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# Molecular genetics of the *y* locus in pepper: its relation to capsanthin-capsorubin synthase and to fruit color

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**Abstract** Classical genetic studies have determined that the yellow fruit color in pepper is recessive to red in the locus *y*. We studied the relation of the *y* locus with the gene coding for capsanthin-capsorubin synthase (CCS) that synthesizes the red carotenoid pigments in the mature fruit. Cosegregation of *y* and CCS in populations derived from crosses between plants bearing red×white and red×yellow fruits indicated the correspondence of the two genes. We obtained indications for the occurrence of a deletion in the CCS gene in plants containing the recessive *y* allele. This deletion did not contain the distal 220 bp of the 3′ end of the gene. We used the CCS gene to determine the genotype of peppers with different fruit colors at the *y* locus. In $BC_1$ segregants from a red×white cross, the red and peach-fruited progenies had the wild-type allele at the CCS locus, while the orange, yellow and white-fruited progenies had the mutant allele. Screening orange-fruited cultivars with CCS as well as segregation analysis of CCS in an additional red×white cross indicated two possible genotypes of the orange fruit color in this locus.

**Keywords** *Capsicum* · Fruit color · Capsanthin-capsorubin synthase

## Introduction

Early studies determined that the mature red color of pepper (*Capsicum*) fruits is dominant to yellow and it is controlled by a single gene designated y (Atkins & Sherrard 1915; Shaw & Khan 1928; Kormos & Kormos 1960). Crosses between red and white-fruited parents have indicated that the inheritance of mature fruit color in pepper is controlled by three independent pairs of genes: *c1*, *c2* and *y* (Kormos & Kormos 1960; Hernandez & Smith 1985). The presence of dominant alleles at the three loci results in red mature fruit, while the presence of recessive alleles at the three loci results in white mature fruit. In an $F_2$ cross between red and white, segregation of eight different colors is expected.

The mature fruit color of pepper is determined by carotenoid pigments. The predominant pigments in the red fruit are capsanthin and capsorubin, that are synthesized by the enzyme capsanthin-capsorubin synthase (CCS). This enzyme was purified from a membrane fraction of pepper fruits by Bouvier et al. (1994). Antibodies raised against the enzyme allowed the isolation of its full length cDNA of 1750 bp. Independently, a cDNA of CCS was isolated from a cDNA library made from ripe fruits (Houlne et al. 1994). An intronless genomic clone of CCS was isolated by Deruere et al. (1994). Expression studies indicated that CCS is induced during chromoplast differentiation when the fruit ripens and that it is not expressed in leaves or in green fruits (Bouvier et al. 1994; Houlne et al. 1994; Hugueney et al. 1996). In yellow fruits the ketocarotenoids, capsanthin and capsorubin are absent, which correlates with the absence of the CCS enzyme and its lack of expression in yellow fruits (Bouvier et al. 1994; Houlne et al. 1994). CCS was determined to be functional in tobacco (Kumagai et al. 1998) as well as its promoter in tomato (Kuntz et al. 1998).

Recently, the association between CCS and *y* was determined by segregation analysis in a red×yellow cross (Lefebvre et al. 1998). It has also been shown that the yellow phenotype is probably associated with a deletion of the CCS gene (Lefebvre et al. 1998). CCS was assigned to pepper linkage group Indigo 25 cM away from the RFLP marker CT204 (Lefebvre et al. 1998). In the present paper we provide additional data on the development of CCS as a codominant marker for *y*, on the extent of the deletion at the mutant CCS allele, and on the genotype at the CCS locus of peppers bearing fruits of additional colors. Inconsistencies with the expected fruit color genotypes based on the model suggested by Hernandez

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& Smith (1985) and the effect of the genetic background on fruit color segregation are discussed.

## Materials and methods

### Plant material

Segregation of CCS and *y* was examined in two $BC_1$ populations (a total of 302 plants). These populations were constructed from inter-specific crosses between *C. chinense* PI 152225, a red-fruited accession and two *C. annuum* lines: Kelvin, a yellow-fruited hybrid cultivar, and 4751, a white-fruited inbred line. The population from the red×yellow cross consisted of 128 $BC_1$ progenies, while that from the red×white cross consisted of 174 $BC_1$ progenies. According to Hernandez & Smith (1985), the red parent genotype is $y^+c1^+c2^+$ and the white parent genotype is *yc1c2*. Therefore, the latter population was expected to segregate for *c1* and *c2* in addition to *y*. The plants were grown in the greenhouse in 1997 at Bet Dagan, Israel. Five to ten fruits from each plant were harvested at maturity and were stored in the laboratory for one week before color determination. Fruit color was determined by matching the fruits to Munsell color standards (Munsell book of color, 1966).

In the present study, 18 commercial or experimental sweet bell-type $F_1$ hybrids grown in the Besor Experimental Station were used for RFLP and PCR analyses. Red cultivars comprised: Cuby (Sluis & Groot), Mazurka (Rijk Zwaan), Torkal (De Ruiter), 35–48 (Rijk Zwaan), Cumbia (Rijk Zwaan), Carisma (Fito), 207693 (Seminis), 208693 (Seminis) and 244691 (Seminis). Yellow cultivars comprised: Taranto (Rijk Zwaan), Oberon (Enza Zaden), Cadia (Enza Zaden), Bossanova (Rijk Zwaan) and Fiesta (Enza Zaden). Orange cultivars comprised: Eagle (Enza Zaden), 35–34 (Rijk Zwaan), Nayrobi (De Ruiter) and 0789 (Enza Zaden).

### PCR analysis

Two 21-mer oligonucleotides (synthesized by Biotechnology General Ltd, Israel) designated CCS-524 (containing the ATG codon) and CCS-525 (containing the stop codon) were used to amplify the CCS gene with DNA from the red-fruited Maor cultivar used as a template for PCR. The sequences for the oligonucleotides were obtained from the CCS gene in the GenBank (accession number X77289). CCS-524: 5′ CTAATGGAAACCCTTCTAAAGC. CCS-525: 5′ AATTCAAAGGCTCTCTATTGCT. PCR was performed with genomic DNA for template in an MJ Research PTC-100 thermocycler under the following conditions: 30 cycles, each comprising 30 s. at 94°C, 1 min. at 62°C and 2 min. at 72°C. The 30 cycles were followed by a 5 min. extension at 72°C.

### RFLP analysis

DNA was extracted either from young fruits by the CTAB method as described by Lefebvre et al. (1993) or from leaves as described by Prince et al. (1997). For RFLP analysis, 10 µg of genomic DNA was digested with 20 units of restriction enzymes (NEB), electrophoresed in a 1% agarose gel and blotted onto a Hybond $N^+$ membrane (Amersham) according to the manufacturer's protocol. The CCS probe was amplified by PCR and was labeled by the random hexamer method. After hybridization, the membranes were washed to a high stringency of 0.1×SSC at 65°C. Membranes were exposed and developed in a Fuji phosphor imager BAS 1500 for few hours to overnight.

## Results

The CCS amplified product (a 1490-bp fragment) was probed to membranes containing DNA from the two segregating populations. Complete cosegregation of CCS and fruit color was observed in two segregating populations: in the red×yellow population all the 68 red-fruited plants were heterozygotes for CCS, while all the 60 yellow-fruited plants were homozygous for the *C. annuum* CCS allele (Fig. 1). Fruit color in the progenies of the red×white cross segregated in a continuous fashion. Only six distinct color classes could be clearly recognized, compared with the eight classes expected according to the model suggested by Hernandez & Smith (1985). The six color classes observed were red (Munsell color: 7.5R 3/12), peach (pale orange; 7.5YR 8/8), orange (5YR 7/16), orange-yellow (10YR 8/16), lemon-yellow (5Y 8.5/10) and white (7.5Y 8.5/8). Red and light red fruits were often indistinguishable from each other and therefore were pooled together. Similarly, orange-yellow and pale orange-yellow fruits were pooled together. All the plants with red or peach fruits (a total of 85) were heterozygous for CCS. All the plants with orange, orange-yellow, lemon-yellow and white fruits (a total of 89) were homozygous for the *C. annuum* CCS allele. The joint segregation of CCS in the two populations fitted a 1:1 ratio for a single gene (Chi square=0.05, $0.75<P<0.9$).

In order to determine whether the polymorphism at the *y* locus is associated with a rearrangement in the recessive allele, we probed the CCS gene to Southern blots containing DNA from the red and the white-fruited parents (PI 152225 and 4751, respectively) digested by different restriction enzymes (Fig. 2). For all the eight restriction enzymes that were tested, a polymorphism between the red and the white-fruited plants was observed. Similar polymorphisms were obtained between the red and the yellow-fruited parents. These results strongly indicate that the polymorphism in this locus is caused by a deletion in the non-red-fruited plants. We could not determine the exact size of the deletion, because the size difference between the two alleles differed among all the

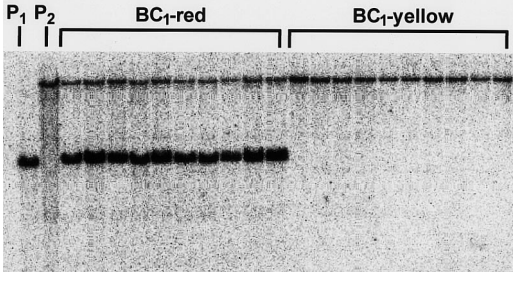


**Fig. 1** Hybridization of CCS to a Southern blot containing DNA digested by *Eco*RI from $BC_1$ plants segregating for fruit color. $P_1$=a red-fruited parent PI 152225 (*C. chinense*), $P_2$=a yellow-fruited parent Cv. Kelvin (*C. annuum*). $BC_1$-red and $BC_1$-yellow represent DNA from plants that are heterozygote $y^+y$ and homozygotes yy respectively

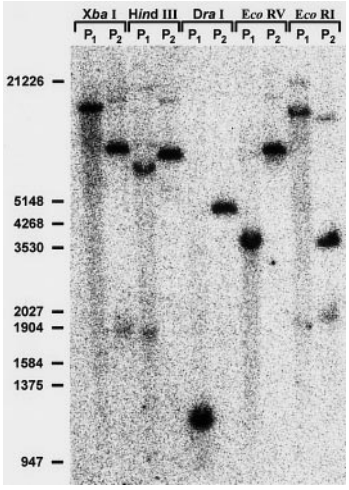


**Fig. 2** Hybridization of CCS to a Southern blot containing DNA from white ($P_1$=*C. annuum* 4751) and red ($P_2$=*C. chinense* PI152225) fruited cultivars digested with five restriction enzymes. Numbers to the left of the blot represent lambda *Eco*RI/*Hin*dIII DNA in bp

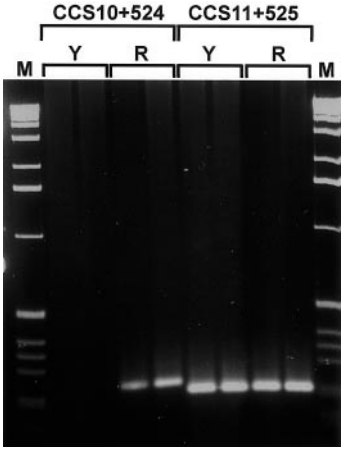


**Fig. 3** PCR analysis of CCS using primers from the 5′ and 3′ ends of the gene (CCS 10+524 and CCS 11+525 respectively). Genomic DNA from two red-fruited cultivars (3548 and 244691) and two yellow-fruited cultivars (Fiesta and Bossanova) was used as a template. M-1-kb bp ladder

digestions. The smallest difference between the two alleles was 3.7 kb in the *DraI* digestion.

The RFLP analysis indicated that part of the CCS gene is maintained in yellow or white-fruited plants, since we observed hybridization signals in these parents. In order to determine which part of the gene was maintained in the yellow phenotype, we designed primers (CCS 10: 5′-GTCCAGATCAGTATCAACCCA; CCS 11: 5′-GGAAGGTACTAGGAGATTGTT) that allowed the amplification of the 5′ and 3′ ends of the coding region of CCS. The use of primers CCS 524 and CCS 10, enabled us to detect a 240-bp amplified product from the 5′ end of CCS in red lines, which was absent from yellow lines (Fig. 3). By using primers CCS 525 and CCS

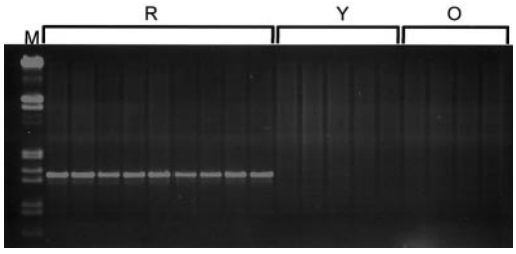


**Fig. 4** Distinguishing among red (R), yellow (Y) and orange (O)-fruited bell-type pepper cultivars by PCR analysis using primers CCS-524 and CCS-525. The eighteen cultivars are listed in Materials and methods. The first lane is a lambda *Eco*RI/*Hin*dIII size marker

11, we detected a 220-bp amplified product from the 3′ end of CCS in both the red and the yellow lines, indicating that the 3′ end of the CCS gene is not deleted in the yellow phenotype.

To verify the association of CCS and *y* further, we analyzed PCR products of CCS using DNA from red, yellow and orange-fruited commercial hybrid cultivars (Fig. 4). By means of primers CCS-524 and CCS-525, a 1490-bp fragment was amplified for all the red-fruited cultivars, while no amplification was observed for either the yellow or orange-fruited cultivars.

In addition to the bell-type orange-fruited cultivars, we analyzed two orange-fruited chile inbreds. The first one was a paprika line (1215) that was identified as a spontaneous mutation of the red paprika cultivar Shalhevet (Levy et al. 1998). The second line was the ornamental cultivar NuMex Sunset (Bosland et al. 1990). In contrast to the bell type orange-fruited cultivars, that had a mutant allele at the CCS locus, PCR and RFLP analyses of both orange lines indicated that they had a wild-type allele at the CCS locus (data not shown).

## Discussion

We obtained genetic evidence that CCS corresponds to the morphological locus *y* that determines the red fruit color in pepper. We cannot completely rule out the possibility that CCS differs from *y*, as the two genes may be very closely linked to each other. Conversion of the yellow color to red by complementation of CCS, either by stable transformation or by transient expression, would provide the ultimate proof for the identity of these two genes.

Breeding for sweet blocky-type peppers is mostly focused on red cultivars, however, the increasing demand for additional colored fruits such as yellow and orange has enhanced breeding for colored peppers. CCS can be used as a codominant RFLP marker or dominant PCR-based marker for fruit color selection at the seedling stage. The distinction between red and yellow fruits by using CCS was consistent for all the cultivars tested in the present study and by Lefebvre et al. (1998). The distinction between red and orange or between yellow

and orange by using CCS should be determined in each case because of the two possible genotypes of the orange fruit color which was observed for the orange cultivars tested in the present study.

In a similar study, by Lefebvre et al. (1998), CCS was used as a dominant RFLP marker because the probe used for hybridization was a truncated cDNA and did not include the last 220 bases at the 3′ end of the coding region. As shown in Fig. 3, this part of the gene is not deleted in the yellow phenotype and, therefore, allows signal detection of the mutant allele by Southern analysis.

The literature on color classification and genotypes is somewhat confusing. Kormos & Kormos (1960) reported a three-genes model in a red×white cross, but they reported only six color classes in the progenies (red, salmon-red and pink being dominant for *y* and orange, lemon-yellow and white being recessive for *y*). In contrast, Hernandez & Smith (1985) reported the expected eight color classes in a red×white cross: red, light-red, orange and pale-orange, were interpreted as being dominant for *y*, while orange-yellow, pale-orange yellow, lemon-yellow and white, were interpreted as being recessive for *y*.

The results from the present study were not consistent with the model suggested by Hernandez & Smith (1985). While the red phenotype in the progenies of the red×white cross had the $y^+$ genotype in both studies, the genotype of the orange fruit was $y^+$ in the study of Hernandez & Smith and was *y* in the present study. Our HPLC analysis supported this conclusion, since the red pigments, capsanthin and capsorubin which were detected in red and peach fruits (although in much smaller quantities than in red fruits), were not detected in orange fruits (data not shown). The genotypic differences at the *y* locus in orange-fruited plants between the present study and the study performed by Hernandez & Smith (1985) may be explained by the use of parents with different genetic background in the two crosses.

Since the red and white parents which were used by Hernandez & Smith (1985) were not listed in their study, we could not repeat their cross. However, in order to examine the effect of the genetic background on fruit color segregation, we analyzed a second red×white population. In this latter population, we crossed the same white-fruited parent (4751) to the red-fruited *C.chinense* accession PI 159234 to create a $BC_1$ population of 107 plants. Compared to PI 152225 that had a light red fruit, PI 159234 had a darker red fruit. We could classify four distinct color classes in the PI 159234×4751 cross. These classes included: red (Munsell color: 7.5R 3/12), orange (10R 5/18), orange-yellow (5YR 7/16) and white (5Y 8.5/12). CCS was scored in these $BC_1$ plants and it was determined that all the red and orange plants (a total of 53) were heterozygous, while all the orange-yellow and white plants (a total of 54) were homozygous for the 4751 allele. The comparison of fruit color and CCS segregation in the two red×white crosses indicated that the peach-fruited phenotype in the PI 152225×4751 cross had a darker orange color in the PI 159234×4751 cross. Therefore, orange-fruited plants were recessive for CCS in the PI 152225×4751 cross and dominant for CCS in the PI 159234×4751 cross. These results demonstrate the effect of the genetic background on fruit color segregation and provide additional evidence for the multiple genotypes of the orange fruit color in pepper.

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