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Heritabilities and minimum gene number estimates of carrot carotenoids*

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Received 30 November 2005; accepted 15 February 2006

Key words: carotenes, *Daucus carota*, HPLC

Summary

Broad sense heritabilities and gene numbers were estimated for the production of total carotenoids and the major component carotenoids of carrot storage roots: phytoene, ζ -carotene, β -carotene, α -carotene, and lycopene. Two crosses with different backgrounds were evaluated: orange B493 \times white QAL and orange Brasília \times dark orange HCM. The HCM (high carotene mass selection), Brasília and B493 parents had both α -carotene and β -carotene, but HCM had proportionally more α -carotene. Carotene content in F_2 populations ranged from 522 ppm to 1714 ppm in Brasília \times HCM and from 0 to 695 ppm in B493 \times QAL progeny. F_2 plants segregating for absence of α -carotene were identified in B493 \times QAL. Broad-sense heritabilities ranged from 28% to 48% for all carotenes except lycopene and phytoene where estimates were 44% to 89% in the Brasília \times HCM cross. All heritability values exceeded 88% for the B493 \times QAL cross, except one estimate for lycopene. The estimated number of genes was 4 conditioning α -carotene, 2 to 3 each for β -carotene and total carotenes and one each for ζ -carotene, lycopene and phytoene in the orange \times dark orange cross. In the orange \times white cross, the estimates were 4 genes for α -carotene, 1 to 2 each for lycopene and total carotenes and 1 for each of the other carotenes. These results are in general agreement with QTL studies and they provided evidence for continuous inheritance of α -carotene, β -carotene and total carotenoids in the orange \times dark orange cross and discrete inheritance for β -carotene and total carotenoids in the orange \times white cross.

The biosynthesis of carotenoids is a well established biochemical pathway that has been studied in many plants (Cunningham & Gantt 1998; Sandmann 1994), fungi and microorganisms (Sandmann 1994). Vertebrates do not synthesize carotenoids and they ultimately depend on plant carotenoids in their diet for synthesizing their retinoids, such as retinal (the main visual pigment), retinol (vitamin A) and retinoic acid (a substance controlling morphogenesis) (Giuliano et al.,

2000). Although more than 40 carotenoids have been found to be vitamin A precursors, the main precursor of retinoids is β -carotene (Simpson 1983).

According to the World Health Organization—WHO (2001), vitamin A deficiency is a public health problem in 118 countries, especially in Africa and South-East Asia, resulting in an estimated 100 to 140 million vitamin A-deficient children, of which 250,000 to 500,000 become blind every year and half of these die within 12 months of losing their sight. Nearly 600,000 women die from childbirth-related causes each year, the vast majority of them attributable to vitamin A deficiency. The challenge of this continuing nutritional health problem emphasizes the need to increase and sustain the dietary sources of provitamin A carotenoid

* Part of thesis "Biometrical studies and quantitative trait loci associated with major products of the carotenoids pathway of carrot (*Daucus carota* L.)" presented by the first author as partial fulfillment of the requirement of the PhD degree in Plant Breeding and Plant Genetics, UW-Madison, USA, 2001.

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2006 SP-PP-00430



CPATSA-34303-1



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sources. Biotechnological approaches have been employed to achieve this goal (Ye et al., 2000) and numerous horticultural crops are already good sources of provitamin A carotenoids, but outside of heritability estimates for carotenoid content in a very few crops such as chickpea (Abbo et al., 2005) and tomato (Saliba-Colombani et al., 2001; Stevens, 1986), genetic and environmental variables affecting carotene content have not been studied in most of these crops (Simon, 1992).

The storage root of modern carrot contains carotenoids that account for their orange, yellow, and red color which varies widely in intensity (Buishand & Gabelman 1979; Simon et al., 1989, Surles et al., 2004). Total carotenoid content (fresh weight basis) ranges from 64 to up 600 $\mu\text{g/g}$ in orange carrot, 15 to 71 $\mu\text{g/g}$ in yellow carrot, 0 to 1.5 $\mu\text{g/g}$ in white carrot and around 100 $\mu\text{g/g}$ in red carrot (Buishand & Gabelman 1979; Laferriere & Gabelman, 1968; Surles et al., 2004).

Since the original observations of carrot storage root color variation in progeny of white-rooted wild carrot by Vilnorin in 1859, the inheritance of this trait has been the subject of several studies. Laferriere and Gabelman (1968) inferred a complex pattern of inheritance and Buishand and Gabelman (1979) investigated carrot color inheritance by crossing uniformly colored roots to various roots for which the xylem differs in color from the phloem. They concluded that 1) a single major gene (Y) was responsible for the differences in progenies of orange \times orange (phloem)-white (xylem) color and 2) another major gene was responsible for the difference between yellow \times orange or between orange(phloem)-yellow(xylem) \times orange crosses (Y_2). Simon (1996) and Bradeen and Simon (1998) studied the inheritance of Y_2 in diverse genetic backgrounds and concluded it was the single major locus accounting for the color differences described by Buishand and Gabelman. While Buishand and Gabelman (1979) attempted to categorize root color into discrete intensity classes, continuous variation was observed. More recently we have identified QTL for the major component carotenoids in carrot storage roots (Santos & Simon 2002), but their heritabilities have not been reported.

In this study we utilized two crosses of carrot with very different backgrounds to estimate the broad-sense heritabilities and number of genes involved in the production of phytoene, lycopene, ζ -carotene, β -carotene, α -carotene and total carotenoids. These are important estimations to help carrot breeding programs, to understand the evolution of this pathway and to provide a basis for comparison with quantitative trait loci studies.

Material and methods

Plant material

The experimental populations were developed from two unrelated crosses, B493 \times Queen Anne's Lace (QAL) and Brasilia \times High Mass Carotene (HCM). Individual plants from each parental line or population were intercrossed and single F_1 plants were self-pollinated to generate the F_2 populations evaluated for both crosses.

B493 \times QAL

This population was included to study the factors that condition the accumulation of carotenoids (orange parent) in the presence of factors that result in no carotenoid production (white parent). B493 is an inbred dark orange carrot developed with carotene content ranging from 180 to 210 ppm fresh weight basis (Simon et al., 1990). QAL is a white (no carotenoids accumulated) wild carrot (*D. carota* var. *carota*) well distributed in temperate regions of North and South America, and from the Atlantic coast of Eastern Europe to western China (Rubatzky et al., 1999). The population evaluated was derived from a single F_1 plant from a cross between B493 and QAL from Madison, WI, USA, in 1989.

Brasilia \times HCM

This population was included to study the effects of loci contributing to carotenoid accumulation when both parents accumulate carotenoids but in significantly different quantities. HCM is a population with an average carotene content ranging from 460 to 499 ppm that was developed from a cross between European and Asian carrot germplasm (Simon et al., 1989). Brasilia is a typical orange population adapted to warmer Brazilian production areas (Vieira et al., 1983; Hamerschmidt, 1993) with a carotene content ranging from 35 to 85 ppm (P.W. Simon, personal communication).

Parental lines B493, QAL, Brasilia and HCM, and the F_1 and F_2 progeny of B493 \times QAL and Brasilia \times HCM were grown at Palmyra, Wisconsin, USA. From 12 to 48 parental plants, 3 to 10 F_1 plants and 157 to 178 F_2 plants were harvested and stored at 2C until sampling for carotenoid type and amount (Table 1). Roots were vernalized and seed produced to advance to next generation. Slices for carotenoid quantification around 2 g were sampled from the middle portion of freshly washed root, weighed, wrapped in cheesecloth and frozen for at least 48 hrs at -80°C . Frozen-slices

Table 1. Means, standard error of means, maximum and minimum values, in ppm or $\mu\text{g/g}$ (dry weight), for ζ -carotene, α -carotene, β -carotene, phytoene, lycopene and total carotenoids of the parents, F_1 and F_2 plants of crosses B493 \times QAL and Brasília \times HCM.

Character/Population	Mean	n	Means standard error	Range	
				Low	High
ζ -carotene					
Brasília	64.6	19	7.21	29.0	123.7
HCM	117.3	12	18.3	41.1	233.6
Brasília \times HCM - F_1	88.0	10	20.0	25.5	233.7
Brasília \times HCM - F_2	125.0	157	6.7	4.4	417.1
B493	187.6	48	12.1	27.6	370.3
QAL	0.0	34	0.0	0.0	0.0
B493 \times QAL - F_1	0.0	3	0.0	0.0	0.0
B493 \times QAL - F_2	8.0	178	2.5	0.0	271.1
α -carotene					
Brasília	133.2	19	17.1	19.1	320.1
HCM	697.7	12	95.6	255.9	1224.4
Brasília \times HCM - F_1	318.5	10	55.5	113.5	561.3
Brasília \times HCM - F_2	452.8	157	16.6	10.5	1100.4
B493	266.4	48	11.2	31.0	390.8
QAL	0.0	34	0.0	0.0	0.0
B493 \times QAL - F_1	0.0	3	0.0	0.0	0.0
B493 \times QAL - F_2	4.7	178	2.3	0.0	285.5
β -carotene					
Brasília	232.1	19	27.6	83.4	552.0
HCM	584.5	12	74.9	211.2	999.4
Brasília \times HCM - F_1	278.2	10	41.9	109.2	500.0
Brasília \times HCM - F_2	366.3	157	16.2	4.4	911.6
B493	460.9	48	19.6	147.7	704.8
QAL	0.0	34	0.0	0.0	0.0
B493 \times QAL - F_1	0.0	3	0.0	0.0	0.0
B493 \times QAL - F_2	13.8	178	3.6	0.0	299.0
Phytoene					
Brasília	157.3	19	26.0	62.3	438.9
HCM	412.4	12	56.0	147.2	788.4
Brasília \times HCM - F_1	172.0	10	14.0	114.8	256.5
Brasília \times HCM - F_2	303.1	157	17.5	7.3	1049.5
B493	500.4	48	24.3	246.1	796.1
QAL	0.0	34	0.0	0.0	0.0
B493 \times QAL - F_1	0.0	3	0.0	0.0	0.0
B493 \times QAL - F_2	16.1	178	4.4	0.0	406.9
Lycopene					
Brasília	8.9	19	1.6	2.5	29.5
HCM	31.9	12	6.7	7.2	77.1
Brasília \times HCM - F_1	19.5	10	4.3	48.1	3.7
Brasília \times HCM - F_2	35.2	157	1.5	0.0	130.4
B493	18.1	48	2.0	2.4	66.2
QAL	0.0	34	0.0	0.0	0.0
B493 \times QAL - F_1	0.0	3	0.0	0.0	0.0
B493 \times QAL - F_2	1.8	178	0.8	0.0	103.5
Total carotenoids					
Brasília	454.5	19	37.1	194.6	882.0
HCM	1619.5	12	139.5	955.6	2346.4
Brasília \times HCM - F_1	781.2	9	87.8	1163.1	450.3
Brasília \times HCM - F_2	942.7	160	27.1	98.0	1714.1
B493	946.5	48	33.9	414.0	1490.1
QAL	12.9	34	4.0	0.0	129.5
B493 \times QAL - F_1	2.9	3	0.8	1.3	3.8
B493 \times QAL - F_2	64.2	182	8.6	0.0	695.5

were placed into a Virtis Lyophilizer (Model No. 10-146MR-BA) and freeze-dried for 72 hrs.

Quantification of total carotene

The dry weight of lyophilized carrot slices was recorded and samples were processed as described by Simon and Wolff (1987). Briefly, individual samples were chopped with a razor blade, suspended in pure and water free hexane, and ground with Virtis blender at 65,000 rpm for three minutes while chilled in ice to minimize isomerization. The solution was filtered twice and any trace of water was removed through an additional filter with sodium sulfate. The purified solution was then brought to volume of a 100 ml and quantified at the 450 nm wavelength in a Gilford Spectrophotometer. An estimate of "total carotenoid" concentration was obtained by comparing mean sample absorbance at 450 nm of each individual sample to a standard curve of β -carotene.

Quantification of major carotenoids by high-performance liquid chromatography

To prepare the samples for high-performance liquid chromatography (HPLC) analysis, 4.0 ml of each hexane-carotenoid solution was transferred to a 5.0 ml brown glass vial and stored in the refrigerator at 4 °C.

The HPLC equipment used consisted of a Waters 6000A pump, a Waters WISP 710B autosampler, and a Waters 996 Photodiode Array Detector (Waters Associates, Milford, MA). Separations were performed with the system of Khachik et al. (1992), using a Rainin Microsorb-MV 5 μ m C18 25 cm \times 4.6 mm 100 A column, a 2.5 cm \times 4.6 mm Bondapak C18/Corasil guard column, and a mobile phase of acetonitrile: methylene chloride: methanol, 55:23:22 at a flow rate of 1 ml/min. Data were collected from 192 to 600 nm (1.2 nm resolution), at 0.5 s intervals, and peaks were quantified using Millennium version 2.1 software (Waters Corporation, 1994). Resulting data were used to estimate phytoene, lycopene, ζ -carotene, β -carotene and α -carotene contents. Synthetic β -carotene (Sigma C-9750) was used as a reference standard in separate sampling and the identity of individual compounds was confirmed by an evaluation of the absorption spectra. Lycopene, ζ -carotene, β -carotene and α -carotene were quantified at wavelength of 404 nm absorbance with retention times of around 12, 14, 16 and 17 min, respec-

tively (Figure 1A, 1B and 1C). Phytoene was quantified at 287 nm with a peak around 20 min (Figure 1D) as reported by Simon and Wolff (1987) and Khachik et al. (1989).

Statistical analysis

Means, variances, standard error of means and minimum and maximum values for parental lines, F_1 and F_2 populations for total carotenoid, phytoene, ζ -carotene, β -carotene, lycopene and α -carotene were estimated using the Univariate procedure (SAS 1989). All carotenoid concentrations are given on a dry weight basis in this study. Tests for normality of all traits in the F_2 populations were obtained with kurtosis and the Wilk-Shapiro test in the Univariate procedure.

Estimates of the minimum number of genes influencing total carotenoid, phytoene, ζ -carotene, β -carotene, lycopene and α -carotene were determined by the methods of first moments, also called Sewall Wright's estimator, and applying the generalization presented by Lande (1981). Two estimates were obtained from the available data with the equations presented by Lande (1981): Effective number of factors (n_E) = $(\mu_{F_2} - \mu_{P_1})^2 / (8\sigma_S^2) \leq$ the actual number of factor (n). The two estimates for σ_S^2 were obtained with $\sigma_S^2 = \sigma_{F_2}^2 - \sigma_{F_1}^2$ (estimate 1) and $\sigma_S^2 = \sigma_{F_2}^2 - [\frac{1}{2}\sigma_{F_1}^2 + \frac{1}{4}\sigma_{P_1}^2 + \frac{1}{4}\sigma_{P_2}^2]$ (estimate 2), where σ_S^2 is the genetic variance segregating in the F_2 population, $\sigma_{F_2}^2$ is the total variance of F_2 due to the genetic and the environment variances, $\sigma_{F_1}^2$ is the environmental variance of F_1 and $\sigma_{P_1}^2$ and $\sigma_{P_2}^2$ are environmental variances of both parent lines. To estimate the minimum numbers of loci or factors, both datasets were transformed to the logarithmic scale plus one in order to make the genetic variance additive, as recommended by Lande (1981). Standard errors of minimum number of factors estimates were performed as described by Lande (1981).

An estimate of heritability for total carotenoid, phytoene, ζ -carotene, β -carotene, lycopene and α -carotene was calculated with $h^2 = (\sigma_{F_2}^2 - \sigma_{F_1}^2) / \sigma_{F_2}^2$ formula. According to Burton (1951) this formula gives upward bias and the estimates so obtained should be considered as the maximum heritability since the genetic variance in $F_2 - F_1$ will include total genetic variance (due to additive effects, dominance deviations and the interaction of nonallelic genes) and variance due to interaction of genotypes and the environment.

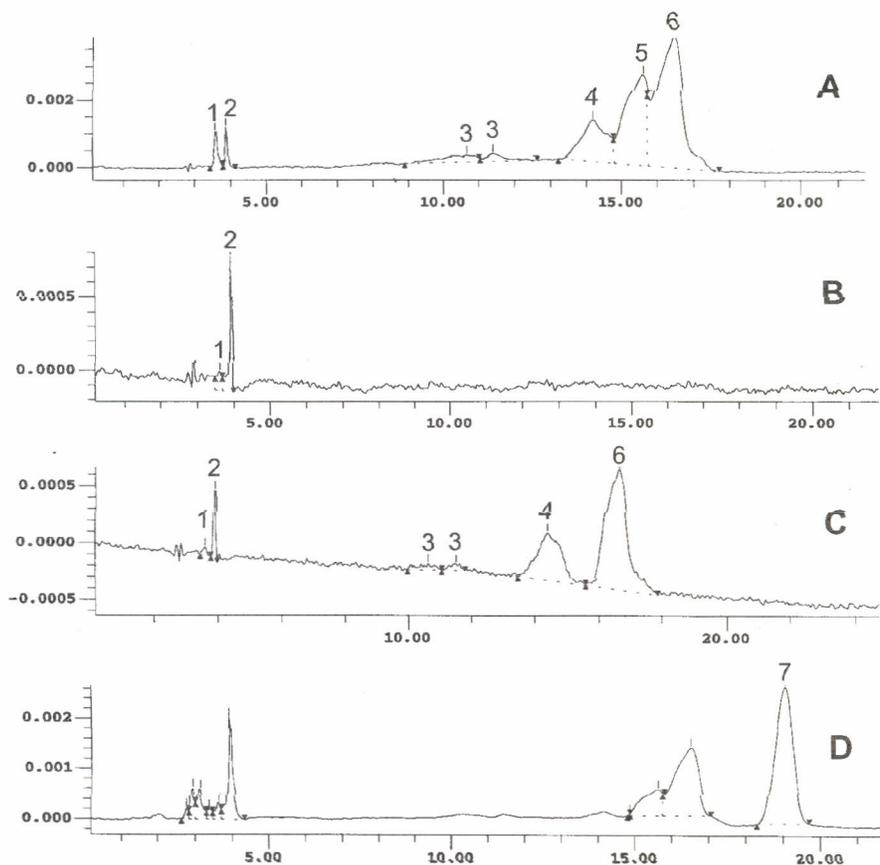


Figure 1. Representative HPLC chromatograms at 404 nm absorbance from roots of carrots: A) a typical sample of 'Brasília'; B) a white sample of QAL; C) a F_2 plant segregating for absence of α -carotene in the cross B493 \times QAL; and D) a representative HPLC chromatogram at 287 nm absorbance from a sample of 'Brasília'. Peaks correspond to (1) lutein or zeaxanthin, (2) hexane noise, (3), lycopene, (4) ζ -carotene, (5) α -carotene, (6) β -carotene and (7) phytoene.

Results and discussion

Identification of major carotenoid products by HPLC chromatograms

All major carotenenes were present in the parental lines B493, Brasília and HCM, and absent in QAL, as expected (Figure 1). Chromatograms revealed a higher relative content of α -carotene, compared to β -carotene, in the HCM line, suggesting that much of the total carotene increase realized in selection of HCM resulted in an increase in α -carotene. Early selection on HCM population had presented β -carotene and α -carotene accounting for 50% and 40% of total carotenenes, respectively (Simon & Wolf, 1987).

F_2 plants segregating for absence of α -carotene peak were observed in the cross B493 \times QAL F_2 (Figure 1). In the case of α -carotene, with one β and one ϵ ring, two different enzymes, lycopene β -cyclase and lycopene ϵ -cyclase, are involved (Sandmann 1994). Whether the route to α -carotene is only via ϵ ring first or can also proceed via β ring first has not been determined (Cunningham & Gantt, 1998). Our data support the hypothesis that α -carotene is produced after the synthesis of β -carotene in carrot. Lines free of α -carotene are being developed not only for genetics studies, but also to improve the provitamin A content, since, according Sharman (1983) and van der Berg et al. (2000), α -carotene has less provitamin A activity than β -carotene.

Table 2. Estimation of broad sense heritability and minimum number of genes or factors, with standard errors of estimates, controlling the inheritance of ζ -carotene, α -carotene, β -carotene, phytoene, lycopene and total carotenoids ("total caro") in two F_2 populations of carrot.

Character	Brasilia \times HCM				B493 \times QAL			
	Heritability		Number of factors and standard error		Heritability		Number of factors and standard error	
	1 ¹	2 ²	1 ¹	2 ²	1 ¹	2 ²	1 ¹	2 ²
ζ -carotene	0.46	0.48	0.17 \pm 0.1	0.18 \pm 0.1	0.92	0.98	1.13 \pm 0.2	1.05 \pm 0.2
α -carotene	0.32	0.36	4.00 \pm 3.2	3.50 \pm 3.0	0.83	0.95	3.93 \pm 0.8	3.47 \pm 0.7
β -carotene	0.28	0.42	1.67 \pm 1.3	2.52 \pm 2.3	0.97	0.99	1.12 \pm 0.2	1.10 \pm 0.2
Phytoene	0.53	0.89	0.22 \pm 0.2	0.60 \pm 0.4	0.97	0.99	1.12 \pm 0.2	1.10 \pm 0.2
Lycopene	0.44	0.66	0.36 \pm 0.2	0.53 \pm 0.4	0.51	0.88	1.83 \pm 0.4	1.27 \pm 0.3
Total caro	0.38	0.45	2.92 \pm 2.0	3.45 \pm 2.4	0.98	0.89	1.68 \pm 0.3	0.90 \pm 0.2

Based on ^{1/} $\sigma_S^2 = \sigma_{F_2}^2 - \sigma_{F_1}^2$ and ^{2/} $\sigma_S^2 = \sigma_{F_2}^2 - \left[\frac{1}{2}\sigma_{F_1}^2 + \frac{1}{4}\sigma_{P_1}^2 + \frac{1}{4}\sigma_{P_2}^2 \right]$.

Quantification and descriptive statistics of total carotenoids and major carotenoids

Parental lines of each cross presented large differences among them for all carotenoids products analyzed (Table 1). Significant differences among parental lines is a requirement for the accurate estimation of the number of genes involved in the control of characters, as it is for mapping and quantitative traits loci (QTL) studies. Carotene concentrations observed in the parental lines in this study were comparable to those reported in previous studies.

Brasilia \times HCM F_2 plants with values higher than the higher value parent were observed in characters ζ -carotene, phytoene and lycopene, and plants segregating with less carotene than the lower parent value were observed in all characters. Transgressive segregation was not observed in the B493 \times QAL cross, except for lycopene, indicating that selection in the carotenoid pathway is a long-term selection process, with several genes interacting to increase the content of each carotenoid. These basic descriptive statistics are very important to estimate the broad sense heritability and the number of loci involved in the inheritance of the carotenoid enzyme pathway and this study is the first to report comprehensive estimates of them.

Since the storage roots of most plants contained no carotenoids in the B493 \times QAL F_2 population, all characters deviated strongly from the normal distribution, with p -values less than 0.0001 and kurtosis values greater than 12. In the Brasilia \times HCM F_2 population, the normal distribution fit very well for α -carotene and total carotenoids, with moderate fit for β -carotene. Non-significant Wilk-Shapiro test and kurtosis values around zero suggests the normal distribution probab-

ity of the dataset (SAS 1989). Lycopene, phytoene and ζ -carotene, in the Brasilia \times HCM cross had kurtosis values less than 3.5 and Shapiro-Wilk tests with p -values less than 0.0001, indicating departure from the normal distribution.

Broad-sense heritability and minimum number of genes involved in the inheritance of major carotenoids in carrot

Heritability values ranged from 28% to 48% for α -carotene, ζ -carotene, β -carotene and total carotenoids, from 44% to 66% for lycopene, and from 53% to 89% for phytoene in the cross Brasilia \times HCM (Table 2). The heritability values were higher for all characters in the cross B493 \times QAL and exceeded 90% for most estimates. Since we found almost no products of the carotenoid pathway accumulated in the orange \times white cross, as also reported early by Laferriere and Gabelman (1968), it is likely that the small number of F_1 plants evaluated did not bias the estimates of heritabilities and minimum number of genes. To date there are very few reports on heritabilities in carrot and none with carotenoid pathway products so comparisons to other studies are not possible. The broad sense heritabilities estimated here should be considered as the maximum values, regardless of how many plants were evaluated.

The estimated number of factors was 4 for α -carotene, 2 to 3 each for β -carotene and total carotenoids and one each for ζ -carotene, lycopene and phytoene in the Brasilia \times HCM cross, whereas only 1 or 2 genes condition all characters except for α -carotene where 3 or 4 genes are estimated in the B493 \times QAL cross (Table 2). The two methods used to estimate minimum

numbers of genes provided similar values for all traits in both populations. No standard errors of minimum number of factors (Table 2) exceeded the estimated minimum number of factors and these two diverse populations gave quite similar estimates, so it is valid to infer that these numbers are accurate. The standard error estimates were similar to the minimum number of factors in the Brasília × HCM cross. The higher estimate for genes conditioning α -carotene in the B493 × QAL cross may be expected as we observed a single gene controlling its presence among orange-rooted segregants in this cross. Our estimates are similar to those by Laferriere and Gabelman (1968) who reported that at least three major genes separate orange storage root color from white.

Considering total carotenoid as an indirect measure of color, the present study suggests two major loci as the minimum number that are separating orange from white carrot in this study. Based on the estimation from ζ -carotene, phytoene and lycopene accumulation, it is hypothesized that the primary difference between white and orange carrots results from an enzymatic change blocking carotenoid accumulation early in the pathway.

This study presents evidence that β -carotene and α -carotene quantity, the two major products of the carotenoid pathway in carrot, varied in a continuous pattern fitting a normal distribution among progeny of a cross between orange × dark orange carrots, while discrete segregation with significant difference from a normal distribution was observed in a cross of orange × white carrots. A larger number of genes was estimated in orange × dark orange than in orange × white cross, reinforcing this hypothesis of continuous and discrete inheritance of final products of the carotenoid pathway in carrot. Wild white-rooted carrots have been used as an important source of genetic variation for reproductive and disease resistance traits by carrot breeders, and further screening of wild carrot germplasm indicates it to be a source for more traits of interest (Simon, 2000). These results of this study indicate that retrieval of orange-rooted progeny in crosses with wild white-rooted carrots can be achieved with relative ease.

Acknowledgments

This research was supported by Initiative for Future Agriculture and Food Systems Grant no. 2000-4258 from the USDA Cooperative State Research, Education, and Extension Service and by the California Fresh Carrot Advisory Board.

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