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Molecular identification of the yellow fruit color (*c*) locus in diploid strawberry: a candidate gene approach

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Abstract A candidate gene approach was used to determine the likely molecular identity of the *c* locus (yellow fruit color) in *Fragaria vesca*, a diploid (2n=2*x*=14) strawberry. Using PCR with degenerate primer pairs, intron-containing segments of structural genes coding for chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS) and one *Del*-like regulatory gene in the anthocyanin biosynthetic pathway, were amplified, cloned and sequenced. Intron length polymorphisms for each of these genes were detected among three diploid varieties: *F. vesca* Alpine variety 'Yellow Wonder' (YW) (Europe); DN1C, a *F. vesca* clone collected from Northern California; and *Fragaria nubicola* FRA520, a U.S.D.A. accession collected in Pakistan. Using F_2 generations of the crosses DN1C×YW and YW×FRA520 as mapping populations, the six candidate genes were mapped in relation to previously mapped randomly amplified polymorphic DNA (RAPD) markers and morphological markers. The *F3H* gene was linked without recombination to the *c* locus in linkage group I, while the other five candidate genes mapped to different linkage groups. These results suggest that the wild-type allele (*C*) of the *c* (yellow fruit color) locus encodes an F3H necessary for red fruit color in *F. vesca*.

Keywords *Fragaria* · Anthocyanin · Fruit color · Mapping · Intron length polymorphism

Introduction

Anthocyanins, the largest flavonoid subclass, are well known for the coloration they provide as blue (delphinidins), red (cyanidins) and purple (pelargonidins) pigments of flowers and fruits in higher plants. Anthocyanins in fruits and flowers serve as visual signals that attract insects and animals, thus playing an important role in the ecology of pollination and seed dispersal (Holton and Cornish 1995; Shirley 1996; Mol et al. 1998). The anthocyanin biosynthetic pathway has been well established and many genes encoding anthocyanin biosynthetic enzymes have been characterized and cloned (Dooner et al. 1991).

The enzymes in the core linear pathway of anthocyanin biosynthesis are chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase (ANS). In addition, flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′5′-hydroxylase (F3′5′H) catalyze the hydroxylation of flavanones, dihydroflavonols and leucoanthocyanidins in the 3′ position only or in both of the 3′ and 5′ positions, thus making different anthocyanins (Stotz et al. 1985; Holton et al. 1993). Species lacking F3′5′H activity, such as strawberry and rose, can not synthesize the blue delphinidin derivatives (Elomao and Holton 1994). Other enzymes, such as 3- and 5-glucosyl transferase (3GT, 5GT), rhamnosyl transferase (RT) and O-methyl transferase (OMT) may be required for modification of anthocyanins, and anthocyanin glutathione S-transferase (GST) is required for the modification and transfer of anthocyanins into the vacuole (Shirley 1996; Mol et al. 1998).

Regulatory genes that control the expression of genes for anthocyanin biosynthesis have also been identified in many plants (Dooner et al. 1991; Mol et al. 1996). Two families of regulatory genes, the *R/B* and *C1/Pl* gene families, have been found in maize. The members of the *R* family, which share homology with the basic helix-loop-helix (bHLH) motif, encode products related to the *myc* family of transcription factors, while members of the *C1* family encode *myb*-type transcription factors (Quattrocchio et al. 1998). In snapdragon, the *Delila* (*Del*) gene shares extensive homology with the *R* gene family of maize (Goodrich et al. 1992). These regulatory

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genes regulate various subsets of anthocyanin biosynthetic genes in different plant species, conditioning the spatial and temporal accumulation of pigments (Dooner et al. 1991; Deboo et al. 1995).

Fruit color is an important component of fruit quality in the octoploid (2n=8*x*=56) cultivated strawberry, *Fragaria×ananassa* Duch.. In both *F.×ananassa* and its diploid (2n=2*x*=14) relative *Fragaria vesca* L., the red pigmentation of fruit has been attributed to the 3-glucosides of pelargonidin and cyanidin (Sondheimer and Karash 1956). Sistrunk and Morris (1985) have described the external color of different *F.×ananassa* cultivars as varying from a "light orange red" to "dark purple red" when the fruit is ripe. These authors described internal fruit color as varying from white-centered fruit with a dark purple-red cortex to a uniform color throughout, and covering the entire range of color stated above. Although genotypic variation in the anthocyanin pigmentation of commercial strawberry fruit has been the subject of several studies (Shaw 1991), no QTL loci or Mendelian genes affecting this trait have been described in *F.×ananassa*.

As a self-pollinating diploid with a small (<200 mb) genome size (Nehra et al. 1991; Nyman and Wallin 1992), *F. vesca* is an attractive "surrogate system" for genetic investigations of economically important traits in strawberry, including fruit color. Several varieties of *F. vesca*, including 'Yellow Wonder', bear yellow or white fruit. The locus governing red (*C*) versus yellow/white (*c*) fruit color in *F. vesca* was named by Brown and Wareing (1965). Using a cross between *F. vesca* 'Alpine' varieties 'Yellow Wonder' (YW) and 'Baron Solemacher' (BS), Williamson et al. (1995) showed that the *c* locus was closely linked (1.1 cM) to a shikimate dehydrogenase (SDH) isozyme locus. Subsequently, using a cross between BS and *F. vesca* WC6 (a wild plant collected in New Hampshire), Davis and Yu (1997) constructed a *F. vesca* genetic linkage map of seven linkage groups. The SDH locus and, by inference, the *c* locus (which was not segregating in the BS×WC6 cross), were located at the bottom of linkage group I.

We hypothesized that yellow fruit color in *F. vesca* is conditioned by a mutation in a structural or regulatory gene of the anthocyanin biosynthetic pathway. To test this hypothesis, six candidate genes were mapped to determine their linkage relationships with the *c* locus. These candidate genes were the *CHS*, *CHI*, *F3H*, *DFR* and *ANS* structural genes, and a *Del*-like regulatory gene we hereafter refer to as *RAN* (regulation of anthocyanin biosynthetic pathway). Each candidate gene was mapped on the basis of PCR-detectable intron length polymorphism, an approach made feasible by the availability of mapping populations generated from wide crosses.

Among the known anthocyanin genes, only a full-length *DFR* cDNA and partial *CHS* and *F3H* cDNA sequences have been reported for *F.×ananassa* (Manning 1998; Moyano et al. 1998), and no *F. vesca* anthocyanin biosynthetic gene sequences had been determined prior to the present study. Our primary goal was to determine the molecular identity of the *c* locus of *F. vesca*. Additionally, an expected by-product of this research was the generation of new, strawberry specific sequence information as well as primer sets useful for future study of fruit color variation in the commercial strawberry, *F.×ananassa*.

Material and methods

Plant material

'Alpine' *F. vesca* L. ssp. *vesca*, variety 'Yellow Wonder' (YW) (Europe) (commercially available from W. Atlee Burpee and Co., Warminster, Pa.), *F. vesca* ssp. *bracteata* DN1C (collected from the wild in Northern California) and *Fragaria nubicola* Lindl. FRA520 (Pakistan – obtained from National Clonal Germplasm Repository, Corvallis, Ore.) were used in the present study. All are diploid (2n=2*x*=14). YW has the recessive mutant traits, yellow fruit (*c*) and runnerless (*r*), while DN1C and FRA520 have the wild-type traits, red fruit and runnering. F_1 plants from the crosses DN1C×YW and YW×FRA520 were allowed to naturally self-pollinate in the greenhouse, and two respective F_2 mapping populations of 40 individuals each were established and maintained in the field.

DNA isolation, basic PCR techniques and gels

Genomic DNA was isolated from young, unexpanded leaf tissue using a protocol (Davis et al. 1995) modified from that of Torres et al. (1993). PCR reaction mixes (25 µl) were prepared as previously described (Davis and Yu 1997), except when modified for use with degenerate primer pairs as described later. For all PCR reactions, total template DNA amount and individual primer concentrations were 100 ng and 0.4 µM, respectively. The amplification profile for RAPD markers was as previously described (Davis et al. 1995). For intron polymorphisms, the general amplification profile began with 2 min denaturation at 94°C, followed by 36 cycles of 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C, followed by a final 5 min at 72°C. Annealing temperatures were adjusted experimentally in the range of 48°C to 65°C to get the best result for each primer pair. Mixed template reactions (Davis et al. 1995) were used when heteroduplex analysis was required to determine the allelic composition of plants in segregating populations. To analyze PCR results, 12 µl of PCR reaction products were loaded on a 2% agarose TBE gel.

Degenerate primers

For each of the six candidate genes, heterologous DNA and protein sequences from various plant species, and a full-length *F.×ananassa DFR* cDNA (Accession No. AF029685), were obtained from the NCBI GenBank. For each candidate gene, the computer program Lasergene MegAlign (DNAStar, Inc.) was used to compare genomic and cDNA sequences to determine the locations of introns and to align protein sequences to identify highly conserved coding regions. Sequences from 20 different plant genera were used for these purposes. Details are available from the authors upon request.

Degenerate PCR primer pairs (Table 1) were designed to target conserved exon regions flanking intron sites (Fig. 1). Primers CHSF and CHSR flank the single intron in the *CHS* gene. The primers CHID2 and CHID4 flank intron II of the *CHI* gene. For the *F3H* gene, primers F3 and F4 flank intron II. In the *DFR* gene, primers D1 and D2 flank intron I. The A2 and A3 primers flank the single intron in the *ANS* gene. For the *Del*-like regulatory gene, in *Arabidopsis* there are five introns at conserved locations in the *Atmyc-146* and *Atmyc-1* genes and two additional introns

Table 1 Primer pairs used to clone segments from anthocyanin biosynthesis genes and/or to map these genes in diploid strawberry. Primers were designed using Lasergene Primer Select (DNAStar, Inc.). Annealing temperatures used for PCR reactions are given. Mixed base site code: R=A,G; Y=C,T; M=A,C; K=G,T; S=C,G; W=A,T; H=A,C,T; B=C,G,T; V=A,C,G; D=A,G,T; N=A,C,G,T

Gene	Primer pair	Sequence	Annealing temperature
CHS	CHSF	TAY CCN GAY TWY TAY TTC	54°C
	CHSR	GYT GNC CCC ATT CYT T	
	CHSNF	TAT CCC GAC TAC TAC TTC	56°C
	CHSNR	GCT GGC CCC ATT CCT TAA	
CHI	CHID2	AAR TGG AAR GGH AAR AC	50°C
	CHID4	GAR GCV CCN GGW GGR AA	
	CHIF	AAG ACG GCC GAG GAG TTG	56°C
	CHIR	CGT CAG CGG TAG TAT CAT TGT C	
F3H Int. I	FS1	ATG GCC CCT ACT CCT ACT ACT CTG	56°C
	FS2	GTC ACA ATC TCG CGC CAA TCC T	
F3H Int. II	F3	TGG ATY ACB GTK CAR CC	56°C
	F4	GAG TTC ACY ACB GCY TG	
	FC	AAC TGT TCC ATC ACC CAA ATA AT	56°C
	FD	CGA CCC AGA AAC TCA TAC TCA A	
DFR	D1	CGW GCM ACY GTB CGW GA	56°C
	D2	CAT RGG NGT KGC SAC RT	
ANS	A2	TGG GAR GAY TAY TTY TT	52°C
	A3	ATG TTG TGK AGD ATR AA	
RAN	DG1	TAY GSM MTH TTY TGG TC	56°C
	DG2	AGA GAB TCR TAR AGY TC	
	R1	TTC ACC AAA ACA ACC AG	56°C
	R2	GTA CCC ATC ACC CCA CTC	

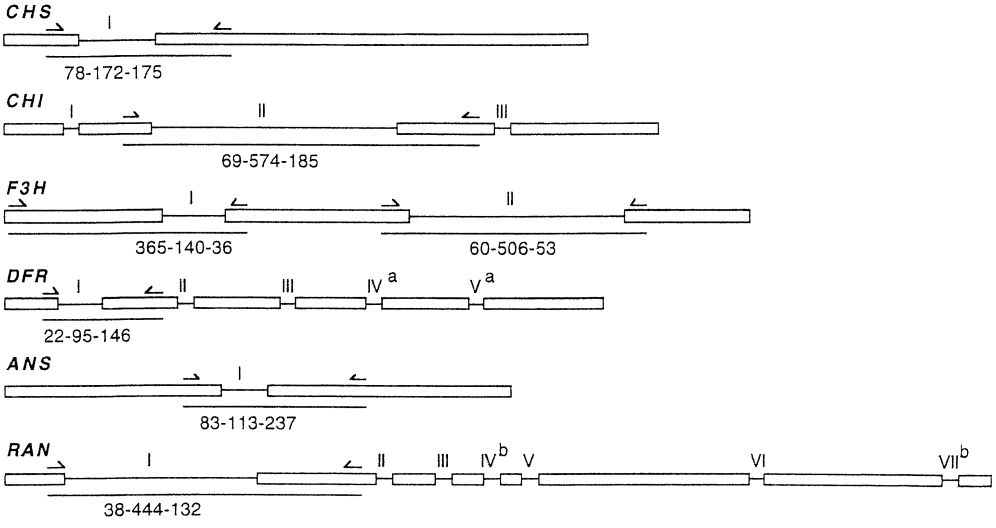

Fig. 1 Genomic structures of anthocyanin genes. The *Roman numerals* indicate introns. The *numbers* below the gene structures were the lengths in base pairs of exon-intron-exon regions of cloned gene segments. Exons and amplified introns are drawn to scale, while all unamplified introns (lengths unknown in *F. vesca*) are drawn to equal (small) size. *a* Introns present in snapdragon, petunia, arabidopsis, but not in maize and barley. *b* Introns present in Atmyc-1 of *Arabidopsis*, but not in Atmyc-146 of the same species

present in *Atmyc-1* only. The degenerate primers, DG1 and DG2, flank the first common intron (*Arabidopsis* intron I) of this gene family.

For PCR with degenerate primers, the primer concentrations were varied according to their degree of degeneracy and were adjusted empirically to get optimized PCR results (i.e., a strong target band and minimal secondary bands). Control reactions containing only the forward or the reverse member of a primer pair were also employed for purposes of comparison to help distinguish the target product from non-target products.

Cloning, sequencing and new strawberry specific primers

After PCR, bands of the approximate expected sizes (assuming intron lengths of less than 600 bp) were excised from 2% agarose TAE gels, purified with a Geneclean kit (Bio101, Inc.), and cloned using a TA cloning kit (Invitrogen, Inc.). Cloned PCR products were sequenced at the University of New Hampshire DNA sequencing facility using an automatic sequencer model 373 A (Applied Biosystems). DNA sequences were analyzed using the SeqEd program (Applied Biosystems). To confirm product identity, the GenBank Blast application was used to compare DNA sequences, and the amino-acid sequences predicted from them, with corresponding GenBank sequences. To determine the basis for polymorphisms in each gene, homologous sequences from crossing parents were compared using Lasergene MegAlign (DNAStar, Inc). Based upon cloned PCR product sequences, and upon the *F3H* cDNA sequence acquired in a related investigation (C. Deng, unpublished), strawberry specific, non-degenerate primer pairs were designed for the *CHS*, *CHI*, *F3H*, and *RAN* genes (Table 1).

Mapping candidate genes using intron length polymorphisms

The strawberry specific primer pairs, and the original degenerate primer pairs for *DFR* and *ANS*, were used to detect intron length polymorphisms between YW and the other two mapping parents. Intron length polymorphisms were detected on the basis of electrophoretic band-mobility shifts and/or heteroduplex band formation in heterozygotes and mixed template reactions, using 2% agarose TBE gels. These polymorphisms were then used to map the candidate genes with respect to RAPD markers and morphological markers, including the *c* gene.

RAPD and morphological markers

RAPD markers were amplified using individual decamer primers employed in construction of the original *F. vesca* linkage map (Davis and Yu 1997). Two steps were taken in the selection of RAPD primers and the markers used in this study. Initially, it was necessary to determine which of the previously mapped RAPD markers were conserved (detectable and polymorphic) in either of the two new mapping populations. Each of 28 previously used RAPD primers were tested on the mapping parents (YW, DN1C and FRA520), and banding patterns were compared with those generated in the previous study employing mapping parents BS and WC6 (Davis and Yu 1997). Conserved RAPD markers representing the seven *F. vesca* linkage groups were then amplified and mapped in the two new mapping populations. One previously mapped RAPD marker, PX18AB, was converted (by cloning, sequencing and specific primer pair design) into a site-specific marker PX18SS, and detected by the following primer pair:

PX18–25: 5′-TGGGGTGAATATTGGGGTAGA-3′
PX18–13: 5′-AGGTGGGGTAGGAGAAAAGAAAG-3′

The two morphological traits, yellow fruit versus red fruit, and runnering versus runnerless, were classified unambiguously in the F_2 mapping populations based on 1 year of visual observation in greenhouse and field environments.

Data analysis

Segregation data for anthocyanin genes, RAPD and morphological markers were collected from the mapping populations and analyzed with the aid of the Mapmaker Program (Lander et al. 1987). Linkages detected between anthocyanin genes, RAPD and morphological markers were used to assign the anthocyanin genes to map positions.

Results

Cloning of intron-containing gene fragments

Initially, PCR amplification with degenerate primers was used to isolate and clone an intron-containing segment of each candidate gene. In each case, PCR amplification with degenerate primer pairs (Table 1) yielded one or more product bands (data not shown). If multiple bands were initially present, the adjustment of primer concentrations (and concentration ratios between primers) and annealing temperatures was often sufficient to eliminate non-specific products. Putative target bands were of approximately the expected size based upon the sum of the primer-primer distance (as determined from known cDNA sequences) and an expected intron size of less than 600 bp, and were absent in respective single primer control reactions.

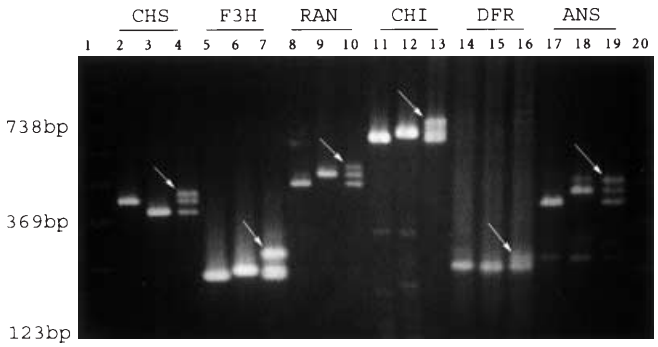

Fig. 2 Detection of intron length polymorphisms between YW and either DN1C (CHS, F3H) or FRA520 (RAN, CHI, DFR and ANS). For each target gene, the PCR templates were (left to right) YW, DN1C or FRA520, mixed template (YW plus either DN1C or FRA520). Heteroduplex bands, such as those seen (*arrows*) in all mixed template reactions, were useful indicators of marker heterozygosity for genotyping the mapping populations. *Lanes 1 and 20* are a 123-bp ladder (Gibco-BRL)

Following cloning of PCR products from both of the crossing parents, the identities of the putative target segments were determined by sequencing, followed by a Blast search and by alignment with heterologous GenBank sequences. In each case, the identity of the cloned PCR product was as intended, confirming that a segment of each of the six candidate genes had been successfully amplified from genomic DNA. Clone insert (PCR product) sizes ranged from 263 bp (*DFR*) to 835 bp (*CHI*). The sequences have been deposited in GenBank under the following accession numbers: CHS/YW, AY017477; CHI/YW, AY017478; F3H/YW, AY017479; DFR/YW, AY017480; ANS/YW, AY017481; RAN/YW, AY017482; CHS/DN1C, AY017483; CHI/FRA520, AY017484; F3H/DN1C, AY017485; DFR/FRA520, AY017486; ANS/FRA520, AY017487; RAN/FRA520, AY017488.

For each of the six candidate genes, the cloned sequence contained an intron at the expected position (Fig. 1). Intron sizes ranged from 95 bp (*DFR*) to 574 bp (*CHI*) (Fig. 1). Conjoined sequences of flanking exons in each case consisted of open reading frames, from which predicted amino-acid sequences were derived and compared with corresponding GenBank sequences using the Blast program. The amino-acid percent identities of the heterologous GenBank sequences most closely matching the six candidate genes ranged from 100% for F3H to 78% for the *RAN* gene.

Intron length polymorphisms

For each of the six candidate genes, an intron length polymorphism was detected between YW and one or both of the other crossing parents (Fig. 2). Intron length polymorphisms were detected on the basis of band mobility shifts, and/or heteroduplex band formation. The indels, described here as insertions or deletions in comparison to the YW sequence, were as follows: in *CHS*, a 30-bp deletion in DN1C (intron I); in *CHI*, a 16-bp de-

Table 2 RAPD and morphological markers conserved between original and new mapping populations. Non-conserved markers were either not amplified (NA), or were amplified but monomorphic (M) in the respective populations. Change in dominance states (B102C→B102CC′, B102A→B102AA′, PZ04CD→PZ04D, B194BD→B194B) or to site-specific marker (PX18AB→PX18SS) are described in the text

Linkage group	Original[a]	YW×FRA520	DN1C×YW
I	B102DE	NA	B102DE
	B191A	NA	B191A
	c	*c*	*c*
II	B102C	B102CC′	B102CC′
	B104C	NA	B104C
	B194A	NA	B194A
	r	*r*	*r*
III	B102B	NA	B102B
	B194E	NA	B194E
	PA07C	PA07C	PA07C
IV	B186D	B186D	M
	B85C	B85C	M
	B84A	NA	B84A
	B102F	NA	B102F
V	B102A	B102A	B102AA′
	PZ04CD	PZ04D	M
VI	PX18AB	M	PX18SS
VII	B194BD	B194B	B194BD
	PO20C	PO20C	PO20 C

[a] BS×WC6 F_2 population (Davis and Yu 1997)

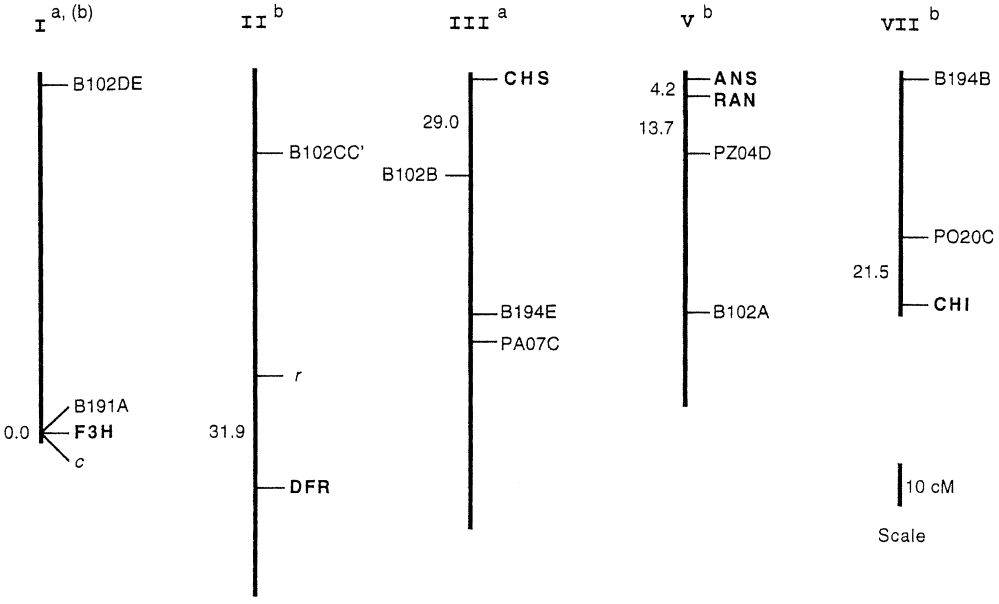

Fig. 3 Map locations of anthocyanin genes. Only linkage groups containing anthocyanin genes (*bold type*) are shown. Map distances (cM) between anthocyanin genes and adjacent markers were calculated using segregation data from the crosses (**a**) DN1C×YW (linkage groups I and III) and (**b**) YW×FRA520 (linkage groups I, II, V and VII). Marker dominant alleles from YW are shown on the right, and from the alternate parent (DN1C or FRA520) on the left, of each linkage group axis. Codominant markers (all anthocyanin genes, and RAPD markers B102DE and B102CC′) are shown on the right. The map scale and scale bar are based on the original *F. vesca* map (Davis and Yu 1997)

letion and a 32-bp insertion in FRA520 (intron II); in *F3H*, a 6-bp and an 8-bp insertion in DN1C (intron II), and a 18-bp insertion in FRA520 (intron I); in *DFR*, a 5-base deletion as well as numerous base substitutions (sufficient to produce a detectable heteroduplex band) (intron I); in *ANS*, a 12-bp and a 26-bp insertion in FRA520 (intron I); and in *RAN*, a 30-bp insertion in FRA520 (intron I).

Mapping

RAPD markers of previously determined map position (Davis and Yu 1997) were used to define the seven *F. vesca* linkage groups in the two mapping populations used here. Of 28 RAPD primers tested, a total of ten primers detected eight and 13 previously mapped markers segregating in the F_2 populations of YW×FRA520 and DN1C×YW, respectively. With the original OPX18 RAPD primer, the previously mapped RAPD marker PX18AB was not polymorphic in either population; however, a corresponding polymorphism PX18SS was detected in DN1C×YW using a site-specific primer pair. Five RAPD markers were segregating in both populations, eight RAPD markers were segregating only in DN1C×YW, and three RAPD markers were segregating only in YW×FRA520 (Table 2). Certain markers were converted from codominant (variant band mobility) to

dominant (band present/absent) markers, or vice versa, as compared with their dominance status in the previous BS×WC6 cross. For instance, the previously mapped dominant marker B102C (linkage group II) was detected as a codominant marker (B102CC′) in both new populations, while previously dominant B102A (linkage group V) was detected as a dominant marker in YW×FRA520 but as a codominant marker (B102AA′) in DN1C×YW. Previously codominant marker B194BD was also detected as codominant in DN1C×YW, but as dominant (B194B) in YW×FRA520. Finally, previously codominant marker PZ04CD was detected as dominant in YW×FRA520, but was monomorphic in DN1C×YW (Table 2).

The two morphological traits, yellow fruit versus red fruit (*c*, linkage group I), and runnering versus runnerless (*r*, linkage group II), were segregating according to expected monohybrid ratios in both mapping populations. Linkage relationships among the RAPD and morphological markers (Fig. 3) were as expected, based upon the existing *F. vesca* map.

Using intron length polymorphisms as markers, we assigned the six candidate genes to linkage groups by mapping them in relation to the RAPD and morphological markers. The *F3H* and *CHS* genes were mapped in the DN1C×YW F_2 population, and the *F3H*, *CHI*, *DFR*, *ANS* and *RAN* genes were mapped in the YW×FRA520 F_2 population (Fig. 3). The *F3H* gene was located in linkage group I, where it cosegregated with the *c* locus. The remaining five candidate genes segregated independently of the *c* locus. The *CHS* gene was linked with RAPD marker B102B, placing it into group III. The *DFR* gene was linked with the *r* gene in linkage group II. *CHI* was linked with RAPD markers PO20C and B194B in linkage group VII. The *ANS* and *RAN* genes were closely linked to each other and to RAPD marker PZ04D in linkage group V.

Discussion

In the research described here, a candidate-gene approach was used to determine the molecular identity of the *F. vesca c* (yellow fruit) locus. The segregation patterns of six anthocyanin candidate genes, detected on the basis of intron length polymorphisms, were determined in two mapping populations also segregating for red (C_) versus yellow (cc) fruit color. Fruit color cosegregated with the *F3H* polymorphism and segregated independently of the other five candidate gene polymorphisms. These results show that the *c* locus and the *F3H* gene are tightly linked (no recombination detected), indicating that they may be identical. The results also eliminate *CHS*, *CHI*, *DFR*, *ANS* and *RAN* as *c* gene candidates.

Mapping of the candidate genes as intron length polymorphisms was made possible by the use of crossing parents that were genetically highly divergent. Five intron length polymorphisms (for *F3H*, *CHI*, *DFR*, *ANS* and *RAN*) were detected in the inter-specific cross, YW×FRA520 (*F. vesca*×*F. nubicola*), while only two such polymorphisms (for *F3H* and *CHS*) were detected in the wide, intra-specific cross, DN1C×YW (*F. vesca* ssp. *bracteata*×*F. vesca* ssp. *vesca*). In a preliminary study (C. Deng, unpublished data), no candidate gene intron length polymorphisms were detected in the BS×WC6 (*F. vesca* ssp. *vesca*×*F. vesca* ssp. *americana*) population used to establish the original *F. vesca* map. BS closely resembles YW; both are day-neutral, ‘Alpine’ varieties of European origin, and both are runnerless, but BS has red fruit and YW has yellow fruit. Thus, in crosses involving ‘Alpine’ *F. vesca* ssp. *vesca* as one parent, the extent of intron length polymorphism was greatest with *F. nubicola*, intermediate with *F. vesca* ssp. *bracteata*, and least with *F. vesca* ssp. *americana*, as the alternate parent.

Our mapping approach also relied upon the conservation of RAPD markers between the BS×WC6 mapping population used to construct the original *F. vesca* map (Davis and Yu 1997) and the two mapping populations used here. Using the 28 decamer primers that had been used to detect 75 RAPD markers in construction of the previous map, we detected 13 conserved RAPDs in the DN1C×YW (intra-specific) mapping population. Only eight previously mapped RAPDs were conserved in the YW×FRA520 (inter-specific) mapping population. Five previously mapped RAPD markers were conserved in both new populations, of which three were detected as codominant markers in at least one of the three mapping populations. Five dominant RAPD markers (B104C, B194A, B102B, B84A and B102F) were conserved between WC6 and DN1C, while no dominant RAPD markers were conserved between WC6 and FRA520. The *r* (runnerless) locus was segregating in the original and both new mapping populations, each of which involved a cross between a runnering wild-type and a runnerless (mutant) ‘Alpine’ type plant.

The coding regions of the cloned candidate gene segments closely resembled corresponding heterologous genes in terms of predicted amino-acid sequences. Interestingly, the cloned *F. vesca DFR* gene segment had a higher degree of predicted polypeptide sequence identity to rose (94%; Accession No. BAA12723) and apple (91%; Accession No. AAD26204) (also members of the Rosaceae), than to *F.×ananassa* (87%; Accession No. AAC25960). Importantly, the target sites of the degenerate primer pairs developed in this study were chosen because they were particularly well conserved among comparative species. Although not “universal”, these primer pairs should be useful in a wide range of Angiosperm species.

Although strongly implicating *F3H* as the molecular identity of the *c* locus, further investigations are needed to confirm this conclusion. We are currently producing genetically transformed yellow-fruited *F. vesca* to see whether a functional *F3H* gene can complement the *c* gene mutation. Comparative sequencing of the complete *F3H* gene in various wild-type and yellow-fruited mutant plants is also in progress, as are complementation

crosses between YW and other yellow-fruited *F. vesca*. The strawberry specific sequence data and primer pairs generated for six genes of the anthocyanin biosynthetic pathway by the present investigation have also provided a foundation for future genetic analysis and manipulation of fruit color in the commercial strawberry, *F.×ananassa*.

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