Structure and Expression Levels of Alleles of *Citrus* Zeaxanthin Epoxidase Genes

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The carotenoid metabolism enzyme zeaxanthin epoxidase (ZEP) is a key regulator of carotenoid accumulation in *Citrus* fruit. The expression level of the gene encoding this enzyme, *ZEP*, influences varietal differences in the conversion of zeaxanthin to violaxanthin during fruit development. To determine how this gene is regulated, we investigated the structure of its alleles and analyzed allele-specific *ZEP* expression in heterozygous *Citrus* fruit. Four independent genomic sequences were isolated from a BAC library of Satsuma mandarin (*Citrus unshiu* Marc.). All *ZEP* gene sequences in Satsuma mandarin consisted of 16 exons and 15 introns. Genetic analyses of hybrid populations and analyses of sequence variations identified that two of the sequences were alleles from the *ZEP-1/-2* locus, and others were from different locus/loci, *ZEP-3/-4*, for which allelism has not been confirmed. The *ZEP-4* allele was expressed in young-stage fruit, but not in mature-stage fruit, while *ZEP-1/-2* alleles were expressed as fruit matured. Our results showed that the newly identified isoform, *ZEP-4*, did not contribute to the accumulation of violaxanthin during fruit maturation. Expression levels of *ZEP-1/-2* alleles in the fruit of three heterozygous *Citrus* cultivars were compared using allele-specific RT-PCR. Transcripts of *ZEP-1* alleles were more abundant than those of *ZEP-2* alleles in all three cultivars. Sequence diversity among the 5' UTRs of *ZEP* alleles was also analyzed. The implications of sequence diversity with respect to diversity of the expression and phylogenetic relationships among *ZEP* genes in *Citrus* are discussed.

Key Words: alleles-specific transcript ratio, carotenoid, *cis*-element, fruit maturation, isoform.

Introduction

Altering the accumulation and composition of carotenoids in *Citrus* fruit is one of the major objectives of *Citrus* breeding programs. Carotenoids determine fruit color, and have roles as bio-functional compounds, for example, vitamin A and various compounds with anti-cancer activity (Nishino et al., 2000; Olson, 1989).

Carotenoid content and composition in *Citrus* fruit varies greatly among varieties, and depends on growing conditions (Gross, 1987; Ikoma et al., 2001; Kato et al., 2004). Satsuma mandarin (*Citrus unshiu* Marc.) and other mandarins accumulate β -cryptoxanthin predominantly in the flavedo and juice sacs in mature fruit (Goodner et al., 2001; Ikoma et al., 2001). In contrast, sweet orange (*Citrus sinensis* Osbeck) fruit accumulates violaxanthins, predominantly 9-*cis*-violaxanthin (Lee

and Castle, 2001; Molnár and Szabolcs, 1980).

Understanding how carotenoid metabolism is regulated in fruit is key to increasing carotenoid content or altering its composition. Therefore, carotenoid biosynthesis and its regulation have been studied extensively in *Citrus*. Recently, expression profiles of genes encoding various carotenoid biosynthetic enzymes in *Citrus* fruit have been reported. Phytoene synthase (PSY) and β -ring hydroxylase (HYb) are the major enzymes in the metabolic pathway. Transcript levels of *PSY* increased in the peel and juice sacs at the onset of rind coloration in Satsuma mandarin (Ikoma et al., 2001; Kim et al., 2001).

Kato et al. (2004) compared the expression level of genes affecting carotenoid content between Satsuma mandarin and 'Valencia' orange during fruit maturation. Their results showed that simultaneous increases in expression levels of *PSY*, phytoene desaturase (*PDS*), zeta-carotene desaturase (*ZDS*), lycopene beta-cyclase (*Lcyb*), and zeaxanthin epoxidase (*ZEP*) were correlated

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with the massive accumulation of β,β -xanthophyll (β -cryptoxanthin, zeaxanthin, and violaxanthin) in both the flavedo and juice sacs. In addition, accumulation of phytoene was initiated along with a decrease in the transcript level of *PDS* in the flavedo of Satsuma mandarin. Together, these results suggested that types and levels of carotenoids are highly regulated by the expression of carotenoid biosynthetic genes during fruit maturation (Kato et al., 2004).

Carotenoid metabolism has been investigated at the gene expression level in many plant species aside from *Citrus*, such as *Arabidopsis* (Park et al., 2002; Pogson et al., 1996), tomato (*Solanum lycopersicum*; Fraser et al., 1994; Giuliano et al., 1993; Isaacson et al., 2002; Ronen et al., 1999), pepper (*Capsicum annuum*; Bouvier et al., 1996, 1998), and tobacco (*Nicotiana tabacum*; Busch et al., 2002); however, only a few studies have focused on the regulation of carotenoid metabolism genes. Welsch et al. (2003) reported that, in *Arabidopsis*, there are two main regions in the *PSY* promoter that regulate transcriptional activity. They also discovered a transcription factor gene, *RAP2.2*, which interacts with the promoter motifs found in both *PSY* and *PDS* (Welsch et al., 2008). These analyses suggested that diversity among *cis*-elements as well as transcription factor genes would contribute to the genotype-dependent expression of genes involved in carotenogenesis.

Whereas some plants are genetically homozygous, such as *Arabidopsis* and rice, *Citrus* cultivars are usually highly heterozygous at many EST loci (Omura et al., 2000); therefore, the transcript levels in fruit represent the total expression from all alleles. For example, allele-specific expression profiles of the UDP-glucose: limonoid glucosyltransferase gene have been determined during fruit development in heterozygous Satsuma mandarin (Kita et al., 2003).

Here, to understand the role of *cis*-elements in expression, we investigated the allele-specific expression of *ZEP* in *Citrus* cultivars. *ZEP* catalyzes the conversion of zeaxanthin to violaxanthin in the carotenoid pathway, and possibly influences the accumulation of violaxanthin, which accumulates at high levels in fruit of *C. sinensis* but low levels in *C. unshiu* and 'Kiyomi' tangor (*C. unshiu* $\times$ *C. sinensis*).

Differences in the genomic structure of alleles associated with the expression level could be utilized to generate functionally defined DNA markers, which could be employed in *Citrus* breeding programs to alter carotenoid content. We investigated the genomic structure of *ZEP* genes, and examined diversity among alleles and its effects on gene expression in *Citrus*. To confirm the genetic background of allelism in *ZEP* genes, we analyzed gene sequences and expressions in three *Citrus* cultivars; 'Miyagawa wase' (*C. unshiu*) and 'Trovita' orange (*C. sinensis*), which differ in their carotenoid accumulation profile and in the expression profiles of carotenoid metabolism genes, and their hybrid

'Kiyomi' tangor accumulates violaxanthin at intermediate levels of the parents.

Materials and Methods

Plant materials

'Miyagawa wase' Satsuma mandarin (*C. unshiu* Marc.), 'Trovita' orange (*C. sinensis* Osbeck), and 'Kiyomi' tangor (*C. unshiu* $\times$ *C. sinensis*) were used for genomic sequence and expression analyses. The cultivars shown in Figure 1B were also used to analyze the polymorphism of the *ZEP* gene among cultivars. All cultivars used in this study were cultivated at the National Institute of Fruit Tree Science (NIFTS), Okitsu (Shizuoka, Japan). For segregation analyses, we also used hybrids between 'Kiyomi' tangor $\times$ 'Miyagawa wase', 'Jutaro unshiu' (*C. unshiu* Marc.) $\times$ 'Trovita' orange, 'Kiyomi' tangor $\times$ 'Clementine' (*C. clementina* hort. ex Tanaka), and 'Hirakisuyu' (*C. kinokuni* hort. ex Tanaka) $\times$ 'Miyagawa wase'. Genomic DNA was isolated from leaves of the cultivars and hybrids using a Nucleon Phytopure extraction kit (GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, England). For expression analyses, in harvested fruits juice sacs were separated from rind, and juice sacs were immediately frozen in liquid nitrogen and kept at -80°C until RNA extraction.

Isolation and sequence analysis of *ZEP*

The cDNA sequence of the *ZEP* gene in *Citrus* (DDBJ accession number AB075547) was used to design the following primer pair: Forward, 5'-TTTATTGGGCCA CAGGAAGC; Reverse, 5'-TCCCCAAGCAAAGTAAC ACGA. These primers were used to genotype and screen the BAC library derived from 'Miyagawa wase' (*C. unshiu*) (Shimada et al., 2005) by PCR, with DNAs isolated from BAC clones using a QIAGEN Large-Construct Kit (Qiagen, Hilden, Germany). An insert from BAC screening was sequenced by primer walking, and primer sets designed from this sequence (Table 1) were used for primer walking of genomic DNAs of *Citrus* cultivars.

For genomic DNA and BAC DNA, PCR amplification was performed using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) protocols at an annealing temperature of 56°C for 35 cycles. Amplified PCR products were cloned into a pGEM[®]-T Easy vector (Promega, WI, USA), transformed into *Escherichia coli* strain XL-1 Blue, and sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Genetyx-Win ver. 8 (Software Development Co., Tokyo, Japan) and ATGC ver. 3.0.3 (Software Development Co.) were used for alignment analysis. A phylogenetic tree was constructed using MEGA4 Build#: 4026 (<http://evolgen.biol.metro-u.ac.jp/MEGA/>) by the NJ method.

Fig. 1. PCR amplification patterns of *Citrus* cultivars and hybrid populations using ZEP primers. (A) Amplification pattern of each ZEP. These three ZEP PCR fragments were derived from each 'Miyagawa wase' BAC clone. The amplicon sizes of ZEP-1, ZEP-2 and ZEP-3 are 887 bp, 1166 bp and 1396 bp, respectively. PCR fragment in right lane was derived from 'Miyagawa wase' genome. (B) *Citrus* cultivars, from left to right: Kumquat, Citron, Satsuma mandarin ('Miyagawa wase', Mg), 'Trovita' orange (Tr), 'Kiyomi' tangor (Km), Tachibana, Koji, 'Hirakisyu' (Ky), 'Mato' pumello, Kunenbo, 'King' mandarin, 'Ohta' ponkan, 'Dancy' tangerin, Mediterranean mandarin, 'Clementine' (Cl), 'Seminor', 'Seiho' (Sh), 'Akemi'. All cultivars could be classified as ZEP-1 type, ZEP-2 type and ZEP-1/-2 type. As an example, 'Seiho' is ZEP-1 type, 'Hirakisyu' and 'Clementine' are ZEP-2 type, and ZEP-1/-2 type is 'Miyagawa wase', 'Trovita' orange, and 'Kiyomi' tangor. (C) Segregation of fragment patterns in 'Kiyomi' (left), 'Miyagawa wase', and their progeny. Numbers in parentheses indicate segregation in the population analyzed. (D) Segregation of fragment patterns in 'Kiyomi' (left), 'Clementine', and their progeny. Numbers in parentheses indicate segregation in the population analyzed.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from juice sacs separated from fruits sampled at 30, 60, 120, and 180 days after flowering (DAF) from 'Miyagawa wase' and 'Washington' navel orange (*C. sinensis*). Total RNA was extracted as described by Ikoma et al. (1996) and purified

with an RNeasy Minikit (Qiagen) with on-column DNase digestion. First-strand cDNAs were synthesized from 5 µg total RNA by reverse transcriptase with Oligo-(dT) primers using a Ready-To-Go You-Primed First Strand Kit (GE Healthcare). The ZEP-1/-2 sequence was used to design primers (Forward, 5'-AGATTTTGTTCGGCT GATAT; Reverse, 5'-CCGAGTCCCCAAGCAA), not to

Table 1. Primers used for direct sequencing and RT-PCR expression analyses, locus-specific TaqMan MGB probes, and allele-discriminating TaqMan probes/primers for *Citrus ZEP* genes.

Purpose of primers	Intended locus/alleles	Primer and probe sequence (5' to 3')	Orientation
Sequencing	<i>ZEP-1/-2</i> and <i>ZEP-3/-4</i>	TTTATTTGGGCCACAGGAAGC	Sense read
		TCCCCAAGCAAAGTAACACGA	Antisense read
		AAGGGCGAAGGTATAGGGTAT	Sense read
		AGAGCTAGGAGACTGCGAGTG	Sense read
		TTGGCTGCTAAAAGGAAAGGA	Sense read
		CCCTATACCTTCGCCCTTCAT	Antisense read
	<i>ZEP-1/-2</i>	AAGAGTAATTAGCCGAATG	Sense read
		GTTCCTTGTAATGCGTACC	Antisense read
		GCAGAGCGAACATGGAACCTA	Sense read
		GGAGTCCGATCACCTTAACC	Antisense read
		GATACTTTCACCTCTGCAGCAGAA	Sense read
		CTTCGGGGTCATCCACTCCA	Antisense read
	<i>ZEP-3/-4</i>	TAAGTGTAGGACTTGGACCAC	Sense read
		GCTATTGGATGGACGAA	Antisense read
Allele-specific RT-PCRs	<i>ZEP-1m/-2m</i> of 'Miyagawa wase'	TCGAGTGTTTCTAGGACACAAACAGT	Sense read
		TTTCAGCAACCTTTCCTTTTACC	Antisense read
		AGTGGATG <u>AC</u> CCCCGAA	TaqMan MGB probe (FAM probe)
		AGTGGATG <u>GC</u> CCCCGA	TaqMan MGB probe (VIC probe)
	<i>ZEP-1o/-2o</i> or <i>-2m</i> of 'Trovita' orange and 'Kiyomi' tangor	GGGCTGGAAAAATGCAGTGGA	Sense read
		GGCCATCCCACTCCACCTG	Antisense read
		CTGGTTCCTTGTTAAAT	TaqMan MGB probe (FAM probe)
		CTGGTTCCTTGTTAAAT	TaqMan MGB probe (VIC probe)
<i>ZEP-1/-2</i> locus specific RT-PCR	<i>ZEP-1/-2</i> of all cultivars	TTCAGCGCAGGTGTCCAA	Sense read
		AATCAGGTAGAAGGCACCATCTTT	Antisense read
		ATGCATGCTCGTATCAG	TaqMan MGB probe

amplify *ZEP-3/-4*; in contrast, the *ZEP-3/-4* sequence was used to design primers (Forward, 5'-TAAGTGTAGG ACTTGGACCAC; Reverse, 5'-GCTATTGGATGGAC GAA), not to amplify *ZEP-1/-2*, and RT-PCR was conducted using the AmpliTaq Gold (Applied Biosystems) protocol in a 20 µL reaction volume for 25 PCR cycles. The PCR reaction mixtures were separated by electrophoresis on a 1.5% agarose gel and stained with EtBr solution. Amplified fragments were detected on a UV trans-illuminator.

Quantification of expression for allele-specific transcripts using TaqMan allele discrimination probe/primer system

Total RNA was extracted from 'Miyagawa wase', 'Trovita' orange, and 'Kiyomi' tangor fruit harvested in November 2005, January 2006, and January 2008. Total RNA (0.2 µg) was used to synthesize cDNAs with random hexamers at 37°C for 60 min using TaqMan reverse transcription reagents (Applied Biosystems).

First, the relative quantity of total *ZEP* expression was evaluated using a TaqMan MGB probe with a set of primers/probe. We used Primer Express software (Applied Biosystems) to design primers from common sequences in *ZEP* cDNA among the three *Citrus* varieties. The primers were as follows; Forward, 5'-

TTCAGCGCAGGTGTCCAA; Reverse, 5'-AATCAGGT AGAAGGCACCATCTTT; and the MGB-Probe, 5'-ATGCATGCTCGTATCAG. TaqMan Ribosomal RNA Control Reagents VIC Probe (Applied Biosystems) was used as an endogenous control. Each reaction contained 900 nM primers, 250 nM TaqMan MGB Probe, and 2.5 µL template cDNA. 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. The levels of gene expression were analyzed with ABI PRISM 7300 Sequence Detection System Software (Applied Biosystems) and relative quantities of *ZEP* expression were normalized against the expression of 18S ribosomal RNA.

Next, Primer Express software (Applied Biosystems) was used to design TaqMan MGB probe sets labeled with VIC or FAM to discriminate single nucleotide polymorphisms (SNPs) among *ZEP* alleles in 'Miyagawa wase' and 'Trovita' orange. To evaluate the ratio of the expression of *ZEP* alleles, TaqMan allele discrimination PCR was carried out using the TaqMan Universal PCR Master Mix (Applied Biosystems) and an ABI PRISM 7300 (Applied Biosystems). Each reaction mixture contained 900 nM primers, 200 nM TaqMan FAM or VIC probes, and 2 µL template cDNA. The thermal cycling conditions were the same as described for the total *ZEP* expression RT-PCR assay. The relative

fluorescence levels of VIC and FAM were calibrated against the cDNA ratio. A calibration curve for each SNP detection probe was constructed as described by Katsumoto et al. (2005). For all alleles with an SNP site, partial *ZEP* cDNA sequences of the same length were cloned and purified. Two concentrations of cDNA, 10^{-5} ng· μ L $^{-1}$ and 10^{-6} ng· μ L $^{-1}$, were prepared for each clone. Then, homozygous samples were mixed to obtain mixtures with the following allele ratios: 10 : 0, 8 : 2, 6 : 4, 4 : 6, 2 : 8, and 0 : 10. Allele-specific expression ratios were normalized against each calibration curve. Real-time quantitative RT-PCR was performed for three replicates for each sample. Mean values $\pm$ SD ($n=3$) are shown. For ‘Kiyomi’ tangor, probes and sets of primers were the same as those used for ‘Trovita’ orange.

Results

1. Analysis of genome sequences of *ZEP* genes in *Citrus*

1) Three types of *ZEP* gene sequences in ‘Miyagawa wase’

A set of primers was designed from the conserved region of previously published *ZEP* cDNA sequences (accession numbers AB114654 and AB075547). These primers produced three PCR fragments from ‘Miyagawa wase’ genomic DNA. The 887 bp, 1166 bp, and 1396 bp fragments were designated as *ZEP*-1, *ZEP*-2 and *ZEP*-3, respectively (Fig. 1A). As shown in Figure 1B, the *ZEP*-3 fragment was amplified from all cultivars, but *ZEP*-1 and *ZEP*-2 were not present in all citrus cultivars. Cultivars were classed as *ZEP*-1 type, *ZEP*-2 type, or *ZEP*-1/-2 type on the basis of the presence or absence of *ZEP*-1 and *ZEP*-2. For example, *ZEP*-2 was present and *ZEP*-1 was absent in ‘Clementine’ (*C. clementina*) and ‘Hirakisyu’ (*C. kinokuni*); hence, they were classed as *ZEP*-2 types. The *ZEP*-1 type ‘Seiho’ (a hybrid of ‘Kiyomi’ $\times$ ‘Mineola’) contained *ZEP*-1 but lacked *ZEP*-2. To determine whether the fragments were derived from the same locus, we examined segregation in hybrid progenies derived from four different crosses. Progeny derived from ‘Kiyomi’ tangor (*ZEP*-1/-2) and ‘Miyagawa wase’ (*ZEP*-1/-2), segregated into three types: 4 individuals had *ZEP*-1 only; 6 had *ZEP*-2 only, and 11 had both *ZEP*-1 and *ZEP*-2 (Fig. 1C). Hybrid progeny derived from ‘Kiyomi’ tangor (*ZEP*-1/-2) and

‘Clementine’ (*ZEP*-2) segregated into *ZEP*-1/-2 and *ZEP*-2 types at 12 : 9 (Fig. 1D). Progeny derived from the other two crosses was also segregated into *ZEP*-1/-2 and *ZEP*-2 or *ZEP*-1, *ZEP*-1/-2 and *ZEP*-2 groups (data not shown).

These segregation patterns suggest that *ZEP*-1 and *ZEP*-2 are amplification products derived from alleles at one locus (designated as *ZEP*-1/-2) and that because *ZEP*-3 amplification products are detected in all progeny, *ZEP*-3 fragments are derived from another locus in the *Citrus* genome.

To confirm whether the PCR fragments were derived from *ZEP* genes in the ‘Miyagawa wase’ genome, BAC clones containing the *ZEP* gene were screened by PCR with the same primer pairs used for the ‘Miyagawa wase’ genomic PCR. Of the six PCR-positive clones, two contained *ZEP*-1 (BAC113-9G and BAC131-6G), one contained *ZEP*-2 (BAC150-3F), and two contained *ZEP*-3 (BAC180-4G and BAC198-3A). In addition, one PCR-positive clone was obtained but the fragment size was approximately 2000 bp (BAC212-6B); these clones were used for sequence analyses. To distinguish these genomic sequences from those of other *Citrus* cultivars, *ZEP*-1, *ZEP*-2, and *ZEP*-3 from ‘Miyagawa wase’ BAC clones were designated as *ZEP*-1m, *ZEP*-2m, and *ZEP*-3m, respectively.

The BAC clones were sequenced by primer extension from the *ZEP* genomic region and read over 7995 bp for *ZEP*-1m (AB548570) and 8274 bp for *ZEP*-2m (AB548571), which fully covered the *ZEP* gene coding regions. The predicted coding sequences of *ZEP*-1m and *ZEP*-2m were identical to previously published cDNA sequences of *Citrus* (AB075547 and AB114654, respectively).

Like the *Arabidopsis* *ZEP* gene, the genomic sequences of *ZEP*-1m and *ZEP*-2m consisted of 16 exons and 15 introns (Fig. 2). The coding region of *ZEP*-1m had high sequence identity with that of *ZEP*-2m (>98% in deduced amino acids). The main difference between *ZEP*-1m and *ZEP*-2m was in the 10th exon, where a frameshift caused by an indel variation resulted in a different amino acid sequence in *ZEP*-1m (Fig. 3).

Sequence analyses showed a low level of nucleotide substitution among the coding sequences. We detected

Fig. 2. Genome structure of *ZEP* alleles of ‘Miyagawa wase’. Boxes show exons predicted by cDNA sequence alignment. Numbers shown at or between boxes indicate lengths of exons/introns. Numbers in parentheses indicate percentage identity compared with *ZEP*-1m sequence.

ZEP-1m	1: MVSSHFVNSVNLSTAVFSRTHFPVVPVYKHSICIEFSRYDHCINVKFRGTGTSQSKNPTQMK	60
ZEP-1o	1: MVSSHFVNSVNLSTAVFSRTHFPVVPVYKHSICIEFSRYDHCINVKFRGTGTSQSKNPTQMK	60
ZEP-2m	1: MVSSHFVNSVNLSTAVFSRTHFPVVPVYKHSICIEFSRYDHCINVKFRGTGTSQSKNPTQMK	60
ZEP-2o	1: MVSSHFVNSVNLSTAVFSRTHFPVVPVYKHSICIEFSRYDHCINVKFRGTGTSQSKNPTQMK	60
ZEP-3m	1: NASTHFVNSVNPSTAFSPRTHFPVVPVYKHSICIEFSRYDHCINCKFRTRITGSRNLQYK	60
ZEP-4m	1: NASTHFVNSVNPSTAFSPRTHFPVVPVYKHSICIEFSRYDHCINCKFRTRITGSRNLQYK	60

ZEP-1m	61: AAVAESPTNNSDENKKLRILVAGGGIGGLVFLAALKRKGFEVLVFEKDHSAIRGEQYR	120
ZEP-1o	61: AAVAESPTNNSDENKKLRILVAGGGIGGLVFLAALKRKGFEVLVFEKDHSAIRGEQYR	120
ZEP-2m	61: AAVAESPTNNSDENKKLRILVAGGGIGGLVFLAALKRKGFEVLVFEKDHSAIRGEQYR	120
ZEP-2o	61: AAVAESPTNNSDENKKLRILVAGGGIGGLVFLAALKRKGFEVLVFEKDHSAIRGEQYR	120
ZEP-3m	61: AAVAESLTNNGDSEKKLRILVAGGGIGGLVFLAALKRKGFEVLVFEKDHSAIRGEGLYR	120
ZEP-4m	61: AAVAESLTNNGDSEKKLRILVAGGGIGGLVFLAALKRKGFEVLVFEKDHSAIRGEGLYR	120

ZEP-1m	121: GPIQIQSNALAAEIDLVAEEVHRAGCVTGRINGLVDSIGSUYIKFDTFTPAAEKG	180
ZEP-1o	121: GPIQIQSNALAAEIDLVAEEVHRAGCVTGRINGLVDSIGSUYIKFDTFTPAAEKG	180
ZEP-2m	121: GPIQIQSNALAAEIDLVAEEVHRAGCVTGRINGLVDSIGSUYIKFDTFTPAAEKG	180
ZEP-2o	121: GPIQIQSNALAAEIDLVAEEVHRAGCVTGRINGLVDSIGSUYIKFDTFTPAAEKG	180
ZEP-3m	121: GPIQIQSNALAAEIDLVAEEVHRAGCVTGRINGLVDSIGSUYIKFDTFTPAAEKG	180
ZEP-4m	121: GPIQIQSNALAAEIDLVAEEVHRAGCVTGRINGLVDSIGSUYIKFDTFTPAAEKG	180

ZEP-1m	181: LPVTRVISRNTLOQLAKAVGDEIILNESNVDFDKHGDVSVVLENGQCYAGDLLVGAD	240
ZEP-1o	181: LPVTRVISRNTLOQLAKAVGDEIILNESNVDFDKHGDVSVVLENGQCYAGDLLVGAD	240
ZEP-2m	181: LPVTRVISRNTLOQLAKAVGDEIILNESNVDFDKHGDVSVVLENGQCYAGDLLVGAD	240
ZEP-2o	181: LPVTRVISRNTLOQLAKAVGDEIILNESNVDFDKHGDVSVVLENGQCYAGDLLVGAD	240
ZEP-3m	181: LPVTRVISRNTLOQLAKAVGDEIILNESNVDFDKHGDVSVVLENGQCYAGDLLVGAD	240
ZEP-4m	181: LPVTRVISRNTLOQLAKAVGDEIILNESNVDFDKHGDVSVVLENGQCYAGDLLVGAD	240

Exon 2		
ZEP-1m	241: GUSKVRKNLFGPQEAISVGYTCYTGTADVPADIESVGYRVFLGHKKYFVSSDVGAGKM	300
ZEP-1o	241: GUSKVRKNLFGPQEAISVGYTCYTGTADVPADIESVGYRVFLGHKKYFVSSDVGAGKM	300
ZEP-2m	241: GUSKVRKNLFGPQEAISVGYTCYTGTADVPADIESVGYRVFLGHKKYFVSSDVGAGKM	300
ZEP-2o	241: GUSKVRKNLFGPQEAISVGYTCYTGTADVPADIESVGYRVFLGHKKYFVSSDVGAGKM	300
ZEP-3m	238: GUSKVRKNLFGPQEAISVGYTCYTGTADVPADIESVGYRVFLGHKKYFVSSDVGAGKM	297
ZEP-4m	241: GUSKVRKNLFGPQEAISVGYTCYTGTADVPADIESVGYRVFLGHKKYFVSSDVGAGKM	300

ZEP-1m	301: QWYAFHKEPAGGVDPGEGKERLLIFEGGCDNVVDLILATDEEAILRRDIYDRPTIFTT	360
ZEP-1o	301: QWYAFHKEPAGGVDPGEGKERLLIFEGGCDNVVDLILATDEEAILRRDIYDRPTIFTT	360
ZEP-2m	301: QWYAFHKEPAGGVDPGEGKERLLIFEGGCDNVVDLILATDEEAILRRDIYDRPTIFTT	360
ZEP-2o	301: QWYAFHKEPAGGVDPGEGKERLLIFEGGCDNVVDLILATDEEAILRRDIYDRPTIFTT	360
ZEP-3m	298: QWYAFHKEPAGGVDPGEGKERLLIFEGGCDNVVDLILATDEEAILRRDIYDRPTIFTT	357
ZEP-4m	301: QWYAFHKEPAGGVDPGEGKERLLIFEGGCDNVVDLILATDEEAILRRDIYDRPTIFTT	360

ZEP-1m	361: GRGRVTLGDSVHMQPNLGGGGCHATEDGQVLALEKACKSKNESKPTIDVSAKSY	420
ZEP-1o	361: GRGRVTLGDSVHMQPNLGGGGCHATEDGQVLALEKACKSKNESKPTIDVSAKSY	420
ZEP-2m	361: GRGRVTLGDSVHMQPNLGGGGCHATEDGQVLALEKACKSKNESKPTIDVSAKSY	420
ZEP-2o	361: GRGRVTLGDSVHMQPNLGGGGCHATEDGQVLALEKACKSKNESKPTIDVSAKSY	420
ZEP-3m	358: GRGRVTLGDSVHMQPNLGGGGCHATEDGQVLALEKACKSKNESKPTIDVSAKSY	417
ZEP-4m	361: GRGRVTLGDSVHMQPNLGGGGCHATEDGQVLALEKACKSKNESKPTIDVSAKSY	420

ZEP-1m	421: ERARLRVAVIHGLARSAVMASTYKAYLGVLGPLSFLTKFRIPHPGRVGRFFIDLAM	480
ZEP-1o	421: ERARLRVAVIHGLARSAVMASTYKAYLGVLGPLSFLTKFRIPHPGRVGRFFIDLAM	480
ZEP-2m	421: ERARLRVAVIHGLARSAVMASTYKAYLGVLGPLSFLTKFRIPHPGRVGRFFIDLAM	480
ZEP-2o	421: ERARLRVAVIHGLARSAVMASTYKAYLGVLGPLSFLTKFRIPHPGRVGRFFIDLAM	480
ZEP-3m	418: ERARLRVAVIHGLARSAVMASTYKAYLGVLGPLSFLTKFRIPHPGRVGRFFIDLAM	477
ZEP-4m	421: ERARLRVAVIHGLARSAVMASTYKAYLGVLGPLSFLTKFRIPHPGRVGRFFIDLAM	480

ZEP-1m	481: PLMLSVVLGNSSKLEGRSPCKLSDKASDNLRTVFRDDALERAMNGEVLVPSGSENV	540
ZEP-1o	481: PLMLSVVLGNSSKLEGRSPCKLSDKASDNLRTVFRDDALERAMNGEVLVPSGSENV	540
ZEP-2m	481: PLMLSVVLGNSSKLKGVRHVASSTKASDNLRTVFRDDALERAMNGEVLVPSGSENV	540
ZEP-2o	481: PLMLSVVLGNSSKLKGVRHVASSTKASDNLRTVFRDDALERAMNGEVLVPSGSENV	540
ZEP-3m	478: PLMLSVVLGNSSKLEGRSPCKLSDKASDNLRTVFRDDALERAMNGEVLVPSGSENV	520
ZEP-4m	481: PLMLSVVLGNSSKLEGRSPCKLSDKASDNLRTVFRDDALERAMNGEVLVPSGSENV	540

Exon 10		
ZEP-1m	541: VSQPIYLSVSHNEPFIYLGSESHDFPRTSIVIPSAQVSKNHARISYKDGAFYLDLQSE	600
ZEP-1o	541: VSQPIYLSVSHNEPFIYLGSESHDFPRTSIVIPSAQVSKNHARISYKDGAFYLDLQSE	600
ZEP-2m	541: VSQPIYLSVSHNEPFIYLGSESHDFPRTSIVIPSAQVSKNHARISYKDGAFYLDLQSE	600
ZEP-2o	541: VSQPIYLSVSHNEPFIYLGSESHDFPRTSIVIPSAQVSKNHARISYKDGAFYLDLQSE	600
ZEP-3m	520: I	520
ZEP-4m	541: VSQPIYLSRSDNEPFIYLGSESHDFPRTSIVIPSAQVSKNHARISYKDGAFYLDLQSE	600

ZEP-1m	601: HGTVTVDNENGRYRVSNFPAFRPSPDITFEGSDKKAIFRVKVIPTPNNSERKEAGEI	660
ZEP-1o	601: HGTVTVDNENGRYRVSNFPAFRPSPDITFEGSDKKAIFRVKVIPTPNNSERKEAGEI	660
ZEP-2m	601: HGTVTVDNENGRYRVSNFPAFRPSPDITFEGSDKKAIFRVKVIPTPNNSERKEAGEI	660
ZEP-2o	601: HGTVTVDNENGRYRVSNFPAFRPSPDITFEGSDKKAIFRVKVIPTPNNSERKEAGEI	660
ZEP-3m	520: I	520
ZEP-4m	601: HGTVTVDNENGRYRVSNFPAFRPSPDITFEGSDKKAIFRVKVIPTPNNSERKEAGEI	660

ZEP-1m	661: LQAV---	664
ZEP-1o	661: LQAV---	664
ZEP-2m	661: LQAV---	664
ZEP-2o	661: LQAV---	664
ZEP-3m	520: I	520
ZEP-4m	661: LQAVQKI	667

Exon 11		

Fig. 3. Alignment of deduced amino acid sequence of ZEP-1, ZEP-2, ZEP-3m and ZEP-4m. These six sequences are ZEP-1 and ZEP-2 from 'Miyagawa wase' (suffix m) and 'Trovita' orange (suffix o), ZEP-3m and ZEP-4m from 'Miyagawa wase'. These six haplotypes showed high identity; however, in 10th exon, a frameshift caused by an indel variation resulted in a different amino acid sequence between ZEP-1 and ZEP-2. Although ZEP-3m and ZEP-4m sequences also showed high identities at CDSs, ZEP-3m had 9 bp and 7 bp deletion in 2th exon and 11th exon. This 7 bp deletion at 11th exon in ZEP-3m showed the appearance of a stop codon, which suggests a possible loss of function of ZEP-3m.

36 SNPs in the 3609 bp of the sequences. The 5th intron was 279 bp longer in ZEP-2m than in ZEP-1m, corresponding to the differences in PCR fragment sizes. Compared with the transcribed region, there was much

greater sequence variation in the 1736 bp 5' UTR sequences between ZEP-1 and ZEP-2 of 'Miyagawa wase'. Sequence identity between the 1736 bp 5' UTR sequences was 93.6% at the nucleotide level.

To confirm sequence diversity between ZEP-1 and ZEP-2 in other *Citrus* cultivars, genomic sequences for ZEP-1 and ZEP-2 were investigated in 'Trovita' orange, which was also heterozygous at the ZEP-1/-2 locus. Since then, ZEP-1 and ZEP-2, derived from 'Trovita' orange, have been described as ZEP-1o (AB548572) and ZEP-2o (AB548573). The PCR fragment length of ZEP-1o from 'Trovita' orange was similar to that of ZEP-1m. The entire gene was sequenced, including 1736 bp of the 5' UTR and 669 bp of the 3' UTR. Within the 8010 bp region there were 4 indel sites, 20 SNP sites, and 2 base substitutions in the coding region as compared with ZEP-1m. ZEP-2o from 'Trovita' orange was a typical ZEP-2 type gene. An 8259 bp region was sequenced, and 8 indels and 21 SNPs were observed in the coding region, as compared with ZEP-2m; however, as shown in Figure 3, amino acid sequences were highly conserved within ZEP-1 types and ZEP-2 types. Genomic sequence analysis of ZEP-1/-2 in a heterozygous cultivar 'Kiyomi' tangor (hybrid of 'Miyagawa wase' and 'Trovita' orange) clarified that 'Kiyomi' was inherited ZEP-1o from 'Trovita' orange, and ZEP-2m from 'Miyagawa wase'. For ZEP-3m, we sequenced a 7983 bp region, including 5' and 3' UTRs on two BAC clones. The sequences of ZEP-3m (BAC180-4G and BAC198-3A) were very similar to ZEP-1/-2; however, to date, no sequences corresponding to this type have been registered in public databases. To confirm whether the BAC clone (BAC212-6B) with 2239 bp of PCR fragment has a ZEP-like sequence, the sequence covering 7744 bp including 5' and 3' UTRs was determined. The sequence was very similar to ZEP-3m and was therefore new sequence designated tentatively as ZEP-4m (Fig. 2).

To investigate the genomic structure of ZEP-3m and ZEP-4m, a cDNA clone from young fruit (30 days after anthesis) of 'Miyagawa wase' was isolated and sequenced to confirm the coding regions. Sequence alignment of the cDNA sequence and ZEP-3m or ZEP-4m showed that the coding sequences of ZEP-3m and ZEP-4m were divided into 16 exons, such as ZEP-1m and ZEP-2m. The ZEP-3m genomic sequence differed from that of ZEP-4m mainly at the 2th and 11th exons. In ZEP-3m, the 11th exon contained a 7 bp deletion, resulting in generation of a stop codon, which suggests the possible loss of function for ZEP-3m (Fig. 3). The predicted coding sequences of ZEP-4m showed 90.0% identity to ZEP-1m over the 16 exons, and 89.9% identity to ZEP-2m (Fig. 3). The 4th introns of ZEP-3m were longer than those of ZEP-1m and ZEP-2m, corresponding to the longest PCR products amplified from *Citrus* genomic DNA (Fig. 1B).

Furthermore, the 5th intron of ZEP-4m was 843 bp longer than that of ZEP-3m, suggesting that because this

insertion made it difficult to amplify the fragment of ZEP-4m, the longest genomic PCR fragment of ZEP-4m was not detected (Fig. 1). ZEP-3m and ZEP-4m had very high sequence identity in the possible coding sequences, but whether ZEP-3m and ZEP-4m were in allelism has not been confirmed yet.

Cluster analysis using the NJ method showed that ZEP-3m/-4m clustered more tightly with ZEP-1m and ZEP-2m of *Citrus* than with ZEPs from other plants. This suggested that ZEP genes might have arisen from relatively recent duplication events in *Citrus* (Fig. 4).

2. Expression profiles of ZEP genes during fruit development in *Citrus*

1) ZEP-1/-2 and ZEP-3/-4 expression in *Citrus* fruit

The expression profiles of ZEP genes in ‘Miyagawa wase’ and ‘Washington navel’ orange (*C. sinensis*) were evaluated by RT-PCR using specific primers that discriminated ZEP-1/-2 from ZEP-3/-4 but were common to ZEP-1 and ZEP-2 groups; in addition, the specific primers could not discriminate ZEP-3m from ZEP-4m. As shown in Figure 5A, a low level of ZEP-4m expression was detected in young ‘Miyagawa wase’ fruit (DAF30 and DAF60), but expression decreased below detection limits at DAF120 and at maturity (DAF180), while ZEP-3m, which has a stop codon at the 11th exon, was not detected at any stage of fruit development (data not shown). RT-PCR using specific primers that discriminate between ZEP-1/-2 and ZEP-3/-

-4 was carried out to evaluate the expression of ZEP-3/-4. The results showed that only ZEP-4m was expressed.

The expression patterns of ZEP-1m/-2m differed from that of ZEP-4m (Fig. 5B). Expressions of both ZEP-1m and ZEP-2m increased from immature (DAF60) to mature fruit (DAF180) in ‘Miyagawa wase’ fruit, but peaked level in DAF180 fruit. The expression profiles of ZEP-1/-2 and ZEP-3/-4 in ‘Washington navel’ fruit were similar to those in ‘Miyagawa wase’ (data not shown). These results suggest that ZEP-1/-2 have important roles in determining the ZEP expression level in mature *Citrus* fruit, but ZEP-3/-4 does not.

Next, total transcript levels of ZEP-1/-2 were estimated quantitatively by RT-PCR using a TaqMan probe that simultaneously detected the expression of ZEP-1 and ZEP-2 in maturing fruit (November 2005 to January 2006). Levels of ZEP-1/-2 transcripts decreased from November (DAF180) to January (DAF240) in ‘Miyagawa wase’ and ‘Trovita’ orange fruit. The decrease was more rapid in ‘Miyagawa wase’, in which the transcript level in January was 62.7% of that in November. In ‘Trovita’ orange, the transcript level in January was 88.2% of that in November; therefore, the transcript level in ‘Miyagawa wase’ was lower than that in ‘Trovita’ orange in January, although that in ‘Miyagawa wase’ was higher than that in ‘Trovita’

Fig. 4. Phylogenetic tree based on deduced amino acid sequences of ZEP genes constructed using MEGA4 Build#: 4026 by the NJ method. Scale bar indicates distances (branch length) among compared sequences. Compared sequences: ZEP-1m (AB548570), ZEP-2m (AB548571), ZEP-3m, and ZEP-4m from *C. unshiu*, ZEP-1o (AB548572) and ZEP-2o (AB548573) from *C. sinensis*, and previously published sequences from *A. thaliana* (*AtZEP*, accession no. AF281655), grape (*VvZEP1*, XM_002282507; *VvZEP2*, AY337615), *Zea mays* (*ZmZEP*, EU970775), carrot (*DcZEP*, DQ192197), and tomato (*LeZEP1*, EF581828; *LeZEP2*, Z83835).

Fig. 5. RT-PCR analysis of ZEP-4m expression during fruit development in ‘Miyagawa wase’. (A) ZEP-4m expression during fruit development. ZEP-4m was expressed in immature fruit and, as fruit matured, ZEP-4m expression progressively weakened. (B) Comparison of ZEP-1m/-2m and ZEP-4m expression intensity. This showed that the expression levels of ZEP-4m were much weaker than ZEP-1m/-2m. This result was common during fruit development.

orange in November (Fig. 6).

2) Allelic differences of *ZEP-1/-2* expression in mature-stage fruit

To determine the transcript ratio of *ZEP-1* : *ZEP-2* at fruit maturity, we estimated expression levels in the fruit of three heterozygous cultivars, 'Miyagawa wase' (*ZEP-1m/ZEP-2m*), 'Trovita' orange (*ZEP-1o/ZEP-2o*), and their hybrid 'Kiyomi' (*ZEP-1o/ZEP-2m*) using SNP discrimination. The TaqMan probe set was allele-specific, and detected both *ZEP-1* and *ZEP-2*. As shown in Table 2, expression levels of the two alleles differed among the three cultivars in maturing fruit collected in November 2005, January 2006, and January 2008. In 'Miyagawa wase' fruit harvested in November 2005, *ZEP-1m* accounted for 59% of total *ZEP-1/-2* gene expression, while *ZEP-2m* accounted for the remaining 41%. The *ZEP-1/-2* ratio was very similar in January 2006 (*ZEP-1m*: 60%, *ZEP-2m*: 40%; Table 2). In 'Trovita' orange fruit, *ZEP-1o* was expressed at higher levels than *ZEP-2o* (69 and 31%, respectively, in November 2005; and 72 and 28%, respectively, in January 2006). The heterozygous 'Kiyomi' tangor containing both *ZEP-1o* and *ZEP-2m* showed large differences in expression levels between the alleles. In

Fig. 6. Total expression of *ZEP-1* and *ZEP-2* in 'Miyagawa wase' and 'Trovita' orange at 2 months. Levels of allele-specific expression were analyzed by TaqMan real-time quantitative RT-PCR. Probes and sets of primers used for analyses are shown in Table 1. Horizontal bars indicate SD ($n=3$). In these two varieties, the expressions of *ZEP-1* and *ZEP-2* declined in January compared with in November, while, the decline in expression in 'Miyagawa wase' was greater than in 'Trovita' orange.

'Kiyomi' fruit harvested in November 2005, *ZEP-1o* derived from 'Trovita' orange accounted for 75.5% of total expression and *ZEP-2m* from 'Miyagawa wase' accounted for the remaining 24.5%. A similar expression rate was observed in fruit harvested in January, 2006 (*ZEP-1o*: 76.6%, *ZEP-2m*: 23.4%, Table 2). Differences in expression levels between alleles were confirmed in fruit harvested in 2008 (67 : 33 *ZEP-1o* : *ZEP-2o* expression ratio in 'Trovita' orange fruit; 74 : 26 *ZEP-1o* : *ZEP-2m* expression ratio in 'Kiyomi' tangor fruit). Therefore, the expression ratio of *ZEP-1/-2* alleles in *Citrus* cultivars was maintained at similar levels in different fruiting seasons, although the total transcript levels of *ZEP-1/-2* changed as fruit matured.

3. Sequence diversity of 5' untranslated regions (UTRs) at *ZEP-1/-2*

To examine structural differences in *cis*-element regions in relation to expression levels of *ZEP* genes, two 1783 bp genomic sequences located in the 5' UTR of *ZEP-1/-2* genes from 'Trovita' orange were compared with those from 'Miyagawa wase' BAC sequences. Within this region, the lowest identity was between *ZEP-1m* and *ZEP-2o* (93.5%). The four haplotype sequences had relatively high identity in this region, but there were many SNPs, 1–30 bp indels, and replacements at two or more nucleotides. The sequence-level variations among the four haplotypes resulted in different motif structures of possible *cis*-regions.

Using PLACE (<http://www.dna.affrc.go.jp/PLACE/index.html>), the possible motifs were surveyed for the four haplotype sequences and annotated as shown in Figure 7. We observed (TAA) and (TAATGA) repeats in the region from –489 to –540 bp from ATG in *ZEP-1m*. These repeated motif sequences are known as POLASIG3, IBOXCORE and GATABOX, respectively (Fig. 7). The repeat structure of the two motifs in the various alleles was as follows: (TAA)₅ (TAATGA)₄ for *ZEP-1m*; (TAA)₆ (TAATGA)₁ for *ZEP-2m*; (TAA)₇ (TAATGA)₅ for *ZEP-1o*; and (TAA)₇ (TAATGA)₁ for *ZEP-2o*. Compared with *ZEP-1o*, the sequence of this repeat domain was 12 bp shorter in *ZEP-1m*, 24 bp shorter in *ZEP-2m*, and 30 bp shorter in *ZEP-2o*.

There were many variations in the presence or absence of motif sequences among the four haplotypes. Almost

Table 2. Allele-specific expression ratio (expressed as percentage) of *ZEP-1* and *ZEP-2* in three *Citrus* varieties. Levels of allele-specific expression were analyzed by TaqMan real-time quantitative RT-PCR. Probes and sets of primers used for analyses are shown in Table 1. Data are means $\pm$ SD ($n=3$).

	'Miyagawa wase'		'Trovita' orange		'Kiyomi'	
	<i>ZEP-1m</i>	<i>ZEP-2m</i>	<i>ZEP-1o</i>	<i>ZEP-2o</i>	<i>ZEP-1o</i>	<i>ZEP-2m</i>
2005 November	59.1	40.9	69.1	30.9	75.5	24.5
2006 January	60.3	39.7	72.0	28.0	76.6	23.4
2008 January	—	—	67.4	32.6	74.4	25.6
Average	59.7	40.3	69.5	30.5	75.5	24.5
SD	0.6	0.6	1.3	0.9	0.6	0.6

Fig. 7. Diagram of upstream regions of *ZEP* genes (–600 bp to +1 from translation start site). Nucleotide position shown relative to the translation start site. GARE1OSREP1 had three haplotypes other than *ZEP-2m*. This motif is “Gibberellin-responsive element (GARE)” found in the promoter region of a cysteine proteinase (REP-1) gene in rice. PYRIMIDINEBOX found in rice alpha-amylase gene. ACGTTBOX is an ACGT element. ACGATERD1 is ACGT sequence (from –155 to –152) required for etiolation-induced expression of *erd1* (early responsive to dehydration) in *Arabidopsis*. MYBPZM motif is the core region of the consensus maize P binding site. Maize P gene encodes red pigmentation of kernel pericarp, cob, and other floral organs. Number of (TAA)_x (TAATGA)_y repeats differed among the four haplotypes in the repeat sequence region, and, POLASIG3, IBOXCORE and GATABOX motifs were confirmed. The number of these three motifs in the repeat sequence region differed among haplotypes. Sequences were registered in the DDBJ: *ZEP-1m*, AB548570; *ZEP-2m*, AB548571; *ZEP-1o*, AB548572; *ZEP-2o*, AB548573.

all variations were haplotype-specific, but not allele-type-specific; however, some features were restricted to either *ZEP-1* type or *ZEP-2* type alleles. In *ZEP-1m* and *ZEP-1o*, MYB, DOF and PYRIMIDINEBOXOSRAMY1A motifs were commonly detected around –500 bp from the ATG codon, while *ZEP-2m* and *ZEP-2o* alleles lacked these motifs. On the other hand, *ZEP-2* alleles had ACGTTBOX and ACGATERD1 motifs (Fig. 7). Furthermore, *ZEP-1* alleles had an MYBPZM motif, which is the core of a consensus maize P (Myb homolog) binding site, at –442 bp, while *ZEP-2* alleles did not. In contrast, *ZEP-2* type alleles had a WRKY710 motif located at –272 bp.

Discussion

ZEP is a carotenoid biosynthetic gene encoding an enzyme with crucial roles in determining the level and composition of carotenoids. In grape and tomato, there are two *ZEP* sequences in public databases but they did not seem to be paralogs because they showed very high identities with each other, with more than 99% identity at the amino acid level. In *Citrus* fruit, Kato et al. (2004) showed that the expression of *ZEP* increased during fruit maturation in Satsuma mandarin and ‘Valencia’ orange, and that expression levels differed between the two cultivars; however, not only cultivar-specific or genotype-specific mechanisms controlling *ZEP* expression, but also, from our results, the presence of a possible paralogous *ZEP* gene with sequence diversity in plants, remain unclear.

Here, we analyzed in detail the allelic structure and expression of *ZEP* genes in *Citrus*. Our results provide new information about the control of *ZEP* gene expression.

1. Newly identified *ZEP* gene in *Citrus*

In *Arabidopsis*, two genomic DNA sequences with 99.7% identity were identified from the DDBJ database (AF134577 and AF283761). To date, only one *ZEP* gene located at one locus on chromosome 5 (At5g67030) has been identified in *Arabidopsis*, and no other *ZEP* gene homologs have been reported.

In this study, four different genomic sequences were isolated from the BAC library of ‘Miyagawa wase’ (*C. unshiu*) and sequenced. Two of these sequences (*ZEP-1m* and *ZEP-2m*) were similar to cDNA sequences registered in the DDBJ database (AB114654 and AB075547), and were very similar; however, the other two sequences showed a conserved exon/intron structure of the *ZEP* gene, but had longer 4th introns than other *ZEP* genes. Their predicted CDSs had 90% nucleotide identity with previously published cDNA sequences.

Progeny tests and comparisons of PCR fragment size from the 4th to 6th exon among cultivars suggested that the former two genomic sequences were alleles at the *ZEP-1/-2* locus, which had identical coding sequences with previously registered cDNA in the DDBJ. On the other hand, whether *ZEP-3/-4* were in allelism in the second locus of *ZEP* has not been confirmed although they had very similar coding sequences. Phylogenetic

analyses of four *ZEP* sequences in *Citrus* suggested that *ZEP-3/-4* were separated from *ZEP-1/-2* before *ZEP-1* and *ZEP-2* speciated; however, both *ZEP-3/-4* and *ZEP-1/-2* sequences in *Citrus* were clustered more tightly than *ZEP* sequences from other plants. Since both Satsuma mandarin and sweet orange were heterozygous in the *ZEP-1/-2* locus, *ZEP-3/-4* should be located in other loci. While *ZEP-1/-2* were well characterized by sequencing and expression analysis in *Citrus* (Kato et al., 2004), there is information on *ZEP-3/-4* in *Citrus*. *ZEP-4* was weakly expressed only at early stages of fruit development (DAF30 to DAF120) in contrast with *ZEP-1/-2* expression. Compared with *ZEP-1/-2*, *ZEP-3/-4* may contribute differently to carotenoid metabolism in *Citrus*, but it may have a minor contribution to violaxanthin accumulation in fruit. Komatsu et al. (1999) reported the differential expression of three sucrose-phosphate synthase (SPS) isoforms during sucrose accumulation in developing citrus fruit. Their result was that one of three genes showed a differential expression pattern compared with the other two SPS genes; in addition, the transcription levels of the two SPS genes paralleled the activity of SPS. It would be interesting to know whether the two classes of *ZEP* genes with different expression profiles were generated similarly to the SPS isoforms.

These results suggest that *ZEP-4* is an isoform with a different expression pattern from those of *ZEP-1/-2* as well as sequence similarity. How *ZEP-3/-4* were generated in *Citrus* should be clarified in the future in addition to the contribution to carotenoid metabolism in whole plant organs, such as leaf, stem or root.

2. Uneven transcript levels of *ZEP-1/-2* alleles

As reported previously (Kato et al., 2004), the expression of *ZEP* varied throughout fruit development and from year to year. In this study, we observed that *ZEP* expression in 'Miyagawa wase' fruit was higher than in 'Trovita' orange from November to January, which was not consistent with results reported previously by Kato et al. (2004); however, the tendency for a decreased expression level toward the maturity of *ZEP* in all cultivars was consistent with their results. In addition to the variation among cultivars in the expression of *ZEP* during fruit development and maturation, the results detected that allelic combination is one of the factors affecting variable *ZEP* expression. The results showed that *ZEP-1* alleles were expressed at 1.5- to 3-fold higher levels than *ZEP-2* alleles in all three heterozygous cultivars examined. The expression ratios were fairly constant as fruit matured; and in different fruit growing seasons. To date, there have been no reports on the differential expression of *ZEP-1/-2* gene alleles in *Citrus*. There are some reports on different expression patterns between alleles in the other genes of plants. In *C. unshiu*, alleles of a heterozygous UDP-glucose, limonoid glucosyl transferase gene, were expressed differently (Kita et al., 2003). In *F₁* hybrids

of poplar, 17 out of 30 analyzed genes showed 1.5-fold differences between expressions of the two alleles (Yan and Adams, 2007). Similarly, 43–53% genes showed unequal allelic expression in maize (Springer and Stupar, 2007a, 2007b); however, the mechanisms that regulate the differential expression of the two alleles remain unclear.

The genotype of the *Citrus* hybrid cultivar 'Kiyomi' was a combination of *ZEP-1o*, the stronger allele from 'Trovita' orange, and *ZEP-2m*, the weaker allele from 'Miyagawa wase'. Compared with either of its parents, 'Kiyomi' showed much larger differences between the expression levels of the two alleles. This result suggests that the differences between the expression levels of the two alleles were caused at least partly by differences in *cis*-structures located in the 5' UTR of *ZEP* genes, as indicated below.

3. Comparison of 5' untranslated regions of *ZEP* genes

For carotenoid metabolism genes, *RAP2.2*, a member of the AP2/ethylene-responsive element-binding protein gene, was only reported to act on the promoter region of *PSY* and *PDS* (Welsch et al., 2008). The report suggested that diversity among *cis*-elements as well as transcription factor genes would contribute to the genotype-dependent expression of genes involved in carotenogenesis; however, there has been no such analysis in the *ZEP* gene in *Citrus*.

The 5' UTRs of *ZEP* genes from 'Miyagawa wase' and 'Trovita' orange were compared to associate the allelic sequence differences to the expression in heterozygous plants. *ZEP-1* alleles were transcribed at higher levels than *ZEP-2* alleles; therefore, we selected sequence motifs that were common within *ZEP-1*s but differed from *ZEP-2*s. As a *ZEP-1*-specific motif, we detected motifs related to Myb-binding sites, including the Myb-related MYBPZM motif in *ZEP-1* type haplotypes at –442 bp. The MYBPZM motif was absent from *ZEP-2* type alleles. A Myb-transcriptional factor is involved in regulating the gene expression in grape (Kobayashi et al., 2004) and apple (Ban et al., 2007) during anthocyanin biosynthesis, but it is still unknown whether Myb-like factors have roles in regulating the expression of *ZEP* or carotenoid biosynthesis genes, and how the sequence variations in Myb-sites in 5' UTRs are correlated with expression levels among alleles of the *Citrus ZEP* gene; however, the sequence structure of the possible *cis*-region and its variation would provide important information to investigate the regulatory mechanism in the near future.

In addition to the potential of molecular analysis to improve the regulation of *ZEP* gene expression, sequence diversity would be utilized to generate molecular markers to manipulate total *ZEP* expression and carotenoid components for breeding. Hybrids having weak allele combination might be selected by identifying the presence or absence of candidate elements, such as Myb

sites, to accumulate zeaxanthin or β -cryptoxanthin in fruit. The functional site was discovered on a *Myb* promoter region to regulate fruit rind color in grape (Kobayashi et al., 2004) and on an ACC synthase gene promoter to discriminate ethylene production in apple and Japanese pear fruit (Harada et al., 1997; Itai et al., 1999). However, beside *cis*-regulation, it is thought that various factors are involved in the gene expression, and the existence of trans-regulation or regulation factors of mRNA cannot be disregarded. This approach only clarified part of the mechanism of gene expression, and although the expression levels of *ZEP* are regulated in a quantitative manner in *Citrus*, further statistical analyses should be performed to confirm the effect of the functional sites in the *cis*-region on carotenoid metabolism, and other regulation factors should be investigated; that is, *cis*-element, transcription factors, regulation factor of mRNA and so on. When considering differences in expression levels, various factors should be considered comprehensively. From this result, because haplotypes are thought to be factors affecting gene expressions, they should be considered at breeding selection.

Literature Cited

- Ban, Y., C. Honda, Y. Hatsuyama, M. Igarashi, H. Bessho and T. Moriguchi. 2007. Isolation and functional analysis of a MYB transcription factor gene that is a key regulator for the development of red coloration in apple skin. *Plant Cell Physiol.* 48: 958–970.
- Bouvier, F., R. A. Backhaus and B. Camara. 1998. Induction and control of chromoplast-specific carotenoid genes by oxidative stress. *J. Biol. Chem.* 273: 30651–30659.
- Bouvier, F., A. d’Harlingue, P. Hugueney, E. Marin, A. Marion-Poll and B. Camara. 1996. Xanthophyll biosynthesis. Cloning, expression, functional reconstitution, and regulation of beta-cyclohexenyl carotenoid epoxidase from pepper (*Capsicum annuum*). *J. Biol. Chem.* 271: 28861–28867.
- Busch, M., A. Seuter and R. Hain. 2002. Functional analysis of the early steps of carotenoid biosynthesis in tobacco. *Plant Physiol.* 128: 439–453.
- Fraser, P. D., M. R. Truesdale, C. R. Bird, W. Schuch and P. M. Bramley. 1994. Carotenoid biosynthesis during tomato fruit development: Evidence for tissue-specific gene expression. *Plant Physiol.* 105: 405–413.
- Giuliano, G., G. E. Bartley and P. A. Scolnik. 1993. Regulation of carotenoid biosynthesis during tomato development. *Plant Cell* 5: 379–387.
- Goodner, K. L., R. L. Rouseff and H. J. Hofsommer. 2001. Orange, mandarin, and hybrid classification using multivariate statistics based on carotenoid profiles. *J. Agric. Food Chem.* 49: 1146–1150.
- Gross, J. 1987. Carotenoid: pigment in fruit. Academic Press, London.
- Harada, T., T. Sunako, W. Sakuraba, S. Goto, M. Senda, S. Akada and M. Niizeki. 1997. Genomic nucleotide sequence of a ripening-related 1-aminocyclopropane-1-carboxylate synthase gene (MdACS-1) in apple. *Plant Physiol.* 113: 1465.
- Ikoma, Y., A. Komatsu, M. Kita, K. Ogawa, M. Omura, M. Yano and T. Moriguchi. 2001. Expression of a phytoene synthase gene and characteristic carotenoid accumulation during citrus fruit development. *Physiol. Plant.* 111: 232–238.
- Ikoma, Y., M. Yano, K. Ogawa, T. Yoshioka, Z. C. Xu, S. Hisada, M. Omura and T. Moriguchi. 1996. Isolation and evaluation of RNA from polysaccharide-rich tissues in fruit for quality by cDNA library construction and RT-PCR. *J. Japan. Soc. Hort. Sci.* 64: 809–814.
- Isaacson, T., G. Ronen, D. Zamir and J. Hirschberg. 2002. Cloning of tangerine from tomato reveals a carotenoid isomerase essential for the production of β -carotene and xanthophylls in plants. *Plant Cell* 14: 333–342.
- Itai, A., T. Kawata, K. Tanabe, F. Tamura, M. Uchiyama, M. Tomomitsu and N. Shiraiwa. 1999. Identification of 1-aminocyclopropane-1-carboxylic acid synthase genes controlling the ethylene level of ripening fruit in Japanese pear (*Pyrus pyrifolia* Nakai). *Mol. General Genet.* 262: 42–49.
- Kato, M., Y. Ikoma, H. Matsumoto, M. Sugiura, H. Hyodo and M. Yano. 2004. Accumulation of carotenoids and expression of carotenoid biosynthetic genes during maturation in citrus fruit. *Plant Physiol.* 134: 824–837.
- Katsumoto, H., M. Ito, O. Sakatsume and I. Ieiri. 2005. Allele-specific transcript quantification using TaqMan® PCR method. The 28th Annual Meeting of the Molecular Biology Society of Japan 3P-1142 (In Japanese).
- Kita, M., T. Endo, T. Shimada, T. Moriguchi, Y. Hirata, S. Hasegawa and M. Omura. 2003. Allelic structure of UDP-glucose: limonoid glucosyltransferase affect limonoid bitterness in *Citrus unshiu* and *C. sinensis*. *Euphytica* 132: 87–94.
- Kim, I. J., K. C. Ko, C. S. Kim and W. I. Chung. 2001. Isolation and characterization of cDNAs encoding β -carotene hydroxylase in Citrus. *Plant Sci.* 161: 1005–1010.
- Kobayashi, S., N. Goto-Yamamoto and H. Hirochika. 2004. Retrotransposon-induced mutations in grape skin color. *Science* 304: 982.
- Komatsu, A., Y. Takanokura, T. Moriguchi, M. Omura and T. Akihama. 1999. Differential expression of three sucrose-phosphate synthase isoforms during sucrose accumulation in citrus fruits (*Citrus unshiu* Marc.). *Plant Sci.* 140: 169–178.
- Lee, H. S. and W. S. Castle. 2001. Seasonal changes of carotenoid pigments and color in Hamlin, Earlygold, and Budd Blood orange juices. *J. Agric. Food Chem.* 49: 877–882.
- Molnár, P. and J. Szabolcs. 1980. β -Citraurin epoxide, a new carotenoid from Valencia orange peel. *Phytochemistry* 19: 633–637.
- Nishino, H., H. Tokuda, M. Murakoshi, Y. Satomi, M. Masuda, M. Onozuka, S. Yamaguchi, J. Takayasu, J. Tsuruta, M. Okuda, F. Khachik, T. Narisawa, N. Takasuka and M. Yano. 2000. Cancer prevention by natural carotenoids. *Biofactors* 2000 13: 89–94.
- Olson, J. A. 1989. Provitamin-A function of carotenoids: the conversion of β -carotene into vitamin-A. *J. Nutr.* 119: 105–108.
- Omura, M., T. Ueda, M. Kita, A. Komatsu, Y. Takanokura, T. Shimada, T. Endo-Inagaki, H. Nesumi and T. Yoshida. 2000. EST mapping of *Citrus*. *Proc. Intl. Soc. Citriculture for IX Congr.* 2000: 71–74.
- Park, H., S. Kreunen, A. J. Cuttriss, D. DellaPenna and B. J. Pogson. 2002. Identification of the carotenoid isomerase provides insight into carotenoid biosynthesis, prolamellar body formation, and photomorphogenesis. *Plant Cell* 14: 321–332.
- Pogson, B., K. A. McDonald, M. Truong, G. Britton and D. DellaPenna. 1996. Arabidopsis carotenoid mutants demonstrate that lutein is not essential for photosynthesis in higher plants. *Plant Cell* 8: 1627–1639.

- Ronen, G., M. Cohen, D. Zamir and J. Hirschberg. 1999. Regulation of carotenoid biosynthesis during tomato fruit development: expression of the gene for lycopene epsilon-cyclase is down-regulated during ripening and is elevated in the mutant *Delta*. *Plant J.* 17: 341–351.
- Shimada, T., F. Nishikawa, T. Endo, H. Fujii, Y. Nobata, T. Shimizu and M. Omura. 2005. Construction and initial evaluation of BAC library for *Citrus unshiu* Marc. 'Miyagawa wase'. Abstr. Japan. Soc. Hort. Sci. Spring Meet. 74: 187 (In Japanese).
- Springer, N. M. and R. M. Stupar. 2007a. Allele-specific expression patterns reveal biases and embryo-specific parent-of-origin effects in hybrid maize. *Plant Cell* 19: 2391–2402.
- Springer, N. M. and R. M. Stupar. 2007b. Allelic variation and heterosis in maize: how do two halves make more than a whole? *Genome Res.* 17: 264–275.
- Welsch, R., J. Medina, G. Giuliano, P. Beyer and J. von Lintig. 2003. Structural and functional characterization of the phytoene synthase promoter from *Arabidopsis thaliana*. *Planta* 216: 523–534.
- Welsch, R., D. Maass, T. Voegel, D. Dellapenna and P. Beyer. 2008. Transcription factor RAP2.2 and its interacting partner SINAT2: stable elements in the carotenogenesis of *Arabidopsis* leaves. *Plant Physiol.* 145: 1073–1085.
- Yan, Z. and K. L. Adams. 2007. Extensive allelic variation in gene expression in populus F₁ hybrids. *Genetics* 177: 1987–1996.