

Inheritance of beta-carotene-associated flesh color in cucumber (*Cucumis sativus* L.) fruit

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Abstract The nutritional value of cucumber (*Cucumis sativus* L.) can be improved by the introgression of β -carotene (i.e., provitamin A and/or orange flesh) genes from “Xishuangbanna gourd” (XIS; *Cucumis sativus* var. *xishuangbannanensis* Qi et Yuan) into US pickling cucumber. However, the genetics of β -carotene content has not been clearly defined in this US market type. Thus, three previous populations

derived from a US pickling cucumber (‘Addis’) \times XIS mating were evaluated for β -carotene content, from which the high β -carotene inbred line (S₄), ‘EOM 402-10’, was developed. A cross was then made between the US pickling cucumber inbred line ‘Gy7’ [gynoecious, no β -carotene, white flesh; P₁] and ‘EOM 402-10’ [monoecious, possessing β -carotene, orange flesh; P₂] to determine the inheritance of β -carotene in fruit mesocarp and endocarp tissue. Parents and derived cross-progenies (F₁, F₂, BC₁P₁, and BC₁P₂) were evaluated for β -carotene content in a greenhouse in Madison, Wisconsin. While F₁ and BC₁P₁ progeny produced mature fruits possessing white, light-green, and green (0.01–0.02 $\mu\text{g g}^{-1}$ β -carotene) mesocarp, the F₂ and BC₁P₂ progeny mesocarp segregated in various hues of white, green, yellow (0.01–0.34 $\mu\text{g g}^{-1}$ β -carotene), and orange (1.90–2.72 $\mu\text{g g}^{-1}$ β -carotene). Mesocarp and endocarp F₂ segregation adequately fit a 15:1 [low- β -carotene (0.01–0.34 $\mu\text{g g}^{-1}$): high- β -carotene (1.90–2.72 $\mu\text{g g}^{-1}$)] and 3:1 (low- β -carotene: high- β -carotene) ratio, respectively. Likewise, segregation of carotene concentration in mesocarp and endocarp tissues in BC₁P₂ progeny adequately fit a 3:1 (low- β -carotene: high- β -carotene) and 1:1 (low- β -carotene: high- β -carotene) ratio, respectively. Progeny segregations indicate that two recessive genes control the β -carotene content in the mesocarp, while one recessive gene controls β -carotene content in the endocarp. Single marker analysis of F₂ progeny using the carotenoid biosynthesis gene *Phytoene synthase*

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determined that there was no association between this gene and the observed β -carotene variation in either fruit mesocarp or endocarp.

Keywords Carotenoid · Beta-carotene · Exotic germplasm · Vitamin A · Nutraceutical

Introduction

Carotenoids play indispensable roles in plants which includes phyto-hormone precursor regulation (Schwartz et al. 2003) and modulation of biosynthetic pathways for environmental adaptation (Demming-Adams and Adams 2002). Strong evidence suggests that diets rich in β -carotene (synom., pro-vitamin A; precursor of retinol) and other carotenoids (e.g., lycopene) can prevent the onset of some chronic diseases such as phrynoderma, anemia (Mares-Perlman et al. 2002) and certain cancers (e.g., prostate; Giovannucci 2002). The United States recommended dietary allowance (RDA) of retinol is 1 mg day⁻¹ equivalent (i.e., approximately 6 mg of β -carotene equivalents). However, practical implementation of this recommendation is often not achieved in rural and underdeveloped areas of the world. As a result, vitamin A deficiency (VAD) is a major public problem in over 75 countries in the developing world (West et al. 2002).

The white to green-colored mesocarp of commercial cucumber (*Cucumis sativus* L.; $2n = 2x = 14$) has been of culinary importance to humans for millennia. Several major types of cucumber are cultivated worldwide, including American processing and fresh market classes, European gherkin and glasshouse types, the German Schalgurken, the Mideast Beit Alpha form, and Oriental trellis (burpless) varieties (Staub et al. 2008). Although cucumber has a narrow genetic base (i.e., low diversity), plant introductions (i.e., PIs) have contributed significantly to the improvement of all major market types (Tatlioglu 1993).

Several unique, exotic germplasms that could potentially enhance cucumber production and nutritional quality have not been widely utilized in breeding programs. For instance, the “Xishuangbanna gourd” (XIS; *Cucumis sativus* var. *xishuangbannanesis* Qi et Yuan; $2n = 2x = 14$) is cultivated in the mountain regions (800–1,200 m elevation) of southern Yunnan

province, P.R. China. This exotic germplasm was introduced into the US between 1987 and 1988 (Navazio 1994), produces fruit that possess an orange-colored endocarp/mesocarp (i.e., β -carotene content), and is cross-compatible with cultivated cucumber (Yang et al. 1991).

Simon and Navazio (1997) made crosses between XIS (PI 509549) and 10 US pickling cucumber cultigens (i.e., cultivars, breeding lines, and accessions) to produce progeny segregating for yellow and orange internal fruit (mesocarp/endocarp) color. For instance, half-sib families derived from backcross families incorporating two elite breeding lines and XIS {[('SMR 18' × XIS) × XIS] and [(‘Addis’ × XIS) × XIS]} exhibited intensive yellow-orange endocarp/mesocarp pigmentation. Subsequently, Navazio and Simon (2001) determine that ‘SMR 18’ × XIS hybrid progeny exhibited good general combining ability (GCA) for expression of orange flesh color in mature fruit and ‘Addis’ × XIS progeny exhibited superior GCA for expression of orange flesh color in immature fruits. Additionally, they found that additive genetic effects condition carotenoid accumulation in immature fruits, and additive and non-additive factors are important for orange color expression in mature fruits. However, growing environment has a dramatic influence on the expression of carotenoid pigmentation in immature fruits, while it does not appreciably influence the internal color of mature fruit.

Yang et al. (1991) indicated that orange-flesh fruit coloration (β -carotene) was conditioned by a dominant genetic factor(s) in F₁ progeny derived from crosses between XIS and a line that produced fruit without mesocarp coloration (i.e., white-light green). In contrast Navazio (1994), explained segregation in F₂ progeny ($n = 46$) originating from a cross between ‘SMR18’ and XIS using a recessive, two-gene model. Since the inheritance of β -carotene accumulation in cucumber is equivocal and the development of cucumber germplasm with enhanced provitamin A content would have worldwide nutritional importance, a study was design to elucidate the genetics of β -carotene accumulation (flesh color) in fruit of germplasm derived from XIS. This study provides information and germplasm for the subsequent development of elite US processing cucumber lines which can accumulate high (>3.00 $\mu\text{g g}^{-1}$) β -carotene concentrations in immature (USDA 2B-3A grade; 25–30 mm in diameter) and mature fruit.

Materials and methods

Plant material

Three “high β -carotene” cucumber populations [Early Orange Mass 400 (EOM 400), Early Orange Mass 402 (EOM 402) and Late Orange Mass 404 (LOM 404)] derived from the crosses between US pickling cucumber lines [‘SMR 18’ and ‘Addis’; (*C. sativus* L. var. *sativus*; white-green flesh color) and XIS (*C. sativus* L. var. *xishuangbannanesis* Qi et Yuan PI 509549; yellow to orange flesh color; Simon and Navazio 1997) were utilized herein. The fruit of these populations segregated for differing hues of yellow and orange color in endocarp and mesocarp tissue. Mature fruit of 50 plants from each of these populations were evaluated visually at Hancock, Wisconsin in Summer 2004 for the expression of orange flesh color (i.e., β -carotene accumulation).

Eleven plants from the EOM 402 population with high β -carotene accumulation were selected, and meristem cuttings were taken and transferred to a greenhouse in Madison, Wisc. Plants obtained from these cuttings were self-pollinated, and mature fruits (i.e., 35–40 days after pollination) were evaluated visually for relative degree of mesocarp and endocarp pigmentation. Individual plants bearing fruits with orange interiors were selected and self-pollinated with continued selection for orange pigmentation and inbreeding for four generations to produce an S₄ line designated ‘EOM 402-10’ (in 2007) that produced true-breeding orange-pigmented fruit (i.e., orange flesh color).

The gynococious inbred line ‘Gy7’ (P₁; white endocarp/mesocarp; Fazio et al. 2003) was crossed to the monoecious line ‘EOM 402-10’ (P₂; orange endocarp/mesocarp). A single F₁ plant from this initial mating was self-pollinated to produce F₂ individuals, and backcrossed to yield BC₁P₁ (F₁ × P₁) and BC₁P₂ (P₂ × F₁) progeny for examination.

Experimental design

Seeds from P₁, P₂, F₁, F₂, BC₁P₁, and BC₁P₂ cross-progenies were sown (Growing Mix No. 2; Conrad Fafard, Inc., Agawam, Mass.) in a greenhouse (University of Wisconsin-Madison, Wisc.) in the Spring of 2008. Seedlings were subsequently transplanted into 3.8 l pots at the three-leaf stage in each of two

greenhouses (designated GH1 and GH2), and grown under a 14 h photoperiod supplied by high pressure sodium lighting [1,000 W; ~500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at mid-plant height (1.82 m)].

The experiment was arranged in a randomized complete block design (RCBD), consisting of two blocks (one block per greenhouse). Each of the two experimental blocks consisted of parents (two P₁ and P₂ plants per block), F₁ (two plants per block), and 86 and 61 F₂ progeny in GH1 and GH2, respectively, and 62 and 40 progeny of each backcross (BC₁P₁, and BC₁P₂, respectively) in GH1 and GH2, respectively. All fruits were set by manual self-pollination or by backcrossing to ‘EOM 402-10’ (P₂), hence, 111 F₂, 81 BC₁P₁, and 51 BC₁P₂ fruits were used to determine the inheritance of fruit mesocarp and endocarp color.

Data collection

Mesocarp and endocarp color classification

One mature fruit (40 days after pollination; USDA 4 grade) was harvested from each plant and cut in longitudinal section to determine mesocarp and endocarp color by visual inspection. Mesocarp and endocarp were categorized into nine color groupings using the Royal Horticultural Society (RHS) mini-color chart (2005; Fig. 1). Mesocarp/endocarp color groupings were 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).

Analyses of β -carotene

Biochemical analyses determined the average β -carotene content within each mesocarp and endocarp visual color classification. Eight-nine mesocarp and 46 endocarp samples (~5.0 g fresh weight) were analyzed for β -carotene content to define the color classes (9) assigned during visual examination. Samples were held at –80°C prior to lyophilization, 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).

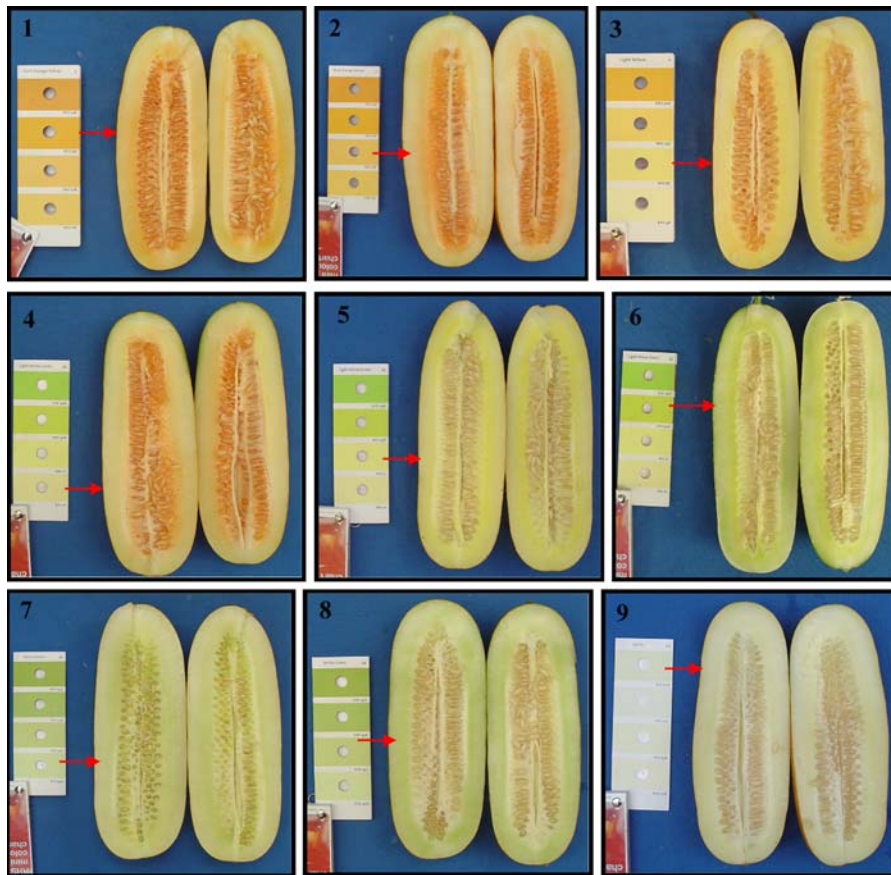


Fig. 1 Fruit mesocarp and endocarp color classification used to characterized cucumber (*Cucumis sativus* L.) cultivars, parental lines, and segregating generations (F_2 , BC_1P_1 and BC_1P_2) derived from a cross between cucumber line ‘Gy7’ (P_1 ; white flesh) and ‘EOM 402-10 (S_4)’ (P_2 ; orange flesh) as evaluated at Walnut Greenhouse of University of Wisconsin, Madison, WI in 2008. Color grouping of mesocarp according

to the Royal Horticultural Society (RHS) Mini-color Chart (2005), where 1 = Orange (RHS 21B), 2 = Light orange (RHS 20B), 3 = Yellow (RHS 8C), 4 = Light yellow (RHS 2D), 5 = Yellow green (RHS 2C), 6 = Green yellow (RHS 150B), 7 = Light green (RHS 149D), 8 = Green (RHS 145C) and, 9 = White flesh (RHS 157B)

Means and standard errors (SE) of fruits within each mesocarp and endocarp color category were calculated. Since variances among color categories were not homogenous, data were transformed (square root) to increase variance homogeneity prior to further statistical appraisals. Subsequently, transformed data were used in single degree of freedom contrasts (SAS 1999) to establish β -carotene groups. Untransformed data are presented herein for convenience of discussion and as a basis for future comparisons.

Genetic ratio analysis

Mesocarp and endocarp color groupings of classified F_2 and BC_1P_2 individuals were analyzed by Chi-square

(χ^2) tests for goodness of fit to standard segregation ratios to determine the segregation pattern in each generation (SAS 1999). Initially, the nine different color categories were grouped in two classes based on HPLC results (Table 1): (1) orange or high β -carotene (1.90–2.72 and 3.05–7.54 $\mu\text{g g}^{-1}$ for mesocarp and endocarp respectively) which consists of light orange (*LORG*), and orange (*ORG*), and (2) non-orange or low β -carotene (0.01–0.34 and 0.16–0.73 $\mu\text{g g}^{-1}$ for mesocarp and endocarp, respectively), which consisted of white (*WH*), light green (*LGR*), green (*GR*), green-yellow (*GRY*), yellow-green (*YGR*), light yellow (*LY*), and yellow (*Y*). The genetic ratios tested were 15:1 and 3:1 (low- β -carotene: high- β -carotene) for mesocarp and endocarp, respectively, using F_2

Table 1 Number of samples, means and standard errors (SE) for β -carotene content ($\mu\text{g g}^{-1}$) of fruit mesocarp and endocarp classification used to characterized cucumber (*Cucumis sativus* L.) flesh color of parental lines and segregating generations (F_2 , BC_1P_1 , and BC_1P_2) derived from unpigmented (white) interior flesh cucumber inbred line ‘Gy7’ (P_1) and the pigmented (orange) interior flesh inbred line ‘EOM 402-10’ (P_2) as evaluated in two greenhouses in Madison, Wisc. in 2008

Color segregation ^a	Mesocarp		Endocarp	
	n^b	Means ^c \pm SE	n^b	Means ^c \pm SE
Orange (<i>ORG</i>)	4	2.72 \pm 1.15 ^a	26	7.54 \pm 0.68 ^a
Light orange (<i>LORG</i>)	4	1.90 \pm 0.83 ^a	1	3.05 ^a
Yellow (<i>Y</i>)	11	0.34 \pm 0.17 ^b	5	0.73 \pm 0.18 ^b
Light yellow (<i>LY</i>)	6	0.06 \pm 0.04 ^b	2	0.33 \pm 0.11 ^b
Yellow green (<i>YGR</i>)	7	0.10 \pm 0.07 ^b	–	–
Green yellow (<i>GRY</i>)	5	0.07 \pm 0.05 ^b	2	0.19 \pm 0.18 ^b
Light green (<i>LGR</i>)	5	0.01 \pm 0.00 ^b	2	0.19 \pm 0.17 ^b
Green (<i>GR</i>)	5	0.01 \pm 0.00 ^b	3	0.37 \pm 0.32 ^b
White (<i>WH</i>)	42	0.02 \pm 0.00 ^b	5	0.16 \pm 0.08 ^b

^a Color grouping according to the English Royal Horticultural Society (RHS) Mini-color Chart (2005), where *ORG* RHS 21B, *LORG* RHS 20B, *Y* RHS 8C, *LY* RHS 2D, *YGR* RHS 2C, *GRY* RHS 150B, *LGR* RHS 149D, *GR* RHS 145C, and *WH* RHS 157B

^b Number of samples (fruits) from segregating generations (F_2 , BC_1P_1 , and BC_1P_2) utilized to determine β -carotene content in each color class. The total numbers of progeny used herein are not represented

^c Means and SE are presented as non-transformed data. Different letter indicates color class significantly different at $P \leq 0.05$ by single degree of freedom contrasts using transformed data (root square)

progeny segregation; and 3:1 and 1:1 (low- β -carotene: high- β -carotene) for mesocarp and endocarp, respectively, using BC_1P_2 progeny segregation. Subsequently, the segregation of mesocarp fruit color in F_2 progeny and BC_1P_2 segregation data were grouped in three color classes by visual inspection (Tables 2, 3); where 1 = white and consisted of white (*WH*), light green (*LGR*) and green (*GR*) fruit, 2 = yellow and consisted of green yellow (*GRY*), yellow green (*YGR*), light yellow (*LY*) and yellow (*Y*) fruit, and 3 = orange and consisted of orange (*ORG*) and light orange (*LOR*) fruit. The genetic ratios tested were 9:6:1 and 1:2:1 (white: yellow: orange) for F_2 and BC_1P_2 , respectively. Finally, the co-segregation of mesocarp and endocarp was evaluated using the three (white, yellow and orange) and two (orange and non-orange) color categories, respectively. The genetic ratios tested were

27:18:3:9:6:1 and 1:2:1:1:2:1 (white/non-orange: yellow/non-orange: orange/non-orange: white/orange: yellow/orange: orange/orange) for F_2 and BC_1P_2 progeny segregations, respectively.

Carotenoid related genes

Previously published degenerative primers for five carotenoid structural genes [*Phytoene synthase* (*PS*), *β -carotene hydroxylase* (*BOH*), *Lycopene β -cyclase* (*LycB*), *Carotenoid isomerase* (*CRTISO*), and *Violaxanthin de-epoxidase* (*VDE*)] and one carotenoid-related gene [*Orange gene* (*Or*; Lu et al. 2006)] (Cuevas et al. 2008) were utilized to amplify parental DNA (‘Gy7’). Polymerase chain reactions and cycling conditions were performed according to Cuevas et al. (2008). After gel electrophoresis, unique amplicons were physically isolated, purified using the Wizard SV Gel clean-up system (Promega Corp., Madison, Wisc.), sequenced via BigDye terminator chemistry, and subsequently evaluated with other homologous species using the NCBI Basic Alignment Search Tool (BLAST; <http://www.ncbi.nlm.nih.gov/BLAST>). Putative genes were declared if the BLAST results contained matched regions not covered by the original degenerative primers. This genomic sequence information was then utilized to design cucumber-specific primers (Table 4) and amplified parental DNA. Sequence polymorphisms between parental line sequences were examined using the Staden Package (Staden et al. 1999), and then more specific primers sets flanking each polymorphism were designed.

Single marker analysis

The degree of segregation distortion associated with newly identified single nucleotide polymorphic (SNP) markers was determined by marker data comparison against the expected 1:2:1 ratio for F_2 progeny using chi-square tests, where significant distortion was declared at $P < 0.01$ (Vuylsteke et al. 1999).

Phenotypic fruit color data obtained from segregating F_2 individuals were converted into metric values using the β -carotene values ($\mu\text{g g}^{-1}$) characteristic of each color grouping (e.g., orange = $2.72 \mu\text{g g}^{-1}$, yellow = $0.34 \mu\text{g g}^{-1}$; Table 1). Subsequently, a linear regression model was employed using the *Proc Reg* procedure of SAS (SAS 1999)

Table 2 Fruit mesocarp and endocarp color segregation in cucumber (*Cucumis sativus* L.) fruit from the segregating progeny (F₂ and BC) derived from a cross between line ‘Gy7’ (P₁; no pigmentation, white flesh) and ‘EOM 402-10 (S₄)’ (P₂; pigmentation, orange flesh) evaluated in a greenhouse in Madison, Wisc. in 2008

	No carotenoids ^a										Total		Segregation	
	White ^b Green ^b	Light Green ^b	Green yellow ^b	Yellow green ^b	Light yellow ^b	Yellow ^b orange ^b	Light orange ^b	Orange ^b	No carotenoids ^a	Carotenoids ^a	Test ratio	X ²	P	
F ₂	55	7	7	11	7	12	4	3	104	7	15:1	0.00	0.98	
Endocarp	56	3	4	2	3	7	1	29	81	30	3:1	0.24	0.62	
BC ₁ P ₁	79	1	0	0	0	0	0	0	81	0	–	–	–	
Endocarp	76	0	0	0	0	0	0	0	81	0	–	–	–	
BC ₁ P ₂	14	3	11	7	5	4	5	1	45	6	3:1	4.8	0.03	
Endocarp	24	0	1	1	3	2	4	14	33	18	1:1	4.4	0.04	

^a Refers to the presence of β-carotene in fruit color tissues evaluated by HPLC analysis

^b Color grouping according to the Royal Horticultural Society (RHS) Mini-color Chart (2005), where white interior tissue RHS 157B, green RHS 145C, light green, RHS 149D, green-yellow RHS 150B, yellow-green RHS 2C, light yellow RHS 2D, yellow RHS 8C, light orange RHS 20B, and orange RHS 21B

Table 3 Co-segregation of fruit mesocarp and endocarp color in cucumber (*Cucumis sativus* L.) fruit from segregating progeny (F₂ and BC₁P₂) derived from a cross between line ‘Gy7’ (P₁; no pigmentation, white flesh) and ‘EOM 402-10 (S₄)’ (P₂; pigmentation, orange flesh) evaluated in a greenhouse in Madison, Wisc. in 2008

	Fruit flesh color ^a						Segregation		
	White ^b	Yellow ^b	White ^c	Orange ^b	White ^b	Yellow ^b	Orange ^b	Test ratio	X ²
Mesocarp	58	23	0	9	14	7	27:18:3:9:6:1	30.1	0.00
Endocarp	18	15	0	0	12	6	1:2:1:1:2:1	34.4	0.00

^a Color grouping according to the Royal Horticultural Society (RHS) Mini-color Chart (2005)

^b White group refer to white (RHS 157B), green (RHS 145C) and light green (RHS 149D) mesocarp tissue; Yellow group refer to green-yellow (RHS 150B), yellow-green (RHS 2C), light yellow (RHS 2D) and yellow (RHS 8C); and Orange group refer to light orange (RHS 20B), and orange (RHS 21B) mesocarp tissue

^c White group refer to white (RHS 157B), green (RHS 145C), light green (RHS 149D), green-yellow (RHS 150B), yellow-green (RHS 2C), light yellow (RHS 2D) and yellow (RHS 8C), and Orange group refer to light orange (RHS 20B), and orange (RHS 21B) endocarp tissue

Table 4 Characteristics of candidate putative carotenoid structural and related genes evaluated in cucumber (*Cucumis sativus* L.) using the F₂ population derived from a cross between 'Gy7' (P₁; non-pigmented, white flesh) and 'EOM 402-10' (P₂; pigmented, orange flesh) as evaluated in a greenhouse in Madison, Wisc. in 2008

Gene symbol ^a	Sequence of primers	Annealing temp. (°C)		Fragment size (bp)		Accession number of closest putative ortholog and % identity ^b
		Exon	Intron	Exon	Intron	
<i>PS</i>	F1- TTGGGTTGATGAGTGTCTCT	60	805 (2)	342	1147	ABK55717 (100)
	R1- AAACATTACCGGCCAATCTG					
	F2- TGAGGACATAATTTGCTGGAAG					
	R2- GCGAAAAACATAATTCCTTTGA					
<i>LycB</i>	F1- GCTGTTGTGTTACCAATGA	60	–	884	884	ABM90918 (98)
	R1- TCCCTCTGTCTCCTCCTTC					
<i>BOH</i>	F1- AATATAACGAAGGTGTACATACAGAA	50	553 (2)	123	676	ABB52074 (75)
	R1- AGCCACGTCATACAAAAAGC					
<i>CRTISO</i>	F1- TCAAGCCCTTCCAGATTCTA	60	169 (2)	256	425	NP172167 (94)
	R1- TGAAGAATATGACGTCCATCG					
<i>VDE</i>	F1- ACAATGGCAACTTGTGTGGT	60	504 (1)	271	775	ABB70514 (73)
	R1- TCAATTGCCCTTTCTCAATC					
<i>Or</i>	F1- GCTGATTGAGCCAGTATCGAC	60	654 (1)	128	782	ABH07408 (66)
	R1- ACAAAGACAGTAGGGCACA					

Gene symbol ^a	Cucumber EST database	NCBI	Sequence of primers used to amplify fragment used for genotyping	Annealing temp. (°C)	Fragment size (bp)	Polymorphism
<i>PS</i>	CU2624	GQ203104	F- CTTTGTCTGGTGAAGATGG R- CACGCCCTGTCAATTTGTTG	60	110	INDEL (13 bp) ^c
<i>LycB</i>	CU2635	GQ203103	–	–	–	–
<i>BOH</i>	–	GQ203101	–	–	–	–
<i>CRTISO</i>	–	GQ203102	–	–	–	–
<i>VDE</i>	–	GQ203105	–	–	–	–
<i>Or</i>	–	GQ203106	–	–	–	–

^a Carotenoid biosynthesis gene *phytoene synthase* (*PS*), *lycopene beta-cyclase* (*Lycβ*), *beta-carotene hydroxylase* (*BOH*), *carotenoid isomerase* (*CRTISO*), *violaxanthin de-epoxidase* (*VDE*), and the *Orange* gene (*Or*)

^b Sequence similarity at the protein level based on tblastx hits. Percent similarity is shown for longest matching stretch of best hit sequence labeled as a desired ortholog gene

^c Insertion/deletion (INDEL) used to construct a co-dominant marker visualized by agarose gel electrophoresis

and the F_2 progeny segregation model according to Liu (1998), in order to determine associations between marker genotype and β -carotene variation.

Results

β -carotene content and fruit color classification

Differences in fruit mesocarp and endocarp color classification were associated with differences in their β -carotene content (Table 1). The two orange mesocarp color categories (*ORG* and *LORG*) and their associated β -carotene values (2.72 and 1.90 $\mu\text{g g}^{-1}$, respectively) were designated the “high- β -carotene” group. In contrast, the other seven color categories (*Y*, *LY*, *YGR*, *GRY*, *LGR*, *GR*, and *WH*) reflected comparatively lower mean mesocarp β -carotene values (0.34, 0.06, 0.10, 0.07, 0.01, 0.01, 0.02 $\mu\text{g g}^{-1}$, respectively) and were designated the “low- β -carotene” group. These groups were significantly different by single degree of freedom contrasts (transformed data; $P < 0.05$). Similar group assortment (high/low- β -carotene) was designed for endocarp color categories. The β -carotene values of *ORG* and *LORG* endocarp (“high- β -carotene”; 7.54 and 3.05 $\mu\text{g g}^{-1}$, respectively) tissues were significantly different from *Y*, *LY*, *GRY*, *LGR*, *GR*, and *WH* endocarp value (“low- β -carotene”; 0.73, 0.33, 0.19, 0.19, 0.37, 0.16 $\mu\text{g g}^{-1}$, respectively) by single degree of freedom contrasts (transformed data; $P < 0.05$).

Phenotypic segregation

The F_1 hybrid progeny derived from crossing line ‘Gy7’ (P_1 ; white flesh) and ‘EOM 402-10’ (P_2 ; orange flesh) as well as BC_1P_1 progeny produced fruit whose mesocarp and endocarp pigmentation possessed low- β -carotene (i.e., white, light green and green flesh; Table 2). In contrast, the pigmentation of mesocarp and endocarp of mature fruit harvested from F_2 and BC_1P_2 progeny varied widely (i.e., segregated for low and high β -carotene content).

The color segregation of fruit of F_2 progeny adequately fit a 15:1 (low- β -carotene: high- β -carotene; Table 2) ratio in mesocarp tissue ($X^2 = 0.00$, $P = 0.98$) and 3:1 ratio in endocarp tissue ($X^2 = 0.24$, $P = 0.62$), which is indicative of the action of two genes for the mesocarp and one for the endocarp.

Likewise, BC_1P_2 progeny mesocarp and endocarp fruit color segregation adequately fit a 3:1 (low- β -carotene: high- β -carotene; Table 2) ratio ($X^2 = 4.8$, $P = 0.03$) and a 1:1 ratio ($X^2 = 4.4$, $P = 0.04$), respectively. The evaluation of 111 F_2 and 51 BC_1P_2 individuals allowed for the identification of three fruits and one fruit, respectively, which developed orange color (*ORG*; high β -carotene content) in both mesocarp and endocarp tissues.

Since the BC_1P_1 mesocarp segregation included *WH*, *LGR* and *GR* pigmentation classes, they were grouped together for analysis. Subsequently, the assessment of segregation of mesocarp fruit color in F_2 progeny indicated adequate fit to a 9:6:1 [(*WH*, *LGR*, *GR*): (*GRY*, *YGR*, *LY*, *Y*): (*LORG*, *ORG*); $X^2 = 1.4$, $P = 0.50$] ratio, and segregation in BC_1P_2 progeny for these classes fit a 1:2:1 ratio ($X^2 = 5.8$, $P = 0.05$), which is indicative of the action of two independent genes. Fruit pigment segregations in F_2 and BC_1P_2 progeny were consistent in each of the two greenhouses used [e.g., in F_2 progeny mesocarp (9:6:1); GH1, $X^2 = 2.45$, $P = 0.29$; GH2, $X^2 = 1.11$, $P = 0.57$, and endocarp (3:1); GH1, $X^2 = 0.12$, $P = 0.73$; GH2, $X^2 = 1.56$, $P = 0.21$; in BC_1P_2 progeny mesocarp (3:1); GH1, $X^2 = 2.80$, $P = 0.09$; GH2, $X^2 = 2.10$, $P = 0.15$, and endocarp (1:1); GH1, $X^2 = 5.2$, $P = 0.02$; GH2, $X^2 = 0.70$, $P = 0.78$].

The co-segregation of fruit mesocarp and endocarp tissue color involved three genes and six different phenotypes. However, orange mesocarp/non-orange endocarp and the white mesocarp/orange endocarp classes were not observed in BC_1P_2 progenies, and individuals possessing orange mesocarp/non-orange endocarp were absent in the F_2 progeny (Table 4). Thus, the segregation of mesocarp/endocarp fruit color in F_2 and BC_1P_2 progeny did not adequately fit the predicted 27:18:3:9:6:1 and 1:2:1:1:2:1 (white/non-orange: yellow/non-orange: orange/non-orange: white/orange: yellow/orange: orange/orange) ratios, respectively, for a three-gene model ($X^2 = 30.10$ and 34.40, $P < 0.00$, respectively).

PCR amplification of putative carotenoid structural and related genes

Genomic sequences from five structural carotenoid genes [*Phytoene synthase* (*PS*), *Lycopene β -cyclase* (*LycB*), *Violaxanthin de-epoxidase* (*VDE*), *β -carotene hydroxylase* (*BOH*) and *Carotenoid isomerase*

(*CRITSO*) and one carotenoid related gene (*Orange*; Lu et al. 2006) were generated using previously published degenerative primers (Cuevas et al. 2008). One insertion/deletion (INDEL) was identified in the intron sequence of the *PS* gene, and this was in turn used to create a co-dominant marker for genotyping by agarose electrophoresis. Gene sequence information regarding *BOH*, *CRTISO*, and *VDE* is not currently present in the only publicly released EST cucumber library (Cucurbit Genomic Resources; access: June 2009, <http://cucurbit.bti.cornell.edu/>).

Single marker analysis

No distorted segregation was detected in the *PS* marker evaluated by chi-square segregation analysis ($\chi^2 = 4.5$, $P = 0.11$). Moreover, linear regression analysis using the *PS* marker did not explain β -carotene variation observed in the mesocarp ($P = 0.22$) and endocarp ($P = 0.20$) of the F_2 progeny.

Discussion

Historically, carotenoid inheritance in cucumber fruit has not been rigorously examined. Likewise, breeding for orange-fleshed cucumbers has not been pursued since germplasm that accumulates β -carotene (i.e., orange flesh) is not widely known to consumers. However, the recent rise in interest by international food organizations for sources of dietary vitamin A and by consumers in nutraceuticals as they relate to health benefits has resulted in the concomitant interest in foods with enhanced nutritional value [e.g., vitamin A (β -carotene)]. The development of vegetables with enhanced β -carotene benefits from a complete understanding of the genetics of plant pigmentation and β -carotene accumulation. The data reported herein establishes a genetic basis for the development of breeding strategies to increase the nutritional value of cucumber.

Previous inheritance studies of carotene-associated color pigmentation in cucumber have reported contrasting results. This might not be expected since all such studies have used XIS germplasm as the source of orange fruit pigmentation. In particular, Yang et al. (1991) indicated that orange mesocarp fruit pigmentation is dominant to white while Navazio (1994) presented a two gene model where one gene controls

expression in mesocarp and the other in endocarp, where orange mesocarp fruit pigmentation is recessive to white. In this latter model, when one gene is homozygous recessive it produces an orange endocarp and an unpigmented mesocarp, and when the other gene is homozygous recessive an orange mesocarp and an unpigmented endocarp is observed. When these genes are homozygous recessive, both the endocarp and mesocarp are orange colored.

The results obtained herein agree in principle with the recessive inheritance model of orange color [9:3:3:1 (non-carotenoid: pigmented mesocarp: pigmented endocarp: pigmented mesocarp and endocarp)] presented by Navazio (1994). Both of these more recent studies indicated that β -carotene expression in endocarp and mesocarp is recessive (i.e., recessive homozygosity conditions orange color in both mesocarp and endocarp tissue). However, the F_2 and BC_1P_2 progeny segregation for mesocarp color reported herein (9:6:1; 3:1, respectively; Table 2) provides evidence that two recessive genes control the mesocarp pigmentation (i.e., β -carotene accumulation). The disparity between these studies is likely attributable to the population sizes employed (Navazio (1994) = 46 F_2 progenies versus this study = 111 F_2 and 51 BC_1P_2 progenies) or environmental influences varying between the studies. Likewise, the small population size utilized herein resulted in co-segregation of mesocarp and endocarp flesh color that did not fit expected ratios (i.e., some predicted phenotypes were not observed). The evaluation of larger populations and/or the examination of populations having different structures (e.g., recombinant inbred lines) would likely be useful for clarification of β -carotene inheritance in cucumber.

The genetic base of cucumber is relatively narrow (i.e., polymorphic markers = 3–12%; Dijkhuizen et al. 1996), and thus genetic map construction and the identification of quantitative trait loci (QTL) have been difficult (Serquen et al. 1997). However, a moderately saturated genetic linkage map has been constructed in cucumber using recombinant inbred lines (RIL) derived from a cross between inbred line ‘Gy7’ and ‘H19’ (Fazio et al. 2003). The fact that ‘Gy7’ is also a parental line in this inheritance study will assist in the molecular mapping of β -carotene in cucumber. Bulk segregant analysis (Michelmore et al. 1991) might be used to identify molecular markers linked to β -carotene genes using contrast F_5 families

(i.e., + and β -carotene in mesocarp and endocarp tissues) derived from F₂ progeny examined herein.

The inheritance of β -carotene in cucumber and melon is recessive and controlled by at least two genes, which in the case of melon, are themselves influenced by epistatic interactions (Clayberg 1992; Cuevas et al. 2009). In melon, one major QTL (putative *Orange* gene; Lu et al. 2006) interacts with two minor QTL to control the expression of β -carotene in mesocarp and endocarp tissues (Cuevas et al. 2009). In cucumber, it is likely that the *Orange* gene is associated with the inheritance of β -carotene accumulation (i.e., orange flesh color). As cucumber and melon maps become more saturated, the genome homology (synteny) between the melon and cucumber could be examined (Staub et al. 2007), and economically important candidate genes identified.

A previous diallel analysis of XIS \times US pickling cucumber hybrids suggested that parental genotype dramatically influenced horticultural trait performance including the carotenoid expression in fruit (Navazio 1994; Navazio and Simon 2001). The results described herein (i.e., orange pigmentation) using progeny derived from the inbred lines ‘Gy7’ [US pickling cucumber (white flesh)] and ‘EOM 402-10’ [unrefined US pickling cucumber line (orange flesh)] demonstrated the genetic basis for the expression of carotenoid in cucumber fruit. Although the breeding germplasm developed by Simon and Navazio (1997) and those used herein produce fruit with orange mesocarp and endocarp tissue (e.g., line ‘EOM 402-10’), some commercially undesired traits (e.g., poor fruit quality including fruit shape, length, and spine color) are apparent in this germplasm.

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