

A New Nonsense Mutation in *Capsanthin/Capsorubin Synthase* Controlling Orange Pepper Fruit

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Abstract

Carotenoids are plant pigments that play a major role in conferring fruit color. Carotenoid content is often controlled by genetic variation in the biosynthetic genes. The color of mature pepper fruit is mainly classified as red, orange, and yellow. Orange and yellow fruit colors are determined by mutations in *phytoene synthase (Psy)* and *capsanthin-capsorubin synthase (Ccs)*, respectively. In contrast to the current fruit color model, we hypothesized that genetic variation in *Ccs* also controls orange fruit color in pepper. Ripe fruit of *Capsicum annuum* ‘K146465’ is orange, and its carotenoid profile obtained by HPLC analysis showed a lack of the major pepper carotenoid capsanthin but an abundance of lutein, zeaxanthin, and β -carotene compared to red pepper. cDNA cloning and sequencing analysis detected a new nonsense mutation due to a T¹⁰²⁶ insertion in the coding region of *Ccs*, but no DNA sequence variation in *Psy*. We developed a derived cleaved amplified polymorphic sequence (dCAPS) marker to distinguish the nonsense mutation in *Ccs*. Genetic analysis of the F₂ population derived from *C. annuum* ‘Sweet Banana’ (red fruit color) \times *C. annuum* ‘K146465’ revealed that orange fruit color is determined by a single recessive gene. The nonsense mutation in *Ccs* distinguished by the dCAPS marker co-segregated with orange fruit color in the F₂ population. This germplasm, coupled with the dCAPS marker and carotenoid profiling, will facilitate marker-assisted breeding to select orange fruit color and improve lutein, zeaxanthin, and β -carotene levels in pepper.

Additional key words: carotenoid, fruit quality, marker-assisted selection, metabolite profiling, molecular marker

Introduction

Fruit color, a trait that attracts the consumer, is associated with differential pigment levels. Carotenoids, the major pigments of fruits and flowers, are synthesized in plastids (Bartley and Scolnik, 1995). They play a crucial role in photo-protection of photosynthesis and act as a precursor of the plant hormone abscisic acid (ABA) (Tanaka et al., 2008). In humans, they are important dietary nutrients as they provide many health benefits. For instance, β -carotene, α -carotene, γ -carotene, and β -cryptoxanthin are the precursors of

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vitamin A (Yonekura and Nagao, 2007); lutein and zeaxanthin have a significant role in delaying and preventing cataract and age-related macular degeneration, both of which are the causes of blindness in 56% of cases worldwide (Nwachukwu et al., 2016); capsanthin acts as an antioxidant (Taylor and Ramsay, 2005). Although carotenoids are essential nutrients, they cannot be synthesized by the human body (Fiedor and Burda, 2014), and therefore their intake from fruits and vegetables is important for improving human health.

Diverse fruit colors often are due to variation in the functionality and expression of carotenoid biosynthetic genes. Pepper fruits (*Capsicum* spp.) are typically red, but in some cultivars their color varies such as orange, yellow and brown (Lightbourn et al., 2008). A three-locus model has been proposed to explain variation in pepper fruit colors, in which three independent genes encoded by the *y*, *c1*, and *c2* loci control the ripe fruit color (Hurtado-Hernandez and Smith, 1985). The *y* locus is responsible for yellow fruit color and is controlled by *capsanthin-capsorubin synthase* (*Ccs*) (Lefebvre et al., 1998); the *c2* locus is responsible for orange fruit color and is controlled by *phytoene synthase* (*Psy*) (Huh et al., 2001; Kim et al., 2010) the role of the *c1* locus is still unknown. Different combinations of alleles in these three loci can generate additional fruit colors, such as white and lemon (Thorup et al., 2000).

Among the various colors of pepper fruits, orange fruits are known to be associated with three independent genes that are involved in carotenoid biosynthesis. The first gene is *Psy*, which is involved in the early steps of the carotenoid biosynthesis pathway (Bartley and Scolnik, 1995). Because *Psy* encodes a rate-limiting enzyme, impaired activity of *Psy* decreases the amount of total carotenoids (Cazzonelli and Pogson, 2010), resulting in orange fruit color of ripe peppers; a typical example is *C. chinense* ‘Habanero’ (Huh et al., 2001; Kim et al., 2010). The second gene is *Ccs*, which synthesizes the red carotenoid compounds capsanthin and capsorubin in the last step of the carotenoid biosynthesis pathway. Interestingly, impaired activity of *Ccs* is associated with not only yellow fruit color (Lefebvre et al., 1998), but also orange fruit color (Lang et al., 2004). The third gene is *β -carotene hydroxylase2* (*Chy2*), which catalyzes the conversion of α - and β -carotene into lutein and zeaxanthin, respectively. A study using ethyl methane sulfonate (EMS) mutagenesis of the wild type red pepper *C. annuum* ‘Maor’ identified a mutant *C. annuum* ‘E-172-3’ with orange fruit color and high levels of β -carotene accumulation, which resulted from a mutation in *Chy2* (Borovsky et al., 2013).

In the present study, we explored the genetic variation associated with fruit color and carotenoid variation in the orange pepper *C. annuum* ‘K146465’. This germplasm and the new causal variation discovered will facilitate marker-assisted breeding for selection of orange fruit color and improvement of carotenoid levels in pepper.

Materials and Methods

Plant Material

Two pepper accessions, *C. annuum* ‘Sweet Banana’ (‘Sweet Banana’) and *C. annuum* ‘K146465’ (‘K146465’), were obtained from the National Agro Biodiversity Center, Rural Development Administration, Korea. A mapping population was constructed from a cross between ‘Sweet Banana’ and ‘K146465’. An F₂ population of 217 individuals was used to conduct co-segregation analysis of fruit color and candidate genotypes. All plant material was grown under greenhouse conditions at Kyungpook National University, Daegu, Korea. Pericarp from ripe fruits of ‘Sweet Banana’ and ‘K146465’ were collected, immediately frozen in liquid nitrogen, and ground with a chilled mortar and pestle for total RNA and carotenoid extractions.

cDNA Cloning and Sequencing of *Psy*, *Chy2*, and *Ccs*

Total RNA from the ripe fruit pericarp was extracted using a TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA), and cDNA was synthesized using a DiaStar™ RT Kit (SolGent Co., Ltd., Daejeon, Korea). Primers for *Psy*, *Chy2*, and *Ccs* cloning were designed and synthesized by Neoprobe (Daejeon, Korea). The *Psy* (CA04g04080) cDNA cloning was conducted using two sets of primers, *Psy*-1 and *Psy*-2, and primer sequences are as follows. *Psy*-1 forward: 5'-ATGTCTGTTGCCTTGTTATGGGTT-3', reverse: 5'-TCTGTATCTTGACTTCCTCAAGTC-3' and *Psy*-2 forward: 5'-GAAGACAGGCTAGAAAGATGTTTC-3', reverse: 5'-TCATGTTCTGTAGAAGGCACAAG-3'. The *Chy2* (CA03g25820) primer sequences included the forward primer: 5'-ATGGCTGCTGAAATTTCAATCTC-3', and reverse primer: 5'-CATAATCTCTTCGAACTTTTAATTCTTC-3'. The *Ccs* (CA06g22860) cDNA cloning was also conducted using two sets of primers, *Ccs*-1 and *Ccs*-2, and primer sequences are as follows. *Ccs*-1 forward: 5'-ATGGAACCCCTTCTAAAGCC TTTTC-3', reverse: 5'-CCAAATTCCTATCAAATGGCATTGC-3' and *Ccs*-2 forward: 5'-TGGAGGGATTCTCATTAG GTAATG-3', reverse: 5'-TCAAAGGCTCTCTATTGCTAGATTG-3'.

PCR amplification was carried out using *e-Taq* DNA polymerase (SolGent Co., Ltd) according to the manufacturer's instructions and under the following conditions: 95°C for 3 min, followed by 35 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min, and a final extension step at 72°C for 5 min. All cDNA amplicons were cloned using a T-blunt PCR Cloning Kit (SolGent Co., Ltd). Plasmid DNA was isolated using a HiGene™ Plasmid Mini Prep Kit (BIOFACT Co., Ltd., Daejeon, Korea), and three clones per each gene were sequenced to verify sequence fidelity. Sequencing was conducted bi-directionally (SolGent Co., Ltd), and sequence alignment was performed in Cluster-X (<http://www.cluster-x.org/>) and GeneDoc (Nicholas, 1997), whereas protein translation was conducted using ExPASy Translate tool (<http://www.expasy.org/resources>).

Ccs-based Drived Cleaved Amplified Polymorphic Sequence (dCAPS) Marker for Selecting 'K146465'

dCAPS marker was generated based on the cDNA variation in *Ccs* between 'Sweet Banana' and 'K146465' (Neff et al., 1998); we designed one *Ccs*/dCAPS primer pair and the sequences are as follows. Forward primer: 5'-GAAGAGAAGTGTGTGATC ACTATGGGAGGACGAATT-3' and reverse primer: 5'-CATTCTCTAACACGTCTTCTATCC-3'. The PCR product obtained was 255-bp. The underlined nucleotides in the primer sequence were mutations introduced to create an artificial restriction site. They are 32 C>G and 34 C>A. Genomic DNA was used as a template for amplification, and was extracted from young leaves of 'Sweet Banana', 'K146465', and their F₂ progeny by the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980). The purity and concentration of extracted genomic DNA were monitored by a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). PCR amplification was carried out using *e-Taq* DNA polymerase (SolGent Co., Ltd., Daejeon, Korea) according to the manufacturer's instructions with the following conditions: 95°C for 3 min, followed by 35 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min, with a final extension step at 72°C for 5 min. The PCR amplicons obtained by the dCAPS marker were digested with *EcoRI* (New England Biolabs, Ipswich, MA, USA) for 4 h and electrophoresed on a 2.5% agarose gel.

Carotenoid Extraction and High Performance Liquid Chromatography (HPLC) Analysis

Approximately 100 mg of frozen pericarp powder from five biological replicates was used for carotenoid extraction. Carotenoid extraction and quantification were performed according to the method described by Yoo et al. (2017), except for the saponification procedure.

For saponification, 300 μ L of 20% KOH were added to the dried sample and incubated for 1 h at room temperature. Next, 250 μ L of methyl t-butyl ether were added to the saponified sample and incubated for 15 min. Finally, 242.5 μ L of MeOH were added, samples were vortexed vigorously, then filtered through a 0.45 μ m Smart-Por nylon filter (Woongki Science, Seoul, Korea) and transferred to a HPLC vial (Agilent Technologies, Santa Clara, CA, USA).

After the extraction, carotenoids were analyzed by HPLC (Agilent 1260 Infinity) using a C₃₀ YMC Carotenoid S-5 (4.6 \times 250 mm) and Agilent ChemStation software (Agilent Technologies). Carotenoids were separated using two solvents [MeOH including 0.1% ammonium acetate 100% (A) and methyl tert-butyl ether (B)]. All chemical reagents were HPLC grade. Carotenoids were detected under five spectrums (286, 348, 434, 450, and 471 nm), and each carotenoid was identified by the absorption maxima spectrum (Britton, 1985; Gupta et al., 2015). The concentration of carotenoid compounds were calculated using β -carotene (Sigma-Aldrich, St. Louis, MO, USA) and capsanthin (Sigma-Aldrich) calibration curves. Statistically significant differences were determined by t-test.

Results

New Nonsense Mutation in *Ccs* in Orange Pepper *C. annuum* 'K146465'

To identify genetic variation regulating orange fruit color in 'K146465' (Fig. 1), the cDNAs of *Psy*, *Chy2*, and *Ccs* in ripe fruits of 'K146465' and 'Sweet Banana' were cloned and sequenced. The cDNA sequences of *Psy* and *Chy2* in 'K146465' and 'Sweet Banana' were identical, whereas three single-nucleotide polymorphisms (SNPs) were found in the *Ccs* sequence of 'K146465' when compared to the sequence of 'Sweet Banana'. The SNP (G to C) at 235 bp from the start codon is a missense mutation (glycine to arginine), and the SNP (C to T) at 630 bp from the start codon is a silence mutation. A T insertion at 1026 bp from the start codon results in a premature termination codon at the 370 th position of the amino acid sequence (Fig. 2). These results suggest that 'K146465' carries a new type of nonsense mutation in *Ccs*.



Fig. 1. Ripe fruits of *Capsicum annuum* 'Sweet Banana' (left) and 'K146465' (right). The scale bars indicate 1 cm.

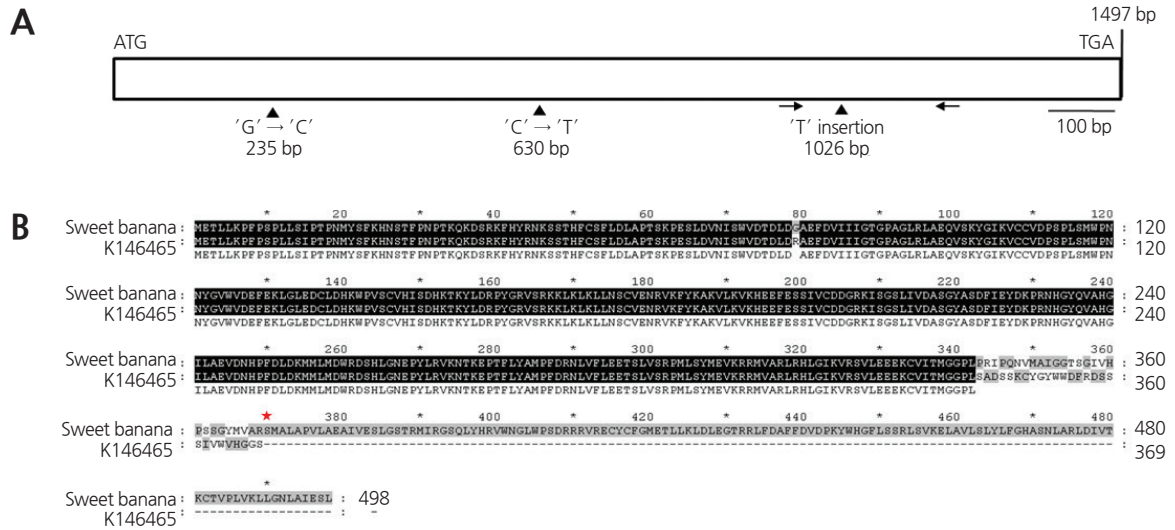


Fig. 2. Allelic variation in *Ccs* in *Capsicum annuum* ‘K146465’. (A) Gene structure of *Ccs*. It consists of a 1497-bp intronless exon. ‘ATG’, start codon and ‘TGA’, stop codon. In ‘K146465’, the *Ccs* coding sequence showed a G (guanine) to C (cytosine) substitution at 235 bp, a C (cytosine) to T (thymine) substitution at 630 bp, and a T insertion at 1026 bp. A pair of arrows indicates the *Ccs*/dCAPS primer site. The bar represents 100 bp. (B) Alignment of the *Ccs* amino acid sequences from *C. annuum* ‘Sweet Banana’ and ‘K146465’. A missense mutation, glycine to arginine, and a premature termination codon are induced at positions 79 and 362 of the amino acid sequence by the G-to-C substitution and T insertion in the *Ccs* sequence in ‘K146465’. A red asterisk indicates the premature termination codon.

Co-segregation Analysis between Orange Fruit Color and *Ccs* Mutation

All fruits of F_1 plants (‘Sweet Banana’ × ‘K146465’) were red, indicating that orange fruit color is a recessive trait. We hypothesized that orange fruit color in ‘K146465’ is controlled by a nonsense mutation in *Ccs*, unlike the previous fruit color models in pepper (Lefebvre et al., 1998; Thorup et al., 2000). To verify this, we conducted a co-segregation analysis between the trait and the mutation in 217 individuals of the F_2 population derived from a cross between ‘Sweet Banana’ and ‘K146465’. Among the F_2 progeny, 164 individuals had red fruits and 53 individuals had orange fruits, thus fitting the expected phenotypic ratio of 3:1 (Table 1).

To distinguish the nonsense mutation in *Ccs*, a dCAPS marker was developed based on the T^{1026} insertion and used to amplify the sequences in ‘Sweet Banana’ and ‘K146465’. Agarose gel electrophoresis of the resulting PCR amplicons digested by *EcoRI* detected a polymorphism. The alleles from Sweet Banana were digested by *EcoRI*, whereas the alleles from ‘K146465’ were not. The co-dominant dCAPS marker was used to identify the *Ccs* genotypes of 217 F_2 progenies. We identified 49 homozygous

Table 1. Segregation of *Ccs* genotypes and ripe fruit color from 217 F_2 progeny derived from a cross between *Capsicum annuum* ‘Sweet Banana’ and ‘K146465’

	Expected ratio	Observed frequency	χ^2 test	p-value
Genotype	1:2:1	49 (^y /-):115 (T ^z /-):53 (T/T)	0.93	0.6293 ^{ns}
Phenotype	3:1	164 (red):53 (orange)	0.04	0.8446 ^{ns}

^z‘T’ indicates thymine insertion in the *Ccs* sequence.

^y ‘-’ indicates no insertion.

^{ns} ‘ns’: not significant.

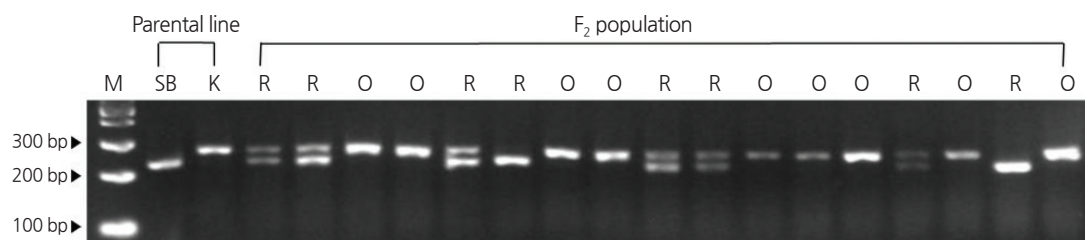


Fig. 3. T^{1026} insertion identified by the dCAPS marker co-segregates with fruit color in an F_2 population derived from a cross between *Capsicum annuum* 'Sweet Banana' (SB) and 'K146465' (K). PCR products amplified by dCAPS primers were digested with *EcoRI* and separated on 2.5% agarose gels. Numbers on the left side indicate the DNA ladder size; M indicates the 1 kb DNA ladder; R and O indicate red and orange fruit color in the F_2 population, respectively.

individuals of 'Sweet Banana' type (red fruits), 115 heterozygous individuals (red fruits), and 53 homozygous individuals of 'K146465' type (orange fruits), fitting the expected genotypic ratio of 1:2:1 (Table 1). Therefore, the nonsense mutation in *Ccs* detected by the dCAPS marker co-segregated with orange fruit color in the 217 individuals of the F_2 progeny (Fig. 3).

Carotenoid Profiling in 'Sweet Banana' and 'K146465' by HPLC

Carotenoid profile was analyzed in ripe fruit pericarps of 'Sweet Banana' and 'K146465' by HPLC. As expected, capsanthin was the most abundant carotenoid in 'Sweet Banana', comprising approximately 74.6% of total carotenoids. Zeaxanthin, β -carotene, β -cryptoxanthin, phytoene, and phytofluene were identified, while lutein and violaxanthin were not detected in 'Sweet Banana'. In contrast, lutein and zeaxanthin were the most abundant carotenoids in 'K146465', while capsanthin was not detected.

Five types of carotenoids were detected in both pepper varieties. Zeaxanthin was detected at the similar levels [$40 \mu\text{g} \cdot \text{g}^{-1}$ fresh weight (FW) in 'Sweet Banana' and $53 \mu\text{g} \cdot \text{g}^{-1}$ FW in 'K146465']. The β -carotene content in 'K146465' was approximately 1.5-fold higher than in 'Sweet Banana'. The levels of phytoene and phytofluene were 10-fold higher in 'K146465' compared to 'Sweet Banana', whereas the amount of β -cryptoxanthin was similar in both pepper varieties (Table 2). These results suggest that the lack of capsanthin and dominance of other carotenoids are metabolic determinants of the orange fruit color in 'K146465'.

Discussion

Pepper fruit color is primarily determined by the concentration and composition of carotenoids (Gómez-García and Ochoa-Alejo, 2013), with capsanthin being the most abundant red carotenoid. Capsanthin and capsorubin are the end products of the carotenoid pathway and are synthesized from antheraxanthin and violaxanthin, respectively. These reactions are catalyzed by

Table 2. Carotenoid quantification ($\mu\text{g} \cdot \text{g}^{-1}$ fresh weight) in ripe fruits of *Capsicum annuum* 'K146465' and 'Sweet Banana' by high performance liquid chromatography analysis

Cultivar	Phytoene	Phytofluene	Lutein	Zeaxanthin	β -carotene	Capsanthin	β -cryptoxanthin	Violaxanthin
K146465	$22.80 \pm 7.11^{*z}$	$10.74 \pm 3.43^*$	$55.77 \pm 7.94^{**}$	53.72 ± 21.24	22.24 ± 6.09	ND ^x	10.58 ± 4.66	4.14 ± 0.53
Sweet Banana	$2.23 \pm 0.62^{*y}$	$0.67 \pm 0.28^*$	ND	40.96 ± 9.62	14.01 ± 3.79	$202.1 \pm 35.8^{**}$	10.79 ± 2.57	ND

^zMean \pm standard error (n = 5).

^ySignificant difference according to the t test (* $p < 0.05$, ** $p < 0.01$).

^x'ND' indicates not detected.

capsanthin-capsorubin synthase (*Ccs*) (Bouvier et al., 1994). Groups of orange (α - and β -carotene, β -cryptoxanthin, antheraxanthin, etc.) and yellow (violaxanthin, lutein, neoxanthin, etc.) carotenoids are minor compounds in pepper fruits (Gómez-García and Ochoa-Alejo, 2013). Red peppers contain high amounts of capsanthin, whereas yellow peppers contain primarily lutein and zeaxanthin and no capsanthin (Kim et al., 2016). Orange peppers accumulate diverse carotenoids, such as capsanthin, lutein, zeaxanthin, and β -carotene, depending on the cultivar (Guzman et al., 2010; Rodriguez-Uribe et al., 2012). Two major loci controlling carotenoid composition in orange peppers were reported as *c2* and *y*, corresponding to genetic variation in *Psy* (Huh et al., 2001) and *Ccs* (Lang et al., 2004), respectively. An EMS mutant in β -carotene hydroxylase also showed orange fruit color (Borovsky et al., 2013). Formerly, it was believed that *Psy* is responsible for orange fruit color (Huh et al., 2001) and *Ccs* for yellow fruit color (Lefebvre et al., 1998). Here, we provide fundamental genetic evidence that *Ccs* can control orange fruit color as well. Two types of genetic variation are related to these recessive alleles: sequence variation and gene expression level variation.

A point mutation was identified in the orange *C. chinense* ‘Habanero’ at the splice-acceptor site of *Psy*; this mutation leads to a frame shift and introduces a premature termination codon (Kim et al., 2010). No expression without coding sequence variation in *Psy* was detected in ripening fruits of the orange *C. annuum* ‘NuMex Sunset’ (Guzman et al., 2010; Rodriguez-Uribe et al., 2012). The distribution of each carotenoid component in the orange pepper *C. chinense* ‘Habanero’ was similar to that of the red pepper. Capsanthin was the major carotenoid, and carotenoid content was 6-fold lower than in red pepper (Huh et al., 2001). Similarly, only small amounts of capsanthin were detected in *C. annuum* ‘NuMex Sunset’, which contains unidentified carotenoids (Rodriguez-Uribe et al., 2012).

An EMS mutant, orange *C. annuum* ‘E-172-3’, carries a missense mutation (glycine 237 > arginine) in *Chy2*, which results in increased levels of β -carotene, a precursor of CHY2 (Borovsky et al., 2013). The orange *C. annuum* ‘msGTY-1’ contains a deletion in the upstream region of *Ccs* (Lang et al., 2004), and the orange *C. annuum* ‘Fogo’ has a single nucleotide deletion in *Ccs*, resulting in a premature termination codon at position 423 of the amino acid sequence (Guzman et al., 2010). No expression without sequence variation in *Ccs* was reported in *C. annuum* ‘Oriole’ and *C. annuum* ‘Orange Grande’ (Guzman et al., 2010; Rodriguez-Uribe et al., 2012).

Carotenoid content variation in these orange peppers was also identified. *C. annuum* ‘Fogo’ accumulates high amounts of violaxanthin, followed by lutein, zeaxanthin, and β -carotene, but no detectable amounts of capsanthin, whereas *C. annuum* ‘Oriole’ and ‘Orange Grande’ contain high amounts of zeaxanthin and lutein, but no capsanthin (Rodriguez-Uribe et al., 2012). Similar to *C. annuum* ‘Fogo’, *C. annuum* ‘K146465’ examined in this study has a point mutation (T insertion) at 1026 bp from the 5’ end, and the carotenoid profile was similar to that of Fogo. Both cultivars accumulate relatively high amounts of lutein, zeaxanthin, and β -carotene and no detectable amounts of capsanthin when compared to red pepper.

Ccs sequence variation regulates both orange and yellow fruit color in pepper. For example, the yellow cultivar *C. annuum* ‘Ramuga’ was reported to have a sequence deletion in *Ccs* (Lefebvre et al., 1998). In addition, two yellow *Capsicum* cultivars show sequence variation in the coding region of *Ccs*; an 8-bp insertion at position 1431 and a single nucleotide substitution at position 599, which introduces premature stop codon (Ha et al., 2007). Carotenoid profiling of these cultivars revealed high levels of lutein and the absence of capsanthin, although the total carotenoid amount was significantly lower than in red peppers. Down regulation of *Ccs* via virus-induced gene silencing also reduced the amount of β -cryptoxanthin, zeaxanthin, and other carotenoids, in particular capsanthin (Tian et al., 2014). The same study reported that the fruits of *Ccs*-silenced peppers were yellow, indicating that *Ccs* can regulate the yellow fruit color in pepper.

β -carotene is one of the most abundant carotenoids in 'K146465' when compared to the red pepper cultivar Sweet Banana. However, it seems that higher β -carotene content may not be a direct effect of the *Ccs* mutation. The lycopene β -cyclase (*Lcy-b*) and lycopene ϵ -cyclase (*Lcy-e*), which are responsible for β -carotene and α -carotene biosynthesis, respectively, evolved from the same ancestor of the lycopene cyclase family. *Ccs* also belongs to this family (Krubasik and Sandmann, 2000). *CCS* showed lycopene β -cyclase activity in vivo (Huguene y et al., 1995) and *Ccs* showed micro-collinearity with the *Beta* locus in tomato (Ronen et al., 2000; Thorup et al., 2000). *Beta* is the overexpressor of the chromoplast-specific lycopene beta cyclase (CYC-B), which accumulates high amounts of β -carotene in tomato fruits (Ronen et al., 2000). The amino acid sequence of *Cyc-b* is very similar to that of *Ccs* (86.1% identity) (Ronen et al., 2000) and neoxanthin synthase (*Nxs*) (99% identity) (Hirschberg, 2001). *Nxs* converts violaxanthin to neoxanthin, which is known as the final product of plant xanthophylls and a precursor of the plant hormone abscisic acid (ABA) (Cutler and Krochko, 1999). In previous studies, neoxanthin was detected equally in yellow and orange *Capsicum* fruits, but not in red peppers (Kim et al., 2016). Despite its detection in *Capsicum* spp., neoxanthin synthase has not been identified yet. In agreement with Guzman et al. (2010), we hypothesize that the truncated *Ccs* in 'K146465' could partly function as *Lcy-b* and *Nxs*, increasing β -carotene and xanthophylls, but does not function as *Ccs*. Additional enzymatic analysis to determine *Lcy-b* and *Nxs* activity of the truncated *Ccs* would be necessary to prove this hypothesis.

Because of differences in carotenoid content, orange peppers carrying the *Ccs* mutation may be nutritionally superior to orange pepper varieties carrying the *Psy* mutation. Although previous reports described *Ccs* mutations in orange peppers with no clear genetic evidence (Guzman et al., 2010; Rodriguez-Urbe et al., 2012), we identified a new nonsense mutation in *Ccs* that determines the orange fruit color by the co-segregation analysis. Furthermore, the use of dCAPS marker will facilitate marker-assisted breeding of *C. annuum* 'K146465' to select for orange fruit color and improve lutein and β -carotene levels in pepper.

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