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Molecular mapping of capsaicinoid biosynthesis genes and quantitative trait loci analysis for capsaicinoid content in *Capsicum*

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Abstract Quantitative variation in the accumulation of two major capsaicinoids responsible for pungency in the fruit of chile peppers, capsaicin and dihydrocapsaicin, was analyzed in a cross between the non-pungent *Capsicum annuum* parent cv. Maor and a pungent *Capsicum frutescens* parent, accession BG 2816. In order to identify quantitative trait loci (QTLs) for capsaicinoid content, we employed the bulked segregant analysis method and screened bulked DNA from F₂ individuals at the extremes of the distribution of capsaicinoid content with RAPD primers. Screening with 400 primers allowed the identification of three loci that were polymorphic between the bulks. These RAPD markers were converted to SCARs and subsequently mapped with additional RFLP markers to chromosome 7 of pepper. QTL interval analysis for individual and total capsaicinoid content identified a major QTL, termed *cap*, which explained 34–38% of the phenotypic variation for this trait in two growing environments. For all measurements, the allele of the pungent parent BG 2816 at *cap* contributed to the increased level of pungency. To determine whether known structural genes in the pathway could define a candidate for this QTL, 12 clones obtained from differentially expressed transcripts from placental tissue in pungent peppers were also mapped. None of them had a

significant effect on this trait, nor did the allelic state at the locus *C*, the on/off switch for pungency in pepper, located on chromosome 2. The identity of *cap* and its effect on capsaicin content in other backgrounds will be addressed in future studies.

Introduction

Capsaicinoids are strongly pungent alkaloids that accumulate in the placenta of maturing *Capsicum* (chile pepper pods). These compounds are used widely in food products, as spice, and in diverse pharmacological applications (<http://www.ars-grin.gov/duke/ethnobot.html>). Pepper genotypes exhibit a wide range of capsaicinoid accumulation (<http://www.chilepepperinstitute.org>) as a consequence of both environmental and genetic variability (Harvell and Bosland 1997; Zewdie and Bosland 2000a, 2000b). Generally, the genetics of this trait remain poorly understood with the important exception of the *C* locus, now mapped on chromosome 2 (Deshpande 1935; Greenleaf 1952; Blum et al. 2002). The dominant allele, *C*, at this locus is essential for capsaicin production. The homozygous recessive condition, *cc*, results in a complete lack of capacity to synthesize capsaicinoids. The inheritance of the quantitative variation of capsaicinoid content was studied by means of biometrical analyses in several crosses (Gill et al. 1973; Zewdie and Bosland 2000a, 2001); however, no information is available on the location and action of specific genes that control the degree of capsaicinoid accumulation in *Capsicum*.

A proposed biosynthetic pathway for capsaicinoid synthesis was first outlined by Bennett and Kirby (1968) and Leete and Loudon (1968), and is depicted in Fig. 1, together with the names of the enzymes corresponding to the structural genes included in this study (Curry et al. 1999; Aluru et al. 2003). Only recently have genes coding for enzymes in the biosynthesis pathway been isolated (Curry et al. 1999; Kim et al. 2001; Aluru et al. 2003). A study of placental transcript levels for the suite of

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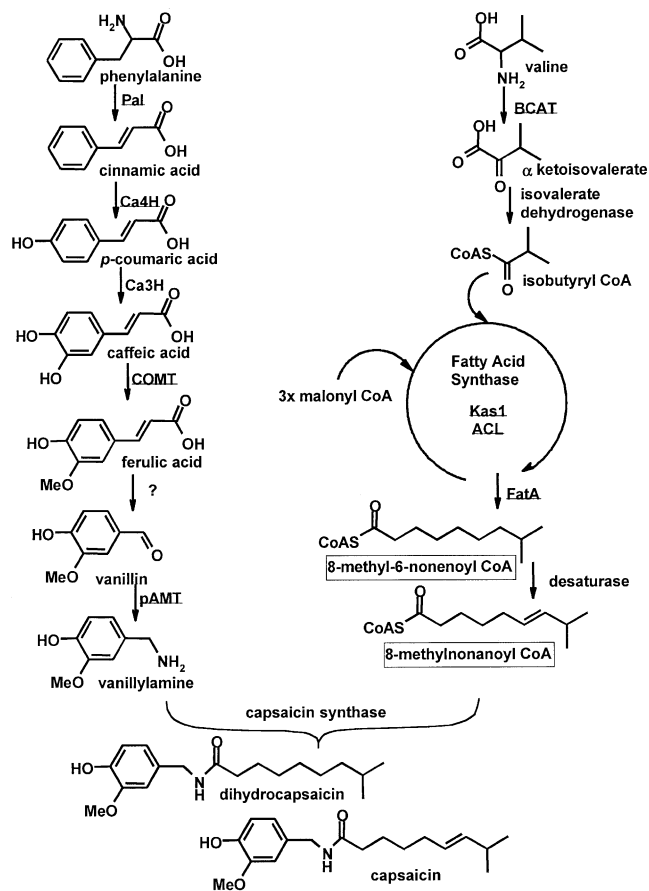


Fig. 1 Proposed pathway for capsaicinoid biosynthesis showing structural genes used in this study. The capsaicinoid biosynthetic pathway combines products from phenylpropanoid metabolism (*left*) with fatty acid synthesis (*right*). Cloned structural enzymes used in this study are indicated as *underlined* and shown adjacent to the reaction they catalyze

structural genes thought to be involved in capsaicin biosynthesis demonstrated a general correlation between transcript level and the degree of pungency, consistent with coordinate regulation (Curry et al. 1999).

Among pungent segregants recovered from the interspecific *Capsicum annuum* × *Capsicum frutescens* F₂ population used to map *C* (Blum et al. 2002), large differences in capsaicinoid content were observed; therefore, we could further screen for major determinants of capsaicinoid accumulation using individuals bulked from the phenotypic extremes of the distribution (Wang and Paterson 1994; Mackay and Caligari 2000). Additionally, we were able to assess the effect of the *C* allele on capsaicinoid accumulation in the heterozygous state. The objectives of the present study were first to identify and map quantitative trait loci (QTLs) for capsaicinoid content; second, to map capsaicinoid biosynthetic genes and other genes with pungency specific transcript accumulation; and finally, to test for possible association between capsaicinoid content QTLs to either the *C* locus or genes related to the capsaicinoid biosynthetic pathway.

Materials and methods

Plant material for capsaicinoid and QTL analysis

The QTL mapping population used in this study was constructed from an F₂ population of 242 plants (177 plants bore pungent fruit) derived from a cross between *C. annuum* cv. Maor, a non-pungent bell inbred variety and *C. frutescens* BG 2816, a line derived from a pungent wild pepper accession (Blum et al. 2002). This population (hereafter termed “AF”) and parents were grown in the open field during summer 2001, in Qiriat Gat, Israel. The AF F₂ plants were classified as pungent and non-pungent as described previously (Blum et al. 2002). For quantification of capsaicin content, ten fruits from each pungent plant were harvested at the color-break stage in August, pedicels were removed, fruit was bulked and dried in an oven at 50°C for 5–7 days. After harvesting, the plants of BG 2816, the F₁ and pungent F₂ plants were trimmed, transferred to 5-l pots, and moved to a net house in the Volcani Center, Bet Dagan. The plants set fruits again during fall 2001, and fruits were harvested in December 2001, for a second pungency determination.

Capsaicinoid content measurement

Dried fruits were ground in a coffee grinder and processed according to a standard protocol provided by Terry Berke (AVRDC, Taiwan). For each determination, 1 g of powder was extracted with 25 ml of acetone shaken at room temperature for 4 h. The extract was filtered through a 0.45- μ m filter (Millex-HN, Millipore) and stored at 4°C until processed. A 10- μ l aliquot was sampled for HPLC and injected via an autosampler for analysis in a Hitachi-Merck 6200. The mobile phase for HPLC was CH₃OH: H₂O=65:35 at a flow rate of 1 ml/min. The solid phase consisted of a LiChrospher 100 RP-18 (5 μ m) column (Merck) and a pre-column guard cartridge.

Detection was at 229 nm and retention times were 11 min for capsaicin and 13 min for dihydrocapsaicin. Capsaicin and dihydrocapsaicin external standards (Sigma, M2028 and M1022, respectively) were prepared as 50 ppm stocks in absolute ethanol. Peak areas of capsaicin and dihydrocapsaicin were converted to ppm as described by Collins et al. (1995).

QTL analysis

DNA was extracted from leaf tissue of each AF plant according to Doyle and Doyle (1990). Tomato cDNA and genomic clones from chromosome 7 were chosen for use as RFLP markers based on the maps of Livingstone et al. (1999), Ben Chaim et al. (2001), and the tomato-*Arabidopsis* synteny map in the Solanaceae Genomics Network Web site (<http://www.sgn.cornell.edu>). The bulked segregant analysis (BSA) technique (Michelmore et al. 1991) was used to screen RAPD primers (The University of British Columbia, kits UBC1–300, 400–500) on bulks comprising pungent plants from the low- and high extremes of the population for capsaicinoid content based on the HPLC data. Each bulk contained DNA from 15 plants representing the lowest and highest 8% of the pungency distribution. Polymorphic RAPD markers were subsequently converted to SCAR markers (Paran and Michelmore 1993) by excising the bands from the gel, cloning into the pDrive cloning vector (QIAGEN), sequencing both ends of the cloned fragment, and designing PCR primers. Mapping these RFLP and SCAR markers was done with MAPMAKER/EXP (Lincoln et al. 1993) as described by Ben Chaim et al. (2001).

For QTL mapping, interval and single-point QTL analyses were performed with QGENE v. 3.04 software (Nelson 1997) using LOD 3.0 and $P \leq 0.001$ as minimum significance levels for QTL detection. The percentages of phenotypic variation explained by the QTL, the degree of dominance and Pearson correlation coefficients were obtained from QGENE. Association between capsaicinoid biosynthesis genes and capsaicinoid content was assessed by selecting a total of 120 plants from the F₂ population

that included the 60 plants with the lowest content and the 60 plants with the highest content. The two parents were screened for RFLP with each of the 12 biosynthetic genes and, subsequently, the clones were hybridized with DNA from the 120 plants representing the phenotypic extremes. Because a complete genome map for the Maor × BG 2816 cross was not available, the significance of the marker/trait association was tested by single-point QTL analysis ($P \leq 0.001$) using the JMP v. 3 software (SAS Institute 1994).

Genetic mapping of capsaicinoid-related clones

The *Capsicum* cDNA clones for candidate capsaicinoid biosynthetic genes used for the mapping were: *BCAT*, branched-chain amino-acid aminotransferase (Entrez accession AY034379); *Fata*, acyl-ACP thioesterase (AF318288); *Acl1*, acyl carrier protein (AF127796); *pAMT*, putative aminotransferase (AF085149); *Kas1*, 3-oxoacyl-[acyl-carrier-protein] synthase (AF085148); *Comt*, caffeic acid-3-O-methyltransferase (AF081214); *Ca4H*, cinnamic acid 4-hydroxylase (AF088847); *Pal*, phenylalanine ammonia lyase (AF081215); *4A1*, acylCoA transferase (AY161301); and 6B11, a differentially expressed unknown open reading frame (CA782116). Two potential regulatory genes were also mapped: the β -ZIP transcription factor (AF127797) and b-ZIP2 (CA782115). Genetic map positions for the cDNA clones were determined according to the procedures of Livingstone et al. (1999) using an existing *Capsicum* map with 1,007 mapped RFLP, AFLP, RAPD, and isozyme loci. The population used in this map construction (hereafter referred to as "AC") consisted of 75 F₂ individuals from, *C. annuum* cv. "NuMex RNaky" (Nakayama and Matta 1985) × *Capsicum chinense* USDA PI 159234. Survey filters used to assess parental polymorphism and mapping filter sets for each enzyme were as described previously (Livingstone et al. 1999).

Probes were generated from plasmid clones by PCR. PCR products were purified by ethanol precipitation, radioactively labeled using Prime-It RmT kits (Stratagene) and hybridized overnight at 65°C in hybridization buffer (7% SDS, 1% BSA, 1 mM EDTA, 0.25 M NaPO₄, pH 7.2). Filters were washed twice at low stringency (2× SSC, 0.1%SDS) and twice at moderate stringency (1× SSC, 0.1%SDS) for 10 min each wash, and exposed on either a PhosphorImager storage screen (Molecular Dynamics) or Kodak XB-1 film with a BioMax MS intensifying screen (NEN).

Segregating bands were scored independently and entered into the MAPMAKER/EXP v 3.0b program (Lincoln et al. 1993). Clones were positioned within the existing AC framework map established for this population using the "assign" command (parameters: LOD > 3, within 20 cM) and the "try" command.

When a probe yielded a band from each parent that co-localized when mapped as a dominant locus, they were re-scored as codominant markers and re-mapped provided there were no other possible band pairs at that locus. "Build" was used to search for the single best order within the framework. Pair-wise distances to the most closely linked marker were also found using the Kosambi function (Kosambi 1944).

Results

Capsaicinoid content distribution

The capsaicinoid content of the fruits of the pungent parent BG 2816, F₁ and pungent AF F₂ plants were measured for the two principal capsaicinoids, capsaicin and dihydrocapsaicin, in two growing environments (Table 1). The total capsaicinoid content of BG 2816 was slightly lower than that of the F₁. In general, capsaicin content was reduced in the cooler season as expected (Cotter 1980). Capsaicin was the main capsaicinoid in the HPLC profile, followed by dihydrocapsaicin; a negligible peak of nordihydrocapsaicin was observed that was not included in estimations of the capsaicinoid content. Although the mean capsaicinoid content for the 177 pungent F₂ plants included in the analysis was lower than that of BG 2816, the F₂ distribution ranged from 11 to 1,144 ppm and 0 to 1,185 ppm for total capsaicinoids in the summer and winter seasons, respectively, indicating transgressive segregation of this trait.

High positive correlations were observed between the content of the two major capsaicinoids, capsaicin and dihydrocapsaicin, in the F₂ population ($r=0.79$ and $r=0.80$

Table 1 Means and standard errors (SE) of capsaicinoids content (ppm) in the pungent parent BG 2816 (P₁), F₁ and AF F₂ generations

Trait	Generation	Season	Mean	SE
Capsaicin	P ₁	Summer	385.7	18.1
		Winter	315.9	9.0
	F ₁	Summer	348.1	3.9
		Winter	323.0	15.7
	F ₂	Summer	203.1	12.5
		Winter	168.4	13.5
Dihydrocapsaicin	P ₁	Summer	139.4	12.0
		Winter	109.7	6.2
	F ₁	Summer	264.8	13.8
		Winter	170.9	5.5
	F ₂	Summer	131.8	8.8
		Winter	73.1	7.1
Total	P ₁	Summer	525.1	18.0
		Winter	425.6	11.2
	F ₁	Summer	612.9	9.9
		Winter	494.0	13.5
	F ₂	Summer	335.8	20.3
		Winter	243.0	20.0

Table 2 Correlations of capsaicinoids content in the AF F₂ generation in summer and winter seasons

Trait	Capsaicin ^s	Dihydrocapsaicin ^s	Total ^s	Capsaicin ^w	Dihydrocapsaicin ^w
Dihydrocapsaicin ^s	0.79	–	–	–	–
Total ^s	0.96	0.93	–	–	–
Capsaicin ^w	0.84	0.76	0.85	–	–
Dihydrocapsaicin ^w	0.65	0.8	0.7	0.8	–
Total ^w	0.8	0.81	0.85	0.9	0.92

^s Indicates summer measurements

^w Indicates winter measurements

Table 3 Genetic linkage mapping results for capsaicinoid biosynthesis related clones in the AC population

Clone	Copy number	Chromosome	LOD	Form ^a	Individuals scored	Nearest framework marker ^b	cM to nearest marker
b-ZIP2	1	1	16.0	Codominant	67	A211 (CT77)	0.0
BCAT	1	4	23.4	Codominant	64	A221	0.8
Ac11	1	1	19.9	Codominant	66	<i>Pgm2</i>	1.3
FatA	1	6	20.3	Codominant	65	TG220a	3.2
bZIP	1	1	17.1	Codominant	61	TG281	4.6
Ca4H	2	6	6.3	<i>chinese</i>	70	TG578	7.3
		6	8.5	<i>annuum</i>	69	TG220a	8.9
		6	14.7	<i>annuum</i>	72	TG220a	4.3
		6	8.9	<i>chinese</i>	72	TG220a	5.1
COMT	2	3	18.2	Codominant	69	TG74 (A430)	4.4
		3	3.9	<i>chinese</i>	63	A430	8.5
pAMT	3	7	23.5	Codominant	67	CD54	0.0
		3	12.7	<i>annuum</i>	67	A105	4.1
		3	17.9	Codominant	64	CD8	3.3
Kas1	>8	12	15.8	<i>annuum</i>	69	PG256	2.4
		1	13.6	<i>annuum</i>	66	CT252	0.2
		4	8.2	<i>annuum</i>	70	TG507	9.7
		12	10.3	<i>annuum</i>	70	A284	20
		2	8.3	<i>chinese</i>	64	A54	2.1
		6	17.4	<i>chinese</i>	65	TG16d	2.6
		3	3.4	<i>annuum</i>	70	CT220	10.7
		1	10.2	<i>chinese</i>	69	TG197	3.8
6B11	2–3	1	27.7	Codominant	64	TG281	0.0
		5	6.7	<i>chinese</i>	70	TG23	8.9
		5	6.2	<i>annuum</i>	57	TG419	10
		5	7.4	<i>chinese</i>	59	TG23	11
Pal	1	9	24.3	Codominant	74	TG18	2.8
4A1	2	7	3.9	Codominant	65	CD74(end)	29.7
		11	23.9	Codominant	65	A70	1.7

^a For dominant markers, the origin of the scored allele is indicated

^b Next nearest flanking marker in parenthesis if order could be established

in the summer and winter, respectively) (Table 2). Similar high positive correlations were observed between the content of the individual capsaicinoids in the summer and winter seasons ($r=0.84$ and $r=0.80$ for capsaicin and dihydrocapsaicin, respectively). Therefore, although the transition to cooler temperatures from the first to second fruit setting resulted in decreased levels of capsaicinoids, genotype \times environment interaction was not observed for this trait in the AF population.

Genetic mapping of cDNA clones of structural genes for capsaicin biosynthesis.

Survey blots bearing *Capsicum* genomic DNA digested with at least nine different restriction enzymes were used to estimate the copy number of the 12 cDNA clones used in this study (Table 3), based on the minimum number of pronounced bands present from any restriction digest. Many clones were present as single-copy genes, but *Kas1* produced many bands, of which only eight were resolved for RFLP mapping. Previously, we published the positions for *Ac11*, *FatA* and *Kas1* (Aluru et al. 2003). In that study, we identified the position of the exact cloned copy

of *Kas1* on the top of chromosome 1 using the 3' untranslated region. For all clones described in this paper, positions were determined within the existing AC map framework and are as presented in Table 3. All pronounced bands segregated on mapping filters.

Use of candidate genes to detect QTLs for capsaicinoid content

In order to test whether any of the 12 cloned structural genes mapped above colocalized with QTLs controlling variation in capsaicinoid content detected in the AF F₂ population, we screened the clones for polymorphism between the parents by RFLP analysis and subsequently scored them in the AF F₂ population. Single-point QTL analysis between each marker and the individual and total capsaicinoid determinations indicated that, except for *FatA* which was monomorphic and could not be scored in this population, none of the genes were significantly associated with capsaicinoid content (data not shown).

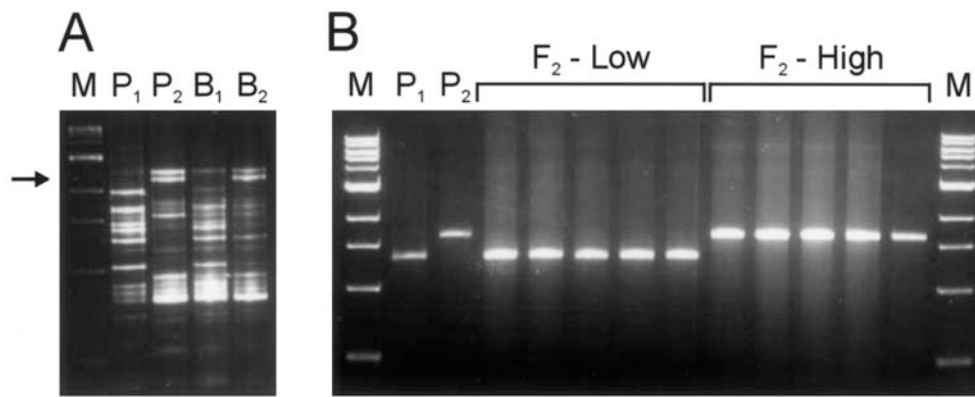


Fig. 2A, B SCAR and RAPD markers linked to *cap* QTLs for elevated levels of capsaicinoids. **A** Bulked segregant analysis with RAPD UBC20. The polymorphic-amplified fragment among the parents and bulks is marked by an *arrow*. P₁=Maor, P₂=BG 2816,

B₁=low-pungency bulk, B₂=high-pungency bulk. **B** SCAR segregation of UBC20 in the FA F₂ population. P₁=Maor, P₂=BG 2816. M=1-kb ladder

Allelic effect of the *C* locus on capsaicinoid content

In order to test the effect of *C* on capsaicinoid content, the mean of pungent F₂ plants homozygous at TG205, an RFLP marker that cosegregates with *C* (Blum et al. 2002), was contrasted with the mean of pungent F₂ plants heterozygous for this marker. There was no significant difference between the *CC* and *Cc* classes (data not shown), indicating complete dominance of *C*. These data suggest that the effect of the *C* allele on capsaicinoid content is qualitative, and that no dose-dependence is observed as a function of the allelic state at this locus.

Use of BSA to detect RAPD markers linked to QTLs for capsaicinoid content

To identify markers linked to capsaicinoid content QTLs, the two bulks representing the extremes of the capsaicin distribution were screened with 400 RAPD primers. Each amplified 5–10 discrete bands of which 1–2 were polymorphic between the parents. Three primers, UBC20 (5'-TCCGGGTTTG-3'), UBC106 (5'-CGTCT-GCCCG-3') and UBC145 (5'-TGTCGGTTGC-3') showed repeated differential PCR amplification between the bulks. UBC20 and UBC145 amplified BG 2816-specific bands of 2,200 bp and 1,600 bp, respectively, while UBC106 amplified a Maor-specific band of 1,500 bp. The genomic amplification of the two parents and bulks by UBC20 is shown in Fig. 2A.

SCAR development

To facilitate reliable mapping of these markers, we converted them to SCARs based on the method of Paran and Michelmore (1993). For the SCARs derived from UBC20 and UBC145, primers that anneal to internal positions of the RAPD fragment were used: UBC20-F: 5'-

TTGTGGGCAAATTCCTCTCAAG-3', UBC20-R: 5'-TCGTGTATGATACAGGCAGTG-3', UBC145-F: 5'-AG-GGAAGCTGAAGAGGAGAT-3' and UBC145-R: 5'-CCTTGATCCCCTCTTTACCAA-3'. For UBC106, the SCAR primers included sequences that extended the sequence of the RAPD primer: UBC106-F: 5'-CGTCT-GCCCGTGAATAGATC-3' and UBC106-R: 5'-CGTCTGCCCCGACTGTTAAGCTG-3'. The SCAR primers for UBC20 amplified a common fragment of 1,700 bp in both parents. A polymorphic restriction site within the amplified region was identified by digestion with *Hind*III. In Maor, two fragments of 1,500 bp and 200 bp were observed after digestion compared to a single undigested fragment in BG 2816 (Fig. 2B). For the SCAR based on UBC145, a monomorphic 600-bp product was obtained for the two parents. After digesting this product by *Cfo*I, a fragment of 450 bp appeared in BG 2816 and was absent in Maor (data not shown). For UBC106, the SCAR primers amplified a Maor-specific band of 1,500 bp identical in size to the band amplified by the corresponding RAPD primer (data not shown). These three markers cosegregated with the RAPD markers amplified by their corresponding primers, indicating that they represent the same loci.

QTL mapping

In order to assign the three SCAR markers to the pepper linkage map, we first scored them in a population derived from an inter-specific cross between *C. annuum* (100/63) and *C. chinense* (PI 152225) for which a complete map is available (Tanyolac and Paran, unpublished). All three markers were linked to each other and were assigned to chromosome 7 (data not shown). We subsequently constructed a map of chromosome 7 in the Maor × BG 2816 cross (Fig. 3). The map included ten markers and spanned 109 cM. The order of the markers was identical to published maps for pepper and tomato (Livingstone et

Table 4 QTLs detected for capsaicinoids content in the AF F₂ generation

Trait	Season	Marker	Mean aa ^a	Mean AA ^b	Direction	P value	R ²	LOD	D/A ^c
Capsaicin	Summer	CT84	57.2	311	BG2816	0	0.36	14.9	0.4
	Winter	CT84	20.4	275	BG2816	0	0.34	14.3	0.6
Dihydrocapsaicin	Summer	CT84	28.0	169	BG2816	0	0.15	8.4	1.0
	Winter	CT84	9.7	127	BG2816	0	0.25	9.2	0.4
Total	Summer	CT84	85.2	480	BG2816	0	0.38	15.9	0.7
	Winter	CT84	30.1	402	BG2816	0	0.34	14.2	0.4

^a Mean homozygous class for the Maor allele

^b Mean homozygous class for the BG 2816 allele

^c Degree of dominance (ratio of dominance to additive effects) of the BG 2816 allele

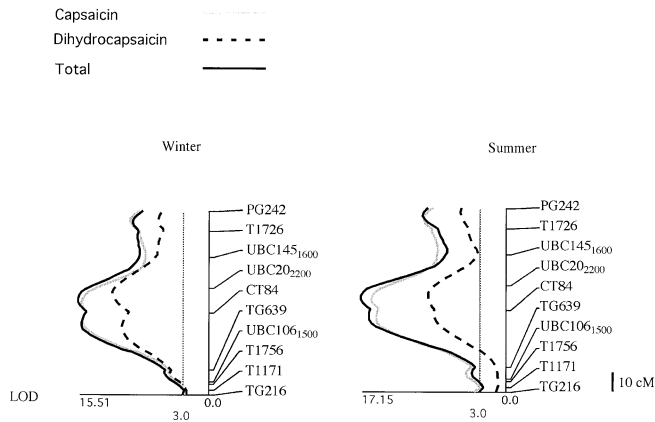


Fig. 3 QTL association with markers on chromosome 7 of the AF population. The LOD score is shown on the x-axis and marker order and scaled distance is shown on the y-axis. Data from both seasons are given

al. 1999; <http://www.sgn.cornell.edu>). In pepper, the interval defined by TG216 and T1726 was 97 cM, a 3.9-fold map expansion relative to tomato (<http://www.sgn.cornell.edu>).

By interval QTL analysis of the capsaicinoid content data from the pungent F₂ and plants, we detected a highly significant QTL we designate *cap* in the interval between UBC20₂₂₀₀ and CT84 (Fig. 3, Table 4). This QTL was detected for both capsaicin and dihydrocapsaicin in both seasons and explained 36% and 34% of the phenotypic variation for total capsaicinoid content in the summer and winter trials, respectively. The degree of dominance (D/A=0.7 and 0.4 for total capsaicinoids in the summer and winter trials, respectively) indicated that the BG 2816 allele at *cap* is partially dominant over the Maor allele, except for dihydrocapsaicin for which complete dominance was observed in the summer season (Table 4). For all measurements, the allele of the pungent parent BG 2816 at *cap* contributed to the increased capsaicinoid content at the QTL. The interval QTL LOD plot of chromosome 7, indicated the occurrence of a possible second minor QTL at PG242. However, composite interval mapping performed by MapQTL v. 4.0 (van Ooijen et al. 2002) revealed that only one QTL at CT84 exists.

Discussion

This study reports the first analysis of the genetics that underlie quantitative variation in capsaicinoid content in pepper by means of molecular mapping. By employing the BSA method (Michelmore et al. 1991), we identified a single major QTL, *cap*, which controls the amount of capsaicinoids in a cross between non-pungent and pungent parents. The most-parsimonious explanation for quantitative differences of flux through biochemical pathways implicates varying expression levels or sequence polymorphism in structural genes of the pathway. We tested this hypothesis for capsaicinoid QTLs by mapping regulatory and structural genes and testing these positions for association with variation in capsaicinoid accumulation. While the position of no known structural or regulatory genes correlated with *cap*, it remains a possibility that a gene in the pathway that has yet to be isolated will correspond to *cap*. Also, we were unable to test the effect of *FatA*. Alternately, *cap* could be involved in a more-complex level of regulation, controlling the movement and availability of substrates or partitioning of metabolites between capsaicin biosynthesis and competing metabolisms.

Our success in identifying markers linked to capsaicinoid content QTLs by employing the BSA method is attributed to the existence of a major QTL for this trait that segregated in the mapping population. This result is in accordance with the conclusions of Wang and Paterson (1994), who assessed the utility of the method for finding linkage between markers and QTLs. Although QTL mapping is commonly done by developing genetic maps with complete genome coverage, our detection-method enabled us to identify the major locus that controls capsaicinoid content in this cross. The screening of 400 RAPD primers each amplifying 5–10 distinct loci and scoring of RFLP loci of the capsaicinoid biosynthesis genes as well as other fruit loci (Blum et al. 2002; Rao and Paran 2003) that are distributed in most pepper chromosomes provided a good coverage of the genome in this population. It is likely, however, that additional minor QTLs that control this trait exist that could not be detected in the current study. The transgressive segregation in the AF F₂ generation may indicate that other minor QTLs exist in this cross for which the Maor allele contributes to

increased capsaicinoid content. In order to determine the effect of *cap* in other genetic backgrounds as well as to determine whether other QTLs that regulate capsaicinoid content exist in *Capsicum*, additional crosses with genotypes that segregate for pungency level will need to be evaluated.

Using exotic germplasm as a source for new alleles to modify cultivated varieties is a common practice in plant breeding. The genus *Capsicum* is especially amenable to this sort of allele-mining given the extensive diversity of pungent chiles. A current treatment of the genus enumerates 16 *Capsicum* species, all of which have pungent members (Hunziker 2001). Within, and especially between, each of these species, the range of capsaicinoid accumulation can vary greatly. We expect there to be many alleles affecting both the amount of capsaicinoids in these cultivars and the relative ratios of the various capsaicinoids. These will be both unique to a species, as a result of a recent evolutionary event, or shared between species, if these differences arose before speciation or were produced by convergent evolutionary processes. *cap* may be a unique allele that functions in hybrids between *C. annuum* and *C. frutescens* or may be an allele that would exert an effect on pungency in a number of different genetic backgrounds.

Similar to the lack of allelic interaction between *C* and pungency, the interaction between TG205 that cosegregated with *C* and *cap* was not significant. The lack of detectable effect on pungency when *C* is in a homozygous or heterozygous state should be of interest to chile breeders. This study confirms the anecdotal observations made by breeders that hybrids produced from crosses between pungent and non-pungent parental lines will produce the same amount of capsaicinoid as an equivalent, true-breeding pungent line.

The LOD plot from the interval analysis indicated that there may be two linked QTLs in the *cap* region. Our attempts to shorten the 34 cM gap between CT84 and TG639 using heterologous probes failed, because markers that were mapped within 6 cM from CT84 in the tomato map were separated by a much greater distance in the pepper map. Therefore, additional marker saturation of this region and introgression lines that divide the chromosome to small fragments will be required for further analysis of the *cap* region.

The high positive-correlations between the content of the different capsaicinoids and the consistency of QTL detection for these capsaicinoids indicated that the synthesis of capsaicin and dihydrocapsaicin is controlled by the same genetic mechanism in this cross. These results do not support the conclusions of previous biometrical analyses of capsaicinoid content that suggested that different genes may be controlling the synthesis of the different capsaicinoids (Zewdie and Bosland 2000a).

The mapping of the capsaicinoid biosynthetic clones was comprehensive for all clones but *KasI* in that all discernable bands were accounted for in the linkage map as either the sole-copy of a clone or all closely related homologs. These are effectively excluded as candidates

for the *cap* QTLs. There are still several missing clones that would code for enzymatic activities of capsaicin biosynthesis. These clones fall into four categories. First, Ca3H functions in part of the pathway derived from phenylpropanoid metabolism, and despite its importance in lignin metabolism, has only recently been cloned in any plant system (Schoch et al. 2001; Franke et al. 2002). Second, the steps converting ferulic acid to vanillin remain unknown and more than one biochemical mechanism may be proposed for the reaction(s). Third, several components of the fatty acid synthase remain unknown in pepper. These are likely to be rather specialized in that the enzymes must accommodate branched substrates and terminate elongation of the alkyl chain earlier than the fatty acid synthesis of primary metabolism. The gene for capsaicinoid synthase has not been isolated. Based on crude enzyme activities, this synthase is expected to contain an acyl-CoA transferase activity (Fujiwake et al. 1980). In this study a differentially expressed cDNA for an acyl CoA transferase, 4A1 was mapped and did not colocalize with any loci associated with capsaicinoid phenotypes. Lastly, only a few regulatory factors have been cloned, none of which so far explain the apparent coordinated regulation of the pathway. *cap* could be a candidate for any one of these functions or a fifth unanticipated role.

Currently, selection for the presence or absence of pungency or for a desired level of pungency in breeding programs is done by tasting or by chromatography based methods such as HPLC. Both methods are also not suitable for high-throughput analyses of large breeding populations. The identification of a major QTL for pungency level will allow the development of a PCR-based assay for pungency level. Previously, we reported the development of a PCR-based marker for the *C* allele that determines whether a plant will produce pungent fruit. A similar marker for the QTL reported here would give breeders another tool in the development of new cultivars. This marker would be advantageous for increasing capsaicin production per pod or plant for oleoresin of capsaicin extraction, or for combining flavor characteristics of specific peppers with a desired pungency level.

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