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RAPD and AFLP tagging and mapping of *Beta* (*B*) and *Beta* modifier (Mo_B), two genes which influence β-carotene accumulation in fruit of tomato (*Lycopersicon esculentum* Mill.)

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Abstract The *Beta* (*B)* locus in tomato (*Lycopersicon esculentum*) increases fruit β-carotene content at the expense of lycopene, resulting in orange-pigmented fruit. Expression of *B* is influenced by the beta-modifier (Mo_B) gene which segregates independently of *B.* RAPD and AFLP analyses were performed using near isogenic lines (NILs) unique for *B* and bulked segregant analysis (BSA) of a *L. esculentum*×*L. cheesmanii*-derived F_2 population segregating for *B.* Using 1018 random primers for RAPD analysis and 64 primer pairs for AFLP analysis, we identified polymorphic products which distinguished the NILs and the two bulked DNA samples constructed for BSA. A single 100 bp AFLP amplification product (E-ACA/M-CTG_{100}) which distinguished the NILs cosegregated with Mo_B and was demonstrated to be tightly linked to the locus. E-ACA/M-CTG_{100} exhibited a recombination frequency of 1.7% in the F_2 progeny derived from an initial cross between the isolines. The Mo_B locus was mapped to the long arm of chromosome 6. Two RAPD products ($OPAR18_{1100}$ and $UBC792_{830}$) of 1100 bp and 830 bp, respectively, were polymorphic between orange- and red-fruited bulks constructed from F_2 individuals in the *L. esculentum* and *L. cheesmanii* mating series. $OPAR18_{1100}$ and $UBC792_{830}$ displayed recombination frequencies of 4.2% and 7.6%, respectively, in F_2 progeny. The *B*-linked $OPAR18_{1100}$ marker was also mapped to the long arm of chromosome 6, proximal to Mo_B, and revealed linkage between *B* and Mo_B.

Key words Molecular marker · Bulk segregant analysis · Near-isogenic lines · *Lycopersicon cheesmanii* · *Lycopersicon hirsutum*

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Introduction

Orange-fruited tomato genotypes containing high levels of β-carotene were first identified among transgressive segregants descended from a cross between a red-fruited *Lycopersicon esculentum* genotype and the green-fruited wild tomato species *L. hirsutum* (Kohler et al. 1947; Lincoln et al. 1943). Inheritance studies suggested that high concentrations of β-carotene in orange-pigmented tomatoes was controlled by a single gene, designated *B*, which exhibited incomplete dominance (Lincoln and Porter 1950). Subsequent studies by Tomes et al. (1954) determined that *B* was dominant but subject to influence by a modifier gene, Mo_B, which segregated independently of *B*. Orange-fruited accessions of *L. cheesmanii*, *L. pimpinellifolium*, *L. chilense*, and *L. chmielewskii* containing high concentrations of β-carotene have also been described (Chalukova 1988; Manuelyan et al. 1975; Rick 1956; Stommel and Haynes 1994). The inheritance of β-carotene content in these wild tomato species is consistent with that described for the dominant *B* gene from *L. hirsutum*.

The carotenoids present in ripened tomato fruit are predominantly carotenes, which account for 90–95% of the total carotenoids (Gross 1991). Lycopene is the most abundant carotene in red tomato fruit, accounting for up to 90% of the total carotenoids. Typical red-pigmented tomato fruit also contains lesser amounts of β-carotene, ζ-carotene, γ-carotene, and neurosporene and the colorless precursors phytoene and phytofluene. Numerous tomato mutants have been described in which fruit and/or foliar pigment composition is altered (Stommel 1992). Expression of the dominant *Beta* (*B*) mutant results in orange-pigmented fruit due to an increase in β-carotene accumulation at the expense of lycopene. A modifier gene, Mo_B, influences the expression of the dominant form of *B* and the relative percentages of lycopene and β-carotene present in ripened fruit. In the presence of the homozygous recessive form of the allele, Mo_BMo_B, β-carotene represents more than 90% of the total carotene content, and fruit are orange-pigmented (Tomes et al.

1954). Expression of the dominant Mo_B^+ form of the allele, however, reduces β-carotene content, to more than 50% of the total carotenoids and increases lycopene to less than 50% of the total carotenoids resulting in red-orange fruit pigmentation. In red-fruited tomatoes, β-carotene accounts for only 10–15% of the total carotenoids. β-carotene is the principal provitamin A carotenoid in ripe tomato fruit and is an essential nutrient in the human diet because of its retinoid activity (Tee 1992). β-carotene may also impart health promotive benefits due to its antioxidant properties (Byers and Perry 1992).

This report describes the identification of polymerase chain reaction (PCR)-based molecular markers linked to the *B* and Mo_B genes in tomato and the mapping of these markers to the tomato molecular linkage map. Using bulked segregant analysis (BSA) (Michelmore et al. 1991) and near isogenic lines (NILs), we identified random amplified polymorphic DNA (RAPD) (Williams et al. 1990) and amplified fragment length polymorphism (AFLP) (Vos et al. 1995) markers which cosegregated with *B* and Mo_B. RAPD analysis relies on the amplification of genomic DNA fragments with short arbitrary primers and is a simple, fast, and inexpensive technique which has received widespread use for the development of genetic markers (Fang et al. 1997; Paran and Michelmore 1993; Poulsen et al. 1995; Yen et al. 1997). AFLP analysis is based on the selective amplification of restriction digests of genomic DNA and is also a rapid, powerful, and reliable technique which can be used for DNA fingerprinting, gene tagging, high-density genome mapping, marker-aided selection and positional gene cloning (Becker et al. 1995; Cervera et al. 1996; Hill et al. 1996; Thomas et al. 1995; Vos et al. 1995). Both NILs and BSA of segregating populations have been successfully used to identify target genes in plants (Fang et al. 1997; Michelmore et al. 1991; Paran et al. 1991; Young et al. 1988; Yu et al. 1991, Poulsen et al. 1995, Yen et al. 1997).

Materials and methods

Plant materials

NILs which differed at the *B* locus were developed in the red-fruited cultivar 'Rutgers' and were kindly supplied by E.C. Tigchelaar (Purdue University, West Lafayette, Ind.). These NILs were derived from an original cross between *L. esculentum* cv 'Indiana Baltimore' and the *B* donor parent *L. hirsutum* PI 126445. True-breeding high β-carotene selections were subsequently crossed to 'Rutgers' for NIL development. An F_2 population segregating for *B* was produced from selfed progeny of a cross between 'Rutgers' and its *B* isoline, hereafter designated '*B* iso-Rutgers', and utilized to confirm linkage between *B* and identified markers in the NILs. This F_2 population also segregated for expression of Mo_B. Individual orange- and red-orange-fruited F_2 plants were selfed to generate F_3 individuals which were used in progeny tests to determine the F_2 parental genotypes at the *B* locus. Progeny tests were replicated over two growing seasons.

A second population produced to identify molecular markers linked to *B* was developed at Beltsville as a segregating F_2 generation. This F_2 was derived from an interspecific cross between the red-fruited *L. esculentum* cv 'Floradade' and an orange-fruited high β-carotene *L. cheesmanii* accession, LA 317 (Stommel and Haynes 1994). Ripe fruits of individual plants in NILs and *L. cheesmanii*-derived generations were scored visually in the field for orange or red fruit pigmentation on two occasions to confirm phenotypes. Previous studies confirmed that orange fruit pigmentation can be attributed to β-carotene in these plant materials (Tigchelaar and Tomes 1974; Stommel and Haynes 1994).

NIL and BSA analyses

Total genomic DNA from the NILs, 'Rutgers' and '*B* iso-Rutgers', were screened using a total of 1018 10-mer RAPD primers (sets A, B, D, H, J, K, L, N, O, P, R, S, T, U, V, X, AA, AG, AO, AQ, and AR from Operon Technologies, Alameda, Calif., and sets 1–8 from the University of British Columbia, Vancouver, B.C.) and 64 AFLP primer pairs derived from all possible combinations of eight *Eco*RI +3 and eight *Mse*I +3 primers obtained from GIBCO BRL Life Technologies (Gaithersburg, Md.). Linkage between RAPD or AFLP products and red or orange fruit pigmentation was confirmed via analysis of cosegregation in individual F_2 progeny.

For BSA of the interspecific *L. esculentum* cv 'Floradade'×*L. cheesmanii* LA317 F_2 generation, two bulks were constructed by combining approximately equal amounts of total genomic DNA from each of 7 red-fruited and 7 orange-fruited plants randomly selected from the F_2 plant population. The two bulked DNA samples were initially screened with all RAPD primers and AFLP primer pairs as noted above for the NILs. Polymorphisms detected between the two bulked DNA samples were subsequently evaluated for cosegregation with red or orange fruit pigmentation via analysis of F_2 individuals.

RAPD and AFLP procedures

Total genomic DNA for RAPD and AFLP analyses was extracted from freeze-dried young tomato leaves using the QIAGEN (Santa Clarita, Calif.) DNeasy Plant Mini Kit. The DNA concentration of derived samples was determined by spectrophotometric analysis and by comparison with lambda DNA standards using agarose gel electrophoresis. A modification of the conditions described by Stommel et al. (1997) was utilized for RAPD analysis. PCR amplification reactions for RAPD analysis contained 20 m*M* NaCl, 50 m*M* Tris-HCl (pH 9.0), 125 μ*M* each of dNTP, 0.2 μ*M* 10-mer primer, 0.5 U *Taq* DNA polymerase (Perkin Elmer, Norwalk, Conn.), 1.9 m*M* $MgCl_2$, 0.1% bovine serum albumin, 1% Triton X-100, and 15 ng template DNA in a reaction volume of 12.5 μl. The amplification was performed in a Perkin Elmer GeneAmp PCR System 9600 thermal cycler for 45 cycles of 15 s at 94 C, 15 s at 48 C and 75 s at 72 C followed by 7 min at 72 C. The amplified products were separated via electrophoresis on 1.5% NuSieve 3:1 agarose (FMC, Rockland, Me) gels in 0.5% TBE buffer and stained with ethidium bromide.

AFLP procedures were performed as described in the GIBCO BRL AFLP Analysis System I with minor modifications. Genomic DNA (50 ng) was double-digested with *Eco*RI/*Mse*I restriction enzymes in a total volume of 5 μl at 37 C for 2 h. The digested fragments were ligated with *Eco*RI and *Mse*I adapters at 20 C for 2 h in a 10-μl reaction volume containing 5 μl digested DNA, 4.8 μl adapter ligation solution, and 0.2 μl T4 DNA ligase. Two-step selective PCR amplification of adapter-ligated DNA was performed in a Perkin Elmer 9600 thermal cycler. The first step, a pre-selective PCR (20 cycles of 94 C for 30 s, 56 C for 60 s, and 72 C for 60 s) with *Eco*RI +1 and *Mse*I +1 primers that have one selective nucleotide each, was performed in a 10.2-μl reaction volume consisting of 1 μl tenfold diluted ligated DNA, 8 μl pre-amp primer mix, 1 μl 10× PCR buffer, and 0.2 μl (0.2 U) *Taq* DNA polymerase (Perkin Elmer Cetus). The second step, selective PCR amplification (1 cycle at 94 C for 30 s, 65 C for 30 s, and 72 C for 60 s, followed by 12 cycles of 0.7 C reduced annealing temperature each cycle and 23 cycles of each beginning at 94 C for 30 s, 56 C for 30 s, and 72 C for 60 s) with *Eco*RI +3 and *Mse*I +3 primers that

have three selective nucleotides each, was carried out in a 10-µl volume consisting of 2.5 µl of 50-fold diluted pre-amplified DNA, 0.09 µl *Eco*RI +3 primer, 2.25 µl *Mse*I +3 primer, 1 µl 10× PCR buffer, 4.06 µl sterile ddH_2O, and 0.1 µl (0.5 U) *Taq* DNA polymerase. Amplified products from selective amplification were resolved by electrophoresis on 5% denaturing polyacrylamide gels and visualized via silver staining using the DNA Silver Staining System (Promega, Madison, Wis.).

Segregation and mapping analysis

Chi-square tests were utilized to analyze segregation of fruit pigmentation and polymorphic molecular markers, and their linkage. Chromosomal localization of identified markers was performed using introgression line (IL) analysis (Eshed and Zamir 1994). The ILs consisted of 50 *L. esculentum* cv 'M82' lines each containing a single chromosomal introgression region from the green-fruited wild tomato species *L. pennellii* LA716. Genomic DNA of these ILs was kindly provided by M.A. Mutschler (Cornell University, Ithaca, N.Y.). Individual ILs were analyzed via RAPD or AFLP analysis as previously described. Since these ILs cover the entire wild species genome, polymorphisms elicited between the ILs should be associated with the unique introgressed segment (Eshed and Zamir 1994, 1995).

Results

Segregation analyses

Three phenotypic classes for fruit pigmentation were observed among progeny in the F_2 population derived from a cross between the red-fruited *L. esculentum* cv 'Rutgers' and the orange-fruited, high β-carotene NIL '*B* iso-Rutgers'. In addition to the orange- and red-fruited parental phenotypes, an intermediate class with red-orange-fruited plants was observed. A segregation ratio of 15 orange-fruited : 31 red-orange-fruited : 14 red-fruited plants was observed among F_2 progeny (Table 1). Progeny tests of the 15 orange-fruited F_2 individuals revealed 14 true-breeding orange individuals. Orange, red-orange, and red-fruited progeny were evident among F_3 progeny of red-orange fruited F_2s. F_1 plants derived from a cross of these NILs produced red-orange pigmented fruit. Orange-pigmented fruit were not observed among F_1 progeny. Although F_2 classes are consistent with the proposed (Tomes et al. 1954) 3:9:4 two-gene model (χ^2=1.54, P>0.30) incorporating the action of the dominant *B* gene and an independently segregating modifier gene, Mo_B, orange-fruited genotypes were skewed and favored true-breeding orange individuals. Hence, the observed F_2 segregation was also consistent with a 1:2:1 ratio for a model incorporating linked *B* and Mo_B genes (χ^2=0.10, P>0.95). Based on observed F_1, F_2, and F_3 fruit pigmentation and a two-gene model, the genotypes of the red-fruited 'Rutgers' and orange-fruited '*B* iso-Rutgers' NILs were $bbMo_B{}^+Mo_B{}^+$ and $BBMo_B\ Mo_B$, respectively.

Fruit pigmentation phenotypes in F_2 plants derived from an initial cross between the red-fruited *L. esculentum* cv 'Floradade' and the orange-fruited, high β-carotene *L. cheesmanii* accession LA317 were observed to segregate 104 orange-fruited : 40 red-fruited plants (Table 1). An intermediate red-orange class was not observed. This segregation pattern is consistent with an expected 3:1 ratio for a single-gene model (χ^2=0.59, P>0.30) wherein expression of the dominant *B* gene results in fruit with a high percentage of β-carotene. Fruit of F_1 plants were exclusively orange pigmented. Genotypes of the parental lines 'Floradade' and LA317 were

Table 1 Chi-square tests for segregation of tomato fruit pigment phenotypes and molecular markers and their linkage analyses in two F_2 populations derived from the cross of *L. esculentum* cv 'Floradade'×*L. cheesmanii* accession LA 317 and from the cross of *L. esculentum* cv 'Rutgers'×'*B* iso-Rutgers'

Locus	Observed frequency	Expected ratio	Recombinant		χ^2	*P*
			Number	%		
'Rutgers'×'B iso-Rutgers' F_2 population'						
Pigmentation	15:31:14[a]	3:9:4			1.54	>0.30
		1:2:1			0.10	>0.95
E-ACA/M-CTG_{100}	14:46[b]	4:12	1	1.7	0.09	>0.70
Mo_B/E-ACA/M-CTG_{100}	45:0:1:14[c]	9:3:3:1			52.36	<0.001
'Floradade'×LA317 F_2 population						
Pigmentation	104:40[d]	3:1			0.59	>0.30
$OPAR18_{1100}$	104:40[e]	3:1	6	4.2	0.59	>0.30
$UBC792_{830}$	105:39[e]	3:1	11	7.6	0.33	>0.50
B/$OPAR18_{1100}$	101:3:37[f]	9:3:3:1			134.72	<0.001
B/$UBC792_{830}$	99:5:6:34[f]	9:3:3:1			107.70	<0.001

[a] Observed fruit pigmentation of F_2 individuals (orange:red-orange:red)
[b] Observed absence:presence of the AFLP marker E-ACA/M-CTG_{100} in F_2 individuals
[c] The expected genotype order is: $Mo_B{}^+\_$ and presence of the markers, $Mo_B{}^+\_$ and absence of the marker; $Mo_B\ Mo_B$ and presence of the marker; $Mo_B\ Mo_B$ and absence of the marker
[d] Observed fruit pigmentation of F_2 individuals (orange:red)
[e] Observed presence:absence of the RAPD markers $OPAR18_{1100}$ and $UBC792_{830}$ in F_2 individuals
[f] The phenotype order is: orange-pigmented fruit and presence of the marker; orange-pigmented fruit and absence of the marker; red-pigmented fruit and presence of the marker; red-pigmented fruit and absence of the marker

Table 2 RAPD products linked to the *B* gene in individuals of a population developed from a cross of *L. esculentum* cv 'Floradade'×*L. cheesmanii* accession LA 317 and AFLP products linked to the gene Mo_B in individuals of a population derived from a cross of *L. esculentum* cv 'Rutgers'×'*B* iso-Rutgers'

Marker type	Primer(s)	Primer sequence (5′ to 3′)	Product size[a]	Pigmentation/marker[b]			Linked locus
				O	R	RO	
AFLP	E-ACA M-CTG	GACTGCGTACCAATTCACA GATGAGTCCTGAGTAACTG	100	–	+	+	Mo_B
RAPD	OPAR18	CTACCGGCA	1100	+	–	–	*B*
RAPD	UBC792	CAACCCACAC	830	+	–	–	*B*

[a] Amplified polymorphic product size in base pairs
[b] O, R, and RO denote orange, red, and red-orange fruit pigmentation, respectively; + and –denote marker presence and absence, respectively

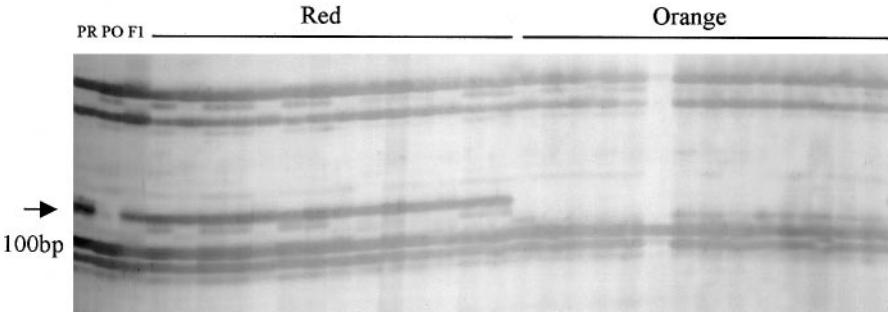

Fig. 1 AFLP marker E-ACA/M-CTG$_{100}$ linked to the Mo_B gene. The marker was identified in lines of the *L. esculentum* cv 'Rutgers' near-isogenic for the *B* gene. *PO* and *PR* Orange-fruited '*B* iso-Rutgers' and red-fruited 'Rutgers' NILs, respectively, *Orange* and *Red* a sample of individual orange- and red-fruited F_2 plants, respectively, generated from an initial cross of the NILs

deduced to be $bbMo_BMo_B$ and $BBMo_BMo_B$, respectively.

NIL marker analysis

'Rutgers' and '*B* iso-Rutgers' NILs were evaluated for polymorphic RAPD and AFLP products linked to the distinguishing *B* locus in these lines. Two RAPD products and three AFLP products were polymorphic between the NILs. These markers were further evaluated for linkage to fruit color genes using a subset of orange- and red-fruited individuals of an F_2 population generated from a cross between the NILs. The 64 AFLP primer pairs generally amplified 40–80 scorable bands ranging in size from 50 to 700 bp in the respective NILs. One primer pair, E-ACA/M-CTG, produced a 100-bp fragment (Table 2) in 'Rutgers' which was absent in '*B* iso-Rutgers'. Preliminary analysis of DNA samples from 14 true-breeding orange-fruited F_2 plants (homozygous $BBMo_BMo_B$ genotypes) and 14 red-fruited F_2 plants with the E-ACA/M-CTG primer pair demonstrated the absence of amplified product in the orange-fruited individuals and presence of the 100-bp fragment in the red-fruited individuals (Fig. 1), suggesting that this marker was linked in repulsion to the *B* locus. Further analysis of 31 red-orange-pigmented individuals revealed the presence of the band in all 31 of these individuals. This was inconsistent with the proposed linkage to *B* since approximately 33% of red-orange-fruited individuals may be represented by homozygous *BB* genotypes and would be expected to lack the marker. Likewise, the marker was not detected in an orange-fruited heterozygous *Bb* genotype which would be expected to exhibit presence of the marker. The recombination frequency for the marker was 1.7%. Only 1 recombinant plant, an orange-fruited individual positive for the marker, was recovered.

The observed 14:46 segregation for absence:presence of the E-ACA/M-CTG 100-bp marker is consistent with linkage in coupling to the *B* modifier gene, Mo_B and was in agreement with the expected 4:12 ratio (χ^2=0.09, P>0.70) for the marker in an interactive two-gene model (Table 1). Failure to accept a two gene model for the marker and Mo_B was indicative of linkage between the marker and Mo_B. Red-fruited $bbMo_BMo_B$ genotypes, which would be expected to lack the 100-bp marker, were not recovered, likely due to the relatively small F_2 population size and the low frequency with which they would be expected to occur. Alternatively, their absence may be explained by the occurrence of recombinants for the marker among red-fruited phenotypes or linkage between *B* and Mo_B. The F_1 individuals derived from a cross of the NILs exhibited red-orange-fruit phenotypes and scored positive for presence of the 100-bp product.

Most RAPD primers amplified 3–12 fragments ranging in size from 200 bp to 4000 bp. Only 2 of 1018 random primers produced polymorphisms between the pair of NILs. Random segregation was observed between polymorphic RAPD products and fruit pigmentation in F_2 individuals, indicating a lack of linkage between these markers and *B* or Mo_B.

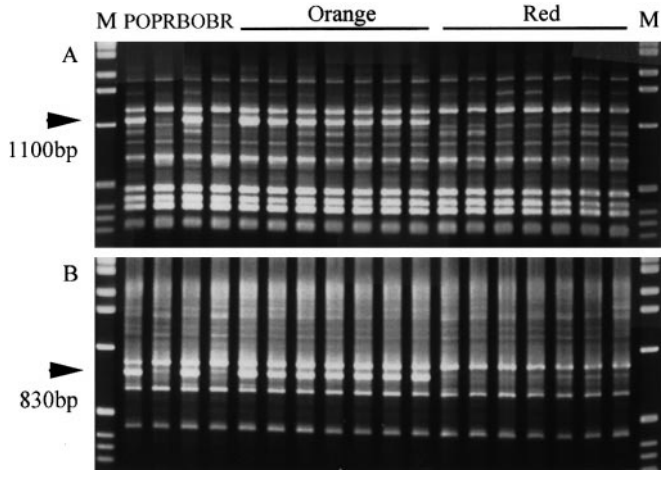

Fig. 2A, B RAPD markers $OPAR18_{1100}$ (**A**) and $UBC792_{830}$ (**B**) linked to the *B* locus. Markers were identified using bulked segregant analysis of an interspecific F_2 population derived from the cross *L. esculentum* cv 'Floradade'×*L. cheesmanii* accession LA 317. *PO* and *PR* Orange- and red-fruited parent lines, respectively, *BO* and *BR* orange- and red-fruited bulks, respectively. *Orange* and *Red* individual F_2 plants from orange- and red-fruited bulks, respectively. *M* 1-kb ladder

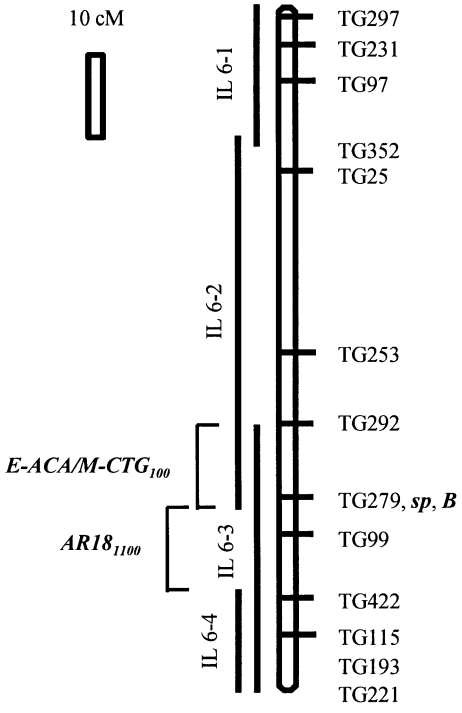

Fig. 3 Linkage map of tomato chromosome 6 (from Eshed and Zamir 1994) showing chromosomal location of mapped markers of $OPAR18_{1100}$ and E-ACA/M-CTG_{100} using introgression line (*IL*) analysis. Bars to the left of the *chromosome* denote the intrgressed region in the IL lines. *Brackets* to the *left* of *IL bars* denote the location of the mapped markers

Bulk segregant marker analysis

RAPD and AFLP analysis of two bulked samples comprised of DNA from 7 orange-fruited and 7 red-fruited plants, respectively, selected at random from the 'Floradade'× LA317 derived F_2 population was performed to identify molecular markers putatively linked to *B*. Four RAPD primers and 3 AFLP primer pairs produced polymorphisms between the two bulks. These markers were subsequently evaluated using individuals of the F_2 population to confirm potential cosegregation with *B*. When random primers and primer pairs which elicited polymorphisms between bulked samples were used, RAPD and AFLP analysis of the 7 individuals which comprised each bulk demonstrated that 2 RAPD primers, OPAR18 and UBC792, produced PCR products of 1100 bp ($OPAR18_{1100}$) and 830 bp ($UBC792_{830}$), respectively (Table 2; Fig. 2), which were present in all of the orange-fruited but not the red-fruited samples. RAPD analysis of all 144 plants in the F_2 population using primers OPAR18 and UBC792 confirmed that these markers cosegregated with orange-pigmented, high β-carotene fruit phenotypes and that these markers were linked in coupling to the *B* locus. Observed segregation ratios of 104:40 and 105:39 for the presence and absence of $OPAR18_{1100}$ and $UBC792_{830}$ (χ^2=0.59, P>0.30 and χ^2=0.33, P>0.50, respectively) approximated the expected 3:1 segregation ratio consistent with that observed for fruit pigmentation phenotypes (Table 1). Six individuals recombinant for $OPAR18_{1100}$ and 11 individuals recombinant for $UBC792_{830}$ were identified, producing recombination frequencies of 4.2% and 7.6%, respectively. Failure to accept two-gene models for the marker and *B* confirmed linkage between the respective markers and *B*. F_1 individuals exhibited orange-pigmented fruit phenotypes and, as expected, scored positive for presence of the markers.

Mapping analysis

To localize these *B*-linked RAPD markers and Mo_B-linked AFLP markers on the tomato molecular linkage map, we used the RAPD primers OPAR18 and UBC792 and the AFLP primer E-ACA/M-CTG, to amplify DNA samples from a set of 50 ILs (Eshed and Zamir 1994). The AFLP marker E-ACA/M-CTG_{100} and the RAPD marker $OPAR18_{1100}$ each amplified a product polymorphic between the IL parents *L. esculentum* cv 'M82' and *L. pennellii* LA716 which was equivalent in size to the polymorphic product produced between parental lines of the respective NIL and 'Floradade'×LA317-derived populations. The marker E-ACA/M-CTG_{100} was mapped within the overlapping region of ILs 6–2 and 6–3 on tomato chromosome 6 between the markers TG292 and TG279, a chromosomal region spanning approximately 7.5 cM (Fig. 3). The marker $OPAR18_{1100}$ was placed within the IL 6–3 introgression located between the markers TG279 and TG422, a region spanning approximately 8 cM (Fig. 3). The RAPD marker $UBC792_{830}$ was not polymorphic between IL parent lines or between individual ILs and so was not positioned on the map.

Discussion

Our fruit pigmentation observations in the NIL and interspecific populations examined are consistent with a two-gene model for fruit β-carotene content wherein the dominant *B* gene conditions increased fruit β-carotene and is influenced by a modifier gene, denoted Mo_B. Our molecular marker mapping data and observed F_1, F_2, and F_2 progeny test data indicate that *B* and Mo_B are linked on chromosome 6 and do not segregate as independent genes as originally proposed (Tomes et al. 1954). The postulated 4:9:3 ratio for two independently segregating genes described by Tomes et al. (1954) is close enough to the 1:2:1 ratio consistent with two linked genes that statistical differentiation of the two models is extremely difficult. Lincoln and Porter (1950) first proposed a single-gene model to explain inheritance of beta-carotene content and postulated the action of incomplete dominance to account for observed 1:2:1 orange:red-orange:red F_2 segregation ratios. Our results and those described by Tomes et al. (1954) clearly discount incomplete dominance to explain inheritance of tomato fruit β-carotene content. Assuming the *B* and Mo_B loci are not tightly linked, individuals with dominant or recessive forms of the modifier gene, as observed in our red-fruited materials, can result via recombination. Tomes et al. (1954) also noted red- and orange-fruited plants with dominant and recessive forms of the modifier.

NILs unique for the *B* gene were screened with more than 1000 RAPD primers which produced more than 5000 amplified fragments, only 2 of which were polymorphic between the NILs. Approximately 3000 amplified fragments were generated in these NILs using 64 AFLP primer pairs, only 3 of which were polymorphic between NILs. Since the NILs were homozygous for the loci of interest, markers in both the *cis* and *trans* orientation with *B* or Mo_B could be identified. A single 100-bp AFLP product was identified which was tightly linked to the *B* modifier gene, Mo_B. A high level of DNA sequence diversity exists between accessions of *L. esculentum* and *L. hirsutum* (Miller and Tanksley 1990), the donor parent for the *B* allele in these NILs. The low level of polymorphism detected between NILs is thus indicative of a relatively small introgression from the *L. hirsutum* donor parent. The introgressed region flanking the *B* locus in these NILs was sufficiently small to preclude detection of RAPD or AFLP markers linked to *B*. In principal, genetic markers which are polymorphic between a pair of NILs are linked with genes influencing the character distinguishing the NIL and its recurrent parent (Muehlbauer et al. 1988). Identification of a linked AFLP marker and failure to detect RAPD-linked products, reflect the fact that AFLP analysis detects large numbers of genetic loci in a single PCR reaction relative to the much smaller number detected in a RAPD analysis, thus increasing the probability of identifying polymorphic loci.

Utilizing BSA in an interspecific F_2 population generated from a *L. esculentum*×*L. cheesmanii* cross, we identified 2 RAPD markers, $OPAR18_{1100}$ and $UBC792_{830}$, which were closely linked to the *B* locus. Despite the greater efficiency with which AFLP analysis may detect polymorphic loci, AFLP products linked to *B* were not detected with the primer pairs evaluated. BSA provided a rapid and simple means of identifying molecular markers linked to specific genes. Unlike NIL analysis wherein NIL development requires a series of time-consuming and labor-intensive backcrosses, the generation of a segregating population is sufficient to proceed with BSA (Michelmore et al. 1991). Similar to NIL analysis, only two DNA samples are utilized for the initial marker screening. This facilitates screening of a relatively large number of primers in a short time period in comparison to the slow pace at which primers can be screened for amplification of polymorphic products in a large F_2 population. When utilizing BSA in segregating populations with minimal gene distortion, the likelihood of identifying falsely linked markers to the target gene is minimized; therefore, few individuals per bulk are required (Michelmore et al. 1991). Within each bulk, the individuals are ideally identical for the gene of interest but arbitrary for all other genes, and markers that detect polymorphism between respective bulks are likely linked to the target gene. The small number of individuals which comprised each of our bulked DNA samples likely contributed to the identification of several polymorphic markers which were not linked to *B*.

The two bulks (orange- and red-fruited individuals, respectively) used in our BSA were constructed based upon fruit phenotypes. Since F_2 individuals comprising the orange-fruited bulk expressed the dominant *B* gene, both homozygous (*BB*) and heterozygous (*Bb*) genotypes are expected in the bulked sample. Genotypes of individuals comprising the red-fruited bulk were uniformly homozygous recessive (*bb*). Because progeny tests were not utilized to distinguish these F_2 *BB* genotypes from *Bb* genotypes in this mating series and because RAPDs are dominant markers, the detection of linked markers was limited to those in *cis* orientation to *B* (Kesseli et al. 1993). Despite this reduced probability of identifying linked markers in our analysis, 2 RAPD products linked to *B* were identified.

The 2 RAPD markers, $OPAR18_{1100}$ and $UBC792_{830}$, exhibited close linkage with *B* in the *L. cheesmanii*-derived mating series. Neither of these markers detected polymorphism between the pair of *L. esculentum* cv 'Rutgers' NILs wherein *L. hirsutum* was the donor species for *B*. Similarly, the AFLP marker E-ACA/M-CTG_{100}, exhibited close linkage with Mo_B in the NILs but was not polymorphic in the *L. cheesmanii*-derived population. For RAPD markers linked to *B*, failure to exhibit polymorphism in the NILs was likely attributed to introgressed regions flanking *B* which are unique to the *L. cheesmanii* donor species. A similar explanation is plausible for the AFLP marker. In any event, since E-

ACA/M-CTG_{100} is linked in coupling to Mo_B, presence of the marker was not anticipated in the *L. cheesmanii* mating series since both parental lines were homozygous recessive at the Mo_B locus.

Molecular markers tightly linked to traits of economic importance are valuable for use in marker-assisted selection breeding programs (Tanksley 1983). The *B* and Mo_B-linked markers identified here are useful in this regard. Development of codominant sequence-characterized amplified regions (SCARs) from these linked markers will enhance their utility in a breeding program by enabling detection at a very early stage of both dominant and recessive forms of the respective loci. In addition to fine mapping of the *B* locus, the linked AFLP marker identified here will facilitate determination of the chromosomal location of the previously unmapped Mo_B locus on the tomato linkage map. In addition to providing relative gene location, tightly linked markers are useful as starting points for gene isolation and cloning (Tanksley et al. 1989). Genes and cDNAs which code for nearly all of the enzymes required for carotenoid biosynthesis in plants have been characterized (Cunningham and Gantt 1998). In tomato, additional knowledge of *B* and Mo_B gene structure and function will facilitate characterization of the molecular mechanisms of carotenoid biosynthesis and regulation of the biosynthetic pathway, in particular, regulation of the conversion of lycopene to β-carotene in ripening fruit.

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