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Physical location of the carotenoid biosynthesis genes *Psy* and β-*Lcy* in *Capsicum annuum* (Solanaceae) using heterologous probes from *Citrus sinensis* (Rutaceae)

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ABSTRACT. Carotenoids are responsible for a range of fruit colors in different hot pepper (*Capsicum*) varieties, from white to deep red. Color traits are genetically determined by three loci, *Y*, *C1*, and *C2*, which are associated with carotenogenic genes. Although such genes have been localized on genetic maps of *Capsicum* and anchored in *Lycopersicon* and *Solanum*, physical mapping in *Capsicum* has been restricted to only a few clusters of some multiple copy genes. Heterologous probes from single copy genes have been rarely used. Fluorescent *in situ* hybridization was performed in *Capsicum annuum* varieties with different fruit colors, using heterologous probes of *Psy* and β-*Lcy* genes obtained from a BAC library of the sweet orange (*Citrus sinensis*). The probes hybridized in the terminal portion of a chromosome pair, confirming the location of these genes in

previous genetic maps. The hybridized segments showed variation in size in both chromosomes.

Key words: Bacterial artificial chromosomes; *In situ* hybridization; Chromosome variation; Pepper

INTRODUCTION

Carotenoids are the most widely distributed pigments in photosynthetic organisms (Fraser and Bramley, 2004). They act as photoprotectors and accessory pigments in photosynthesis, and play an important role in attracting pollinators and dispersing seeds. Carotenoids also can exhibit antioxidant activity, and some are precursors of vitamin A (Thorup et al., 2000; Quirós and Costa, 2006).

In *Capsicum*, carotenoid content varies with fruit development. Correlation between gene expression and variation in fruit color has been investigated in this genus. These genes, *Psy* (phytoene synthase), *Pds* (phytoene desaturase), *Bch* (β-carotene hydroxylases), and *Ccs* (capsanthin/capsorubin synthase), show high levels of expression during fruit ripening in red pepper (*Capsicum annuum*), whereas β-*Lcy* (lycopene β-cyclase) and *Vde* (violaxanthin deepoxidase) show minimum levels of expression. However, all carotenoid biosynthesis genes appear to be required for the accumulation of high levels of carotenoid during ripening in red peppers (Ha et al., 2007).

Ripe fruit of *Capsicum* can be classified into eight classes of colors, ranging from white to deep red. Color variation seems to be determined by three independent gene loci, *Y*, *C1*, and *C2* (Hurtado-Hernandez and Smith, 1985). The *Y* locus corresponds to the *Ccs* gene, whose protein product synthesizes the dominant pigment in red fruit (Lefebvre et al., 1998; Popovsky and Paran, 2000), while the *C2* locus corresponds to the *Psy* gene (Thorup et al., 2000; Huh et al., 2001). The identity of the *C1* locus remains unknown. *C2* and *C1* act in the regulation of amount, rather than type, of carotenoids (Thorup et al., 2000).

Capsicum species show diploid chromosome numbers 2n = 24 or 2n = 26 (Moscone et al., 1993; Pozzobon et al., 2006). *Capsicum annuum* has 10 metacentric pairs, one submetacentric pair with a satellite, and one subtelocentric pair (Moscone et al., 1993). Previous fluorescent *in situ* hybridization (FISH) analysis using heterologous probes in *Capsicum* focused mainly on the location of conserved repetitive ribosomal DNA regions (Park et al., 1999; Kwon and Kim, 2009).

Heterologous probes of single-copy DNA are still not widely used for *in situ* hybridization in plants due to the difficulties posed by the presence of cell wall, cytoplasmic debris or pronounced condensation of metaphase in the chromosome preparations. However, satisfactory results have been obtained with probes cloned in bacterial artificial chromosome (BAC) (Jiang et al., 1995). Such analyses have been successfully applied in conserved genes, either within the same family or among distant families (Zwick et al., 1998), to understand the genome organization and evolution, and to correlate genetic and physical maps (Ning et al., 2000).

In this study, heterologous BAC probes harboring carotenoid biosynthetic genes of *Citrus sinensis* (L.) Osb. were successfully used for *in situ* hybridization with chromosomes of *C. annuum* and comparisons of the hybridization patterns in varieties having distinctively dif-

ferent pigmentation. *Citrus sinensis* has four *Psy* members that were clustered in a single-BAC contig and 11 β-*Lcy* members clustered in three BAC contigs (Chen and Costa, 2010). The *Psy* gene product catalyzes the first committed step in the formation of carotenoids, whereas the β-*Lcy* gene product catalyzes the conversion of lycopene into β-carotene.

MATERIAL AND METHODS

Chromosome preparation

The seeds of *C. annuum* varieties 'Cascadura Ikeda' (green fruit) and 'Ruby Giant' (red fruit) (Feltrin Sementes®) were germinated on wet filter paper on Petri dishes at room temperature. Roots were collected at about 5 mm and pretreated in 8-hydroxyquinoline (2 mM) for 3 h at room temperature, fixed in ethanol and acetic acid (3:1) overnight, and stored at -20°C.

Roots were then washed in distilled water and treated in a solution containing 2% cellulase and 20% pectinase at 37°C for 2 h. Subsequently, the root tips containing the meristems were macerated in 45% acetic acid between slides and coverslips. The coverslips were removed in liquid nitrogen. The best metaphases were selected in a phase-contrast microscope.

Probe labeling

The BAC clones 14I21 (*Psy*) and 37B09 (β-*Lcy*) were obtained from a library of *C. sinensis* var. Ridge Pineapple. The 14I21 clone has two copies of the *Psy* gene, whereas the 37B09 clone contains four copies of β-*Lcy* as determined by Southern blot analysis (data not shown). The DNA of the BACs was isolated by alkaline lysis (Chen and Gmitter, 1999). After purification, DNA was labeled via nick translation with Cy3-dUTP (Amersham Biosciences).

In situ hybridization

FISH procedures were performed according to the protocols of Jiang et al. (1995) at 72% stringency. The hybridization mixture contained 50% (v/v) formamide, 5% (w/v) dextran sulfate, and 2X SSC, 2-5 ng/μL probe. All chromosome preparations were counterstained with DAPI (2 μg/mL) and mounted in Vectashield antifading solution. Images were acquired using a Leica epifluorescence microscope DMRA2 with the aid of the IM50 software and superimposed with Adobe Photoshop 6.0.

RESULTS

Capsicum annuum showed 24 chromosomes in all metaphases analyzed. FISH using heterologous probes of *Citrus* proved to be a useful alternative for mapping homologous genetic loci in *C. annuum* chromosomes. The probes from BACs 37B09 and 14I21, including inserts of β-*Lcy* and *Psy* genes, respectively, hybridized to a pair of chromosomes in the *C. annuum* varieties analyzed. This same number of hybridization marks was also observed in interphase nuclei. However, the hybridized segments showed length heteromorphism, especially within the variety 'Cascadura Ikeda'. The location was always on the chromosome end (Figure 1a-d).

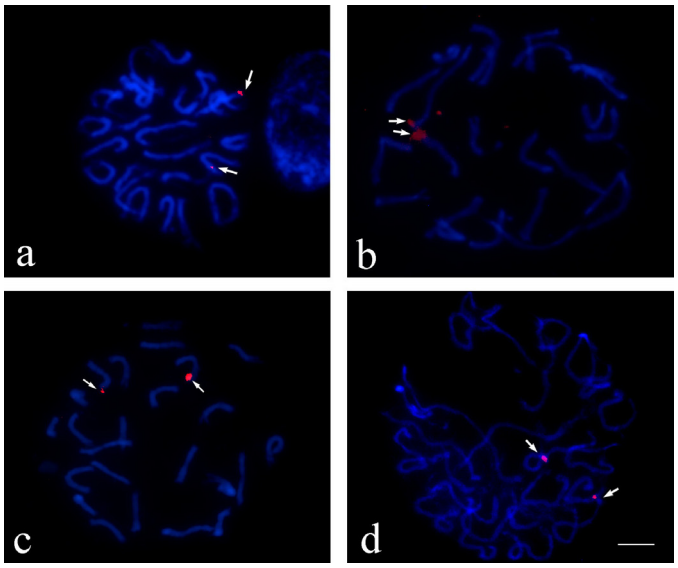

Figure 1. Metaphase of *Capsicum annuum* (a and c) var. 'Ruby Giant' and (b and d) 'Cascadura Ikeda' hybridized with (a and b) *Psy* (BAC 14I21) and (c and d) β-*Lcy* (BAC 37B09) probes. Arrows indicate sites of hybridization. Bar = 10 μm.

A more pronounced difference was observed with the hybridization of probe 37B09 (Figure 1c and d) on chromosomes of the variety 'Ruby Giant', which showed an evident marking in the segment corresponding to the chromosome satellite (Figure 1c). In the variety 'Cascadura Ikeda' markings exhibited the same pattern of differential segment size, but different locations. In this case, the markings occur at greater distance from the ends of chromosomes (Figure 1d), possibly due to positional differences of the genes between varieties.

DISCUSSION

The terminal location of the *Psy* gene (BAC 14I21) was consistent with the available *Capsicum* genetic linkage maps. Huh et al. (2001) estimated the recombination frequencies among the loci coding for orange pigment, red pigment, and other markers in the cross involving *C. annuum* cv. TF68 and *C. chinense* cv. Habanero and inferred that the *Psy* gene is located on one end of the linkage group VII. This result was consistent with the gene mapping done by Kang et al. (2001). Nevertheless, Thorup et al. (2000) estimated the location of this gene to be on the opposite end of linkage group IV. Other genetic markers co-mapped with the *Psy* gene, such as the TG62 RFLP and geranylgeranyl pyrophosphate synthase gene (*Ggps*), were also located on linkage group IV (Livingstone et al., 1999; Thorup et al., 2000; Rao et al., 2003; Wu et al., 2009) or linkage group VII (Kang et al., 2001).

Additional indication of the location of the *Psy* gene on linkage group IV comes from the mapping of the *C2* locus in the same linkage group by Thorup et al. (2000). Molecular genetic analyses in red and orange (*C. annuum* x *C. chinense*) F2 indicate that the *Psy* gene is a likely candidate to match the *C2* locus (Huh et al., 2001), corroborating the location of this gene in *Capsicum*. The *rh4.1* QTL affecting the red color of fruit (Thorup et al., 2000; Ben et

al., 2001) was also mapped to the same linkage group.

Despite the discrepancy found in the previous mapping studies, possibly due to methodological differences, there is agreement about the physical linkage among these genetic markers in the different analyses. Further support for the location of the *Psy* gene on chromosome IV comes from the first genetic mapping of three *Capsicum* species in which the number of linkage groups was assigned to 12 chromosomes (Wu et al., 2009).

The probe from BAC 37B09 containing β-*Lcy* also hybridized to two *C. annuum* chromosomes consistently with the number and pattern of markings in the interphase nuclei (Figure 1c and d). Thorup et al. (2000) detected two β-*Lcy* homologue genes in *Capsicum*, with one of them mapped on the end of chromosome 10. In other maps available, the location of β-*Lcy* gene along with other RAPD and RFLP markers was found in the same linkage group (Livingstone et al., 1999; Kang et al., 2001) and may also correspond to chromosome 10 (Wu et al., 2009). The QTL (*fc10.1* - TG241) correlated with fruit color and the markers for the *Xa* and *l2* mutations, which alter the expression or function of the β-*Lcy* gene, were also mapped in the linkage group X (Thorup et al., 2000; Ben et al., 2001).

The terminal hybridization of the heterologous probes, derived from *Citrus* BAC clones containing *Psy* and β-*Lcy* genes, on a pair of homologous chromosomes in the present analysis is consistent with the previous genetic mapping studies. These results also confirm the typical molecular organization of plant chromosomes that show predominantly expressed genes in the terminal region and repetitive sequences near the centromere (Schmidt and Heslop-Harrison, 1998). High conservation of *Psy* and β-*Lcy* sequences allowed us to successfully hybridize heterologous probes derived from the genomic library of *C. sinensis* to the *C. annuum* chromosomes. *Psy* and β-*Lcy* sequences from *C. sinensis* (Genbank accession Nos. DQ235260 and AY094582) show a similarity as high as 79 and 80%, respectively, with the corresponding genes of *C. annuum*. A high similarity of these and other genes of the carotenoid biosynthetic pathway among species of *Citrus*, *Lycopersicum* and *Arabidopsis* has also been previously reported (Inoue et al., 2006).

Further analyses including other *Capsicum* species and other genes of the carotenoid biosynthetic pathway should be performed to characterize the variation in location and number of gene copies as detected in this analysis. This study can provide support for future investigations by correlating present findings and differences in gene expression in the cells of the *Capsicum annuum* varieties of different colors.

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