus at 57.4 cM of linkage group 4 in AGI map (**Table 1**). Ciclev10018150m.g, Ciclev10018272m.g and Ciclev10015582m.g are annotated as squarene synthase, 15-*cis*-phytoene synthase, squarene/phytoene synthase, respectively and are located from 587,065 to 4,443,386 in the scaffold 2, which was equivalent to the position between Ks9005 and Lp0118 at 11.8–13.6 cM of linkage group 2 in AGI map. These three homologues were located tandemly in clementine mandarin genome and could be interpreted to derive from the same locus in the linkage map. The deduced amino acids from these homologues were aligned and their structures were compared (**Fig. 1**). *PSY* comprises two important functional domains of the chloroplastic transit peptide, which is essential for protein targeting to the plastid compartment (Giorio *et al.* 2008) and trans-isoprenyl diphosphate synthase (trans–IPP–HH) domain (Marchler-Bauer *et al.* 2009). In the deduced amino acids alignment of four clementine mandarin *PSY* homologues,

Table 1. Sequence similarity and location among Clementine *PSY* homologues and *PSY* alleles of A255 and G434

	Gene annotation	Homology in amino acid sequence (%)				Location in Clementine genome sequence			Location in AGI map		
		PSYa-1	*PSYa-2*	*PSYg-1*	*PSYg-2*	Scaffold	Start	End	Linkage group	DNA marker	Position
Ciclev10018150m.g	Squarene synthase	49.9	50.0	50.0	50.0	2	587,065	588,778	2	Ks9005/Lp0118	11.8–13.6 cM
Ciclev10018272m.g*	15-cis-phytoene synthase	34.1	34.2	34.2	33.6	2	593,780	594,691	2	Ks9005/Lp0118	11.8–13.6 cM
Ciclev10015582m.g	Squarene/phytoene synthase	44.2	45.0	45.0	44.4	2	4,441,285	4,443.39	2	Ks9005/Lp0118	11.8–13.6 cM
Ciclev10011841m.g	Chloroplastic phytoene synthase	84.7	85.1	85.9	87.0	6	21,390,477	21,396,087	4	Gn0009	57.4 cM

* Incomplete coding region.

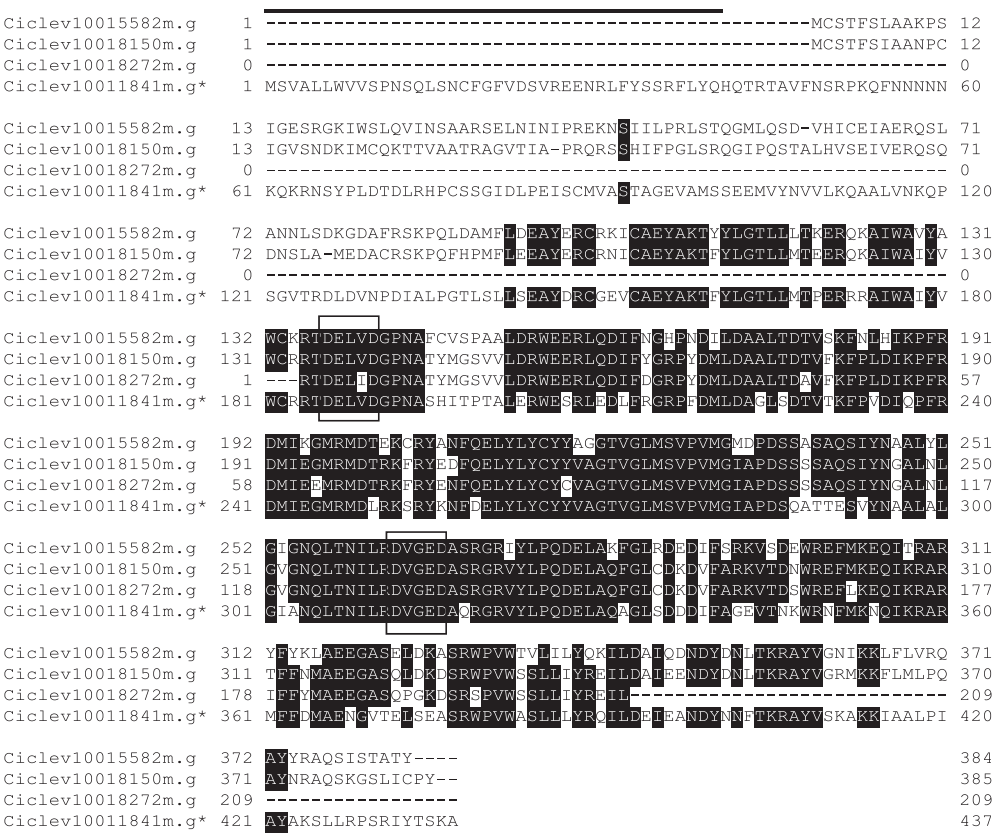

Fig. 1. Multiple alignment of deduced amino acids sequences of four clementine mandarin PSY homologues. Ciclev10011841m.g contains a predicted transient peptide at the N-terminal region (underlined in black) predicted by SignalP 4.1 Server. The putative active site (DXXXD) is boxed in black. The conserved amino acids are shown with black background and these conserved region among four *PSY* homologues is considered as trans-isoprenyl diphosphate synthase (trans–IPP–HH) domain (Marchler-Bauer *et al.* 2009). *: The ORF is properly re-predicted based on the structure of other citrus *PSYs* characterized by Sanger sequencing.

Ciclev10011841m.g possessed two important functional domains, while other three homologues possessed trans–IPP–HH domain but lacked putative transit peptide predicted by SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). In addition, Ciclev10018272m.g lacked N-terminal and C-terminal regions of trans–IPP–HH domain resulting to incomplete coding region. Among four clementine mandarin *PSY* homologues, their trans–IPP–HH domains were well conserved but other regions were divergent.

Phylogenetic analysis with the past reported citrus *PSYs* and *PSYs* in the AG population indicated that Ciclev10011841m.g clustered with them and revealed high homology with them (**Fig. 2**). In contrast, other three *PSY* homologues detected in clementine genome were not clustered with them.

Transcriptional changes of three PSYs *in various tissues of clementine mandarin*

To address the physiological role of clementine mandarin *PSY* homologues during the fruit development, RT-PCR was carried out using various tissues except for Ciclev10018272m.g owing to the incomplete gene structure (**Fig. 3**). The transcript of Ciclev10011841m.g was accumulated in all tissues of clementine mandarin and the transcription level was higher in flower, leave and peel. The transcript of Ciclev10015582m.g was accumulated in flower, young whole fruit at DAF30 and peels at DAF60, DAF120 and DAF180. The transcript of Ciclev10018150m.g was not detected in all tissues even under the 35 PCR cycles and the transcription level would like to be extremely low or none. These results were agreed with the fact that Ciclev10011841m.g would play the important roles in carotenoid biosynthesis of the fruit.

Allelic genotype and gene expression analyses for PSYs *in F_1 individuals of AG population*

In the AG population, there were four alleles with independent nucleotide sequences (*PSY-a1* and *PSY-a2* from A255, *PSY-g1* and *PSY-g2* from G434). Four *PSY* alleles in the AG population showed high identities in amino acid sequence ranging from 97.9% to 100% and the amino acid sequence of *PSYa-1* and *PSYg-1* was identical. The *PSY* sequence genotypes of the 48 F_1 individuals of AG population bearing the fruits were determined using the TaqMan allele discrimination system. The predicted genotypes of the F_1 individuals from the four parental alleles were *PSY-a1/PSY-g1*, *PSY-a2/PSY-g1*, *PSY-a1/PSY-g2* and *PSY-a2/PSY-g2*. The numbers of F_1 individuals with each genotype

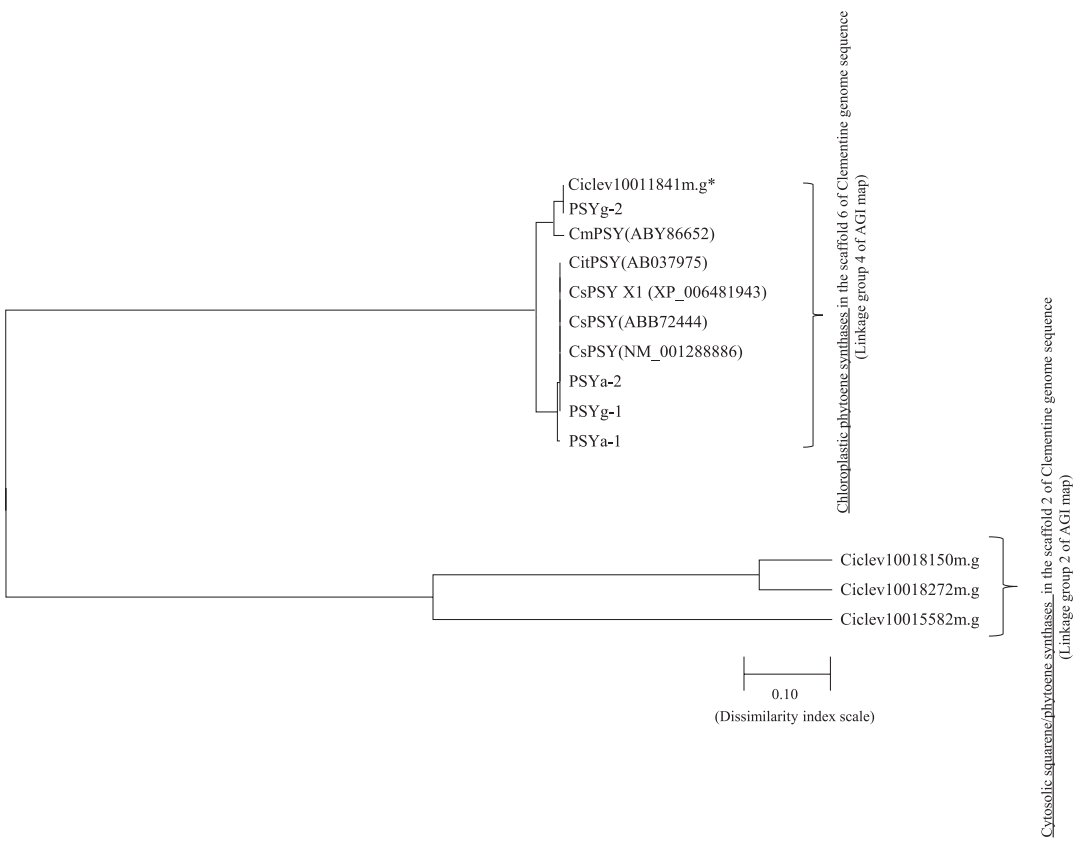

Fig. 2. Phylogenetic tree of citrus phytoene synthases. The tree is constructed using the unweighted pair group maximum average (UPGMA) method based on their amino acid sequences. *PSYs* of satsuma mandarin (*CitPSY*), sweet orange (*CsPSY*) and pummelo (*CmPSY*) are used as reference clones for citrus *PSY*. The examined citrus *PSYs* are divided into two groups of "Chrolopastic phytoene synthases" and "Cytosolic squarene/phytoene synthases". *: The ORF is properly re-predicted based on the structure of other citrus *PSYs* characterized by Sanger sequencing.

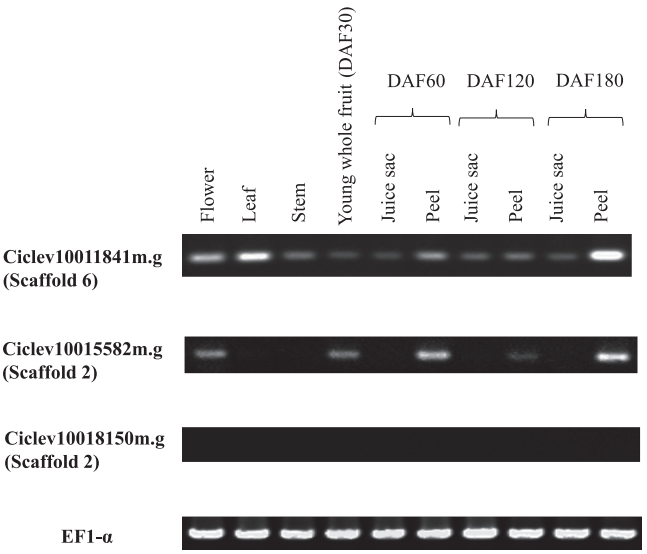

Fig. 3. Gene expression patterns of clementine mandarin *PSY* homologues in flower, leaf, stem, and young whole fruit at 30 days after flowering (DAF), and fruit tissues (juice sac and peel) from 60 to 180 DAF of clementine mandarin by RT-PCR. *EF1-α* is used as a endogenous control gene.

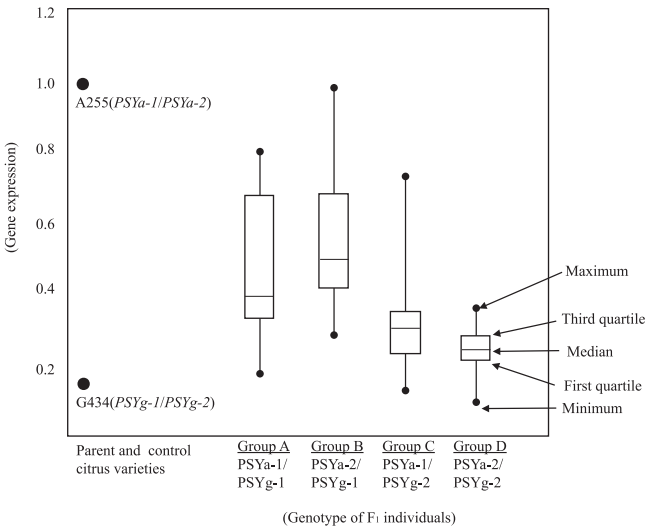

Fig. 4. Box and whisker plot of the distribution of *PSY* transcription levels in F_1 individuals of the AG population. The dots indicate the transcription levels in A255 and G434, having allelic genotypes of *PSYa-1/PSYa-2* and *PSYg-1/PSYg-2*, respectively.

were 13, 12, 13 and 10, respectively. The segregation ratio of these genotypes fitted to the expected Mendelian proportions of 1:1:1:1 in the Chi-square test at the 0.05% level, indicating that these four alleles were derived from a single locus.

The transcription level of *PSY* in the juice sac tissues on middle of November was investigated for 48 F_1 individuals using the TaqMan primers/probes reported by Kato *et al.* (2004) because quantification of the transcriptional amounts of each allele was difficult owing to the high similarity levels in the exon regions of the four alleles. The distribution of the transcription level in each genotype was described using box and whisker plots in **Fig. 4**. The transcription level of A255 with *PSY-a1* and *PSY-a2* was an average of 0.93 and that of G434 with *PSY-g1* and *PSY-g2* was an average of 0.22. The average expression level of F_1 individuals with *PSY-a2* and *PSY-g1* was 0.57, followed by *PSY-a1* and *PSY-g1* individuals with an average of 0.49, *PSY-a1* and *PSY-g2* individuals with an average of 0.35, and *PSY-a2* and *PSY-g2* individuals with an average of 0.26. Thus, F_1 individuals with *PSY-g2* tended to have low transcription level, indicating that *PSY* allelic combination likely influences the transcription level of *PSY*.

Promoter sequence of four PSY *alleles in the AG population*

To understand the structural differences in the promoter regions of four *PSY* alleles, approximately 1.2 kbps of the sequences were compared among four alleles. Their sequences were relatively conserved with the high identities ranging from 99.8% to 93.2%. Among the four alleles, nucleotide substitutions and the indel mutations were observed. The frequency of indel mutation was less than that of nucleotide substitution. Most observed indels were 6–7 nucleotides in length. A homology search using a plant *cis*-acting regulatory DNA element (PLACE, http://www.dna.affrc.go.jp/htdocs/PLACE/) database was carried out against the 1.2 kbps of promoter regions. The representative *cis*-acting regulatory motifs related to carotenoid metabolism are summarized in **Fig. 5**. There are various types of *cis*-regulatory motifs corresponding to light, plant hormones and water stress including GATABOX, which is found in many light-regulated genes (Teakle *et al.* 2002), WRKY710S, which is a transcriptional repressor of the gibberellin signaling pathway (Zhang *et al.* 2004), and ABREATRD22 (Iwasaki *et al.* 1995), DPBFCOREDCDC3 (Kim *et al.* 1997) and ABREZMRAB28 (Guan *et al.* 2000), which are related to abscisic acid (ABA) and dehydration stresses. In addition, several MYB-binding motifs were found in the promoter regions, such as MYBPLANT found in phenylpropanoid biosynthetic genes of *Antirrhinum majus* (Sablowski *et al.* 1994), MYBST1 in *Solanum tuberosum* (Baranowskij *et al.* 1994), and so on. Thus, various *cis*-regulatory motifs were scattered in their promoter regions. The organization of *cis*-regulatory motifs were different in a kind and a copy number among four alleles but the *cis*-regulatory motifs specific to *PSY-g2* were very limited. The structural differences specific to *PSY-g2* redundantly located in the promoter region from –59 bps to –350 bps. There were 15 single nucleotide substitutions and two indels in this region. Out of them, four mutations altered the *cis*-regulatory motifs. The single nucleotide substitution at approximately –340 bps generated a CPBCSPOR motif, which is a specific binding site of the cytokinin-dependent protein of the NADPH-protochlorophyllide oxidoreductase gene in cucumber (*Cucumis sativus* L.) (Fusada *et al.* 2005),

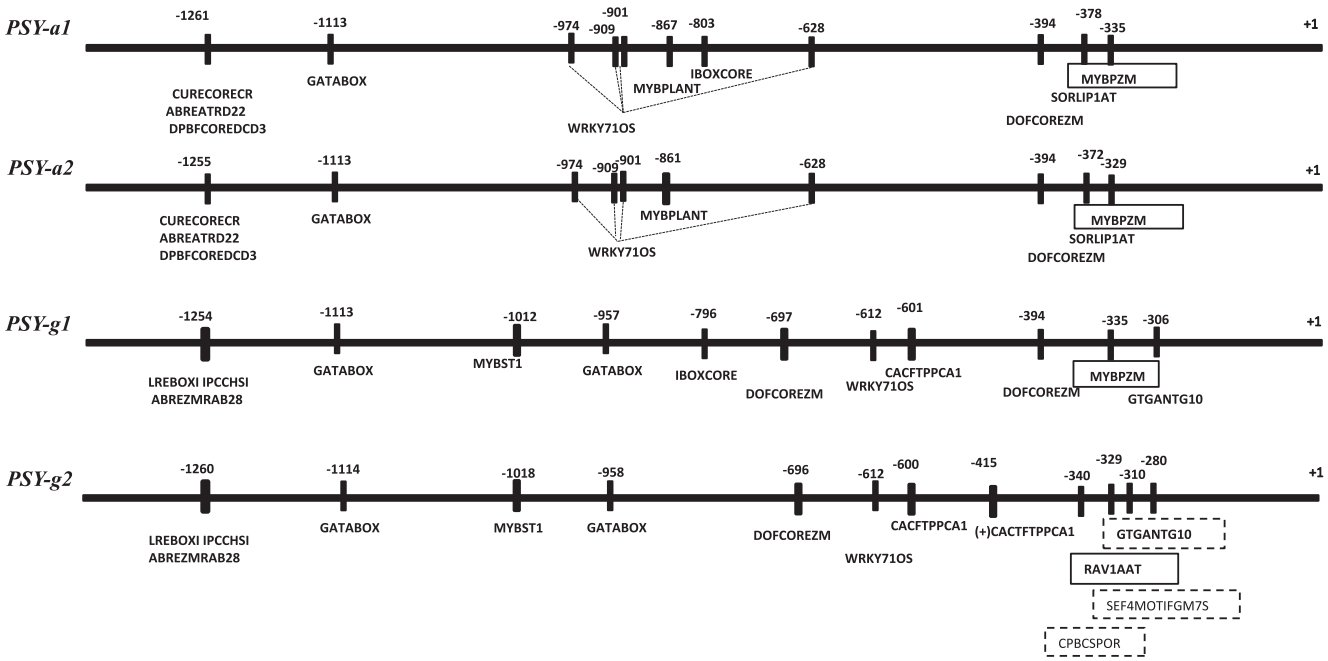

Fig. 5. Diagram of the regions upstream of the *PSY* genes (–1260 bp to +1 from the translational start site). Nucleotide positions are shown relative to the putative translational start site. The box indicated the candidate alteration of *cis*-regulatory motif responsible for low expression of *PSY* in F_1 individuals with *PSYg-2*. The dot box indicated the other *cis*-regulatory motifs which were specifically altered in *PSYg-2*.

while the single nucleotide substitution at approximately –310 bps generated a GTGANTGA motif that is responsible for the pectin lyase of pollen in *Nicotiana tabacum* (Rogers *et al.* 2001). The single nucleotide substitution at approximately –280 bps generated the SEF4MOTIFGM7S motif, which is responsible for the beta-conglycinin gene (Lessard *et al.* 1991). These *cis*-regulatory motifs would like to be not associated with low *PSY* expression because they generally work positively and some of them were located redundantly in the upstream regions of the other alleles. A 6-bps deletion in *PSY-g2* altered the *cis*-regulatory motif from MYBPZM (Grotewold *et al.* 1994) to RAV1AAT (Kagaya *et al.* 1999) at –329 bps. MYBPZM is a core *cis*-regulatory motif for Maize P gene, and is responsible for red pigmentation of the kernel pericarp and flavonoid biosynthesis (Grotewold *et al.* 1994). The RAV1AAT motif is a *cis*-regulatory motif of the RAV1 protein, which is uniquely found in plants. It is involved in the immediate physiological responses and developmental adaptations to environmental stimuli (Kagaya and Hattori 2009).

Genotype of MYBPZM and RAV1AAT motifs in the promoter region influences the transcription level of PSY

To clarify whether the alteration of *cis*-regulatory motif from MYBPZM to RAV1AAT would be major factor to influence the transcription level of *PSY*, the genotype of these motifs in the promoter region of *PSY* was investigated for ten ancestral varieties and two parent lines of AG mapping population. Sequence analysis revealed that most ancestral varieties and G434 have heterozygous genotype of MYBPZM and RAV1AAT motifs in the promoter region, while 'Mukaku kishu ', 'Ueda unshu' and A255 have homozygous genotype of MYBPZM motif (**Fig. 6A**). Except for 'Orlando', 'Sweet spring' and 'Lee', which of the fruits were not available, the transcription level of *PSY* gene in the juice sac tissues on the middle of November was investigated by qRT-PCR (**Fig. 6B**). Interestingly, the transcription level of *PSY* was higher in 'Mukaku kishu', 'Ueda unshu' and A255, which have homozygous genotype of MYBPZM motif in the promoter region of *PSY*. In contrast, the other varieties with heterozygous genotype of MYBPZM and RAV1AAT motifs showed lower transcription level of *PSY*. Thus, the positive relationship was observed between genotype of these motifs and the transcription level of *PSY* in the pedigree of AG mapping population, revealing 0.95 of Pearson's correlation coefficient.

Discussion

PSY regulates the beginning step of the carotenoid metabolic pathway and its expression significantly affects the amount of subsequently produced carotenoids. In *Arabidopsis* genome, *PSY* is a single gene located on chromosome 5, although many *PSY* mRNA sequence entries have been registered. In tomato and rice, two and three *PSYs* have been molecularly characterized and they played different roles and expression profiles during plant development and tissues-specific function or seasonal variation (Bartley and Scolnik 1993, Welsch *et al.* 2008). Based on the gene structure and expression pattern of *PSY* homologues in the clementine

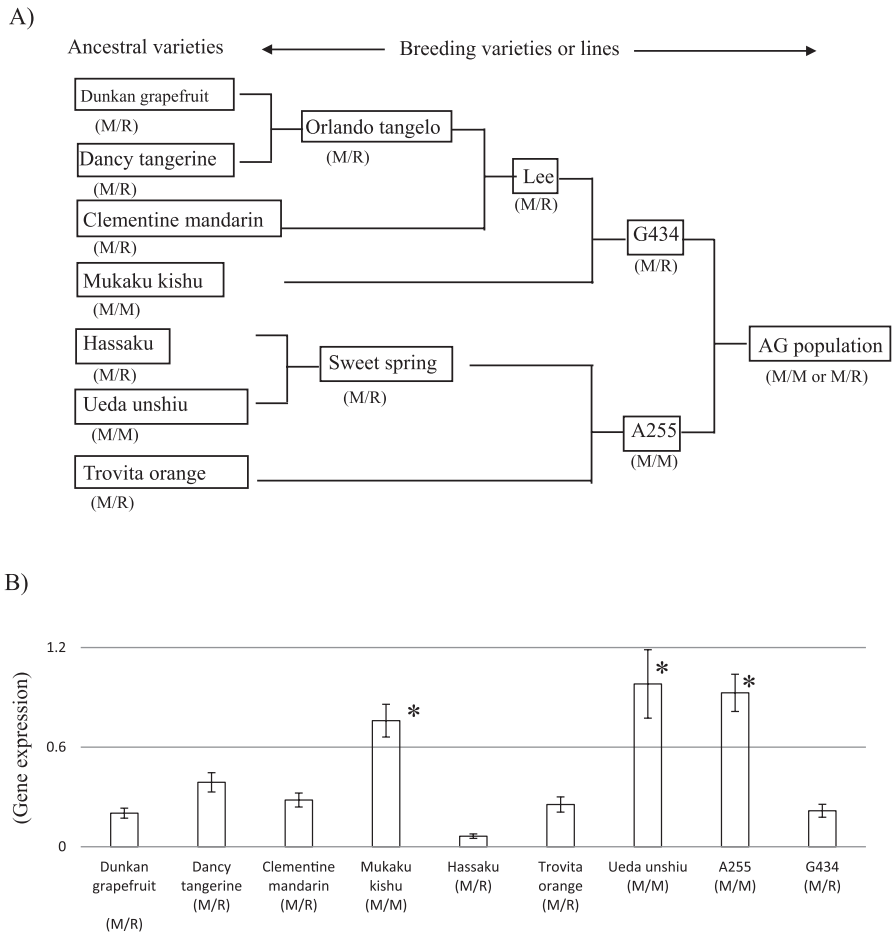

Fig. 6. Pedigree of AG mapping population and their genotypes of MYBPZB and RAV1AAT in the promoter region of *PSY* alleles (A). M/R indicates heterozygous genotypes of these motifs in the promoter region and M/M indicates homozygous genotypes of MYBPBZ motif in the promoter region. Transcription level of *PSY* in the juice sac tissues on the middle of November of seven ancestral varieties and two parent lines of AG mapping population (B). The transcription level of *PSY* in 'Mukaku kishu', 'Ueda unshiu' and A255 with M/M genotype, which are marked with an asterisk, show the significant differences at $P < 0.05$ against those of the other varieties and line with M/R genotype.

mandarin genome sequence, there are two loci for chloroplastic phytoene synthase and cytosolic squarene/phytoene synthase. The *PSY* locus in the scaffold 6, which is mapped by *PsyG-CT* marker on the linkage group 4 of the AGI map, is considered as a principal locus for carotenoid biosynthesis in citrus. The alternative locus comprises three tandem *PSY* homologues without putative transit peptide, which are members of cytosolic squarene/phytoene synthases, and might not contributed to carotenoid biosynthesis. In addition, it was confirmed that four *PSYs* in the AG population have high identities with the past reported citrus *PSYs* and they were derived from the single *PSY* locus in the scaffold 6 from the segregation analysis. Therefore, it is proposed that *PSYs* for carotenoid biosynthesis would be derived from a single locus with various sequence diversity working as multiallelism among citrus varieties.

The promoter structure was very similar among of the examined four *PSY* alleles were but it was found that the slight mutations could alter the organization of the *cis*-regulatory motifs, resulting to influence gene expression level of *PSY*. In a precise comparison of promoter region, we supposed that the motif substitution from MYBPZM to RAV1AAT might be associated with low expression level of *PSY* in AG progenies with *PSY-g2*. MYB family transcription factors are involved in regulating the expression of flavonoid and anthocyanin biosynthesis genes (Aharoni *et al.* 2001, Baudry *et al.* 2004). The loss of functional mutations in *Reduced Carotenoid Pigmentation 1* (*RCP1*), an R2R3 type of MYB transcription factor, lead to the downregulation of all carotenoid biosynthesis genes and a reduced carotenoid content in *Mimulus lewisii* flowers (Sagawa *et al.* 2016). In contrast, RAV1 acts as a negative regulator of ABA in seed germination and green seedling rates (Feng *et al.* 2014). In pepper and cotton, RAV1 increases the tolerance to drought and salt stress (Li *et al.* 2015, Sohn *et al.* 2006). Based on these reports, these two *cis*-regulatory motifs are likely involved in the transcriptional regulation of *PSY*. In the plant kingdom, the antioxidant antagonism between carotenoids and flavonoids is reported to avoid the functional redundancy in the evolutionary mechanism (Han

et al. 2012). In Solanaceae plants, common transcription factors regulating carotenoid and anthocyanin pathways were found to act as initial sensors against various environmental stimuli (Dhar *et al.* 2014). Recently, MYBPZM is reported as one of the candidate *cis*-regulatory motif to influence gene expression of capsanthin-capsorubin synthase, which catalyzes the conversion of antheraxanthin and violaxanthin into capsanthin and capsorubin responsible for red-orange coloration in Pepper (*Capsicum* sp.) (Zheng *et al.* 2013). In citrus, the genetic diversity of *ZEP* alleles among various citrus varieties was characterized and similar results were obtained that the transcription level of *ZEP-1m* allele with MYBPZM was higher than that of *ZEP-2m* allele without it (Sugiyama *et al.* 2010). Therefore, MYBPZM could be one of the important *cis*-regulatory motifs to influence the carotenoid pathway in citrus fruit. A further analysis is required to obtain the direct demonstration whether the lack of MYBPZM in the promoter region of *PSY-g2* is responsible for the low expression level of *PSY* by promoter assay.

Fancicullino *et al.* (2006) reported that the carotenoid composition and content varied among citrus varieties, rather than species, and the carotenoid diversity in cultivated citrus is highly influenced by genetic factors. Considering a single *PSY* locus in the citrus genome, the allelic combinations with different *cis*-regulatory motifs are one of the possible factors to cause a transcriptional variation of *PSY*. There have been a several reports that allelic differences in *PSY* play critical roles in the modulation of carotenogenesis. In wheat grain (*Triticum turgidum*), the allelic divergence of *PSY* may be responsible for the grain's yellow pigment content (Zhang and Dubcovsky 2008). In maize (*Zea mays*), sorghum (*Sorghum bicolor*) and rice (*Oryza sativa*), the three *PSY* genes have overlapping functions in modulating carotenogenesis in different tissues and in response to multiple developmental and/or stress signals (Gallagher *et al.* 2004, Li *et al.* 2008). Out of the two *PSY* alleles in maize, insertions in the *Y1* phytoene synthase gene's promoter increased the expression in endosperm and the carotenoid content of yellow maize (Palaisa *et al.* 2003). Thus, genetic variation within the *cis*-regulatory motifs could affect the transcription rate or tissue specificity of the associated allele and caused phenotypic differentiations through changes in gene expression. On one hand, transcription factors play essential roles controlling gene expression. Various endogenous and exogenous factors affecting carotenoid content and composition during fruit development have been characterized in citrus, such as plant hormones, temperature, light and nutritional factors (Alquézar *et al.* 2008), implicating that numerous transcription factors are involved in regulating carotenoid metabolism in response to environmental and endogenous factors. Therefore, the combination of these transcription factors derived from seven ancestral varieties would also promote the wide variation of the *PSY* transcription level in AG population.

In conclusion, the transcriptional diversity of *PSY* among citrus varieties is affected by *PSY* allelic combination derived from a single locus in the scaffold 6 of clementine genome sequence (linkage group 4 in the AGI map). The genomic sequences on the promoter region of the four *PSY* alleles were very similar at the nucleotide sequence level, but the alteration of *cis*-regulatory motifs would influence the gene expression level of *PSY*. Although the obtained new finding was limited on *PSY* locus in the AG population, it would be applied to the other carotenoid biosynthetic genes. Therefore, it is considered that the allelic diversity in carotenoid biosynthesis genes would be one of the explanations for wide variation in carotenoid composition and content among citrus breeding varieties. Allele mining of carotenoid biosynthetic genes is considered as a suitable approach to promote the molecular breeding for improvements of the carotenoids in citrus fruits through the genome editing and marker assisted selection. To promote the molecular breeding of a desirable carotenoid content in citrus, further research is required to understand the allelic diversity of all carotenoid biosynthesis genes among citrus breeding resources. Also, to characterize the transcription factors in various signaling networks related to carotenoid metabolism.

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