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ORIGINAL PAPER

Expression and mapping of anthocyanin biosynthesis genes in carrot

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Abstract Anthocyanin gene expression has been extensively studied in leaves, fruits and flowers of numerous plants. Little, however, is known about anthocyanin accumulation in roots of carrots or other species. We quantified expression of six anthocyanin biosynthetic genes [phenylalanine ammonia-lyase (*PAL*3), chalcone synthase (*CHS*1), flavanone 3-hydroxylase (*F3H*), dihydroflavonol 4-reductase (*DFR*1), leucoanthocyanidin dioxygenase (*LDOX*2), and UDP-glucose:flavonoid 3-*O*-glucosyltransferase (*UFGT*)] in three carrot inbreds with contrasting root color: solid purple (phloem and xylem); purple outer phloem/orange xylem; and orange phloem and xylem. Transcripts for five of these genes (*CHS*1, *DFR*1, *F3H*, *LDOX*2, *PAL*3) accumulated at high levels in solid purple carrots, less in purple-orange carrot, and low or no transcript in orange carrots. Gene expression coincided with anthocyanin accumulation. In contrast, *UFGT* expression was comparable in purple and orange carrots and relatively unchanged during root development. In addition, five anthocyanin biosynthesis genes [*FLS*1 (flavonol synthase), *F3H, LDOX*2, *PAL*3, and *UFGT*] and three anthocyanin transcription factors (*DcEFR1, DcMYB3* and *DcMYB5*) were mapped in a population segregating for the P_1 locus that conditions purple root color. P_1 mapped to chromosome 3 and of the eight anthocyanin biosynthesis genes, only *F3H* and *FLS*1 were linked to P_1. The gene expression and mapping data suggest a coordinated regulatory control of anthocyanin expression in carrot root and establish a framework for studying the anthocyanin pathway in carrots, and they also suggest that none of the genes evaluated is a candidate for P_1.

Introduction

Anthocyanins, a flavonoid subclass, account for a wide range of colors and are widely spread in many organs of

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higher plants (Holton and Cornish 1995; Mol et al. 1996). They attract animals for pollination and seed dispersal, and given their antioxidant properties, they are believed to protect plant cells against ultraviolet (UV) and high intensity light, wounding, cold temperature, and water stress, and to defend against phytopathogens (McClure 1975; Mo et al. 1992; Koes et al. 1993; Li et al. 1993; Holton and Cornish 1995; Dixon and Palva 1995; Shirley 1996; Chalker-Scott 1999). These same antioxidant properties have been proposed to account for the health benefits associated with the dietary intake of vegetables and fruits high in anthocyanins where they have been suggested to protect against oxidative stress, coronary heart diseases, certain cancers, and other age-related diseases (Ross and Kasum 2002). Two classes of genes are involved in anthocyanin biosynthesis, structural genes encoding the enzymes that directly participate in the formation of anthocyanins and other flavonoids, and regulatory genes that control the transcription of structural genes. Anthocyanin biosynthesis genes have been isolated using a range of methodologies, including protein purification, transposon tagging, differential screening, and PCR amplification. Reverse genetics has also been used to identify gene function (Dooner et al. 1991; Holton and Tanaka 1994; Holton and Cornish 1995; Jaakola et al. 2002; Quattrocchio et al. 1993; Reddy 1996).

Anthocyanin biosynthesis has been most extensively studied in flowers, fruit, and leaves with only a few studies in the underground organs—tubers and storage roots (Jung et al. 2005; Lalusin et al. 2006; Lu and Yang 2006; Mano et al. 2007). Consequently, there is very little known about the control of anthocyanin genes in roots. Carrot (*Daucus carota* L.) is one of the plant species that accumulates large amounts of anthocyanin in the storage root and while the cyanidin derivatives accounting for purple (or "black") root color were characterized by Harborne (1976), and one gene controlling anthocyanin accumulation in carrot roots, P_1, has been described (Simon 1996), there are no published studies on flavonoid biosynthesis in carrots that evaluate the expression or genetic map location of genes in the phenylpropanoid pathway.

The goal of this study was to establish a foundation for understanding anthocyanin biosynthesis in carrot including two approaches: an evaluation of the expression of six anthocyanin biosynthesis genes representing early, middle, and later parts of the pathway (Fig. 1) in purple (P_1P_1) and non-purple (p_1p_1) orange carrots during development; and a determination of the genetic map location of P_1 as well as five anthocyanin biosynthesis genes in the pathway (Fig. 1) and three transcription factors.

Materials and methods

Plant materials

For gene expression studies, three USDA carrot genetic stocks that differ in storage root color and anthocyanin accumulation were compared: inbred B9547, a solid purple (phloem and xylem) derivative of an open-pollinated Turkish cultivar, inbred B7262 which has a purple phloem, but non-purple (orange) xylem (Simon et al. 1997), and inbred B493 (Simon et al. 1990) which is orange and accumulates no anthocyanins. The genotype of both B9547 and B7262 is P_1P_1 whereas that of B493 is p_1p_1 (Simon 1996 and personal communications). Carrot plants were grown in greenhouses at the University of Wisconsin (Madison, USA). Seeds were sown in a mixture of sand and peat and nine plants of each genetic stock were harvested every 3 weeks from the 6th to the 15th week after sowing (Fig. 2). Root slices from the three plants of each inbred were frozen in liquid nitrogen, powdered individually. RNA was extracted and gene expression was analyzed with tissue from each plant evaluated as a biological replicate.

For genetic mapping studies, an F_2 population derived from a heterozygous P_1p_1 F_1 intercross between B1896 (true-breeding p_1p_1 yellow-rooted F_4M inbred derived from the cross PI173687 × B493), and B7262 (P_1P_1 purple exterior with orange inner phloem and xylem inbred from the same cross) was self-pollinated to produce population 10117. Plants of the F_2 population were grown for phenotyping on Imperial silty clay soil at the University of California Desert Research and Extension Center in El Centro, CA, USA (DREC) and on Plainfield loamy sand soil on Miller Farms, a commercial grower's field near Hancock, WI, USA.

Purple root color of B7262 is conditioned by P_1 and phenotyping in the 10117 population (72 F_2 plants) was performed as described by Simon (1996). Yellow versus orange root color is conditioned by Y_2 and while not the focus of this study, was also segregating in the 10117 population, and phenotyping was performed as described by Simon (1996). Total genomic DNA of individual plants for molecular marker analysis was isolated from freeze-dried leaves following the protocol of Doyle and Doyle (1990), with minor modifications tested by Boiteux et al. (1999) for carrots.

Pigment analysis

Root tissue samples were lyophilized from greenhouse-grown plants every 3 weeks on the same schedule as the gene expression analysis. Anthocyanins were extracted with acidified methanol, and analyzed with HPLC and

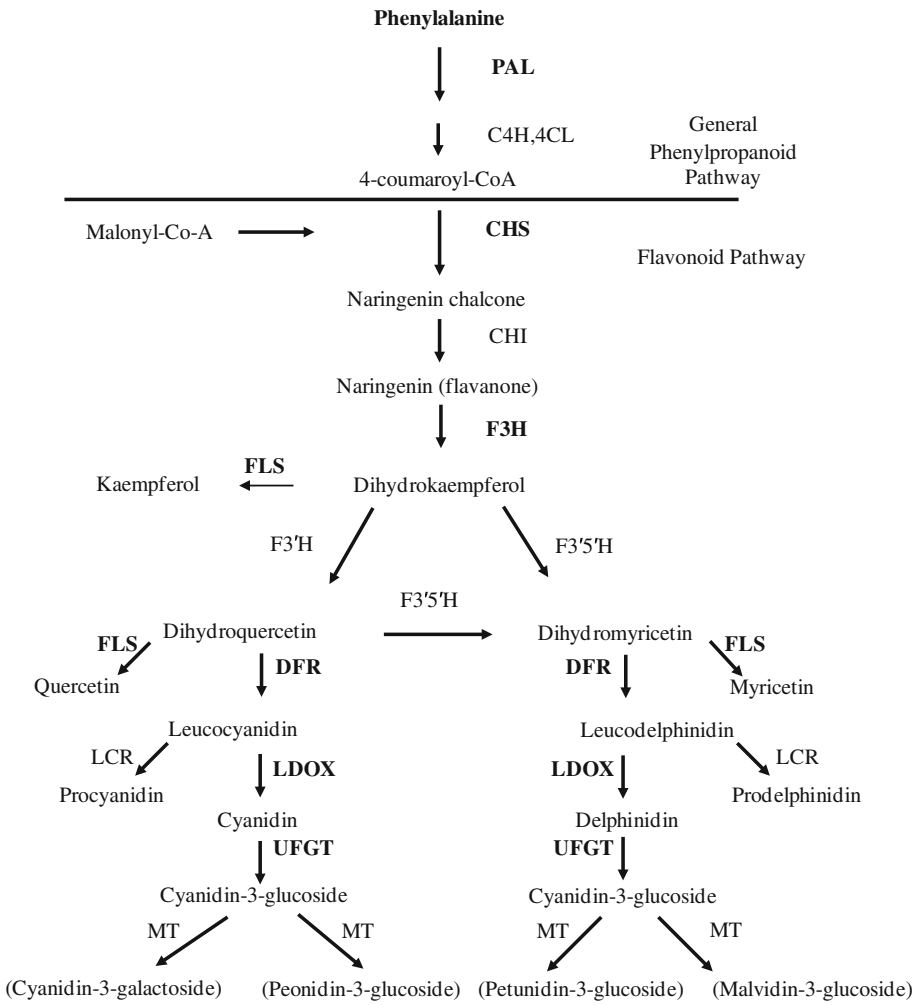


Fig. 1 Schematic representation of the anthocyanin biosynthesis pathway. *PAL* phenylalanine ammonia-lyase (*E* and *M*—expression measured and gene mapped in this study, respectively. *E* and *M* enzymes are in *bold* in the figure), *C4H* cinnamate 4-hydroxylase, *4CL* 4-coumarate CoA ligase, *CHS* chalcone synthase (*E*), *CHI* chalcone isomerase, *F3H* flavanone 3-hydroxylase (*E* & *M*), *FLS* flavonol synthase (*M*), *F3′H* flavonoid 3′-hydroxylase (*E* & *M*), *F3′5′H* flavonoid 3′,5′-hydroxylase, *DFR* dihydroflavonol 4-reductase (*E*), *LDOX2* leucoanthocyanidin dioxygenase (*E* & *M*), *UFGT* UDP-glucose-flavonoid 3-*O*-glucosyl-transferase (*E* & *M*), *MT* methyltransferase (modified from Holton and Cornish 1995)

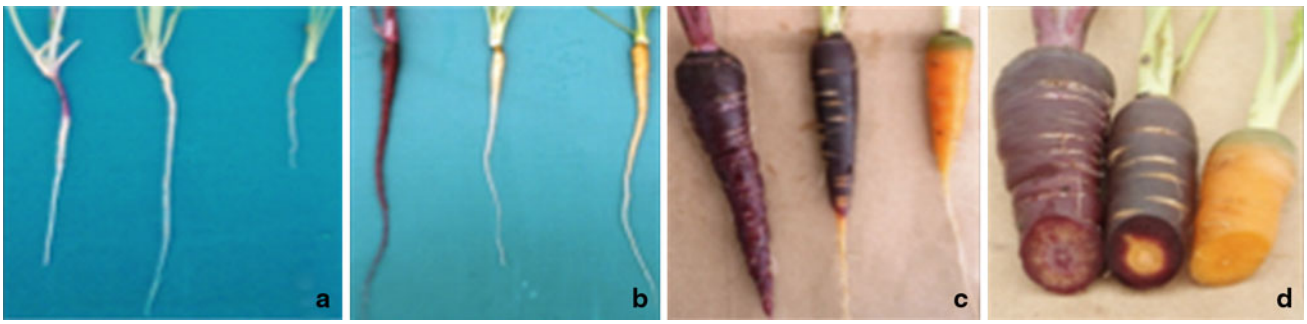


Fig. 2 Three carrot inbreds (B9547, B7262, and B493, respectively) harvested at 6 (**a**), 9 (**b**), 12 (**c**), and 15 (**d**) weeks after sowing

individual pigments quantified as described by Kurilich et al. (2005).

RNA isolation

Total RNA isolation was performed with the plant RNeasy kit (Qiagen). Briefly, 100 mg of root tissue was macerated with a mortar and pestle in liquid nitrogen, vortexed in a 2 ml tube with 600 µl of RTL lysis buffer, and RNA extracted according to manufacturer's protocol. Total RNA was quantified using a micro-spectrophotometer (Nano-Drop Technologies). DNA was removed with Turbo DNAfree (Ambion, Inc.) according to protocol and removal of DNA from the RNA samples was confirmed with real-time qPCR on 100 ng of total RNA using the *Actin* and the *EF*-1 primer set. Those RNA samples found to yield quantification cycle (C_q) values larger than 32 were judged to be sufficiently free of contaminating DNA.

Following removal of DNA contamination, RNA quality was accessed using the Experion microfluidic capillary electrophoresis system (Bio-Rad Laboratories, Inc.). cDNA was generated from 1 μg of total RNA using iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc.) and diluted to a final volume of 400 μl.

Real-time RT-qPCR primer design

Primers for real-time qPCR (RT-qPCR) were designed using the Beacon Designer software package (Premier Biosoft International) for the anthocyanin biosynthesis genes *PAL*3, *CHS1*, *DFR*1, *F3H*, and *LDOX2* based upon carrot mRNA sequences at NCBI (http://www.ncbi.nlm.nih.gov/). Primers for the *UFGT* (UDP glucose: flavonoid-3-*O*-glucosyltransferase) gene were designed using 500 bp of cDNA sequence previously generated by amplification with *UFGT* degenerate primers. Reference gene primers were validated using the BestKeeper software package (Pfaffl et al. 2004) from a pool of four potential reference gene transcripts. We evaluated *Act* (Actin), *Ubi* (Ubiquitin), *EF*1α (Elongation Factor—1α) and *G3PDH* (glyceraldehyde 3-phosphate dehydrogenase) as candidate reference genes and found *EF-1* and *Act* to be stably expressed across all experimental conditions. Real-time RT-qPCR products were sequenced to ensure accuracy of amplification. Primer efficiencies were determined from a dilution curve of target DNA using the formula: $E = 10^{(-1/\text{slope})}$ (Pfaffl 2001; Rasmussen 2001), with the slope determined by the iQ Cycler software. Primer sequences and efficiencies are in Table 1.

Real-time PCR conditions

Two cDNA samples were generated from the total RNA of two roots of each inbred. The target mRNA was quantified by adding 30 μl of iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc.), adjusted to a final concentration of 200 nM with respect to each primer, to 26 μl of diluted cDNA. The reaction mix was vortexed and 25 μl added to two wells in a 96 well plate (technical reps). Reactions were quantified with the iCycler Real-Time PCR Detection system (Bio-Rad Laboratories, Inc.) using the default two-step amplification plus melting curve protocol. The reaction conditions were: enzyme activation and well factor determination at 95 °C for 3 min followed by 40 cycles of 95 °C for 1 min (denaturation) and 55 °C for 45 s (annealing and elongation); the melt curve protocol began immediately after amplification and consisted of 1 min at 55 °C followed by 80–10 s steps with a 0.5 °C increase in temperature at each step. The quantification cycle (C_q) values were generated automatically by the iCycler software. Lack of variation in PCR products and the absence of primer-dimers were ascertained from the melt curve profile of the PCR products.

Analysis of RT-qPCR data

Two iScript reactions were performed on each RNA sample and the iQ SYBR Green reaction mixes for each cDNA sample were run with two technical replicates. This resulted in four real-time RT-qPCR reactions for each RNA sample. Data were expressed as C_q values and treated as follows: the relative abundance of the genes of interest (*PAL3*, *CHS1*, *F3H*, *DFR1*, *LDOX2* and *UFGT*) was determined by first normalizing the target RNA to internal standard RNA (*EF*-1 and *Act*) using the formula: $2^{\Delta C_q\,(\text{target}-\text{reference})}$. To determine the relative expression ratio (RER) of target transcripts, the normalized target RNA value was divided by the average of the normalized values of purple carrot samples. This average was designated as the "calibrator" since the variation of all samples, including the individual purple carrot samples, is determined relative to this value. This method of calculating the RER was derived from the previously published $2^{-\Delta\Delta C_q}$ formula (Pfaffl 2001; Rotenberg et al. 2006). Analysis of variance (ANOVA) was performed on RER values. Analyses were conducted as a split-splitplot factorial design. The statistical model consisted of three main factors (inbreds, genes, and different harvest periods) and all possible two- and three-way interactions. The model was tested using the SAS procedure PROC MIXED.

Molecular marker analysis

A total of 370 SSR primer pairs (156 GSSRs, 144 BSSRs, 70 ESSRs) were evaluated as described by Cavagnaro et al. (2011), and 21 AFLP primer combinations were evaluated (Table 2) as described by Santos and Simon (2002) who utilized the method as described in Vos et al. (1995), with minor adaptations for carrot DNA described by Vivek and Simon (1999). A total of 17 anthocyanin genes (10 structural and seven regulatory genes) were initially screened and eight polymorphic genes used for mapping (Table 1). Primers were developed directly from carrot sequence recorded in NCBI for five anthocyanin biosynthesis structural genes: *F3H*, *FLS*1, *LDOX*2, *PAL*3 and *UFGT*. For the *UFGT* gene, primers were designed from homologous carrot transcriptome sequences (Iorizzo et al. 2011). In addition to biosynthetic structural genes, primers were also designed for three transcription factors: *DcEFR*1, *DcMYB*3, and *DcMYB*5 known to be involved in the anthocyanin pathway (Kimura et al. 2008; Wako et al. 2010). Primer sequence source, type of mapped polymorphism and amplification conditions used to amplify and map polymorphic biosynthetic structural and regulatory

Table 1 Primers used for RT-qPCR and for mapping polymorphic anthocyanin genes

Enzyme name/gene symbol	GenBank accession number	Primer sequence (5′–3′) for RT-qPCR	Efficiency on cDNA for RT-qPCR	Primer sequence used to amplify fragment for genotyping	Annealing temp. (°C) for genotyping	Amplicon size (kb)	Genotyping polymorphism type
Phenylalanine ammonia-lyase 3 (*PAL*3)	AB089813	f-gaacttgagcacttccatc r-gcactccttaatcctgttag	1.94	f-ctaagcaaggtgg cgctcttca r-actgccgaatatt ccagcattcaag	55	1.386	SNP
Chalcone synthase 1 (*CHS*1)	AJ006779	f-ctggatgagatgaggaagg r- cccgaacccgaatagaac	1.90	ND	ND	ND	ND
Flavanone 3-hydroxylase (*F3H*)	AF184270	f- agaaactggctaaagagaaac r- aatgataagatggacagacac	1.99	f-gagacgaaga tgagcgtcctaa r-agagcgtaat tgtgccaggatc	55	0.610	SNP, InDel
Dihydroflavonol 4-reductase 1(*DFR*1)	AF184271	f- gcactatctccgcttactg r- catttctctcttatcaatttcacc	1.85	ND	ND	ND	ND
Leucoanthocyanidin dioxygenase2 (*LDOX* 2)	AF184274	f- cattatctttcggactgggacttg r- ggttgaggacacttcgggtag	1.84	f-gcgatggtggca actacc r-tccataaaaca gttgtagaccag	55	0.878	SNP
UDP-glycose:flavonoid 3-O-glycosyltransferase (*UFGT*)	JX826445 for RT-qPCR/ SRA035376 Contig11088 for genotyping	f- cggtgtcgttgtgtaatcag r- aatcccaatccagtttcaataatc	1.86	f-caaaagca ccataacttgga r-aagcacatc caaacgattagt	55	0.850	Indel
Flavone synthase (*FLS*1)	AY817675	ND	ND	f- ggacttaaaat ggctccaacaac r-cgacaggctgaa cagtaatccaag	55	0.930	SNP
Actin (*Act4*)		f- cacackggtgtgatggtwgg r- gcsawatyttytccatrtcatccc	1.99	ND	ND	ND	ND
Elongation factor 1-alpha (*EF-1*)	DARELF1A	f- ggtgatgctggtttcgttaag r- atccttcttctccacactcttg	1.98	ND	ND	ND	ND
Transcription factor (*DcEFR*1)	AB354581	ND	ND	f- tccaagctctaatt catcca r- tgatatctccttgg ctcaaa	55	0.910	SNP
Transcription factor (*DcMYB*3)	AB298507	ND	ND	f-tcagggaagtt aagtaaaatgaa r-tcaagaactactggaacacga	55	0.600	SNP
Transcription factor (*DcMYB*5)	AB298509	ND	ND	f-ttctttct tccatgcagatg r-caggctca cagaaatgatgt	55	0.910	SNP

ND not determined

genes are listed in Table 1. SNPs were genotyped by amplifying the fragment in each individual followed by direct sequencing as described by Just et al. (2007) for carotenoid biosynthesis genes.

Marker scoring, linkage analysis and map construction

The genotypes of polymorphic markers were recorded according to the symbols required by Mapmaker/EXP

Table 2 Performance of twenty one AFLP primer combinations evaluated for genetic mapping in the F_2 population 10117

AFLP primer combinations[a]	Total number of bands	Number of polymorphic bands	% Polymorphic	Number (and %) of mapped markers
EAAC/MCAA	64	28	43.8	26 (40.6)
EAAC/MCAT	51	26	51.0	25 (49.0)
EAAC/MCTA	54	19	35.2	19 (35.2)
EAAG/MCAA	68	27	38.0	25 (36.8)
EAAG/MCAC	49	25	51.0	24 (49.0)
EAAG/MCAG	40	14	35.0	14 (35.0)
EAAG/MCAT	58	13	22.4	11 (19.0)
EAAG/MCTA	50	9	18.0	9 (18.0)
EAAG/MCTT	48	13	27.1	13 (27.1)
EACA/MCAA	56	11	19.6	10 (17.9)
EACA/MCAG	37	12	32.4	11 (29.7)
EACC/MCAA	36	7	19.4	0 (0)
EACC/MCAC	32	9	28.1	9 (28.1)
EACC/MCAG	11	6	54.5	3 (27.3)
EACC/MCTA	24	4	16.7	3 (12.5)
EACG/MCAT	24	9	37.5	8 (33.3)
EACT/MCAA	48	13	27.1	11 (22.9)
EACT/MCAG	36	14	38.9	13 (36.1)
EACT/MCAT	34	4	11.8	4 (11.8)
EACT/MCTA	39	10	25.6	10 (25.6)
EACT/MCTG	32	10	31.3	10 (31.3)
Total	891	283	–	258
Average	42.4	13.5	31.6	12.2 (27.8)

[a] The primer combinations EAAC/MCAA, EAAC/MCTA, and EACG/MCAT generated three codominant markers (one each) that were mapped

version 3.0 (Lander et al. 1987) as follows: homozygous maternal "A", homozygous paternal "B", heterozygous "H", not A "C", not B "D", and missing data "−". The degree of marker segregation distortion in the F_2 was determined by marker data comparison against the expected 3:1 and 1:2:1 ratio for dominant and codominant markers, respectively, using Chi square tests, where significant distortion was declared at $P < 0.01$ (Vuylsteke et al. 1999).

Because the parental allelic phase was unknown, codominant markers were double-scored by switching the presumed allelic origin. All markers were entered into Mapmaker/EXP version 3.0 for analysis. Markers were grouped with the two-point "*group*" command at LOD = 4.0 and a maximum recombination frequency value of 0.25. Markers within a group were ordered using the *Order* command with LOD of 3.0. The remaining markers were then located with the *Ripple* command. The groups were mapped using the Kosambi (1944) mapping function.

Table 3 Storage root anthocyanin content and relative acylated portion (both on fresh weight basis) of carrot inbreds B7262 and B9547 at 9, 12, and 15 weeks after planting. Inbred B493 had at most trace amounts of anthocyanin and data are not presented

Inbred	Anthocyanin[a]	9 weeks	12 weeks	15 weeks
B7262 (purple-orange)	Total (ppm)	51 ± 3	153 ± 7	513 ± 12
	% Acylated	18 ± 3	12 ± 6	29 ± 4
B9547 (solid purple)	Total (ppm)	714 ± 20	899 ± 18	1,151 ± 27
	% Acylated	15 ± 7	16 ± 3	27 ± 9

Acylated anthocyanins included Cy-3-(2′-xylose-6′-sinapoyl-glucose-galactoside), Cy-3-(2′-xylose-6′-feruloyl-glucose-galactoside, and Cy-3-(2′-xylose-6′-(4-coumaroyl) glucose-galactoside). Remaining percentage consisted of the non-acylated anthocyanins that included Cy-3-(2′-xylose-6-glucose-galactoside) and Cy-3-(2′-xylose-galactoside); (Kurilich et al. 2005)

Cy cyanidin

[a] Values ± SD

Results

Anthocyanin accumulation and expression of carrot anthocyanin biosynthesis genes

Anthocyanin accumulation was minimal early in carrot storage root development with first visually discernible color at 9 weeks in the solid purple carrot and in both the solid purple and purple-orange carrots at 12 weeks (Fig. 2; Table 3). The anthocyanin content of the two purple carrots included in this study differed significantly, although the relative content of acylated anthocyanins as a proportion of the total anthocyanins was similar throughout development (Table 3). Both the total anthocyanin content and percentage acylated anthocyanin were lower in these

greenhouse-grown carrots than reported for field-grown carrots (Kurilich et al. 2005).

The *Act* and *EF*-1 reference transcripts were both validated within each harvest and across all harvests. We established that the reference transcript levels were not variable within a cDNA sample indicating that variation in starting RNA quality or quantity, or in replicated cDNA reactions was not the source of the variation. This allowed us to compare the expression of each target gene not only within each harvest, but also to establish a time course of expression across all four harvests (Figs. 3, 4). As is typical for RT-qPCR, our analysis was characterized by sample-to-sample variation among the various target genes. It should be emphasized that the source of this variation is biological and represents a real variation in target transcript amounts among different carrot roots from the same harvest. Even with observed sample-to-sample variation, significant variation in carrot anthocyanin gene expression was noted for most of the genes analyzed, both during storage root development of the anthocyanin-accumulating inbreds, and between inbreds at a given developmental stage (Figs. 3, 4).

*PAL*3 and *UFGT* transcripts were detectable in solid purple B9547, purple-orange B7262, and orange B493 carrot roots in all harvests (Figs. 3, 4). *PAL*3 had the highest mean RER in purple carrots, less in purple-orange, and even less in the orange B493, but due to sample-to-sample variation the mean was only significantly higher ($P = 0.05$) in the Harvest 2 sample set (Fig. 3). *UFGT* transcript accumulated to roughly equal amounts in storage roots of all three inbreds in the first three harvests and was significantly higher in the purple-orange and orange roots at the fourth harvest. The mean expression level of *PAL3* was lowest in the Harvest 1 samples and increased to a steady state within each carrot color type in the remaining harvests (Fig. 4). The mean RER was consistently lowest in orange, intermediate in purple-orange and highest in purple carrots across all time points, but the differences were only statistically significant for Harvest 2. In contrast to *PAL*3, *UFGT* transcripts accumulated to a higher level in the purple-orange and the orange carrot than in the solid purple carrot, with their highest mean amount at Harvest 1. Means were statistically significant at Harvest 4 for which the RER of *UFGT* increased 4.5- and 4.3-fold in purple-orange and orange carrot relative to purple carrot roots (Figs. 3, 4).

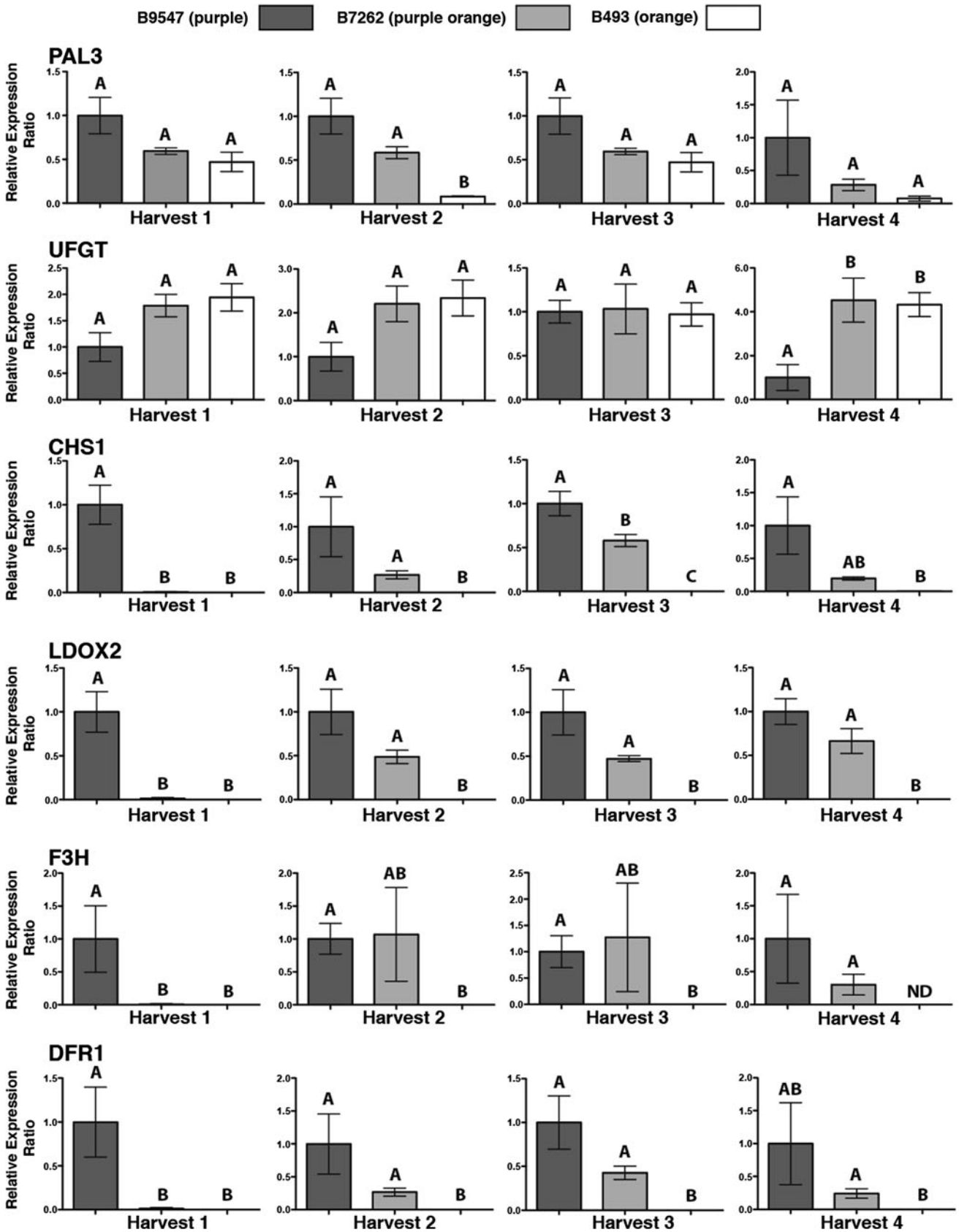
In contrast to *PAL*3 and *UFGT*, transcripts examined with RT-qPCR for *CHS*1, *F3H*, *DFR*1, and *LDOX*2 exhibited large differences in expression among the three carrot inbreds. In general, expression of these genes increased during development in the purple and purple-orange carrots, while the expression of all four genes was extremely low or undetectable in the orange carrot. Expression of these genes in anthocyanin-pigmented carrots (i.e., purple and purple-orange) (Fig. 4) roughly followed the time course for anthocyanin accumulation (Table 3; Fig. 2). *CHS1* transcript was virtually undetectable in the orange carrot roots throughout all developmental stages examined (Fig. 3). It was also not detectable in purple-orange roots from the first harvest (Figs. 3, 4) but in the remaining harvests, the *CHS1* transcript was detectable in both solid purple and purple-orange carrots (Fig. 3) and although the mean RER was consistently higher in solid purple than in purple-orange carrots, expression in the former was only established as significantly greater in samples from Harvests 1 and 3 (Fig. 3). Expression of *F3H*, *DFR*1 and *LDOX*2 transcripts was very similar to the pattern of expression of *CHS1*, with transcripts accumulating at very low levels or not detectable in purple and purple-orange carrots at Harvest 1, with increasing expression in the remaining harvests. Transcripts were expressed at very low or non-detectable levels in orange carrot at all harvests (Figs. 3, 4). In purple and purple-orange carrots, *CHS*1, *DFR*1, and *LDOX*2 expression rose five- to tenfold between harvests 1 and 2, with *CHS*1 continuing to rise in subsequent harvests, but leveling off for *DFR*1, and *LDOX*2. In contrast, *F3H* expression was barely detectable in the first three harvests, rising 10- to 15-fold between harvests 3 and 4 for the solid purple and purple-orange carrots. *PAL*1 and *UFGT* expression, in contrast to the aforementioned genes, was detectable in the orange and purple carrot inbreds with relatively little variation (<3-fold) in expression levels throughout root development (Fig. 4).

Marker and trait analysis in segregating populations

Twenty-one AFLP primer combinations amplified 891 bands of which 283 (31.8 %) were polymorphic in the F_2 population (Table 2). The performance among different primer pairs varied widely (11–68 amplicons; 4–28 polymorphic bands; 11.8–51 % polymorphism). Three codominant AFLP markers were identified on the basis of Chi-square tests significant for segregation ratios of 1(A):2(H):1(B), and co-localization of the individual bands when scored and mapped as dominant markers.

Of the 370 SSR markers initially evaluated for polymorphism and mode of segregation in the F_2 population, 44 (11.9 %) were polymorphic, presented unambigous band patterns, and had segregation ratios consistent with single loci. Only these SSR markers, of which 36 were codominant and 8 were dominant, were included in the linkage analysis.

Primer pairs from five anthocyanin structural genes (*F3H*, *FLS*1, *LDOX*2, *PAL*3, and *UFGT*) and three anthocyanin transcription factors (*DcEFR1*, *DcMYB3*, and


B9547 (purple)
B7262 (purple orange)
B493 (orange)
PAL3
UFGT
CHS1
LDOX2
F3H
DFR1
Relative Expression Ratio
Harvest 1
Harvest 2
Harvest 3
Harvest 4
ND

◀ **Fig. 3** Relative expression ratios of six anthocyanin biosynthesis genes in three carrot inbreds over four different harvest times. *EF-1* and *Actin* reference RNA primers were used for all calculations. The calibrator for each sample set was the mean expression level of the target gene in B9547 (*solid purple*) roots for each harvest. *Error bars* represent standard error. *Columns headed by different letters* are significantly different with P values ≤ 0.05

DcMYB5) were used to generate PCR amplicons of 600–1,386 nt from a subset of the F_2 population (Table 1).

All F_1 hybrids between purple-orange B7262 and yellow-rooted B1896 (which lacks purple) inbreds had purple phloem pigmentation and yellow xylem. Purple root pigmentation was conditioned by a single dominant gene, as indicated by segregation ratios of purple versus non-purple colored roots in the F_2 family ($\chi^2 = 0.296$; $P = 0.586$). Because P_1, the locus conditioning purple root pigmentation in carrot, was originally described in F_2s and BCs derivatives of PI 173,687 (Simon 1996), and the same purple-rooted line was used in the pedigree the 10117 F_2 population used in this study, the genetic evaluations for this trait were highly concordant in both studies, and it was assumed that the P_1 gene also conditions purple root pigmentation in this study.

Genetic evaluation was generally unambigous for purple root color. In $P_1/-$ roots, purple pigmentation was very evident in the outer part of the phloem throughout the entire length of the root except for the tip where reduced pigmentation was observed, like B7262 in Fig. 2. In these roots, tissues of the inner part of the phloem, cambium and xylem had no anthocyanin pigmentation. Instead, these tissues were all either yellow or orange-colored in all roots.

The inner phloem and xylem tissues of all the F_1 hybrids were yellow, whereas yellow versus orange carotenoid pigmentation segregated in the 10117 F_2 population from

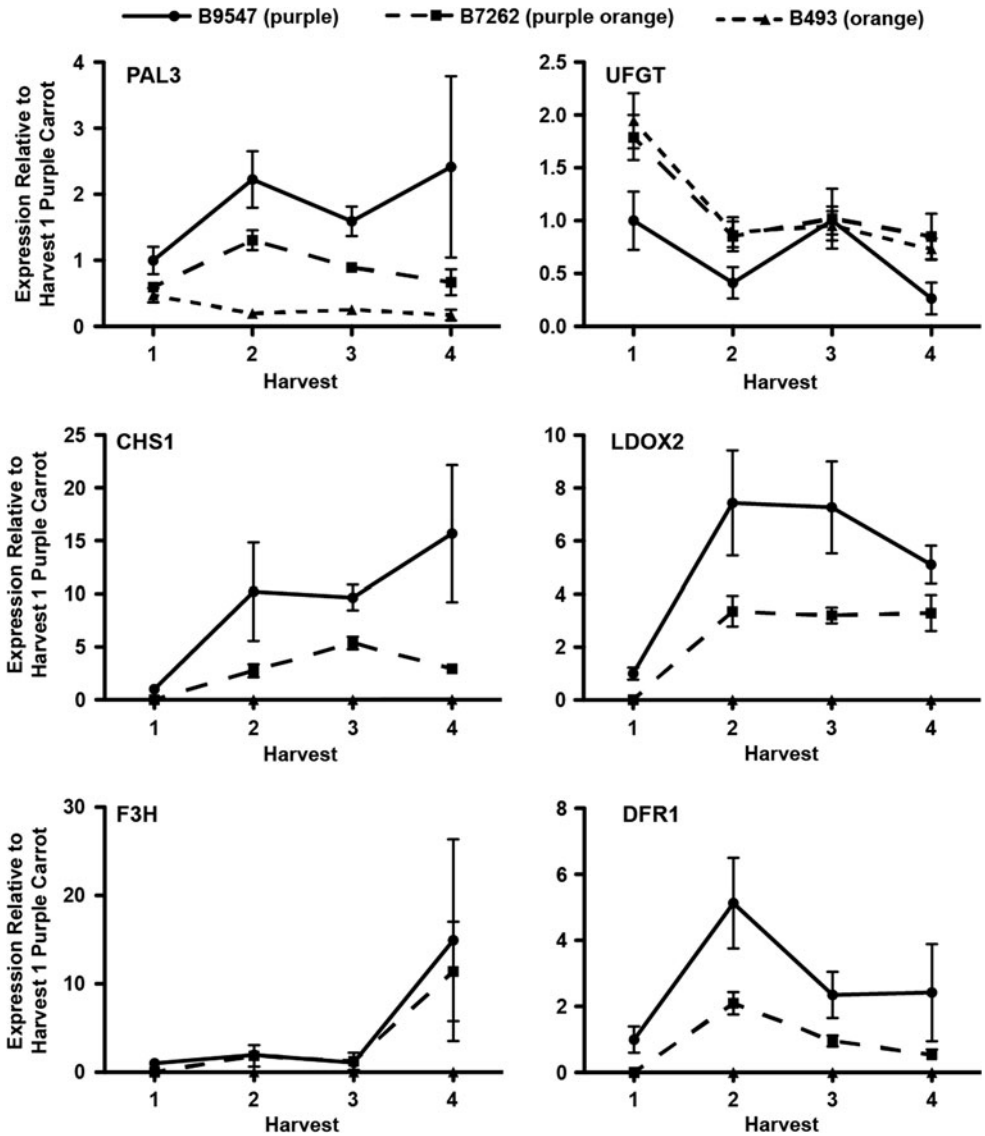


Fig. 4 Relative expression ratios of six anthocyanin biosynthesis genes in three carrot inbreds across four different harvest times. *EF-1* and *Actin* reference RNA primers were validated as stable across all four harvests using the *BestKeeper* program and were used to normalize all target gene values. The calibrator for each sample set was the mean expression level of the target gene in B9547 (*solid purple*) carrot roots at Harvest 1. *Error bars* represent standard error

both locations fitting a 3:1 ratio (yellow:orange) ($\chi^2 = 0.074$; $P = 0.785$) typical for a single dominant gene. This corresponds to the Y_2 locus initially described by Buishand and Gabelman (1979) in various genetic bacgrounds and later identified by Simon (1996) in derivatives of PI 173687, an inbred used for developing 10117 population.

Linkage map

Carrot linkage maps for both parental lines were constructed using 279 marker data points. These included 2 phenotypic loci (P_1 and Y_2), 237 AFLPs, 40 SSRs, 1 SCAR, 5 anthocyanin biosynthesis structural genes (*F3H*, *FLS*1, *LDOX2*, *PAL*3, and *UFGT*), and 3 anthocyanin transcription factors (*DcEFR1*, *DcMYB3*, and *DcMYB5*) with 45 of them (15.7 %) being codominant markers. Additionally, 24 markers (22 AFLPs and 2 SSRs) were unlinked. Based on known chromosome location of molecular (SSR) and phenotypic (*Y2*) markers, LGs were assigned to their respective chromosomes (Cavagnaro et al. 2011; Iovene et al. 2011; Alessandro et al. 2013). Anthocyanin biosynthesis genes and transcription factors we mapped only occurred on carrot chromosomes 2, 3, 4, 7, and 8, and these linkage data and maps are summarized in Table 4 and Fig. 5, respectively.

The P_1 locus controlling root anthocyanin pigmentation mapped to chromosome 3 of the purple-rooted parental (A) map. Two anthocyanin biosynthesis genes, *F3H* and *FLS1*, were closely linked to P_1, at a distance of 6.3 and 6.9 cM, respectively. In addition, this chromosome included seven codominant SSR markers, one of them linked at 21.9 cM from P_1. The other mapped anthocyanin structural and regulatory genes were not positionally associated with P_1 and mapped to chromosomes 2 (*LDOX*2), 4 (*PAL*3 and *DcEFR*1), 7 (*DcMYB*5) and 8 (*DcMYB*3 and *UFGT*). The phenotypic locus Y_2, controlling yellow versus orange root core color mapped to chromosome 7.

Discussion

Gene expression and anthocyanin accumulation

Purple, or sometimes known as black, carrot roots accumulate concentrations of anthocyanins regularly exceeding 1,500 ppm (Kurilich et al. 2005). In this study we found that transcripts of *PAL*3, *CHS*1, *F3H*, *DFR*1, and *LDOX*2 were more abundant in the solid purple carrot inbred B9547, usually less abundant in the purple-orange inbred B7262, and expressed at low to undetectable levels in orange inbred B493 (Fig. 4). Transcript levels roughly followed visual and quantitative variation in anthocyanin

Table 4 Summary of the parental "A" and "B" maps of carrot chromosomes with anthocyanin structural and regulatory genes

LG	Total no. markers mapped	# Markers common to both parental ("A" and "B") maps	Parental "A" map					Parental "B" map					Anthocyanin genes
			No. of markers mapped on the "A" map	No. of mapped markers segregating only from "A"	Total mapped markers[a]	LG size (cM)	Mean marker distance (cM)[b]	No. of markers mapped on the "B" map	No. of mapped markers segregating only from "B"	Total mapped markers[a]	LG size (cM)	Mean marker distance (cM)[b]	
2	47	5	23	18	18A, 4S, 1G	176.8	7.6	29	24	24A, 4S, 1G	175.2	6.0	*LDOX2*
3	31	9	19	10	1Ph, 9A, 7S, 2G	99.2	5.2	21	12	12A, 7S, 2G	165.2	7.8	P_1, *F3H, FLS*
4	40	10	26	16	17A, 7S, 1G, 1TF	155.9	5.9	24	14	15A, 7S, 1G, 1TF	128.5	5.3	*DcEFR1, PAL1*
7	50	6	29	23	1 Ph, 22A, 5S, 1TF	144.4	4.9	27	21	19A, 7S, 1TF	133.1	4.9	Y_2, *DcMYB5*
8	30	7	17	10	10A, 6S, 1TF	75.4	4.4	22	15	1G, 14A, 6S, 1TF	123.6	5.6	*DcMYB3, UFGT*

[a] *Ph* phenotypic trait, *A* AFLP, *S* SSR, *G* gene (anthocyanin)-derived markers, *TF* transcription factor, "A" and "B" refer to the purple-orange- and yellow-rooted parents, respectively

[b] Mean distance between markers is the arithmetic mean of map distances between adjacent markers in Fig. 5

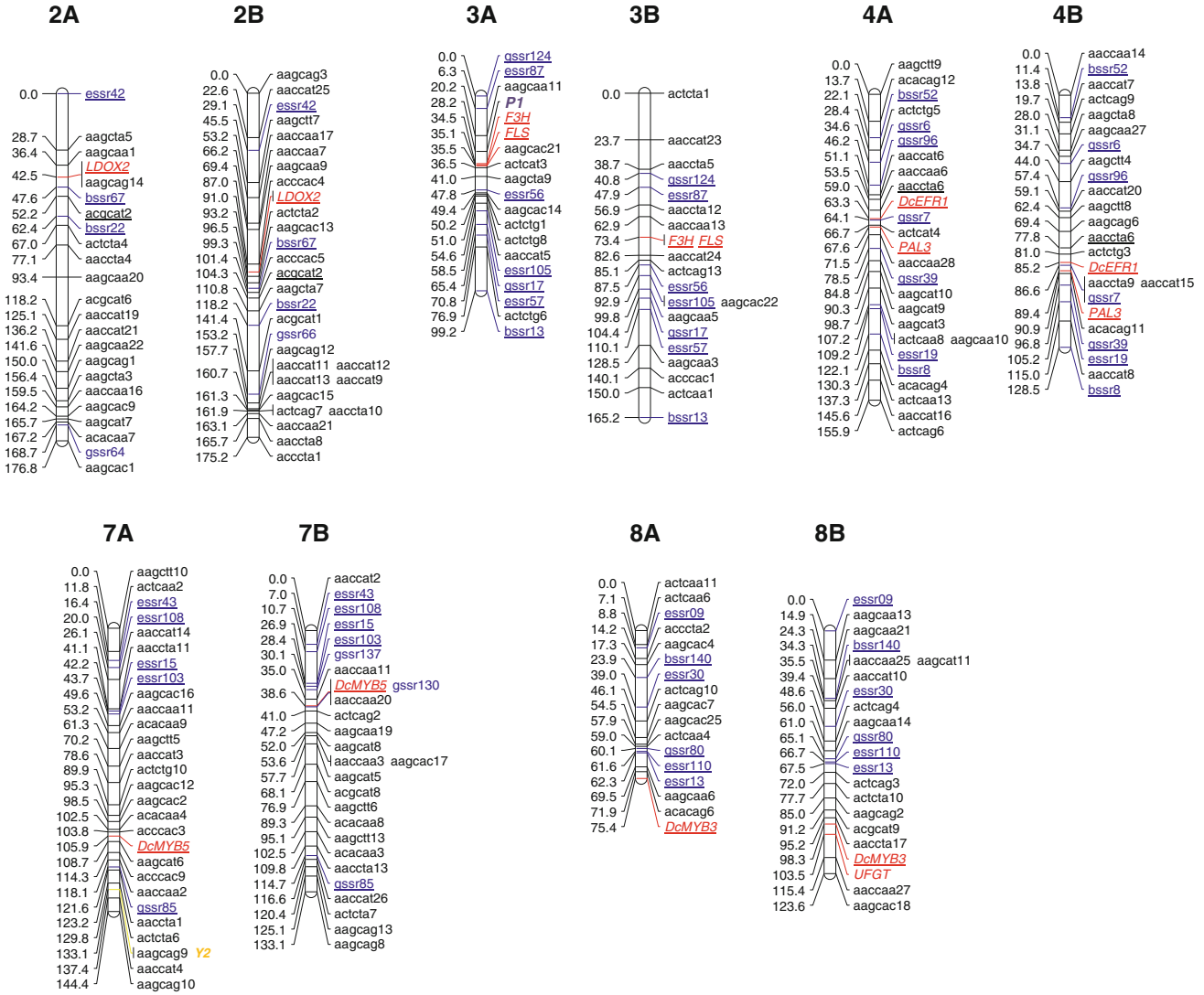


Fig. 5 Genetic linkage maps of chromosomes of the 10117 carrot population that contain anthocyanin structural and regulatory genes. A and B refer to the purple-orange- and yellow-rooted parents, respectively. Anthocyanin genes and microsatellites are denoted by *red and blue letters*, respectively. *P1* (chromosome 3) and *Y2* (chromosome 7) are phenotypic loci denoted by *purple and orange letters*, respectively. Codominant markers present in both parent maps are *underlined*. LGs were oriented and named according to their corresponding physical chromosomes (Iovene et al. 2011)

concentration during carrot storage root development, as is typically observed in studies of anthocyanin accumulation in fruits, flowers and leaves in a wide range of plant species, and in sweet potato storage roots (Mano et al. 2007) suggesting a similar process in all plant tissues. Exposure to light is necessary to stimulate anthocyanin accumulation and biosynthetic gene expression in fruits of grapes, apples, and other plants (e.g. Kondo et al. 2002). Since carrot storage roots are not normally exposed to light, this feature of anthocyanin biosynthesis is apparently absent in roots and likely tubers. Carrots with purple roots often, but not always, have purple leaves, nodes, stems, and petals (Simon 1996). Transcriptional control of leaf anthocyanin biosynthesis differs from that in flower and fruit of pepper, and other plants (e.g. Stommel et al. 2009). Given these diverse observations, comparisons of anthocyanin structural and regulatory gene expression in above-ground carrot tissues in purple-rooted carrots with and without anthocyanin accumulation in above-ground tissues, and with and without exposure to light, will provide an interesting expansion to studies reported here.

Studies of anthocyanin biosynthesis gene expression in plants varying in anthocyanin accumulation have been carried out in many species, and like this study, most evaluated only a subset of the genes in this complex pathway. In some cases, mutations in single genes in the

pathway account for variable anthocyanin accumulation, but in most cases, transcription factors regulate expression of one or more genes in concert (Shirley 2001). A comprehensive evaluation of anthocyanin gene expression is complicated by not only the number of genes in the pathway, but also by the fact that most of these genes occur in multiple copies in a genome (e.g. Durbin et al. 2000; Modolo et al. 2007).

Few previous studies evaluating anthocyanin biosynthesis or accumulation in carrot are reported, although the biochemistry and molecular biology of flavonoid biosynthesis in carrot tissue cultures has been a topic of research in several labs. Relevant to the research we report, Ozeki et al. (1990) reported that Dc*PAL*1 expression was stimulated by stress in carrot suspension cultures, and as we observed in carrot roots, anthocyanin biosynthesis was associated with Dc*PAL*3 expression. It is interesting to note that Hirner et al. (2001) found four of the same genes we studied (*CHS*1, *F3H*, *DFR*1 and *LDOX*2) to be differentially expressed in purple flowers in the center of wild carrot flower umbels, but not in the root, stem, leaves, or white flowers of these plants that otherwise lack anthocyanin pigmentation. While the genetics of purple central umbellets in carrot inflorescences has not been studied in detail, this trait does not co-segregate with storage root or leaf pigmentation (Simon et al. personal communication).

We found *UFGT* expression to be relatively invariable as a function of both storage root color and stage of development, but considering the complexity of anthocyanin biosynthesis, it is not surprising that in plants other than carrot, anthocyanin pigmentation is associated with the expression of *UFGT*. For example, a mutation in *UFGT* accounts for a color sport in grapes (Kobayashi et al. 2001) and *bz1* is a *UFGT* mutation of maize (Sharma et al. 2011). Based upon the extensive genetic variation for anthocyanin biosynthesis genes in maize (Sharma et al. 2011), and similarly broad genetic variation observed at the phenotypic and genomic level in carrots (Iorizzo et al. 2011) it would not be surprising to find anthocyanin-accumulation mutants in carrot conditioned by *UFGT*.

The coincidental rise of several anthocyanin biosynthesis enzymes—concomitantly with an increase in anthocyanin accumulation in the storage root—in purple carrots suggests that a transcription factor is a likely candidate for the P_1 locus and that additional genes likely account for the variable distribution of anthocyanin in root tissue observed in comparing B9547 and B7262 (Fig. 2). To better determine the function of P_1 or other genes modifying anthocyanin accumulation in carrot, differential gene expression must be accompanied by genetic mapping to identify candidate genes involved in phenotypic variation.

Genetic mapping and anthocyanin accumulation

To identify candidate genes that account for variation in anthocyanin accumulation using a genetic mapping approach, a genetic map of molecular markers is essential. Carrot SSR markers have recently been developed from carrot genomic (Cavagnaro et al. 2011) and EST (Iorizzo et al. 2011) sequence data, representing an ideal tool for integrating carrot linkage maps, as these robust PCR-based markers can serve as anchor points across maps. Seventy-one of these SSRs were recently included in two carrot maps (Cavagnaro et al. 2011; Alessandro et al. 2013). The 40 SSRs mapped in this study (32 in linkage groups containing anthocyanin genes; Fig. 5), of which 35 SSRs were codominant, were distributed throughout all chromosomes in both parental maps, thus facilitating comparisons among chromosomes with common markers between the three maps. Of particular interest (for assisting breeding and perhaps map-based cloning) are chromosome 3 and chromosome 7 containing P_1 and Y_2, respectively, since common markers with the reference carrot map were identified (Cavagnaro et al. 2011; Iovene et al. 2011; Alessandro et al. 2013).

A primary thrust of this work was an evaluation of P_1 from the perspective of its positional and functional association with anthocyanin biosynthesis genes. The complete linkage between *F3H* and *FLS* is interesting and we have not found evidence for this in other plants. The close linkage between these genes and P_1 makes this an even more interesting observation. The fact that there is recombination between these anthocyanin structural genes and P_1 suggests these two genes may not be likely candidate genes for P_1. The fact that we found not only *F3H*, but also four other anthocyanin biosynthesis genes to be expressed more in P_1P_1 plants (B7262 and B9547) but not p_1p_1 plants (B493) suggests that P_1 is more likely a transcription factor regulating the expression of several anthocyanin structural genes and, therefore, conditioning anthocyanin pigmentation in carrot roots. The genetic map locations of the *DcEFR1*, *DcMYB3* and *DcMYB5* genes are positionally unrelated to P_1, and this strongly suggests that these are not candidate transcription factors for P_1.

Anthocyanin candidate gene analyses performed in other plant species have reported positional associations of *F3H* or *FLS* genes with loci controlling anthocyanin pigmentation. For example, in diploid strawberry, Deng and Davis (2001) mapped five structural anthocyanin genes (*CHS*, *CHI*, *F3H*, *DFR*, *ANS*), a modifier gene (*Del*), and two morphological characters, *c* (controlling yellow versus red fruit color) and *r* (runnerless), and they found complete linkage between *F3H* and the *c* locus, leading them to conclude that *F3H* plays an important role in pigmentation of *F. vesca* fruits. In petunia, *FLS* was tightly linked

(~2 cM) to *Fl* which controls flavonol biosynthesis (Holton et al. 1993). Relevant to our gene expression observations, Forkmann et al. (1986) also reported that in petunia, *FLS* and *F3′H* transcription is maximum during anthocyanin accumulation.

The expression and mapping of anthocyanin biosynthesis genes that we report represents a foundation for studying this complex pathway in carrots. Primer sequence information is being used to evaluate anthocyanin gene expression and map location in diverse carrot germplasm as well as in related Apiaceae. As the carrot genome is being sequenced, the combination of specific sequences with map locations will assist in sequence assembly and set the stage for more comprehensive identification of homologous and paralogous genes in the carrot genome, and a functional identity of P_1.

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