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Inheritance and mapping of the *ore* gene controlling the quantity of *β*-carotene in cucumber (*Cucumis sativus* L.) endocarp

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Abstract The metabolic precursor of vitamin A, *β*-carotene, is essential for human health. The gene(s) controlling *β*-carotene quantity (Q*β*C) has been introgressed from Xishuangbanna gourd (XIS, possessing *β*-carotene; *Cucumis sativus* L. var. *xishuangbannanesis* Qi et Yuan; $2n = 2x = 14$) into cultivated cucumber (no *β*-carotene; *Cucumis sativus* L.). To determine the inheritance of Q*β*C in cucumber fruit endocarp, F1 progeny and a set of 124 F7 recombinant inbred lines (RILs) derived from the cultivated cucumber line CC3 and XIS line SWCC8 were evaluated for Q*β*C during 2009 and 2010 in Nanjing, China. Segregation analysis revealed that endocarp Q*β*C of greenhouse-grown fruit was controlled by a single recessive gene. Further, marker analysis indicated the gene controlling Q*β*C was linked to seven SSR markers on linkage group 3, where their order was *SSR20710–SSR19511–SSR15419–SSR07706–ore–SSR23231–SSR11633–SSR20270*. These markers and the putative candidate gene were mapped to cucumber chromosome 3DS. An evaluation of 30 genetically diverse cucumber lines indicated that marker *SSR07706* has utility in further genetic analyses of the Q*β*C orange endocarp gene, designated *ore*. Moreover, the markers defined herein may have utility for marker-assisted selection directed towards the development of cucumber germplasm with high fruit *β*-carotene content.

Keywords Xishuangbanna gourd · Carotenoid · Vitamin A nutrition · Marker-assisted selection

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Introduction

Because of its function in vitamin A biosynthesis, *β*-carotene is one of the most important carotenoids related to human health and nutrition (Mares-Perlman et al. 2002). In sub-Saharan Africa and the Americas,

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between 17 and 30% of children under age of five are deficient in vitamin A (Harjes et al. 2008), which results in xerophthalmia (progressive blindness), increased infant morbidity and mortality, and depressed immunological responses (Underwood 2004). Given this reality, breeding to increase β-carotene levels in crop species has been suggested as a feasible approach for eradicating disease caused by dietary vitamin A deficiency (Graham et al. 2001; Fraser and Bramley 2004).

The fruit of commercial cucumber (*Cucumis sativus* L.; $2n = 2x = 14$) possesses only limited amounts of β-carotene (22–48 μg/100 g fresh weight; Navazio and Simon 2001; Kandlakunta et al. 2008). Xishuangbanna gourd (XIS; *Cucumis sativus* L. var. *xishuangbannanesis* Qi et Yuan), however, is a rare botanical variety of cucumber locally cultivated by the Hani people in the Xishuangbanna area of Yunnan Province, China, that develops fruit with substantially high β-carotene content (Qi et al. 1983; Chen et al. 1994). Its mature fruit possesses an orange-colored endocarp/mesocarp, in which the quantity of β-carotene (QβC) can reach ~700 μg β-carotene per 100 g flesh weight (Simon 1992). The XIS gourd is cross-compatible with commercial cucumber, and thus should be considered a source germplasm for increasing its β-carotene (provitamin A) content for improved nutritional quality.

Initial research on orange-fruited cucumber focused primarily on the inheritance of endocarp flesh color without systematic assessment of QβC. Yang et al. (1991) reported that orange color was dominant to white in F1 progeny derived from crosses between XIS and a *C. sativus* line bearing fruit having a white–light green endocarp. In contrast, Navazio (1994) described a two recessive gene model controlling fruit color in F2 progeny originating from a cross between *C. sativus* SWR18 and XIS, where one gene controlled orange color expression in the mesocarp and another orange color in the endocarp. More recently, Navazio and Simon (2001) confirmed that orange color was controlled by recessive genes in several broad-based XIS × commercial hybrids and demonstrated that substantially high QβC was associated with orange mesocarp fruit. In order to elucidate the genetics of QβC in fruit of germplasm derived from XIS, Cuevas et al. (2010) used the US Processing cucumber parental lines Gy7 and EOM 402-10 to create cross-progenies (F1, F2, BC1P1, and BC1P2) to determine the inheritance of QβC in fruit mesocarp and endocarp tissue. Progeny segregations indicated that two recessive genes control mesocarp QβC, while a single recessive gene controlled endocarp QβC. However, the small population size (Cuevas et al. 2010) resulted in co-segregation of mesocarp and endocarp QβC that did not always fit expected ratios. Thus, the inheritance of QβC in cucumber might be more accurately determined using a larger population.

Genes for controlling QβC have been previously reported in *Zea mays* (Harjes et al. 2008), *Solanum lycopersicum* (Fulton et al. 2000; Davuluri et al. 2005), and *Brassica oleracea* L. Botritis Group (Lu et al. 2006), but those in cucumber have not been adequately defined. Moreover, the identification of markers linked to QβC may have utility for marker-assisted selection (MAS) directed towards incorporation of the high QβC from XIS into cultivated cucumber. Therefore, experiments were designed to determine the inheritance of QβC in cucumber fruit endocarp, identify the molecular markers linked to endocarp QβC accumulation, and then map the gene(s) controlling QβC. This required the construction of a high-density, simple sequence repeat (SSR) marker-based genetic linkage map derived from crossing cultivated cucumber line CC3 and the XIS-derived line SWCC8 using the backbone map recently created by Ren et al. (2009) from whole genome shotgun sequencing.

Materials and methods

Plant materials

Experiments employed the cultivated cucumber inbred line CC3 (maternal parent; P1; long-fruited Chinese type) that develops fruit having a white endocarp and a comparatively low quantity of β-carotene (LQβC), and an XIS-derived inbred line SWCC8 (paternal parent; P2; long-fruited Chinese type) that develops fruit with orange endocarp that possesses high quantities of β-carotene (HQβC). To investigate the inheritance of QβC, CC3 was crossed to SWCC8 and then hybrid progeny were self-pollinated to yield 124 F7 RILs that were subsequently evaluated for QβC and the development of an SSR-based map for the identification and mapping of

QβC genes. Additionally, 30 cucumber inbred lines and populations (Electronic Supplementary Material Table 1S) of diverse origins (i.e., growing regions) that developed fruit with a range of endocarp color were used to evaluate the potential utility of SSR markers linked to the putative genes controlling QβC.

Experimental design

The 124 F7 RILs were evaluated during the spring of 2009 and 2010 in a greenhouse at the Nanjing Agricultural University Experimental Farm (NAU-EF), Jiangpu, Nanjing, China. Germinated seeds were planted in 10-cm plastic pots (Nanjing Flower Gardening Co., Ltd.) filled with vermiculite on 10 March and seedlings at three-leaf stage were transplanted into a plastic house on 5 April when the average temperature was about 18/30°C (night/daytime) with a 12-h/12-h photoperiod.

The experimental design employed was a randomized complete block design (RCBD). Ten plants per line were planted to determine the phenotype data. The planting space was 30 cm within row and 70 cm between rows.

Endocarp color classification

At least one fruit was harvested from each plant 40 days after hand pollination. Mature fruit were cut in transverse section to evaluate endocarp color by visual inspection. Endocarp was categorized into nine color groupings using the Royal Horticultural Society (RHS) mini-color chart (2005). These included white (RHS 157B), light green (RHS 149D), green (RHS 145C), green-yellow (RHS 150B), yellow-green (RHS 2C), light yellow (RHS 2D), yellow (RHS 8C), light orange (RHS 20B), and orange (RHS 21B) (Cuevas et al. 2010).

Determination of β-carotene content

Six endocarp samples per line ($\sim$5.0 g fresh weight) were analyzed for QβC from each of the nine color classes assigned during visual examination. Samples were held at -80°C and then lyophilized, after which β-carotene was extracted and quantified by reverse phase high-performance liquid chromatography (HPLC) using a standardized, synthetic β-carotene curve according to Simon and Wolff (1987).

Bulk segregant analysis (BSA)

All RILs were categorized as high β-carotene (HQβC) if fruit color was orange to light orange, or as low β-carotene (LQβC) if fruit color was yellow to white. The bulk DNA pools and parent samples were then used to identify markers with contrasting polymorphisms by bulk segregant analysis (BSA; Michelmore et al. 1991). Total DNA was extracted from young, healthy leaves of the parents and RIL plants using the CTAB method according to Clark (1997), and HQβC and LQβC bulks were constructed using equal amounts of DNA from 10 RIL plants with high β-carotene content (orange endocarp only) and 10 plants with low β-carotene content (white endocarp only), respectively.

SSR marker analysis

The 421 SSR markers employed by Ren et al. (2009) to construct a high-density SSR-based cucumber linkage map were used to identify polymorphisms between parents and to define HQβC and LQβC bulks. The polymorphic SSR markers recovered from BSA and parental analyses were used to genotype RILs to identify marker—trait linkages with putative gene(s) controlling QβC. Table 2S indicates the polymorphic SSR primers used in this study.

PCR and product analysis

The polymerase chain reaction (PCR) for each SSR marker was performed in a TP600 Peltier Thermal Cycler (Takara Bio Inc., Japan) using a total volume of 20 µl containing 10× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 U *Taq* DNA polymerase, 50 ng each primer, and 80–100 ng total DNA (Katzir et al. 1996). PCR was performed at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s; 55 or 60°C (based on primer annealing temperature) for 1 min; and 72°C for 1.5 min, with final incubation at 72°C for 5 min before cooling to 4°C. Each PCR product was mixed with 2 µl loading buffer (98% formamide, 10 mM EDTA, pH 8.0, 0.25% bromophenol blue, and 0.25% xylene cyanol) before loading 4–6 µl on a 7.2% polyacyamide gel (19:1 acrylamide-bis) with 1× TBE buffer (90 mM Tris–borate, pH 8.3, 2 mM EDTA) for electrophoresis. Samples were electrophoresed at

200 V for approximately 1 h, and then visualized by silver staining (Charters et al. 1996).

Chromosome assignment and linkage analysis

The chromosomal locations of the SSR markers linked to putative QβC loci were inferred by their position on the high-density genetic linkage map of Ren et al. (2009). Chi-squared (χ^2) tests for goodness-of-fit were used to test for deviations of observed data from theoretically expected segregations (1:1) where significant distortion was declared at $P < 0.01$. Linkages between DNA markers and the gene controlling QβC were established using Mapmaker/Exp version 3.0b software (Lincoln et al. 1993) with the *group* command at LOD threshold >3.0. Markers within a group were ordered using the *order* command with LOD of 3.0. Map distances were calculated using the Kosambi mapping function (1944) and loci were ordered using the 'sequence' and 'compare' commands, with an LOD threshold score of 3.0.

Evaluation of potential utility of SSR markers linked to the QβC

To evaluate the potential utility of SSR markers closely linked to a gene(s) controlling QβC for breeding, 30 additional cucumber populations and inbred lines (Table 1S) of diverse geographic origins and endocarp fruit coloration were genotyped. The PCR products of those 30 lines, along with mapping parental lines (CC3 and SWCC8), were subjected to electrophoresis (7.2% polyacyamide gels with 1× TBE buffer) and visualized using the methods described above.

Results

Inheritance of QβC

Differences in fruit endocarp color were associated with differences in their QβC (Fig. 1S). These coloration differences and QβC were detected between parental lines (CC3 and SWCC8) and the BSA bulks (HQβC and LQβC; Fig. 1; $P < 0.05$, single degree of freedom contrasts) and among the RILs examined.

The mature fruit endocarp color (40 days post-pollination) in F1 plants (CC3 × SWCC8) was similar to the LQβC (white endocarp) parent (CC3), indicating that QβC is a recessive trait in these cross-progeny, which is in accordance with earlier studies (Navazio and Simon 2001). Endocarp color distribution in the RIL population could be partitioned into two distinct classes, where 64 lines possessed light orange to orange endocarp (HQβC group) and 60 lines were classified as white to yellow (LQβC group) (Table 1). In 2009 and 2010, RIL segregation adequately fitted the 1 HQβC:1 LQβC distribution expected for a single recessive genetic model determining QβC in these RILs ($\chi^2 = 0.036$, $P = 0.85$) (Table 1). These results further support the existence of a single recessive gene that controls endocarp QβC in cucumber (Cuevas et al. 2010), which is designated herein as *ore*.

Identification of SSR markers linked to *ore*

The CC3 × SWCC8 RIL population and associated polymorphic makers described herein were used for mapping *ore*. One hundred and sixteen (27.6%) of the 421 SSR markers chosen for the initial primer screening were polymorphic between parental lines CC3 and SWCC8. Bulk segregant analysis showed that *SSR07706* was possibly linked to *ore*, but a larger population is needed to show that the marker is co-segregated with *ore* (Fig. 2S).

Since *SSR07706* had been previously mapped to the short arm of chromosome 3D (Ren et al. 2009), the 94 SSR primer pairs positioned on that chromosome were assessed using the RILs described herein. This allowed for the further characterization of the genomic region associated with *ore*, where six polymorphic markers (*SSR20710*, *SSR19511*, *SSR15419*, *SSR23231*, *SSR11633*, and *SSR20270*) were found to co-segregate with QβC upon further BSA (Fig. 2). The positions of these markers were subsequently determined using the RIL population, which confirmed their genetic associations with *ore*. The segregation of *SSR07706* (i.e., 195 bp and 200 bp associated with the HQβC and LQβC parents, respectively) is shown in Fig. 3. While the map position of *SSR07706* is proximal to the *ore* gene (1.9 cM), *SSR15419*, *SSR19511*, and *SSR20710* are

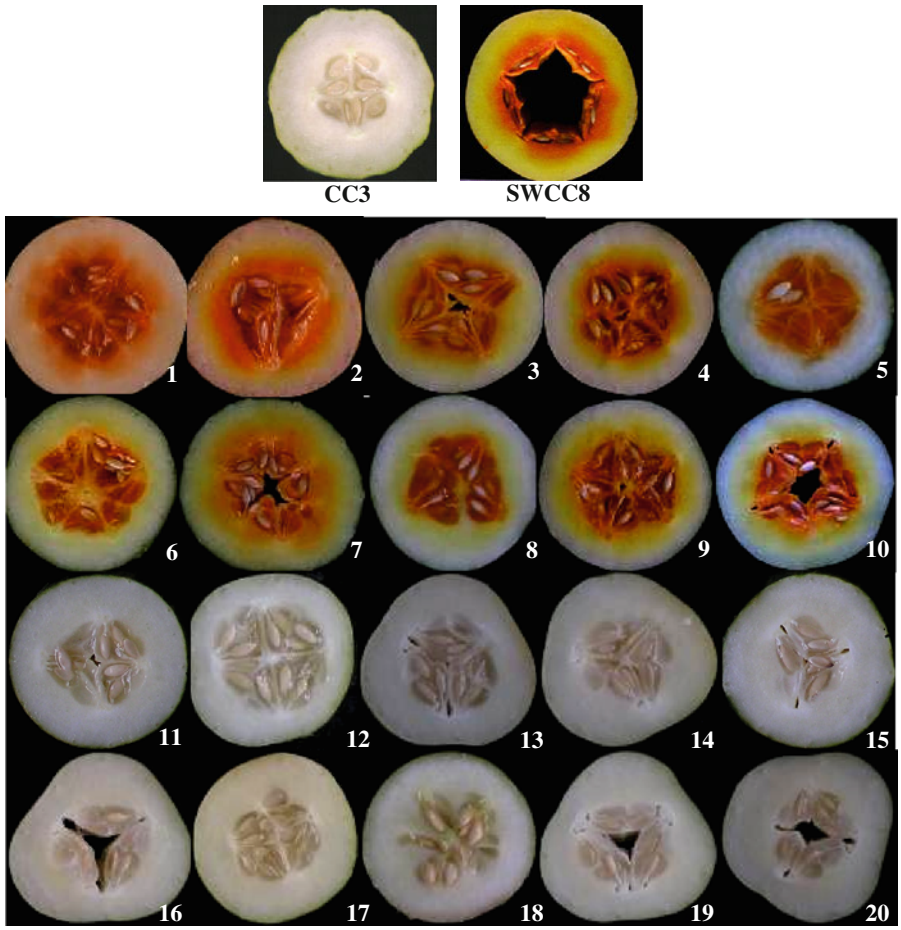

Fig. 1 Endocarp color phenotypes as observed in recombinant inbred lines (RILs) of cucumber (*Cucumis sativus* L.) derived from a CC3 (orange fruit endocarp) × SWCC8 (white fruit endocarp) mating. While samples *1–10* contain comparatively high quantities of *β*-carotene (HQ*β*C; orange endocarp) individuals and were used in the construction of a HQ*β*C pool (10 plants), samples *11–20* possess low quantities of *β*-carotene (LQ*β*C; white fruit endocarp) and were used to develop a LQ*β*C pool (10 plants) for bulk segregant analysis

Table 1 Fit to expected segregation ratios for high (HQ*β*C) and low (LQ*β*C) *β*-carotene fruit endocarp phenotypes among cucumber (*Cucumis sativus* L.) parents, F1, and recombinant inbred lines (RILs) as evaluated in a greenhouse at Nanjing, China, in 2009 and 2010

Parent or cross progeny[a]	Endocarp phenotype[b]		Expected ratio	χ^2 value*	*P* value
	HQ*β*C	LQ*β*C			
CC3	0/0	20/20	0 HQ*β*C:1 LQ*β*C	–	–
SWCC8	20/20	0/0	1 HQ*β*C:0 LQ*β*C	–	–
CC3/SWCC8 F1	0/0	20/20	0 HQ*β*C:1 LQ*β*C	–	–
CC3/SWCC8 RIL	64/64	60/60	1 HQ*β*C:1 LQ*β*C	0.036	0.85

* $P = 0.05$

[a] CC3 = cultivated cucumber inbred line, low *β*-carotene content; SWCC8 = XIS inbred line, high *β*-carotene content; RIL = F7 lines

[b] LQ*β*C = low *β*-carotene content (only white endocarp; 22–48 μg *β*-carotene per 100 g flesh weight), and HQ*β*C = high *β*-carotene content (only orange endocarp; ~700 μg *β*-carotene per 100 g flesh weight)

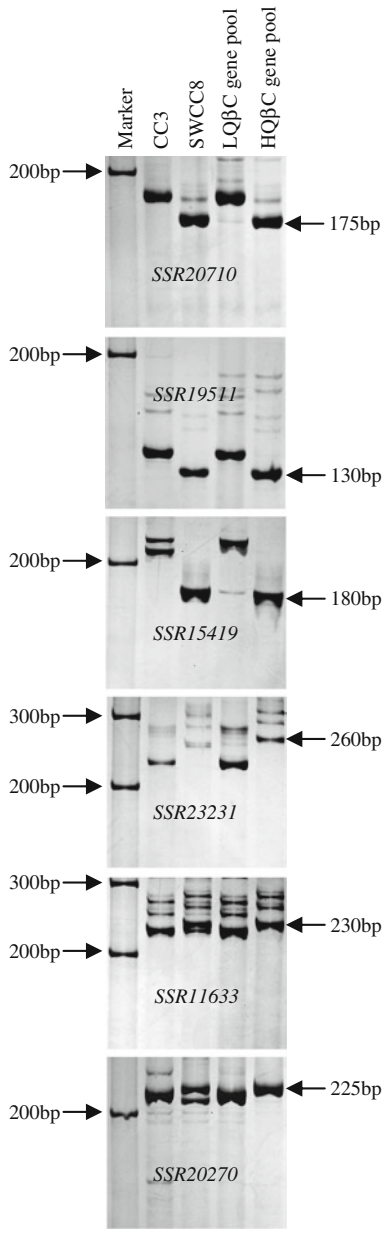

Fig. 2 Amplification products produced by co-dominant SSR markers *SSR20710*, *SSR19511*, *SSR15419*, *SSR23231*, *SSR11633*, *SSR20270* after PCR and electrophoresis

distal to *ore* at 4.4, 8.0, and 11.0 cM, respectively. Although markers *SSR23231*, *SSR11633*, and *SSR20270* were characterized as more distal to *ore* at 4.1, 4.1 and 11.4 cM, respectively (Fig. 4), all linked markers resided on the short arm of chromosome 3D as described by Ren et al. (2009).

Potential utility of the *SSR07706* marker-QβC linkage

The associations of amplicons of marker *SSR07706* [195 bp (HQβC) and 200 bp (LQβC)] in 30 cucumber inbred lines after electrophoresis are shown in Fig. 3S. Although the 195-bp band morphotype was detected in all 15 HQβC lines possessing an orange endocarp, the 15 LQβC lines examined exhibiting a white endocarp did not produce this amplicon.

Discussion

Endocarp color variation in cucumber has been characterized (Simon and Navazio 1997; Navazio and Simon 2001) and the inheritance of this coloration as associated with QβC in XIS has been described (Cuevas et al. 2010). Herein, we report the mapping of the gene associated with QβC, *ore*, and its potential value for improving the nutrition of cucumber and facilitating further map-based cloning of the *ore* gene. Moreover, data presented for the association of *SSR07706* and QβC showed that *SSR07706* has general utility for the development of HQβC germplasm through MAS in cucumber.

Early reports differ as to the mode of inheritance of orange endocarp color in progeny derived from XIS (Yang et al. 1991: orange mesocarp fruit pigmentation was dominant to white; Navazio and Simon 2001: two recessive gene model for control of pigmentation, where one gene controls expression in mesocarp and the other in the endocarp). Recently, Cuevas et al. (2010) confirmed the recessive nature of fruit color (orange dominant to non-pigmentation) in XIS-derived progeny, where two recessive genes control the QβC (orange coloration) in the mesocarp and one recessive gene conditions endocarp QβC. The disparity between the genetic control of mesocarp coloration proposed by Cuevas et al. (2010; two gene model) and Navazio (1994; single gene model) is likely attributable to differences in the population sizes employed [Navazio (1994) = 46 F2 progenies versus Cuevas et al. (2010) = 111 F2 and 51 BC1P2 progenies] and/or to growing environment differences. The results obtained herein from RIL segregation analysis confirm the single gene, recessive inheritance of endocarp QβC (Table 1) as presented by Navazio (1994) and Cuevas et al. (2010).

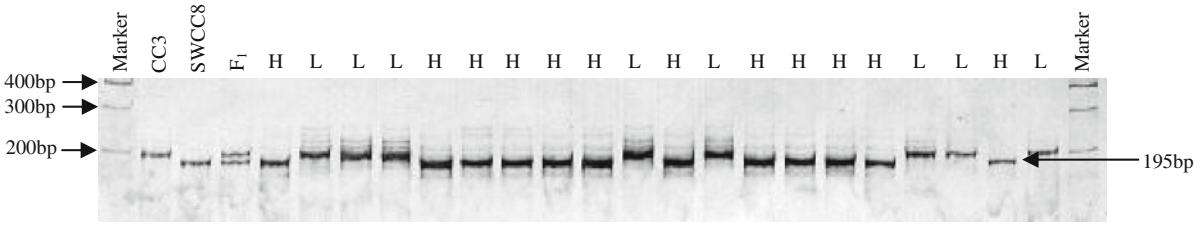

Fig. 3 Amplification products following PCR and electrophoresis of DNA of F7 cucumber (*Cucumis sativus* L.) recombinant inbred lines (RILs) using co-dominant marker *SSR07706* as a primer. RIL originated from CC3 × SWCC8, where fruit of *CC3* have relatively low quantities of *β*-carotene (LQ*β*C; white endocarp; 200 bp) and *SWCC8* have high (HQ*β*C; orange endocarp; 195 bp); *F1* is heterozygous (LQ*β*C; white endocarp); *L* and *H* represent LQ*β*C and HQ*β*C individuals, respectively

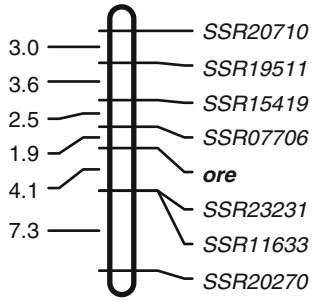

Fig. 4 The map position of *ore*, which is the putative gene conditioning *β*-carotene concentration in cucumber (*Cucumis sativus* L.) fruit, located on chromosome 3DS

The genetic control of Q*β*C in cucumber and melon fruit (endocarp and mesocarp) is controlled by at least two recessive genes (Clayberg 1992; Cuevas et al. 2009). In melon, one major quantitative trait locus (QTL) (putatively, the "orange gene"; Lu et al. 2006) interacts epistatically with two minor QTL to control mesocarp and endocarp Q*β*C (Cuevas et al. 2008). In contrast, control of Q*β*C inheritance (i.e., orange flesh color) in cucumber is simply inherited. Melon and cucumber genome homology and candidate gene analyses of nutritionally important traits (e.g., sugars, *ore*) are becoming increasingly more plausible as genetic maps of these species become highly saturated with common markers (Staub et al. 2007; Ren et al. 2009).

Cuevas et al. (2010) reported that orange and light orange color in cucumber and melon mesocarp/endocarp are rich in *β*-carotene (Cuevas et al. 2008, 2009, 2010). The phenotypic color variation observed in cucumber mesocarp/endocarp during a single season by Cuevas et al. (2010) in F2 progeny was verified by the multi-year study conducted herein (Fig. 1S). The association between fruit endocarp color and the Q*β*C allowed Simon and Navazio (1997) to identify HQ*β*C backcross progeny derived from matings between XIS (PI 509549; donor parent) and 10 US pickling cucumber cultigens (i.e., cultivars, breeding lines, and accessions). These populations permitted Navazio and Simon (2001) to determine lines with the best general combining ability (GCA) for the expression of orange flesh color in mature (SMR × XIS) and immature (Addis × -XIS) fruit. They found that additive genetic effects conditioned carotenoid accumulation in immature fruits, and additive and non-additive factors were important for orange color expression in mature fruits. Although these studies led to the release of HQ*β*C lines with orange mesocarp/endocarp, these germplasms possessed some commercially undesirable traits (e.g., poor fruit quality including fruit shape, length, and spine color). Orange color intensity (hue; Q*β*C) and dispersion (uniform color) in endocarp/mesocarp tissue are important breeding objectives for improved nutritional quality in cucumber that are likely under polygenic control. Based on the studies of Navazio and Simon (2001), Cuevas et al. (2010) and data presented herein, it is likely that gains from selection for HQ*β*C/orange color will likely be complex and require highly replicated, season-specific multiple locations that are under controlled conditions (i.e., greenhouse or hop-house).

Gains from selection for complex cucumber yield and quality traits have enhanced during MAS backcrossing (Fazio et al. 2003; Fan et al. 2006; Robbins and Staub 2009). In this study, BSA and subsequent mapping identified and positioned molecular markers linked to Q*β*C at 2.5–3.6 cM from *ore* on chromosme 3D (Ren et al. 2009), suggesting their utility in MAS (Tanksley 1983; Fazio et al. 2002). Three SSR markers

defined herein are linked tightly to *ore* [*SSR07706* (1.9 cM), *SSR23231* (4.1 cM) and *SSR11633* (4.1 cM)] (Fig. 4). Given the relatively tight linkage between these markers and *ore*, MAS should be considered as a tool in germplasm enhancement of QβC in cucumber.

Deployment of phenotypic and MAS breeding strategies for the development of HQβC germplasm will require the consideration of complex genotype × environment (G × E) and epistatic interactions that affect fruit coloration hue and dispersion (Bohn et al. 2001; Davies et al. 2006; Eathington et al. 1997; Fazio and Staub 2003). The inbred backcross method (IBL; BC2S3) has proven useful for the introgression of complex traits in cucumber (Robbins et al. 2008). When associated with molecular genotyping of progeny in early backcross generations, genetic diversity can be maximized to produce a broad array of phenotypically diverse germplasm (Robbins et al. 2008). A MAS deployment during IBL development of HQβC might employ the use of F1 progeny derived from initial matings between XIS or the HQβC lines identified herein (Table 1S; Fig. 3S) and commercially acceptable germplasm [e.g., SWCC8 (Chinese type), Addis (US processing type)] as recurrent parents in backcrossing. In such cases, BC1 and/or BC2 progeny segregating for color intensity and distribution would be genotyped for markers linked to *ore*, selected for maximum genetic diversity, self-pollinated, and then the resulting HQβC BC2S3 IBL could be evaluated for yield and quality traits, as well as mesocarp/endocarp coloration in replicated, multi-location trials (long-day vs. short-day growing conditions).

Fully characterized HQβC BC2S3 IBL derived from MAS and phenotypic selection could be self-pollinated to derived nearly-isogenic lines (NIL; BC2S6), which, in turn, would allow for direct use in identification of epistatic interactions (Robbins et al. 2008). One NIL analysis could investigate the fruit color differences (orange hue and dispersion) observed in fruit maturing under long-day and short-day growing conditions in the field and greenhouse (Cuevas et al. 2010; unpublished data).

Additionally, NIL coupled with BAC library analysis can be used in the physical mapping and isolation of the *ore* candidate gene. While carotenoid genes in maize (*Zea* spp.; Wong et al. 2004) and wheat (*Triticum* spp.; Pozniak et al. 2007) have been found to be directly associated with β-carotene accumulation, this is not the case with β-carotene accumulation in carrots (*Daucus* spp.; Santos and Simon 2002; Just et al. 2007) and melon (Cuevas et al. 2008). A determination of the relationship between *ore* (or other putative QβC genes) and β-carotene accumulation in cucumber will require use of sequence information recently made available in the cucumber (Cucumber Genome DataBase, http://www.cucumber.genomics.org.cn/cucumber/cucumber/index.jsp). Such information combined with a knowledge of the genomic position of putative QβC genes (e.g., *ore*) could be used unravel the basis of carotenoid accumulation in cucumber.

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