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The relationship between red fruit colour formation and key genes of capsanthin biosynthesis pathway in *Capsicum annuum*

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Abstract

Pepper (*Capsicum annum* L.) fruit colours vary from green, ivory, or yellow at the juvenile stage to red, orange, and yellow at the mature stage. Carotenoid accumulation causes fruit colour formation in ripe *Capsicum* fruits, and capsanthin is a main carotenoid in ripe red pepper fruits. In this study, pepper cultivars with red, yellow, and orange fruits were employed and four key genes named *phytoene synthase* (*Psy*), *lycopene-β-cyclase* (*Lcyb*), *β-carotene hydroxylase* (*Crtz*), and *capsanthin/capsorubin synthase* (*Ccs*) in the capsanthin biosynthetic pathway were analyzed. Results show that a clear expression of the *Ccs* gene in cv. CK7 (yellow) was detected, but the expressions of the *Psy* and *Lcyb* genes were low comparing with the other tested cultivars. In addition, the expression of the *Ccs* gene was not observed in cv. R37-1 (orange). Furthermore, though the four key genes (*Psy*, *Lcyb*, *Crtz*, and *Ccs*) were detected in red cultivar R15, the expressions of them were low. Sequence analysis further indicated that the *Cc*s genes in cv. CK7 and cv. R37-1 had a high sequence identity (97.36 %). Interestingly, amino acid sequences coded by the *Cc*s genes from cv. CK7, cv. R37-1, and cv. R15 were the same. The above results suggest that the genes *Psy*, *Lcyb*, *Crtz*, and *Ccs* were required for capsanthin synthesis in pepper, and their varied expression patterns resulted in the variety of pepper fruit colours.

Additional key words: capsanthin/capsorubin synthase, β-carotene hydroxylase, gene expression, lycopene-β-cyclase, pepper, phytoene synthase.

Introduction

Pepper (*Capsicum annum* L.) is one of the most important vegetable crops grown in tropical and sub-tropical regions (Marín *et al.* 2004, Shah *et al.* 2014). The pepper fruit colours are mainly due to chlorophyll, anthocyanin (Aza-González *et al.* 2013), and carotenoid pigments. Carotenoids are responsible for a variety of colours in fully mature pepper fruits, ranging from yellow to red, and they are stored in chromoplasts (Deli *et al.* 2001). The carotenoid synthesis pathway begins with the synthesis of phytoene by phytoene synthase during development and ripening pepper fruits (Hirschberg 2001). Several desaturation reactions convert phytoene to orange coloured β-carotene, and then β-carotene is oxygenated to form xanthophylls, such as β-cryptoxanthin, zeaxanthin, and antheraxanthin. In *Capsicum* species, capsanthin/capsorubin synthase (Ccs) synthesizes two red pigments, capsanthin and capsorubin (Thorup *et al*. 2000, Shah *et al.* 2014). Phytoene synthase (*Psy*), lycopene-β-cyclase (*Lcyb*), β-carotene hydroxylase (*Crtz*), and capsanthin/capsorubin synthase (*Ccs*) genes are involved in the carotenoid biosynthesis pathway during pepper fruit colour formation (Ronen *et al*. 1999, Dellapenna and Pogson 2006, Guzman *et al*. 2010). Capsanthin accumulates in the thylakoid membranes of chromoplasts in the mature pericarp of red pepper fruits and contributes up to 60 % of total carotenoids (Perez-Galvez *et al*. 2003, Suzuki and Mori 2003).

The red colour in pepper fruits is controlled by *Ccs*

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Abbreviations: *Ccs* - gene coding capsanthin/capsorubin synthase; *Crtz* - β-carotene hydroxylase; DAA - day after anthesis; *Lcyb* - lycopene-β-cyclase; *Psy* - phytoene synthase.
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gene and the red colour is dominant over yellow. The *Ccs* gene expresses during the conversion of antheraxanthin to capsanthin and capsorubin (Moehs *et al*. 2001). Chlorophyll is replaced by capsanthin in red pepper fruits, whereas in yellow fruits, the *Ccs* gene may not be expressed or may be mutated (Lefebvre *et al*. 1998). Li *et al.* (2013) has identified *Ccs* gene in yellow fruit cultivar CK7. They have detected a premature stop codon derived from C to G change, whereas the promoter region has a 176-bp tandem repeat sequence. They have mentioned that the repeat number was three compared with four in the two tested accessions of *Capsicum chinense* and sequence similarity was from 84.8 to 97.7 % among the four types of repeat. But the relations between fruit colour and tandem repeat sequence is not clear. The study of Ha *et al.* (2007) has shown that the yellow colour at the ripening stage is not due to the deletion of *Ccs* gene but because of *Ccs* gene silencing. Sequence analyses of *Ccs* gene further reveal two structural mutations in yellow pepper that may result in either a premature stop-codon or a frame-shift. Lang *et al*. (2004) found out a deletion of an upstream region of *Ccs* gene in orange fruits. The study of Rodriguez-Uribe *et al*. (2012) in cv. Fogo confirmed the transcripts of *Ccs*-3 gene but no *Ccs* protein accumulation, as this cultivar carries a mutant *Ccs*-3 allele. The premature stop codon in *Ccs*-3 blocked the synthesis of capsanthin and capsorubin.

So far, the researchers gave more attention to the molecular mechanism and function of *Ccs* gene, but limited studies are available on its relations with other genes involved in fruit colour formation. The coordination mechanism of key genes during red colour formation and a comparative analysis of *Ccs* gene in yellow and orange pepper fruits were not still reported. We know that fruit colour formation is a complex process and many genes and enzymes are involved in this process. In this experiment, we have studied four key genes important in the capsanthin biosynthesis pathway, *i.e.*, *Psy*, *Lcyb*, *Crtz*, and *Ccs* in red, yellow, and orange coloured *Capsicum* cultivars.

Materials and methods

Plants and cultivation: The seeds of seven different *Capsicum* cultivars (R15, CK4, CK7, F17, F25, CK1-2-1, and R37-1) were provided by the pepper research group of the College of Horticulture, the Northwest A&F University, P.R. China. These are pure lines and at the ripened stage, the fruit colour of cv. R15 is red, of cv. R37-1 is orange, and of cvs. CK4, CK7, F17, F25, and CK1-2-1 is yellow (Fig. 1). The seeds were treated with hot water (55 °C) for 20 min and then soaked in cold water (28 °C) for 5 h to break dormancy. Then, the seeds were covered with wet cotton cloth and put in a growth chamber in the dark. After 4 d when germination started, they were transferred to pots to raise seedlings. The seedlings (with 8 - 10 true leaves) were transplanted to a high plastic tunnel (Tian *et al*. 2013). The plants were grown under natural irradiance from March to July; the temperature range was 16 - 35 °C. Fruits on the 35th day after anthesis (DAA) were harvested, wrapped in tin foil, immediately put in an ice box, and transferred to laboratory for DNA and RNA extraction.

Extraction of genomic DNA and gene expression detection in pepper fruits: Seeds were removed from fruits and five fruit pericarps of the same cultivar were mixed together, triturated in liquid nitrogen, and a cetyltrimethyl ammonium bromide (CTAB) extraction solution was added. The DNA was extracted from samples according to Uddin *et al*. (2014). The DNA samples were dissolved in Tris-EDTA (TE) buffer and placed to a refrigerator (-20 °C) before gene identification. The DNA primers were designed through the *Primer 5* software according to GenBank released pepper genes (*Psy*: GenBank acc. No. GU085278.1; *Lcyb*: GenBank acc. No. GU085272.1; *Crtz*: GenBank acc. No. GU122945.1; *Ccs*: GenBank acc. No. X77289.1) (Table 1 Suppl.). The primers were designed on open reading frames and were synthesized by *GenScript* (Shanghai, China). The DNA samples of different pepper cultivars were used as templates to yield PCR products. The PCR was done in 15 mm^3 of a reaction mixture (11.58 mm^3 of double destilled H_2O, 1.5 mm^3 of a Taq enzyme buffer, 0.3 mm^3 of dNTPs, 0.012 mm^3 of Taq enzyme, 0.5 mm^3 of a forward primer, 0.5 mm^3 of a reverse primer, and 0.5 mm^3 of a template) and thermo cycles were: an initial denaturation at 95 °C for 5 min, 32 cycles (94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s), a final extension at 72 °C for 10 min, and the reaction was hold at 4 °C for 10 min. Then, 2 mm^3 of a loading buffer was added to the PCR products and gene identification was performed on a 1 % (m/v) agarose gel.

Sequencing analysis and alignment of *Ccs* gene: The primers of *Ccs* gene (full-length) for PCR were designed according to the *Ccs* gene sequence. The oligonucleotides were as follows: an upstream primer 5'-CCCAAA AATTTCAGTTAGTTTGG-3' and a downstream primer, 5'-CCCTTAAGGCTTTTAAGAGGGCC-3'. The primers were synthesized by *GenScript*. The DNA samples of cvs. R15, CK7, and R37-1 were used as templates to run PCR amplification according to full-length primers. The PCR products of the three cultivars were linked with pMD-19T vector, transformed into DH5α, and send to *GenScript* for sequencing. For sequence alignment of *Ccs* gene of the three cultivars, *BLASTN* database

(http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) was used (Montañola *et al*. 2013).

Gene expression in pepper fruits: Total RNA was extracted from the pepper pericarp according to Aguilar-Barragán and Ochoa-Alejo (2014) using *TRIZOL* reagent. The concentration and purity of total RNA was checked spectrophotometrically using absorbance ratios A_{260}/A_{280} and A_{260}/A_{230} (a *NanoDrop 2000C* spectrophotometer, *Thermo Scientific*, Wilmington, USA). Gene expression was measured using real-time quantitative PCR. PCR primers (Table 2 Suppl.) were designed from the published mRNA sequences to amplify regions of *Psy*, *Crtz*, *Lcyb*, and *Ccs* genes (Xu *et al*. 2010). For real-time qPCR analysis, first strand cDNA was synthesized using a *PrimeScript*™ kit (*TaKaRa*, Dalian, China) following the manufacturer's protocols. Real-time qPCR was carried out in 20 mm^3 reactions containing 10.0 mm^3 of *SYBR® Premix Ex Taq™ II* (*TaKaRa*), 2.0 mm^3 of diluted cDNA, and 0.8 mm^3 of forward and reverse primers (Demirel *et al*. 2014). Amplification was performed at 95 °C for 1 min, followed by 45 cycles of 95 °C for 10 s, 55 °C for 30 s, and 72 °C for 30 s. The ubiquitin conjugating protein (*Ubi3*) gene (acc. No. AY486137.1) was used as internal control (reference gene) in this study (Wan *et al*. 2011). The primer sequences used for qPCR are shown in Table 2 Suppl. Relative gene expression was determined using the comparative threshold method (Livak and Schmittgen 2001). Each sample had three independent biological replicates and the experiment was repeated thrice.

Results

Flowers were tagged in the different pepper cultivars. Healthy fruits were harvested at the same age of 35 DAA. The fruit colour in cv. R15 was red, in cv. R37-1 orange, and in cvs. CK4, CK7, F17, F25, and CK1-2-1 yellow (Fig. 1).

The DNA from the fruits of the tested cultivars was extracted, and through the PCR reaction and agarose gel electrophoresis, *Ccs* gene was recorded in cv. R15. The DNA fragment bands were about 366 bp. These results show *Ccs* gene existence in the red fruits. Surprisingly, the same size DNA fragment bands were also recorded in cv. CK7 (yellow) and cv. R37-1 (orange) (Fig. 2). This proves the existence of *Ccs* gene also in the non-red fruit cultivars. But in the other yellow cultivars, the same size bands were not observed. It may be possible that a *Ccs* gene mutation or silencing might occur in these cultivars.

The *Psy*, *Lcyb*, and *Crtz* genes were identified in all seven tested pepper cultivars. There was no significant difference among red, yellow, and orange fruit cultivars (Fig. 3).

The sequence analysis of *Ccs* gene in cultivars CK7, R37-1, and R15 was processed because *Ccs* gene was

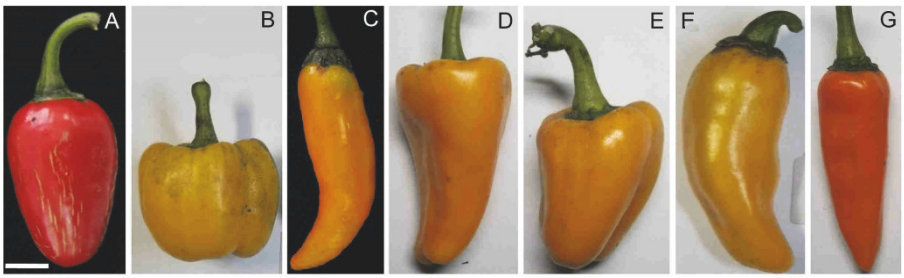

Fig. 1. Seven *Capsicum* cultivars with different colours when their fruits ripen. A red fruit of cv. R15 (*A*), yellow fruits of cvs. CK4, CK7, F17, F25, and CK1-2-1 (*B* - *F*)), and an orange fruit of cv. R37-1 (*G*). *Bar* = 1.5 cm.

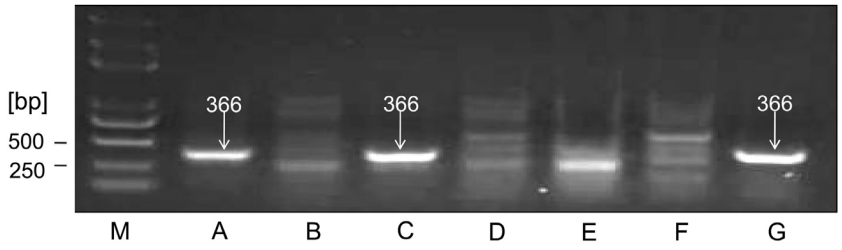

Fig. 2. The identification of capsanthin/capsorubin synthase (*Ccs*) gene in seven *Capsicum* cultivars. *A* - *G* denote the same cvs. as in Fig. 1. M - molecular mass laddder. *Ccs* gene was detected by PCR amplification and agarose gel electrophoresis, and the same size (366 bp) clear bands were found only in cvs. R15, CK7, and R37-1.

detected only in these pepper cultivars. The full-length primers were designed for PCR amplification and *Ccs* gene was extracted from the agarose gel and mixed with pMD-19T vector. The vector was then transferred into the *E. coli* strain, DH5α. The clone was determined to be about 2 160 bp after sequenced (*GenScript*). The *Ccs* gene sequences were highly homologous in cultivars CK7, R37-1, and R15. The similarity rate of *Ccs* gene was 97.36 % among cvs. CK7 and R37-1, whereas among cvs. CK7, R37-1, and R15 it was 98.95 %. These results prove the existence of *Ccs* gene in the yellow (CK7) and orange (R37-1) cultivars, apart from the red pepper cultivars. The similarity ratio of *Ccs* gene in cv. CK7 and cv. R15 was 97.27 %; in cv. CK7, a premature stop codon (TGA) produced a mutation from G to A at 1 176 bp in *Ccs* gene. The similarity ratio of *Ccs* gene in cvs. R15 and R37-1 was 99.63 %; in cv. R37-1 a premature stop codon (TGA) at 1 175 bp changed G to A in *Ccs* gene. The cvs. CK7 and R37-1 sequences were 97.36 % similar; in cv. R37-1, a premature stop codon (TAG) derived from the N to G change at 1309 bp in *Ccs* gene was observed (Fig. 4). The formation of stop codons might be a prime cause of variation in the gene expression in these different cultivars.

The *Ccs* gene was expressed in cv. R15 and cv. CK7. However, it was not expressed in the other yellow fruit cultivars (Fig. 2 and 5*A*). We also detected *Ccs* gene in orange cv. R37-1 (Fig. 2), but its expression was not normal (Fig. 5*A*). It is a strange thing that *Ccs* gene was detected in cv. CK7 (yellow) and its expression was higher than in red cv. R15 (Fig. 5*A*).

The genes *Psy*, *Lcyb*, and *Crtz* were expressed in all tested cultivars (Fig. 5). The expression of *Psy* gene in cvs. R15, F25, and CK1-2-1 was higher than in cvs. CK4, CK7, and F17 (Fig. 5*B*). It was a noteworthy low expression of *Lcyb* gene in cvs. CK7 and R37-1 (Fig. 5*C*). The expression of *Crtz* gene was high in cvs. R15, CK4, and F25, whereas it was lower in cvs. CK7, CK4, and F25, and undetectable in cv. R37-1 (Fig. 5*D*).

The expressions of the target genes of the capsanthin biosynthetic pathway in red cv. R15 were not higher, but they all expressed normally, which is the main cause of the fruit red colour. The *Ccs* gene did not express normally in the yellow fruit cultivars except cv. CK7. The *Ccs* gene in cv. CK7 scored the highest expression, but the expressions of *Psy* and *Lcyb* genes were lower than in the other tested cultivars. The genes expression analysis in orange cv. R37-1 shows that *Ccs* and *Crtz* genes did not express.

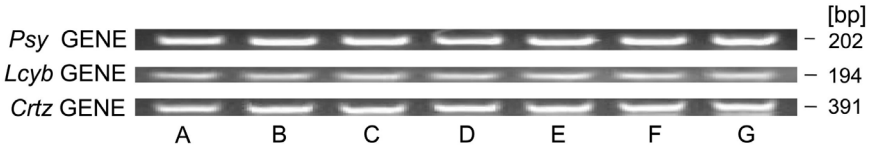

Fig. 3. The identification of *Ccs* upstream genes (*Psy*, *Lcyb*, and *Crtz*) in seven tested *Capsicum* cultivars. *A* - *G* denote the same cvs. as in Fig. 1. The genes were detected by PCR amplification and agarose gel electrophoresis, and there were no difference among tested pepper cultivars.

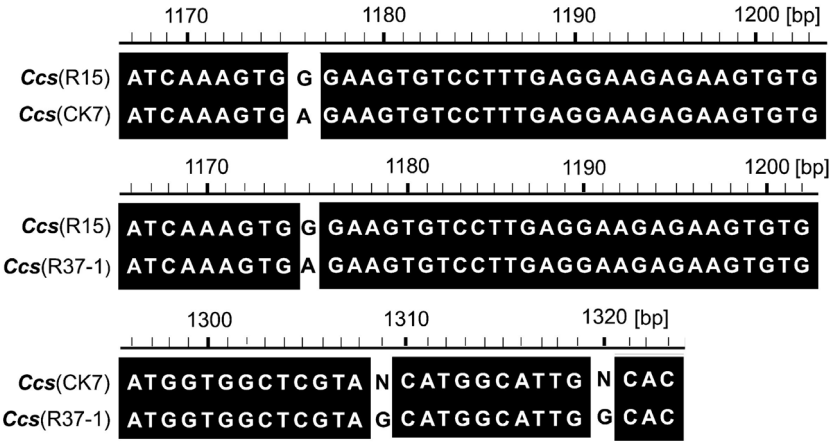

Fig. 4. Stop codons (*white boxes*) of *Ccs* gene from cvs. R15, CK7, and R37-1.

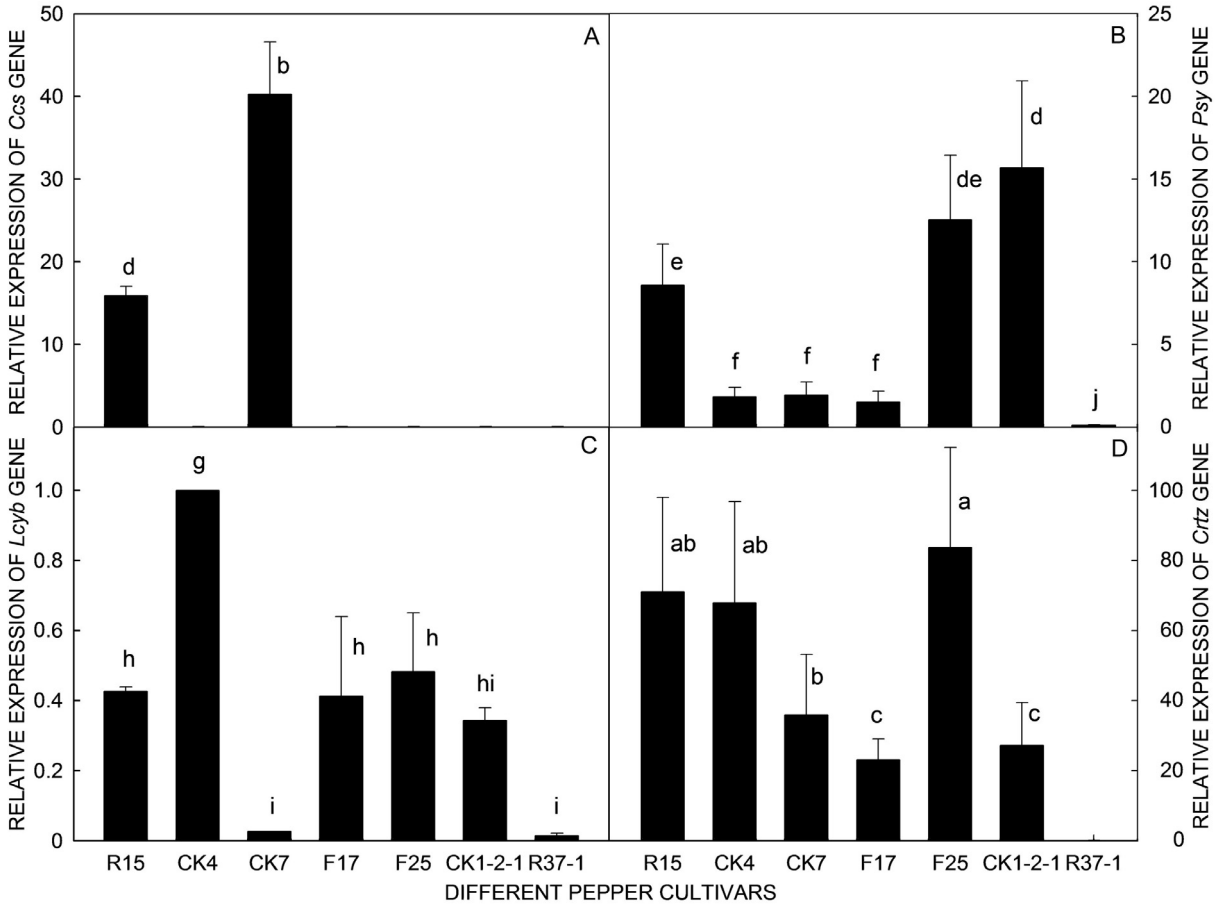

Fig. 5. The analysis of expression of *Ccs*, *Psy*, *Lcyb*, and *Crtz* genes in seven tested *Capsicum* cultivars. Values presented are relative to the *Lcyb* gene expression in cv. CK4 that is considered to have a value of 1. Means ± SD, $n = 3$. Values with different letters denote statistical significant differences among seven tested cvs. according to the Tukey test ($P \leq 0.05$).

Discussion

Many researchers studied pepper fruit colour and their main focus was a role of *Ccs* gene in red colour formation. Previous studies show that the red fruit colour is controlled by *Ccs* gene and the red phenotype is dominant over the yellow phenotype (Thorup *et al*. 2000). The *Ccs* gene is a candidate gene for the y locus controlling the red fruit colour in pepper, which plays an important role in the conversion of antheraxanthin to capsanthin and of violaxanthin to capsorubin. In the F2 segregation from the cross of white and red peppers, three independent loci (y, c1, and c2) are proposed as important for fruit pigmentation. Among the candidate genes in pepper, a single dominant gene corresponding to the y locus has been determined to be *Ccs* gene (Lefebvre *et al*. 1998). The *Ccs* gene is not expressed in orange fruits, which suggests that the orange colour may originate from two possible alleles that are either dominant or recessive for *Ccs* (Popovsky and Paran 2000). Lang *et al*. (2004) thought that the orange fruit colour is due to deletion of *Ccs* gene. A relationship between *Ccs* gene and a fruit colour shows that the normal expression of *Ccs* gene and a sufficient amount of substrates for Ccs are necessary for producing the red colour of pepper fruits, whereas lack of *Ccs* gene expression or deficiency in substrates lead to the orange or yellow colours (Lang *et al*. 2004). Some studies suggested that the deletion of *Ccs* gene is not a prerequisite for the formation of yellow fruits, and indeed, *Ccs* gene was found in two yellow *Capsicum* cultivars (Ha *et al*. 2007). Sequence analyses of *Ccs* gene revealed two structural mutations in these yellow cultivars. These results suggest nonsense-mediated transcriptional silencing *Ccs* gene and not the deletion of *Ccs* gene in yellow fruits (Ha *et al*. 2007). A cv. Fogo carrying a mutant *Ccs*-3 allele shows the transcription of *Ccs* gene but no Ccs protein accumulation. A premature stop codon in *Ccs*-3 prevents Ccs activity to synthesize capsanthin and capsorubin in this cultivar (Rodriguez-Uribe *et al*. 2012).

Here we discuss the research about red colour

formation in *Capsicum* fruits, mutation, non-expression or deletion of *Ccs* gene in yellow or orange cultivars, and *Ccs* impact on red colour formation. In our experiment, we did not find *Ccs* gene expression in yellow cvs. CK4, F17, F25, and CK1-2-1 (Fig. 5*A*). The upstream genes (*Psy*, *Lcyb*, and *Crtz*) were expressed normally, but capsanthin was not synthesized. It is worthwhile to note the existence of *Ccs* gene (Fig. 2) and its normal expression (Fig. 5*A*) in yellow cv. CK7 (Fig. 1). In orange cv. R37-1, *Ccs* gene was detected (Fig. 2) but its expression was very weak (Fig. 5A), which is worth considering for further research.

The similarity ratio of *Ccs* gene sequencing was 97.36 % between cvs. R37-1 and CK7. The sequence comparison of *Ccs* gene in cv. R15 (red) with cv. CK7 (yellow) and cv. R37-1 (orange) shows G→A mutation at 1 176 bp in CK7 and the same mutation at 1 175bp in R37-1; premature stop codons (TGA) of *Ccs* gene was observed in cv. CK7 and cv. R37-1 (Fig. 4). The premature stop codons terminated the normal process although the *Ccs* gene expression was higher in cv. CK7 than in cv. R15 (Fig. 5*A*). The sites of these stop codons were open reading frames in *Ccs* gene (ORF from 201 to 1 686 bp). The base mutation of *Ccs* gene in cv. CK7 and cv. R37-1 affected transcription and expression of the gene.

The expression level of *Ccs* gene was lower in cv. R37-1 than in cv. CK7; logically, the colour should be lighter in cv. R37-1 than in cv. CK7, but in fact, the colour was much darker in cv. R37-1 than in cv. CK7 (Fig. 1). These results reveal that fruit colour formation is a complex process and not only associated with one gene mutation, deletion, or with a stop codon.

The analyses of the other key genes related with the capsanthin biosynthetic pathway shows that expression of *Lcyb* gene in cv. CK7 was much lower than in the other cultivars (Fig. 5*C*). This may be the reason of the red colour in this cultivar, but it still needs confirmation by a future study. We recorded a high similarity ratio (almost 100 %) of *Ccs* gene in cultivars R15, CK7, and R37-1, but there was a great variation among them in the fruit colour.

To sum up, we have explored the functions of the key genes (*Psy*, *Lcyb*, *Crtz*, and *Ccs*) of the capsanthin biosynthesis pathway in the red, yellow, and orange fruit cultivars. Our results reveal that the pepper fruit colour is not associated only with *Ccs* gene. Previous studies and our current study suggest that the red colour is closely associated and indeed inseparable from *Ccs* gene, and it must express normally during red colour formation. However, the current study suggest that *Psy*, *Lcyb*, *Crtz*, and *Ccs* genes were involved in capsanthin synthesis and the abnormal expression of any key gene was the cause of changes in the fruit colour. The previous studies (Rodriguez-Uribe *et al*. 2012, Lang *et al*. 2004) and our current study suggest four requirements for red colour formation in pepper: *1*) *Ccs* gene existence, 2) no deletion or mutation in *Ccs* gene, *3*) the normal expression of *Ccs* gene during pepper fruit colour development, and *4*) the existence and normal expression of *Psy*, *Lcyb*, and *Crtz* genes.

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