Journal of Experimental Botany, Vol. 54, No. 388, pp. 1655–1664, July 2003
DOI: 10.1093/jxb/erg176

Journal of Experimental Botany

RESEARCH PAPER

Differential expression of fatty acid synthase genes, *AcI*, *Fat* and *Kas*, in *Capsicum* fruit

Maneesha R. Aluru[1,*], Michael Mazourek[2], Laurie G. Landry[2], Jeanne Curry[1], Molly Jahn[2] and Mary A. O'Connell[1,†]

[1] *Department of Agronomy and Horticulture, MSC 3Q, PO Box 30003, New Mexico State University, Las Cruces, NM 88003, USA*

[2] *Department of Plant Breeding, Cornell University, Ithaca, NY, USA*

Received 17 December 2002; Accepted 20 March 2003

Abstract

The biosynthesis of capsaicinoids in the placenta of chilli fruit is modelled to require components of the fatty acid synthase (FAS) complex. Three candidate genes for subunits in this complex, *Kas*, *AcI*, and *Fat*, isolated based on differential expression, were characterized. Transcription of these three genes was placental-specific and RNA abundance was positively correlated with degree of pungency. *Kas* and *AcI* were mapped to linkage group 1 and *Fat* to linkage group 6. None of the genes is linked to the pungency locus, *C*, on linkage group 2. KAS accumulation was positively correlated with pungency. Western blots of placental extracts and histological sections both demonstrated that the accumulation of this enzyme was correlated with fruit pungency and KAS was immunolocalized to the expected cell layer, the placental epidermis. Enzyme activity of the recombinant form of the placental-specific KAS was confirmed using crude cell extracts. These FAS components are fruit-specific members of their respective gene families. These genes are predicted to be associated with *Capsicum* fruit traits, for example, capsaicinoid biosynthesis or fatty acid biosynthesis necessary for placental development.

Key words: Branched chain fatty acids, capsaicinoids, genome mapping, pungency.

Introduction

Several different species in the genus *Capsicum* are cultivated as vegetable and spice due to the presence of pungent or 'hot' capsaicinoids that accumulate in vesicles or blisters on the epidermis of the chilli placenta (Suzuki *et al*., 1980; Zamski *et al*., 1987). Capsaicinoids are tasteless, odourless compounds detectable by mammalian pain receptors in solutions of 0.1–1 ppm (~1 Scoville Heat Unit, SHU: Andrews, 1995). Capsaicinoids accumulate in pockets or blisters on the epidermis of the chilli placenta (Suzuki *et al*., 1980; Zamski *et al*., 1987). A single dominant gene at locus *C* controls the presence or absence of pungency in *Capsicum* (Blum *et al*., 2002 and references therein). However, in pungent types the degree of pungency is quantitatively inherited and significantly affected by environment (Zewdie and Bosland, 2000*a*).

Capsaicinoid biosynthesis has been demonstrated in the vacuolar fraction (Fujiwake *et al*., 1980) of placental cells, beginning approximately 20 d post-anthesis and persisting through fruit development (Iwai *et al*., 1979). Capsaicinoids are produced by a condensation reaction catalysed by capsaicin synthase between vanillylamine, derived from phenylalanine, and a branched-chain fatty acid, derived from either valine or leucine (Fig. 1) (Bennett and Kirby, 1968; Leete and Louden, 1968; Ochoa-Alejo and Gomez-Peralta, 1993; Sukrasno and Yeoman, 1993). Members of the capsaicinoid class differ in length of the fatty acid side chain, which varies in the presence of a single desaturation and in chain length from C9 to C11.

Other members of the Solanaceae synthesize branched-chain fatty acids and in each of these cases, the primer for the extension of the alkane is proposed to be a deaminated

* Present address: Department Botany, Iowa State University, Ames, IA 50011-1020, USA.
† To whom correspondence should be addressed. fax: +1 505 646 6041. E-mail: moconnel@nmsu.edu

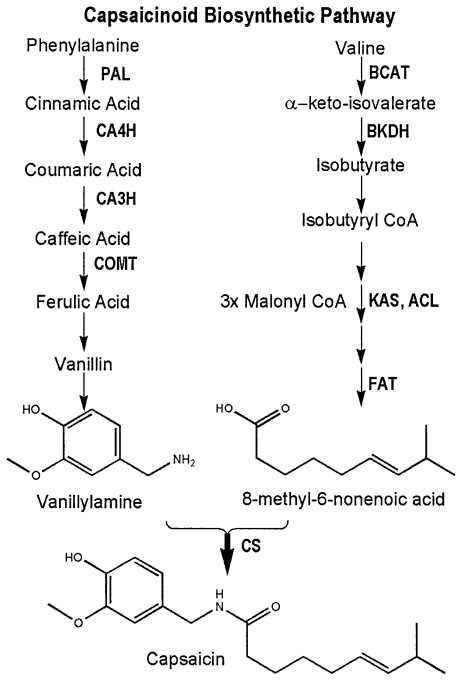

Fig. 1. Proposed capsaicinoid biosynthetic pathway. The enzymes on the pathway are: ACL, acyl carrier protein; BCAT, branched-chain amino acid transferase; BKDH, 3-methyl-2-oxobutanoate dehydrogenase; CA3H, coumaric acid 3-hydroxylase; CA4H cinnamic acid 4-hydroxylase; COMT, caffeic acid *O*-methyltransferase; CS capsaicinoid synthase; FAT, acyl-ACP thioesterase; KAS, β-ketoacyl ACP synthase; PAL, phenylalanine ammonia lyase.

branched-chain amino acid (Burke *et al*., 1987; Kaneda, 1967). Acyl chain growth occurs by the addition of 2-carbon units via the fatty acid synthesis pathway (Markai *et al*., 2002; van der Hoeven and Steffens, 2000). Plants have a type II fatty acid synthase (FAS) similar to prokaryotes, a multi-enzyme complex (Harwood, 1996). Of the FAS component enzymes, β-ketoacyl synthase (KAS) condenses ketoacyl-CoA groups with malonyl-acyl carrier protein (ACL), releasing CO_2. Elongation cycles continue until the fatty acid is released by a thioesterase (FAT), which participates in the regulation of chain length. This model is consistent with results from radiotracer studies and accounts for capsaicinoids with even-numbers of carbons in the acyl chain being extended from iso-butyryl-CoA (from valine) while iso-valeryl-CoA (from leucine) is incorporated into capsaicinoids with odd-number chains (Suzuki *et al*., 1981).

Little is known about the genes or enzymes of the capsaicinoid biosynthetic pathway, or about the role these genes play in the phenotypic variability in capsaicinoid content observed in pepper fruit. In earlier work, differential patterns of transcript accumulation for phenylpropanoid pathway genes, *Pal*, *Ca4h*, and *Comt*, were correlated with fruit pungency (Curry *et al*., 1999). Differential screening of a habanero (*Capsicum chinense*) placental cDNA library recovered three cDNAs with significant sequence similarity to proteins important in fatty acid biosynthesis. In this report the pattern of expression and genomic position of three genes, *Kas*, *Acl*, and *Fat* are described. These gene products are candidates for the biosynthetic enzymes for the branched-chain fatty acids used in capsaicinoid biosynthesis.

Materials and methods

Plant materials

Plant material was propagated by seed in Metro-Mix 360, fertilized with Osmocote (14-14-14) and maintained in a greenhouse on the main campus of NMSU. Plant material included a habanero pepper, *Capsicum chinense*, cv. 'Early Scotch Bonnet' (Stokes Seed); *C. annuum* jalapeño, and a New Mexican pod type, New Mexico 6-4 (P Bosland, NMSU).

RNA blot hybridization

Plant tissues to be used for RNA isolation were collected directly into liquid N_2. The organs used for RNA isolation were from immature (green) fruit for the fruit wall, placenta and seed samples. The roots, stem, leaf, and flower used for RNA isolation were from young habanero plants (~7 months old). Total RNA, and recombinant DNA for probe preparation were isolated as described earlier (Kahn *et al*., 1993). The probes consisted of the entire cDNA insert purified from gels following restriction digestion or, as specifically indicated, the 3′-UTR of *Acl*. This fragment was generated as a 227 bp fragment following digestion of the *Acl* cDNA with *Xcm1* and *Xmn1*. RNA blots were prepared as described previously by Pelle and Murphy (1993). All northern blots were replicated several times using independent sources of RNA and probe.

DNA and protein sequence analyses

Confirmed double-stranded DNA sequences were obtained. Several bioinformatics tolls were used: BLAST at the NCBI web site (www.ncbi.nlm.nih.gov/BLAST, Altschul *et al*., 1997); BLOCKS (www.blocks.fhcrc.org, Henikoff and Henikoff, 1994); PSORT (psort.nibb.ac.jp, Nakai and Kanehisa, 1992), and TargetP v1.01 (www.cbs.dtu.dk/services/TargetP/, Emanuelsson *et al*., 2000). Multiple sequence alignments and dendrograms were generated using a downloaded version of Clustal W (Thompson *et al*., 1994).

Purification of recombinant KAS

Affinity™ LIC cloning kit (Stratagene) was used to express *Kas* in *E. coli*. The entire cDNA minus the signal sequence was cloned into the pCAL-n-EK plasmid. Protein expression was induced in the BL21 (DE3) pLysS cells with 3 mM IPTG for 5 h at 37 °C. After induction, the cell pellet was resuspended in a $CaCl_2$ buffer (Stratagene) and stored at –80 °C until further use. The expressed protein was purified according to the manufacturer's protocol and the eluted protein concentrated in a vivaspin concentrator (ISC BioExpress). To remove the CBP-tag, the concentrated protein was dialysed using BIODIALYSIS dialysis tubing (Sialomed Inc.) against the enterokinase buffer for 24 h at 4 °C and then incubated for 24–48 h at room temperature with enterokinase.

Generation of anti-KAS antibodies and immunoblotting

Discontinuous PAGE was performed with SDS (Laemmli, 1970). Following removal of the CBP tag, the affinity-purified recombinant protein was run on a preparative SDS-PAGE. The KAS band was excised from the polyacrylamide gel to generate polyclonal antibodies in rabbits (Bethyl Laboratories, Montgomery, TX).

Antibodies specific for the pungent habanero proteins (BL21-negative) were made by adsorbing anti KAS-antisera against protein blots of *E. coli* cell lysates prior to use.

Plant samples containing 80–100 μg protein from placental extracts were electrophoresed on discontinuous SDS-PAGE. Western blots were prepared on nitrocellulose membrane (Towbin *et al*., 1979). Immunoblotting was carried out using the Immuno-Blot Assay Kit from BioRad. After blocking, the membranes were incubated with anti-KAS antibody for 16–18 h at room temperature and washed three times (10 mM TRIS-HCl pH 8.0, 150 mM NaCl, 0.1% Tween-20 v/v). Following incubation with goat anti-rabbit IgG conjugated to alkaline phosphatase for 3–5 min at room temperature, colour development proceeded for 3–5 h as recommended in the kit. No staining was detected when the preimmune serum was used as a negative control.

KAS assay

The KAS assay procedure (Shimakata and Stumpf, 1983) measured the production of radiolabelled fatty acids. Phenyl methyl sulphonyl fluoride was added to the cell lysate (1 mM final) and the lysate stored on ice until use. The assay mixture (total volume 0.5 ml) contained 540 μg of total protein, 2.5 mM *E. coli* ACP, 10 μM acetyl CoA, 100 mM potassium phosphate pH 7.4, 15 μM isobutyryl-CoA, 1 mM each of NADH and NADPH, and 15 μM 2-[^{14}C] malonyl-CoA (2.04 GBq mmol^{-1}, 55 mCi mmol^{-1}, Amersham). Reactions were conducted at 37 °C for 25 min and stopped by the addition of 10 ml reducing agent (0.1 M K_2HPO_4, 0.4 M KCl, 30% tetrahydrofuran v/v, 5 mg ml^{-1} $NaBH_4$) with incubation for 30 min at 37 °C. Radiolabelled fatty acids were extracted with toluene, and counted in ScintiVerse E cocktail (Fisher Scientific) in a Packard Liquid Scintillation counter (Packard). To study the effect of the inhibitor cerulenin, cell extracts were preincubated with 20 μM cerulenin for 30 min at 37 °C prior to the assay.

Immunohistochemistry

Fruit were collected directly into fixative, 2.5% (v/v) glutaraldehyde in 0.07 M sodium phosphate, pH 7.4. Samples were dehydrated through 70% (v/v) ethanol and embedded in paraffin. Sections (5 μm) were cut and stained with anti-KAS antibody at 1:500 dilution and Cy5 tagged anti-rabbit goat antibodies. Samples were imaged using a BioRad 1024M confocal scanning ArKr laser and an Olympus microscope. The Cy5 signal was excited with 647 nm and detected with a 680 nm emission filter. The autofluorescence signal was excited with 488 nm and detected with a 540 nm emission filter. A composite image was created with false colour, red for the Cy5 signal and green for the autofluorescence. There was no staining detected when the preimmune sera was used, or when only the secondary antibody was used as a negative control.

Genetic mapping

Genetic map positions for the *Acl*, *Fat*, and *Kas* clones were determined according to the procedures of Livingstone *et al*. (1999) using an existing *Capsicum* map with 1007 mapped RFLP, AFLP, RAPD, and isozyme loci. Three interspecific mapping populations were used, AC, FA and FC. The AC population includes 75 F_2 individuals from *C. annuum* cv. 'NuMex RNaky' (Nakayama and Matta, 1985) × *C. chinense* USDA PI 159234. The FA population includes 47 F_2 individuals genotyped for 218 RFLP markers from *C. frutescens* BG 2814-6 (Zhang, 1997; Grube *et al*., 2000) × *C. annuum* cv. NuMex RNaky. The FC population includes of 55 F_2 plants genotyped for 157 RFLP markers (Zhang, 1997) from *C. frutescens*, BG 2814-6 × *C. chinense* PI159234. Survey filters used to assess parental polymorphism and mapping filter sets for each enzyme were prepared as described previously (Livingstone *et al*., 1999).

Probes were generated from plasmid clones by PCR. For *Acl*, the probe contained the entire transcribed region and flanking polycloning site. The *Fat* probe was a portion from the coding region (bp 35–508). Two *Kas* probes were made: one from the coding region (bp 121–1074) and one from the 3′ untranslated region (bp 1484–1848).

Segregating bands were scored independently and entered into the MAPMAKER/EXPv3.0b program (Lincoln *et al*., 1993). Clones were positioned within the existing AC framework map according to the procedures of Livingstone *et al*. (1999). In the FA, 'group' (LOD >3, within 20 cM) was used to define a linkage group for the *C. frutescens* band that could be visually aligned with the AC map based on shared markers. Pairwise distances to the most closely linked marker were also found using the Kosambi function (Kosambi, 1944).

Results

*Acyl carrier protein (*Acl*)*

The cDNA clone for chilli *Acl* contained a 683 nucleotide transcript: 35 nucleotide 5′-UTR, 399 nucleotide coding region and a 249 nucleotide 3′-UTR (GenBank AF127796). The approved three letter abbreviation for acyl carrier protein is *Acl*, even though in biochemical literature ACP is commonly used, (CPGN, http://genome-www.stanford.edu/Mendel/). The predicted translation product of the coding region was an 132 amino acid protein with greater than 70% sequence identity to ACLs from plants. The amino terminus of the gene product has a plastid targeting sequence as expected (aa 1–49). Transcripts for this gene are abundant in the placenta and fruit wall of immature (green) fruit, with the fruit wall accumulating approximately half the transcript abundance of placenta (Fig. 2a). Transcripts are reduced in abundance in root, stem, leaf, flower, and seed. Transcript abundance for *Acl* in placental tissue was related to capsaicinoid content among varieties differing in capsaicinoid levels (Fig. 2b). *Acl* transcript levels were highest in the most pungent chilli, habanero, then jalapeño and lowest in the mild New Mexico 6-4. The capsaicinoid levels in these varieties range from ~300 000 SHU for habanero to 30 000 for jalapeño to 1500 for New Mexico 6-4. The placental-enhanced pattern of expression observed with the full-length cDNA was also observed when the 3′-UTR portion of the *Acl* cDNA was used as a probe. Transcripts for *Acl* were more abundant early, in placenta from immature or green fruit, and decreased in abundance as the fruit matured (Fig. 2c).

Multiple sequence alignments of the chilli ACL with other plant ACL sequences was performed using only those amino acid residues present in the mature protein, minus the plastid targeting sequence. The chilli ACL sequence aligned with the other plant ACL sequences (Fig. 3). The serine residue at the active site is in a well-conserved sequence motif, GADSLD found in chilli and all other plant ACLs (Kopka *et al*., 1993).

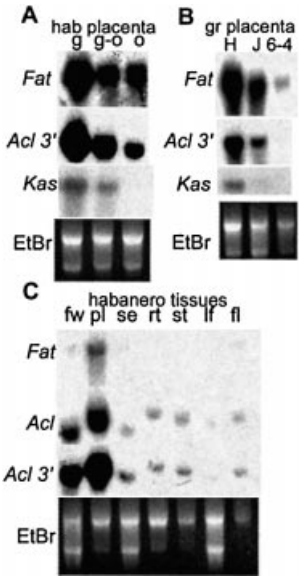

Fig. 2. Expression of *Acl*, *Fat* and *Kas* in chilli tissues. Total RNA (5 μg) was separated by electrophoresis, transferred to nylon membranes and probed with ^{32}P-labelled *Fat*, *Acl*, *Kas*, or the 3′-UTR of *Acl* (Acl 3′). In each panel the RNA load is monitored by the ethidium bromide-stained ribosomal RNA, indicated as EtBr in the figure. (A) RNA from habanero placenta from green (g), green/orange (g/o) or orange (o) fruit; (B) RNA from the placenta of immature (green) fruit of habanero (H), jalapeño (J), New Mexico 6-4, (6-4); (C) RNA was isolated from fruit wall (fw), placenta (pl), seed (se) of immature (green) fruit of habanero; from root (rt), stem, (st), leaf (lf) or flowers (fl) from habanero.

*Thioesterase (*Fat*)*

The cDNA clone for a chilli acyl-ACP thioesterase contained a 1342 nucleotide transcript: 5 nucleotide 5′-UTR, 1116 nucleotide coding region and a 221 nucleotide 3′-UTR (GenBank accession number AF318288). The predicted translation product of the coding region was a 372 amino acid protein with greater than 70% sequence identity to thioesterases from plants. The amino terminus of the predicted gene product has a plastid targeting sequence as expected (aa 1–72). Transcripts for this gene were abundant in the placenta of immature fruit (Fig. 2a). Transcripts were barely detected in the fruit wall, root, stem, leaf, flower, and seed. In Fig. 2b, *Fat* transcript levels were highest in the most pungent chilli, habanero, then jalapeño and lowest in the mild New Mexico 6-4. Transcripts for *Fat* were more abundant early, in placenta from immature or green fruit, and decreased in abundance in mature fruit (Fig. 2c).

Multiple sequence alignments of the amino acid sequence of the chilli FAT with plant FATA sequences were performed using only those amino acid residues present in the mature protein, minus the plastid targeting sequence. In plants, the active site in FATA requires cysteine and histidine (Yuan *et al*., 1996); the chilli FAT contained both of these amino acids in well-conserved sequence motifs: YRRECQ, positions 231–236, and NQHVNN, positions 198–203 (Fig. 4). A larger number of sequences, all plant FATA and FATB sequences available in GenBank (autumn 2000) and Clustal W were used to generate a phylogenetic tree. The thioesterases cluster into two different clades represented on the tree as FATA and FATB; the chilli protein clustered with the FATA group (data not shown).

β-ketoacyl ACP synthase (Kas)

The sequence and pattern of expression of a placental specific chilli *Kas* (GenBank accession number AF085148) has been described (Aluru *et al*., 1998; Curry *et al*., 1999). Transcripts for *Kas* are specific to placental tissue (Curry *et al*., 1999) and have the same differential patterns of placental expression as *Acl* and *Fat* (Fig. 2).

Multiple sequence alignments of the chilli KAS with other plant KAS sequences was performed using only those amino acid residues predicted to be present in the mature protein, i.e. after import into plastids. The PSORT algorithm predicts that the chilli KAS is a plastid targeted protein; the TargetP algorithm predicts only low confidence that the chilli KAS is a plastid targeted protein. The chilli KAS sequence aligned with other plant class 1 KAS sequences. Alanine residues usually flank the cysteine residue at the catalytic site, position ~183, TACAT (Fig. 3C). This amino acid sequence was present in chilli KAS, and there was sequence identity or similarity over extensive stretches of the full-length protein sequence. Using ClustalW, an analysis of a larger number of KAS sequences, all plant KAS sequences (Classes I, II, III) in GenBank (autumn 2000) was also performed. The different classes of KAS clustered into different clades, and as expected, the chilli protein was in the class I clade (data not shown).

Mapping of Kas*,* Fat *and* Acl

All discernible bands, when candidate genes were hybridized with total genomic DNA, were assigned to pepper linkage groups (Table 1). In the AC population, the MAPMAKER/EXP 'build' command did not establish a unique order within the framework at LOD>3. 'Ripple' showed that the only significant orders within the framework placed clones immediately on either side of the most closely linked framework marker for each.

Acl and *Fat* were both single copy loci in pepper based on the number of pronounced bands on survey filters of *C. annuum* and *C. chinense* genomic digests (Fig. 4). *Acl* mapped to a position in the middle of pepper linkage qroup 1 (P1), between TG70 and PG293, 2.7 cM from *Pgm*2. *Fat* mapped to a distal position on P6, 3.2 cM from TG220a (Table 1).

By contrast, the *Kas* probe derived from the coding region hybridized to at least eight bands. Several of the polymorphic bands hybridizing to the *Kas* coding region probe were mapped to eight positions in the genome. To

A. ACL alignments

```
Hv  1 AAMGEAQAKKETVDKVCMIVKKQLAVPDGTPVTAESKFSELGADSLDTVEIVMGLEEEFNITVDETSAQDIATVQDAANLIEKLVTEKTA----
Cl  1 ------...P...A...G.......L..DSE.NGL....A.....................G.S.E.E...S.Q......D.....MEK.GH----
Cc  1 ------A..P.......A.......L.GDSV.NG....AA.................A.G...E.EN..T........D...D..AK.C-----
At  1 ------A..A...Q..SD...E...LAADV.L.......A.............A...K...S.E.SD..N.T.I.E..D...D..QK.P.AETS
So  1 ------A..P.M.T..SD...S...LAEDAK..G.T....I............K.....GV..E.EN..T.T.I.E..DM..A.QQN.------
            ** * * **. ***.***:.    : . :**: :************ *** *:. *:*  ** * *:::**::*: *   *------
```

B. FATA alignments

```
Cs   1 SLAEKLRLGSLTEDGLSYKEKFIVRCYEVGINKTATVETIANLLQEVGGNHAQSVGFSTDGFATTPTMRKLHLIWVTARMHIEIYRYPAW
At   1 T..DQ.................V..S....S.................C.................T...................K....
Bn   1 TP.DR..A.R.M...Y.........S.....................AC..V.KC..........L...................K....
Cc   1 ...DR....TMS...M.............V...V.............C................HS.............Y....K....
Gm   1 G..DR.....................................C.......Y..G..S........R.............K....
         *::** *   *** **.*:*::*.**** ***.****:*******. **.*. *:**.**:** :****.*****:**:****:****

Cs  91 SDVVEIETWCQSEGRIGTRRDWIIKDFATDEVIGRATSKWVMMNQDTRRLQKVSDDVRDEYLVFCPKTPRLSFPEENNKSLKKISKLEDP
At  91 G.....................L..SV.G..T................................QE...A......R.....P.....
Bn  91 .....................LR.S..N.....................R.T.E..........RE...A......S.....P.....
Cc  91 .....................L..C..G..........L.............T.E....LELYF..EL..A......G.....P.....
Gm  91 .......S.G.G..K........LR.Y..GQ.....................DV........H..REL..A......S...........
       .******:* *.**:********::*  * :* *******:***::*****:*  .****  :  *.  **:****** *****.*****

Cs 181 AQHSRLGLSPRRADLDMNQHVNNVAYIGWVLESIPKEVLYTHELETITLDYRRECQHDDVVDSLTSPEV
At 181 ..Y.MI..K..............T..........Q.IVD....QV..........Q........TTTS
Bn 181 ..Y.M.E.K..............T..........Q.IID....QV..........Q..I.....TS.I
Cc 181 .EY.K...V..............T........M.Q.IID....G...I..................V.P
Gm 181 S.Y.K...V..............T........M.Q.IID....Q.....................P
       :::* : * ****************:********:*:*:: **** .**:*******:**:*****:
```

C. KAS alignments

```
Pf   1 RVSAAAAVAAAPKRETDPKKRVVITGMGLVSVFGNDVDAYYEKLLSGESGITLIDRFDASKFPTRFGGQIRGFKAEGYIDGKNDRRLDDC
Gm   1 --------VS..Q.QK.............A.......EG......A......A...................S................
At   1 ---.S.STVS.........................................S.....................SS........E......
Hv   1 ---------...Q.....R..........A....S...TF.DR..A....VGP.......S.....A......SS...............
Cc   1 --------VS...........I.............S.I.NF.N...E.Q...S........SYTV..A....D.SS...............
              :**:*: **:**:**** **.****.*:: :*::** *:**:  *******.:..**.****.*.:********:******

Pf  91 LRYCIVAGKKALEGADLGG--EKLNKIDKIRAGVLVGTGMGGLTVFSDGVKALIEKGHRKITPFFIPYAITNMASALLAIDLGLMGPNYS
Gm  83 .............N...AP--DNHS....E.......S............Q.....................G....G....F......
At  88 .K...........S.N...--D...T...RK................E..QN.......R.S...........G..............
Hv  82 I....LS......S.G..AGSDAHV.L.VG.............S......QN.....Y...S...........G.......V.F......
Cc  83 W...L....R...D.N..Q--QV.DTM..T.I.....SS...SK..A.A.E..VQR.YK..N......S....G.......T.....T..
        :**:::**:*** *.*.   :   .:*  : *****:.*** .**::.*: *:::*:::*.******:****.****.** *:***.**

Pf 179 ISTACATSNYCFYAAANHIRRGEADLMIAGGTEAAMIPIGLGGFVACRALSQRNDDPQTASRPWDKDRDGFVMGEGSGVLVMESLEHAMK
Gm 171 ..................................I.....................K........E.........A.............
At 176 ..........................M.........I.............................A.........A.............
Hv 172 .........................IIV.......I.....................I..C.....E.........A.............
Cc 171 .......A......S..........I.V....D.FISA..V..LI..........EYEK......RN.....I..........N....L.
       *******:******:**********:::****:* : .**:**::**********:  .*.****: *****:***:******.****:*

Pf 269 RGAPIIAEYLGGAVNCDAYHMTDPRADGLGVSSCIQSALEDSGVSPEEVNYINAHATSTIVGDLADVNAIKKVFKNTSGIKINATKSMIG
Gm 261 ........................S......T....S...A.................LA....EI.........D..............
At 266 .....V............H..............ERC...A.................LA....EI.........S..............
Hv 262 .D................................TMS.R.A..A..............LA....E.R...Q....P.E....S......
Cc 261 .............IT...H.I..............VMS.V.A.................LA....E.........D..E..M.G......
       *.***:*******:.***:*:****:******:**  .* *:**:***************:.****::.***:***..* **:*.******

Pf 359 HCLGAAGGLEAIASVKAITTGWLHPTINQFNPEPSIGFDTVANEKQQHEVNVAISNSFGFGGHNSVVAFSAFKP
Gm 351 .............T.....................AVD......V.....I.......................
At 356 .............T....N......S.......QAVD....P...K....D.......................
Hv 352 .............TI.S.....V............EVD.........K......G..............V.AP...
Cc 351 .G...S.......TI..............YDL..QVTI...P.V.K......G..............V.APY..
       * ***:******* :*:*.***:**:***:: *  : :***.* *:***::*.***************.*:.:**
```

Fig. 3. Alignment of ACL. FAT and KAS sequences. Clustal W was used to generate multiple sequence alignments, position 1 is the amino terminus after cleavage of the transit peptide. Amino acid identities with top sequence are represented as dots in the alignments. In the last line, asterisk indicates aa identity, colon indicates highly conservative substitution, and a period indicates a less conservative substitution. The position of the catalytic site is underlined. (A) ACL alignments: Hv, *Hordeum vulgare*, aa 60–149, M24425; Cl, *Cuphea lanceolata*, aa 57–140, X77620; Cc, *Capsicum chinense*, aa 50–131, AF127796; At, *Arabidopsis thaliana*, aa 50–137, AL161562; So, *Spinacia oleracea*, aa 49–130, X52065. (B) FATA alignments: Cs, *Coriandrum sativum* aa 65–313, L20978; At, *Arabidopsis thaliana*, aa 67–315, Z36912; Bn, *Brassica napus*, aa 65–313, X87842; Cc, *Capsicum chinense*, aa 73–321 AF318288; Gm, *Garcinia mangostana*, aa 49–297, U92876. (C) KAS 1 alignments: Pf, *Perilla frutescens*, aa 43–474, AF026148; Gm, *Glycine max*, aa 46–469, AF243182; At, *Arabidopsis thaliana*, aa 45–473, AB010698; Hv, *Hordeum vulgare*, aa 38–462, M60410; Cc, *Capsicum chinense*, aa 65–488, AF085148.

determine which of these polymorphic bands corresponded to the placental *Kas* with putative function in capsaicinoid biosynthesis, a second hybridization was done using a probe generated from only the 3′-UTR, based on the assumption that this region would be less conserved among related gene family members. The *Kas* 3′-UTR probe hybridized to two bands, but only one was polymorphic when DNA was cut with *Bst*NI or *Eco*RV (Fig. 4). All bands from the hybridization of *Kas* 3′-UTR scored in the AC population mapped to the top of P1 within 0.4 cM of CT252. Again, the commands 'build' and 'ripple' did not establish the position of the *Kas* 3′-UTR markers relative to CT252, so the markers are placed on the same line as CT252 (Table 1).

Recombination between markers in three *C. chinense* individuals in *Eco*RV versus *Bst*NI digests suggested that both copies of the placental-specific *Kas* had been identified using the 3′-UTR. To confirm this, two additional mapping populations, the FA and FC populations, were created by crossing each parent of the AC population with a third species, *C. frutescens*. In contrast to the AC population, the upper *Eco*RV band was polymorphic in FA, and both bands were polymorphic and co-segregated in the FC population (Fig. 4). Using data from the FA population, the *C. frutescens* band grouped with and linked to the same marker as those bands mapped in AC. Therefore it is concluded that the two fragments in *C. chinense* that hybridize with the *Kas* 3′-UTR represented two linked copies of placental-specific *Kas* family members.

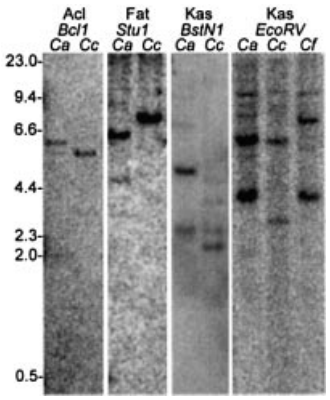

Fig. 4. Survey blots of *Capsicum* mapping parents. Southern blots containing genomic DNA restricted with the indicated enzymes were probed with full-length cDNAs for *Acl*, *Fat*, or the 3′-UTR for *Kas*. The position of size markers (kb) is indicated on the left.

KAS activity of the recombinant protein expressed in E. coli

To test whether *Kas* encodes a protein with KAS activity, the entire coding region of *Kas* except for the first 135 nucleotides (45 amino acids) corresponding to a putative plastid targeting sequence was included in the construction of the recombinant protein. The calculated molecular weight of KAS including the signal sequence was 52.5 kDa. The molecular weight of the protein expressed from the recombinant vector was expected to be 51.5 kDa, 47.5 kDa for the KAS domain plus 4 kDa for the calmodulin-binding-peptide (CBP). On SDS-PAGE, the purified recombinant fusion protein expressed in *E. coli* migrated as a 58 kDa protein (Fig. 5A).

Biochemical assays for KAS were carried out in recombinant *E. coli*; activity was measured as the formation of longer chain fatty acids incorporating ^{14}C-malonyl-CoA. The recombinant KAS from pungent habanero increased the formation of long-chain fatty acids (>C8) as measured by the amount of radioactivity extractable in toluene (Table 2). KAS activity was found to increase approximately 10-fold upon induction of expression of the recombinant protein, and the induced KAS activity was inhibited ~50% by 20 μM cerulenin. This sensitivity to the inhibitor cerulenin was characteristic of class 1 KAS.

Detection of KAS in chilli placental tissues

Immunoblotting was performed with crude placental extracts of green fruit chillies using protein from pungent habanero, jalapeño, and New Mexico 6-4. Anti-KAS antisera reacted with three different proteins, 66, 56 and 47 kDa, in chilli placenta (Fig. 5B). The 66 and 56 kDa proteins were detected in all three chilli types. The 47 kDa protein was detected in the placenta of pungent habanero and jalapeño. The predicted size for the preprotein with plastid transit peptide was 52.5 kDa and for the processed

Table 1. *Mapping summary of chilli fatty acid synthase clones*

	Acl *Bcl*I	Fat *Stu*I	*Kas* *Eco*RV	*Kas* *Eco*RV	*Kas* *Bst*NI	*Kas* *Eco*RV
Number of individuals scored	66	65	66	68	59	45
Marker type	Codom	Codom	*annuum*	*chinense*	*chinense*	*frutescens*
	AC	AC	AC	AC	AC	FA
Assignment	P1	P6	P1	P1	P1	–
(LOD)	(19.9)	(20.3)	(11.6)	(12.3)	(11.1)	
Nearest framework marker	*Pgm2*	TG220a	CT252	CT252	CT252	CT252
cM to nearest marker	2.7	3.2	0.4	0.2	0.4	4.7
(LOD)	(19.9)	(20.3)	(11.6)	(12.3)	(11.1)	(6.6)

form of KAS was 47.5 kDa. Whether the 47 kDa band or the 56 kDa band represents the mature form of the placental KAS is not clear, as the recombinant form of this protein migrated on SDS-PAGE at a molecular weight greater than that predicted from primary amino acid sequence. The 56 kDa form of KAS ran as a doublet in both the *E. coli* preparation and in the placental extracts. This protein from *E. coli* was detected on SDS-PAGE using Coomassie stain; in the placental extracts, the 56 kDa doublet was detected using antibody. No immunostaining was detected on the western blots when the preimmune serum was used (data not shown).

Using the anti-KAS antisera, sections of fruit tissue from different types of chilli were analysed to determine where the protein accumulated. No transcripts for KAS were detected on northern blots containing RNA from fruit wall or seed from habanero, jalapeño, or New Mexico 6-4 (Curry *et al*., 1999). As expected, the anti-KAS antibody did not react with proteins in histological sections of these fruit parts (data not shown). KAS was readily detected in histological sections of placental tissue from immature (green) fruit of habanero and jalapeño and present at lower abundance in New Mexico 6-4 placenta (Fig. 6). No staining was detected when the preimmune serum was used or when only the secondary antibody was used to stain the samples (data not shown). The immunohistochemical signal detected by the laser scanning confocal microscope is expected to reflect the abundance of the sum of all of the bands detected in the western blot (Fig. 5B). KAS appeared to accumulate in the placental epidermal and sub-epidermal cell layers of all three fruit types, with maximal signal detected in habanero. The epidermal receptacles for the accumulation of capsaicinoid oil were adjacent to the cells accumulating KAS in these images.

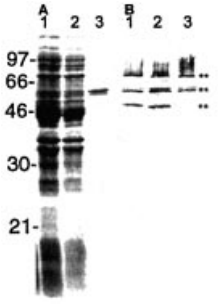

Fig. 5. Expression and detection of KAS. (A) Coomassie Brilliant Blue stained analysis of 1, lysate from BL21 cells induced with IPTG (80 μg); 2, flow-through lysate (80 μg); 3, affinity purified KAS (2 μg) run on 12% SDS-PAGE gels. (B) Western blot detection using anti-KAS polyclonal antibodies and counterstained with goat anti-rabbit antibodies conjugated with alkaline phosphatase for colour development. Placental extracts (100 μg protein) from green fruit: 1, habanero; 2, jalapeño; 3, New Mexico 6-4; were run on 12% SDS-PAGE gels. Arrows mark the position of antigenic proteins at 66, 56 and 47 kDa, relative migration of molecular weight markers in kDa are indicated on left.

The abundance of KAS in these samples was correlated with the degree of pungency in the fruit, habanero having the most KAS and New Mexico 6-4 the least.

Discussion

Capsaicinoids are synthesized via the intersection of the phenylpropanoid pathway and branched-chain fatty acid pathway (Bennett and Kirby, 1968; Leete and Louden, 1968). The phenylpropanoid pathway is ubiquitous in plants and the products of this pathway serve many different functions (Dixon and Paiva, 1995). Enzymes of fatty acid synthesis are highly conserved, and their function has been implicated in the biosynthesis of branched-chain fatty acids within other Solanaceae. The present study identified and partially characterized three fatty acid synthase genes, *Kas*, *Acl*, and *Fat*, from pungent habanero.

Kas was expressed in *E. coli* for protein blot analysis in order to corroborate the expression pattern of this gene in chilli placenta and activity assays confirming the annotation of this gene. Expression of *Acl* and *Fat* was placental-enhanced and developmentally regulated in pungent habanero, and transcript accumulation for these genes was positively correlated with the degree of fruit pungency. These patterns of RNA accumulation were very similar to the patterns of expression for other biosynthetic genes: *Pal*, *Ca4H*, *Comt*, and *pAmt* (Curry *et al*., 1999). Further, immunohistochemical analysis with chilli KAS-specific polyclonal antibodies demonstrated that KAS accumulated in the placenta of pungent chilli fruit, and the greatest abundance of KAS was found in the epidermal cell layers of the placenta near the capsaicinoid receptacles. Since all cells in the plant require expression of FAS genes, the tissue-specific expression profile of the three genes described here is indirect evidence that these genes must be capsaicinoid/placental specific members of their respective gene families. These expression results are consistent with the hypothesis that these FAS components are specific for the branched-chain fatty acids needed for capsaicinoid synthesis. Alternatively, expression of these FAS subunits

Table 2. *KAS activity E. coli* cells (BL21) carrying *Kas* minus the signal peptide domain in the pCAL-n-EK plasmid were assayed for KAS activity. The radioactive products of the KAS assay are collected by partitioning into toluene and counted. Assays were run in triplicate.

Sample	cpm mg^{-1} protein (mean ±sd)
BL21	2048±776
BL21+20 μM cerulenin	573±253
BL21 induced with IPTG	20226±4099
BL21 induced with IPTG+20 μM cerulenin	10403±3533

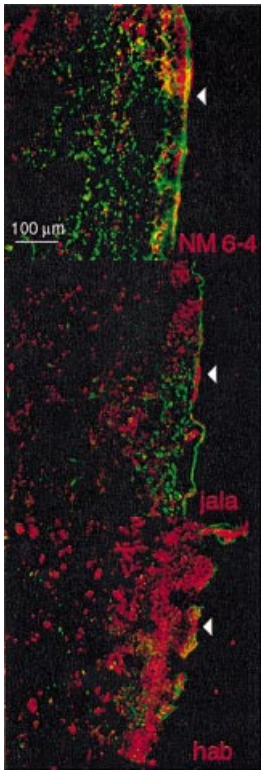

Fig. 6. Immunolocalization of KAS. Sections of placental tissue from green fruit of habanero (hab), jalapeño (jala) or New Mexico 6-4 (NM 6-4) were stained with anti-KAS antibody and counterstained with Cy5 tagged goat anti-rabbit antibody (red signal); autofluorescence is green signal. The sections are oriented with the epidermal layer on the right in each panel (arrowheads at the surface of the epidermis). Images were collected using a BioRad 1024 ArKr laser scanning confocal microscope.

could be associated with other features linked with capsaicinoid accumulation, for example, receptacle or blister structures.

KAS1 is typically a dimer with subunit sizes of 43–46 kDa, usually these are homodimers (Mackintosh *et al*., 1989; Siggaard-Andersen *et al*., 1991). Polyclonal antibodies to habanero KAS detected a protein of molecular size 47 kDa that was present only in pungent chillies and cross-reacted with a protein of same size from jalapeño. In plants, *de novo* fatty acid synthesis occurs in plastids and thus KAS is expected to be targeted to the plastids. Alignment of chilli and barley KAS1 (Siggaard-Andersen *et al*., 1991) sequences indicated the presence of a 46-amino acid transit peptide. The calculated size of chilli KAS without the putative signal sequence was 47.5 kDa, in good agreement with the size of the observed protein band in placental extracts.

The enzyme activity encoded by the chilli *Kas* was determined directly on the induced *E. coli* lysate. Other studies have had mixed results with studying ketoacyl synthases in this manner. Recombinant expression of KAS1 from *Cuphea hookeriana* did not result in an active enzyme (Dehesh *et al*., 1998), while a cDNA for KAS3 from leek expressed in *E. coli* catalysed the expected condensation reaction (Chen and Post-Beittenmiller, 1996). The chilli KAS over-expressed in *E. coli* performed the condensation reaction that results in the elongation of fatty acids and this activity was inhibited by 20 µM cerulenin, thus confirming the annotation of the gene as KAS and suggesting that this gene product is most similar to class I KAS enzymes.

Recent studies in transgenic plants demonstrate that both KAS and thioesterase contribute to the regulation of fatty acid chain length (Dehesh *et al*., 1998; Leonard *et al*., 1998). The role of chilli *Fat* in capsaicinoid biosynthesis would be a novel function for this type of thioesterase. Typically, FATB accepts saturated acyl-ACP substrates of varying length, while FATA is specific to unsaturated fatty acids and acts on C18:1, oleic, acyl-ACPs (Jones *et al*., 1995). In this study, it was found that a clone that clearly groups with FatA genes, based on amino acid sequence, was differentially expressed in pungent tissue. Whether this chilli FAT is a member of a new FAT class or is a member of a FATA subgroup able to recognize capsaicinoid branched-chain acyl groups in place of oleic ones remains to be determined.

The positions of *Acl*, *Fat* and *Kas* were mapped in the chilli genome by RFLP and integrated into the genetic map of Livingstone *et al*. (1999). *Acl* and *Fat* were present as single copies in the genome, while *Kas* occurs as two linked homologues confirmed by the detection of limited recombination in the AC population. None of the possible locations for these three cloned transcripts overlapped with the genomic position of *C* (Blum *et al*., 2002) therefore, these three genes were excluded as candidates for *C*.

There is both a qualitative component and a quantitative component to the heritable variation in capsaicinoid levels in chilli (Blum *et al*., 2002; Zewdie and Bosland, 2000*a*, *b*, *c*). Thorup *et al*. (2000) demonstrated that prior knowledge of QTL candidate genes can be a great advantage in determining the underlying control of quantitative traits within the Solanaceae. Blauth *et al*. (1999) mapped QTLs for short and medium length fatty acids and branched-chain fatty acids in acyl sugars in tomato. An effort is currently underway to map tomato homologues for these chilli genes to define their position relative to QTL that affect fatty acid profiles of acyl sugars in tomato.

Acknowledgements

This work was supported in part by the NM Agricultural Experiment Station and by grants from the NIH (GM S06 08136 and GMO7766724) to M O'C and the United States-Israel Binational Agricultural Research and Development Fund Grant No. IS-3225-01C, USDA IFAFS Award No. 2001-52100-113347 and a gift from Kalsec, Inc., Kalamazoo, MI to MJ. The authors thank Paul Todd for critical review of the manuscript.

References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25,** 3389–3402.

Aluru M, Curry J, O'Connell MA. 1998. Nucleotide sequence of a 3-oxoacyl-[Acyl-Carrier-Protein] synthase (3-ketoacyl-ACP synthase) gene (accession no. AF085148) from habanero chile PGR98–181. *Plant Physiology* **118,** 1102.

Andrews J. 1995. *Peppers: the domesticated capsicums*. Austin: University of Texas Press.

Bennett DJ, Kirby GW. 1968. Constitution and biosynthesis of capsaicin. *Journal of the Chemical Society* **(C),** 442–446.

Blauth SL, Steffens JC, Churchill GA, Mutschler MA. 1999. Identification of QTLs controlling acylsugar fatty acid composition in an intraspecific population *of Lycopersicon pennellii* (Corr.) D'Arcy. *Theoretical and Applied Genetics* **99,** 373–381.

Blum E, Kede L, Mazourek M, Yoo EY, Jahn M, Paran I. 2002. Molecular mapping of the *C* locus for presence of pungency in *Capsicum*. *Genome* **45,** 702–705.

Burke BA, Goldsby G, Mudd JB. 1987. Polar epicuticular lipids of *Lycopersicon pennellii*. *Phytochemistry* **26,** 2567–2571.

Chen J, Post-Beittenmiller D. 1996. Molecular cloning of a cDNA encoding 3-ketoacyl-acyl carrier protein synthase III from leek. *Gene* **182,** 45–52.

Curry J, Aluru M, Mendoza M, Nevarez J, O'Connell MA. 1999. Transcripts for capsaicinoid biosynthetic genes are differentially accumulated in pungent and non-pungent *Capsicum* spp. *Plant Science* **148,** 47–57.

Dehesh K, Edwards P, Fillatti J, Slabaugh M, Byrne J. 1998. KAS IV: a 3-ketoacyl-ACP synthase from *Cuphea* sp. is a medium chain specific condensing enzyme. *The Plant Journal* **15,** 383–390.

Dixon RA, Paiva NL. 1995. Stress-induced phenylpropanoid metabolism. *The Plant Cell* **7,** 1085–1097.

Emanuelsson O, Nielsen H, Brunak S, von Heijne G. 2000. Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *Journal of Molecular Biology* **300,** 1005–1016.

Fujiwake H, Suzuki T. Iwai K. 1980. Intracellular localization of capsaicin and its analogues in *Capsicum* fruit. II. The vacuole as the intracellular accumulation site of capsaicinoid in the protoplast of *Capsicum* fruit. *Plant Cell Physiology* **21,** 1023–1030.

Grube RC, Zhang Y, Murphy JF, Loaiza-Figueroa F, Lackney VK, Providenti R, Jahn MK. 2000. New source of resistance to Cucumber mosaic virus in *Capsicum frutescens*. *Plant Disease* **84,** 885–891.

Harwood JL. 1996. Recent advances in the biosynthesis of plant fatty acids. *Biochimica et Biophysica Acta* **1301,** 7–56.

Henikoff S, Henikoff JG. 1994. Protein family classification based on searching a database of blocks. *Genomics* **19,** 97–107.

Iwai K, Suzuki T, Fujiwake H. 1979. Formation and accumulation of pungent principle of hot pepper fruits, capsaicin and its analogues, in *Capsicum annuum* var. *annuun* cv. karayatsubusa at different growth stages after flowering. *Agricultural Bioliogical Chemistry* **43,** 2493–2498.

Jones A, Davies HM, Voelker TA. 1995. Palmitoyl-acyl carrier protein (ACP) thioesterase and the evolutionary origin of plant acyl-ACP thioesterases. *The Plant Cell* **7,** 359–371.

Kahn TL, Fender SE, Bray EA, O'Connell MA. 1993. Characterization and expression of drought- and ABA-regulated tomato genes in the drought-resistant species *Lycopersicon pennellii*. *Plant Physiology* **103,** 597–605.

Kaneda T. 1967. Biosynthesis of long-chain hydrocarbons. I. Incorporation of L-valine, L-threonine, L-isoleucine and L-leucine into specific branched-chain hydrocarbons in tobacco. *Biochemistry* **6,** 2023–2032.

Kopka J, Robers M, Schuch R, Spener F. 1993. Acyl carrier proteins from developing seeds of *Cuphea lanceolata*. *Planta* **191,** 102–111.

Kosambi DD. 1944. The estimation of map distances from recombination frequency. *Annals of Eugenetics* **12,** 172–175.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Leete E, Louden MCL. 1968. Biosynthesis of capsaicin and dihydrocapsaicin in *Capsicum frutescens*. *Journal of the American Chemical Society* **90,** 6837–6841.

Leonard JM, Knapp SJ, Slaubagh MB. 1998. A *Cuphea* β-ketoacyl -ACP synthase shifts the synthesis of fatty acids towards shorter chains in *Arabidopsis* seeds expressing *Cuphea FatB* thioesterases. *The Plant Journal* **13,** 621–628.

Lincoln SE, Daly MJ, Lander ES. 1993. *Constructing a genetic linkage map with MAPMAKER/EXP v3.0: a tutorial and reference manual*. Cambridge, MA: Whitehead Institute Technical Report.

Livingstone KD, Lackney VK, Blauth JR, van Wijk R, Jahn MK. 1999. Genome mapping in *Capsicum* and the evolution of genome structure in the Solanaceae. *Genetics* **152,** 1183–1202.

Mackintosh RW, Hardie DG, Slabas AR. 1989. A new assay procedure to study the induction of β-ketoacyl-ACP synthase I and II, and the complete purification of β-ketoacyl-ACP synthase I from developing seed of oil seed rape *Brassica napus*. *Biochimica et Biophysica Acta* **1002,** 114–124.

Markai S, Marchand PA, Mabon F, Baguet E, Billault I, Robins RJ. 2002. Natural deuterium distribution in branched chain medium-length fatty acids is non-statistical: a site-specific study by quantitative ^{2}H NMR spectroscopy of the fatty acids of capsaicinoids. *ChemBioChem* **3,** 212–218.

Nakai K, Kanehisa M. 1992. A knowledge base of predicting protein localization sites in eukaryotic cells. *Genomics* **14,** 897–911.

Nakayama RM, Matta FB. 1985. 'NuMex R Naky' chile pepper. *HortScience* **20,** 961–962.

Ochoa-Alejo N, Gomez-Peralta J. 1993. Activity of enzymes involved in capsaicin biosynthesis in callus tissue and fruits of chili pepper (*Capsicum annuum* L.). *Journal of Plant Physiology* **141,** 147–152.

Pelle R, Murphy NB. 1993. Northern hybridization: rapid and simple electrophoretic conditions. *Nucleic Acids Research* **21,** 2783–2784.

Shimakata T, Stumpf PK. 1983. Purification and characterization of β-ketoacyl-ACP synthetases I from *Spinacia oleracea* leaves. *Archives of Biochemistry and Biophysics* **220,** 39–45.

Siggaard-Andersen M, Kauppinen S, Wettstein-Knowles P. 1991. Primary structure of a cerulenin-binding β-ketoacyl-[acyl carrier protein] synthase from barley chloroplasts. *Proceedings of the National Academy of Sciences, USA* **88,** 4114–4118.

Sukrasno N, Yeoman MM. 1993. Phenylpropanoid metabolism during growth and development of *Capsicum frutescens* fruits. *Phytochemistry* **32,** 839–844.

Suzuki T, Fujiwake H, Iwai K. 1980. Intracellular localization of capsaicinoid in *Capsicum* fruit. 1. Microscopic investigation of the structure of the placenta of *Capsicum annuum* var. *annuum* cv. karayatsubusa. *Plant Cell Physiology* **21,** 839–853.

Suzuki T, Kawada T, Iwai K. 1981. Biosynthesis of acyl moieties of capsaicin and its analogues from valine and leucine in *Capsicum* fruits. *Plant Cell Physiology* **22,** 23–32.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence

alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22,** 4673–4680.

Thorup TA, Tanyloc B, Livingstone KD, Popovsky S, Paran I, Jahn M. 2000. Candidate gene analysis of organ pigmentation loci in the Solanaceae. *Proceedings of the National Academy of Sciences, USA* **97,** 11192–11197.

Towbin H, Staehelin T, Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences, USA* **76,** 4350–4354.

van der Hoeven RS, Steffens JC. 2000. Biosynthesis and elongation of short- and medium-chain-length fatty acids. *Plant Physiology* **122,** 275–282.

Yuan L, Nelson BA, Caryl G. 1996. The catalytic cysteine and histidine in the plant acyl-acyl carrier protein thioesterases. *Journal of Biological Chemistry* **271,** 3417–3419.

Zamski E, Shoham O, Palevitch D, Levy A. 1987. Ultrastructure of capsaicinoid-secreting cells in pungent and non-pungent red pepper (*Capsicum annuum* L.) cultivars. *Botanical Gazette* **148,** 1–6.

Zewdie Y, Bosland PW. 2000*a*. Evaluation of genotype, environment and genotype-by-environment interaction for capsaicinoids in *Capsicum annuum* L. *Euphytica* **111,** 185–190.

Zewdie Y, Bosland PW. 2000*b*. Capsaicinoid inheritance in an interspecific hybridization of *Capsicum annuum* × *C. chinense*. *Journal of the American Society of Horticultural Science* **125,** 448–453.

Zewdie Y, Bosland PW. 2000*c*. Pungency of chile (*Capsicum annuum* L.) is affected by node position. *HortScience* **35,** 1174.

Zhang Y. 1997. Detection of restriction fragment length polymorphism, construction of a molecular linkage map, and mapping and tagging cucumber mosaic virus resistance loci in *Capsicum*. PhD thesis, Cornell University. Ithaca, NY.