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The *Pun1* gene for pungency in pepper encodes a putative acyltransferase

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Summary

Pungency in *Capsicum* fruits is due to the accumulation of the alkaloid capsaicin and its analogs. The biosynthesis of capsaicin is restricted to the genus *Capsicum* and results from the acylation of an aromatic moiety, vanillylamine, by a branched-chain fatty acid. Many of the enzymes involved in capsaicin biosynthesis are not well characterized and the regulation of the pathway is not fully understood. Based on the current pathway model, candidate genes were identified in public databases and the literature, and genetically mapped. A published EST co-localized with the *Pun1* locus which is required for the presence of capsaicinoids. This gene, *AT3*, has been isolated and its nucleotide sequence has been determined in an array of genotypes within the genus. AT3 showed significant similarity to acyltransferases in the BAHD superfamily. The recessive allele at this locus contains a deletion spanning the promoter and first exon of the predicted coding region in every non-pungent accession tested. Transcript and protein expression of *AT*3 was tissue-specific and developmentally regulated. Virus-induced gene silencing of *AT3* resulted in a decrease in the accumulation of capsaicinoids, a phenotype consistent with *pun1*. In conclusion, gene mapping, allele sequence data, expression profile and silencing analysis collectively indicate that the *Pun1* locus in pepper encodes a putative acyltransferase, and the *pun1* allele, used in pepper breeding for nearly 50 000 years, results from a large deletion at this locus.

Keywords: *Capsicum*, *Pun1*, capsaicin, pungency, acyltransferase, secondary metabolism.

Introduction

Pepper (*Capsicum*) was among the earliest domesticated plant genera, based upon archeological evidence from Central America dating back at least 7000 years (Basu and Krishna De, 2003). In 1876, the alkaloid capsaicin was identified as the compound responsible for the characteristic pungency in pepper (Suzuki *et al.*, 1980; Thresh, 1876). Within the pepper fruit, capsaicin and its analogs, known collectively as capsaicinoids (Bennett and Kirby, 1968), are synthesized in the epidermal cells of the placental dissepiment beginning approximately 20 days post-anthesis (dpa), and accumulate in pockets or blisters along the epidermis (Iwai *et al.*, 1979; Ohta, 1962; Suzuki *et al.*, 1980; Zamski *et al.*, 1987). Biosynthesis of this group of compounds is unique to the *Capsicum* genus, and has driven the domestication of several species including *C. annuum*, *C. frutescens* and *C. chinense*, which are now valued for use as vegetables, spices, and for medicinal and industrial purposes (Andrews, 1984; Walsh and Hoot, 2001). Although pungency is highly desirable or essential for many uses, non-pungent peppers have also been selected for use as a

vegetable and as the spice known as paprika. Bell peppers were first described nearly 500 years ago; the earliest reference to a bell variety specifically selected for cultivation occurred in 1774 (Boswell, 1937).

Recent years have seen an explosion of information about the directed applications of capsaicin in food and medicine and the evolutionary role these compounds play in *Capsicum*. The nociceptor responsible for perception of these compounds, the vanilloid receptor 1 (VR1), is localized to peripheral pain-sensing nerve fibers (Caterina *et al.*, 1997, 2000). Birds, the primary dispersal agent of *Capsicum* seeds, lack a functional VR1 receptor, hence are oblivious to the presence of capsaicin (Jordt and Julius, 2002; Tewksbury and Nabhan, 2001).

In humans, VR1 is involved in pain sensing pathways. Capsaicin has long been used in topical analgesic preparations and clinical trials have supported capsaicin as an effective treatment for various types of nerve pain and arthritis, although the mechanism is not fully understood (Deal *et al.*, 1991; Watson *et al.*, 1993). Beyond this use as an analgesic, capsaicin has also been described in the treatment of diverse bladder and digestive syndromes, and as a tumorigenesis chemopreventive agent in cancer treatment through its effects on intracellular signaling pathways (Chancellor and De Groat, 1999; Cruz, 2004; Han *et al.*, 2001; Srinivasan, 2005; Surh, 2002, 2003). Evidence also supports an antimicrobial activity. Capsaicin used in spice has been reported to inhibit or kill microorganisms that contribute to food spoilage (Billing and Sherman, 1998). Recently, a physiological basis for the paradoxical attraction between humans and hot peppers has been proposed. Natural capsaicin analogs identified in mammalian brains presumably function as neurotransmitters related to positive sensations (Appendino *et al.*, 2002; Chu *et al.*, 2003; Huang *et al.*, 2002). The ecology and physiology of capsaicin therefore both explains the selective advantage of these compounds, presumably evolved to deter mammalian herbivory, as well as the human desire to consume spicy foods.

Capsaicinoids are produced by the condensation of vanillylamine, derived from phenylalanine, with a branched-chain fatty acid, derived from either valine or leucine (Bennett and Kirby, 1968; Leete and Louden, 1968; Sukrasno and Yeoman, 1993; Suzuki *et al.*, 1981). A vast array of *Capsicum* species and cultivars are now available with varying degrees of pungency (Zewdie-Tarekegen, 1999). Further variation in capsaicinoid content results from the influences of the plant growth environment (Blum *et al.*, 2003; Harvell and Bosland, 1997; Zewdie and Bosland, 2000).

Considering the economic and agricultural importance of this pathway, it is surprising that relatively little is known, particularly at the molecular level, concerning the genetics, biosynthesis, subcellular localization and cellular structures required for pungency accumulation in peppers (Blum *et al.*, 2003). Nearly 100 years ago, Webber reported that the absence of pungency was controlled by a single recessive gene, *pun1* (formerly known as *c*), epistatic to all other pungency-related genes (Boswell, 1937; Webber, 1911). At present, the *pun1* allele is the only confirmed mutation that has a qualitative affect on the presence/absence of capsaicinoids (Blum *et al.*, 2002 and references therein). A cleaved-amplified polymorphic sequence marker linked at a small genetic distance was developed to assist in breeding programs. However, *Pun1*, often presumed to be a master regulator of the pathway, itself has remained elusive (Blum *et al.*, 2002). Here we report the identity of *Pun1* and show that in contrast to earlier predictions, this gene encodes a putative acyltransferase important in capsaicin biosynthesis. We describe the sequence of a very famous allele at this locus that is responsible for the absence of pungency in pepper fruit. Finally, we describe the expression profile of the wild-type transcript and its gene product through fruit development and demonstrate that reducing this transcript and gene product *in planta* reduces accumulation of capsaicinoids in fruit.

Results

Identification of SB2-66 as a candidate gene for Pun1

Systematic screens of genes implicated in capsaicinoid biosynthesis were performed to determine if they co-localized with any previously mapped genomic regions known to affect pungency (e.g., *Pun1*, QTLs) (Blum *et al.*, 2002, 2003). SB2-66, a cDNA clone derived from a published suppression subtraction hybridization library (Kim *et al.*, 2001), co-segregated exactly with pungency and mapped to the same region as *Pun1* in a large F_2 mapping population ($n = 242$) constructed from *C. frutescens* BG2816, a pungent wild pepper, × *C. annuum* 'Maor', a non-pungent inbred Bell pepper (Figure 1a) (Blum *et al.*, 2002). In addition, various pungent and non-pungent genotypes were surveyed to detect polymorphisms in SB2-66. The full-length cDNA was isolated (see below) and used as a probe on a DNA blot of genomic DNA isolated from pungent and non-pungent genotypes. In the pungent genotypes, the probe hybridized to five major fragments in the pepper genome, suggesting the presence of a small gene family. All non-pungent genotypes were missing one major hybridization fragment (Figure 1b). The co-localization of SB2-66 with *Pun1* and the presence/absence of a major hybridization band that showed a pattern consistent with the presence/absence of pungency identified SB2-66 as a strong candidate for *Pun1*.

Isolation of full-length cDNA and genomic sequence of Pun1 *from* C. chinense *and* C. annuum

To obtain full-length cDNA sequence for *Pun1*, 5′-RACE was performed using cDNA reverse transcribed from mRNA

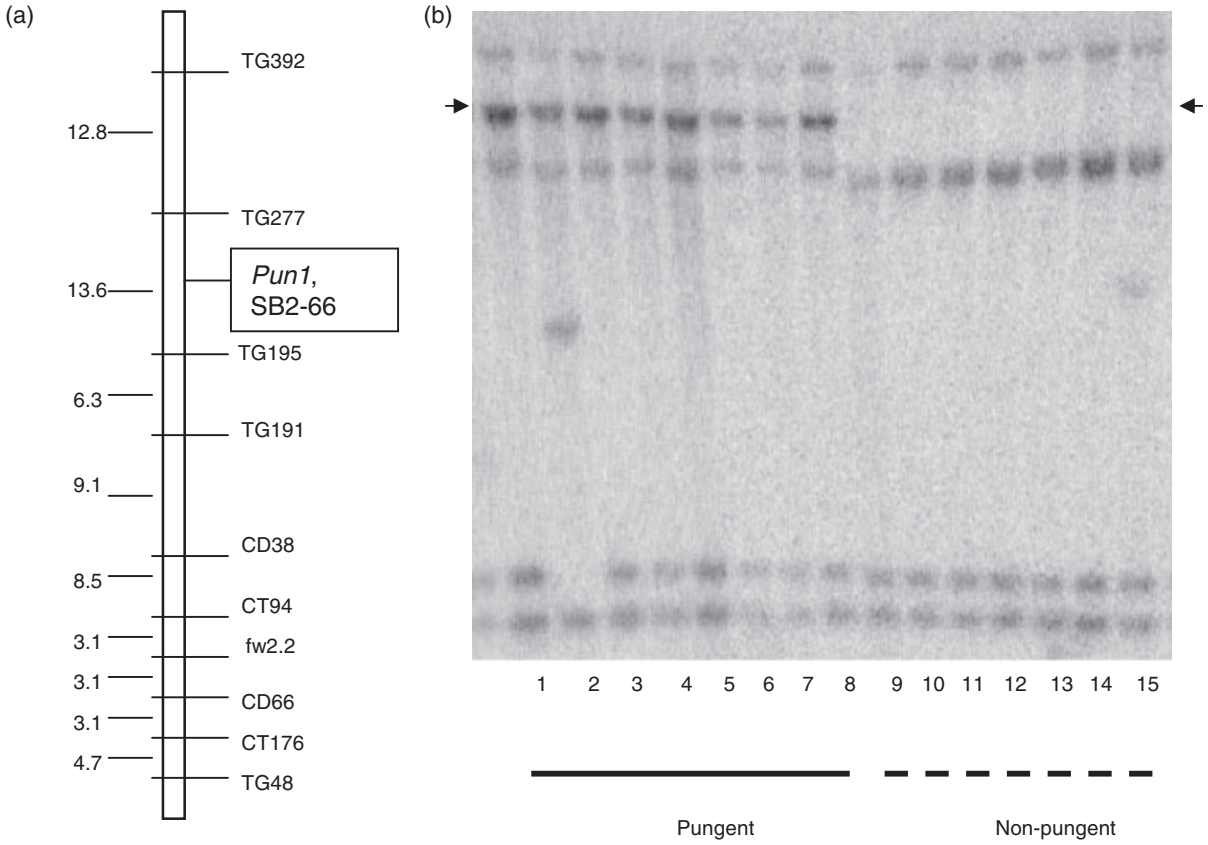

Figure 1. Co-localization of EST SB2-66 with *Pun1* and survey of DNA polymorphism in pungent and non-pungent genotypes.
(a) SB2-66 co-localized with *Pun1* in an F_2 mapping population ($n = 242$) derived from *Capsicum frutescens* BG 2816 (*Pun1/Pun1*, a pungent genotype) × *C. annuum* 'Maor' (*pun1/pun1* a non-pungent genotype). A diagram of the genetic map of *Capsicum* chromosome 2. RFLP markers are to the right and distances in centimorgans are to the left of the linkage bars. Figure adapted from Blum *et al.* (2002).
(b) DNA polymorphism survey of EST clone SB2-66 in pungent and non-pungent genotypes. Genomic DNA was isolated from leaves and digested with the *Bst*NI restriction enzyme and probed with labeled cDNA. Lanes: 1, Habanero (*C. chinense*); 2, BG2814-6 (*C. frutescens*); 3, Tabasco (*C. frutescens*); 4, Chung-Yang (*C. annuum*); 5, Hot 1493 (*C. annuum*); 6, RNaky (*C. annuum*); 7, Santa Fe Grande (*C. annuum*); 8, Thai Hot (*C. annuum*); 9, Cal Wonder (*C. annuum*); 10, Jupiter (*C. annuum*); 11, King of the North (*C. annuum*); 12, Lehava (*C. annuum*); 13, Maor (*C. annuum*); 14, Sweet 3575 (*C. annuum*); 15, Yolo Y (*C. annuum*).

from immature *C. chinense* 'Habanero' fruit at the onset of pungency, approximately 20 dpa. Sequence analysis revealed that the 1547 bp full-length cDNA contained an open-reading frame of 1323 bp, a 5′-UTR of 43 bp and a 3′-UTR of 181 bp. Comparisons between the full-length genomic and cDNA sequence revealed two exons of 738 and 585 bp, respectively, and one intron of 348 bp. The putative promoter was identified by sequencing 1.8 kb upstream of the transcription start site.

Capsicum annuum is the largest, most diverse and the most economically important species within the genus. It includes bell, paprika, cayenne and jalapeno peppers. To investigate the structure of the *Pun1* locus within *C. annuum*, the homologous sequence from *C. annuum* 'Thai Hot', a pungent variety, was amplified using primers based on the Habanero genomic sequence. The two sequences showed 98% nucleotide sequence identity, based on both coding and non-coding regions, and contained the same gene structure.

Survey of allelic diversity of Pun1

The genomic sequence at this locus was determined in several *pun1*/*pun1* genotypes. Consistent with the results from the Southern blot, a large 2.5 kb deletion spanning 1.8 kb of the putative promoter and 0.7 kb of the first exon was observed in the *C. annuum* Bell peppers 'Jupiter' and 'Maor', and the non-pungent Jalapeno 'Sweet 3575' (Figure 2a). The conservation of the deletion between Bell and Jalapeno peppers indicated that the deletion was likely widespread throughout *C. annuum*. This deletion was absent in all pungent (*Pun1*/*Pun1*) genotypes tested (Figure 2b).

Sequence analysis

The simple modular architecture research tool (SMART) program predicted the presence of an acyltransferase domain within *Pun1* (Schultz *et al.*, 1998). BLAST searches using

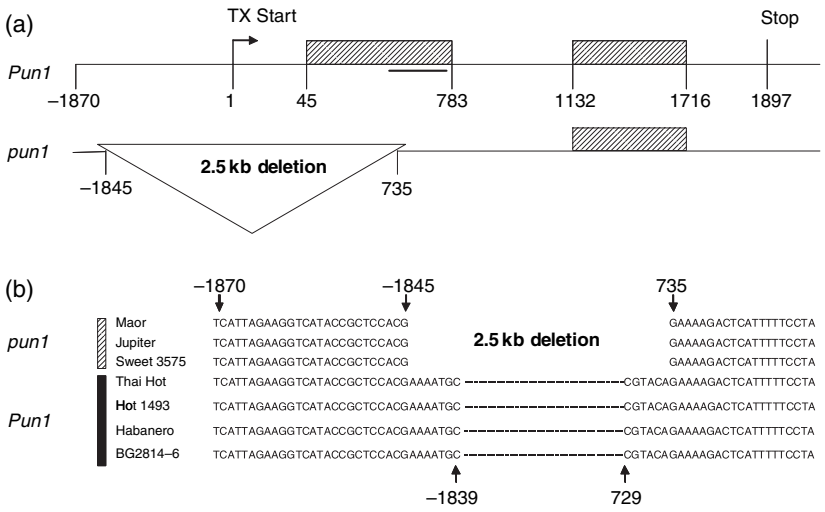

Figure 2. Genomic DNA sequence and structure at the *Pun1* locus from pungent (*Pun1*/*Pun1*) and non-pungent (*pun1*/*pun1*) genotypes.
(a) Schematic diagram of the *Pun1* locus. Exons (closed boxes) were deduced from cDNA sequence. The location of the PCR-amplified fragment used for virus-induced gene silencing is shown by a solid black bar. The deletion in the *pun1* allele is represented with an inverted triangle.
(b) Nucleotide sequences bordering the deletion in *pun1* genotypes (*Capsicum annuum* Maor, Jupiter, Sweet 3575) and *Pun1* genotypes (*C. annuum* Thai Hot, Hot 1493, *C. frutescens* BG2814-6, *C. chinense* Habanero). Non-pungent and pungent genotypes are indicated by open gray and black boxes, respectively.

the predicted amino acid sequence showed that *Pun1* has significant similarity (>40%) to a number of acyltransferase genes from diverse plant species (Figure 3). The presence of the highly conserved active site, HXXXDG, and the DFGWGKP motif indicates that this gene belongs to the BAHD superfamily of acyltransferases (St. Pierre and De Luca, 2000). Several of these genes such as deacetylvindoline 4-*O*-acetyltransferase (*DAT*) and benzylalcohol acetyltransferase (*BEAT*) have been functionally characterized with respect to their involvement in various secondary metabolic pathways (Nam *et al.*, 1999; St. Pierre *et al.*, 1998). Two other acyltransferases isolated from Habanero fruit, Acyltransferase 1 (*AT1*, AAN85435) and Acyltransferase 2 (*AT2*, AAN85436) were also found to show significant amino acid similarity (>40%) using BLAST searches. *AT1* and *AT2* did not map to the chromosome region containing *Pun1* and consequently were eliminated as candidate genes for *Pun1*. Based on its sequence similarity, we will hereafter refer to the gene identified in this study as Acyltransferase 3 (*AT3*).

Regulation of AT3 *expression in pungent and non-pungent genotypes*

In Habanero, *AT3* expression was specific to the placenta dissepiment, the site of capsaicin biosynthesis (Figure 4a). Based on a new model of capsaicinoid biosynthesis shown in Figure 4b, *AT3* and 11 other previously identified capsaicinoid candidate genes were analyzed for their expression through fruit development in *C. chinense* Habanero (*Pun1*/*Pun1*) and *C. annuum* Bell (*pun1*/*pun1*) pepper types (Figure 4c) (Aluru *et al.*, 2003; Blum *et al.*, 2003; Curry *et al.*, 1999). *pAMT*, *Kas*, *FatA* and *AT3* expression initiated strongly at 20 dpa but was not detectable by 40 dpa. *Pal*, *Ca4H*, *Comt* and *6B11* expression was detected as early as 10 dpa and gradually decreased through fruit development. *BCAT* and *Acl* were constitutively expressed though fruit development. *bZip-3B2* and *bZip* expression was detectable at 10 dpa and increased through fruit development. In *pun1*/*pun1* Bell pepper, expression of *AT3* and all other candidate genes, except *BCAT* and *Acl*, was not detectable or significantly reduced during fruit development.

The interspecific comparison of gene expression between *C. chinense* and *C. annuum* shown above was refined to focus specifically on *AT3* expression in different organs and through fruit development in *Pun1*/*Pun1* and *pun1*/*pun1* genotypes within the most familiar and diverse domesticated species, *C. annuum*. Habanero is the most pungent pepper commercially available. However, significant differences in gene content and expression have been noted between the *Capsicum* species (K. Liu and M. M. Jahn, unpublished data). *Capsicum annuum* Thai Hot (*Pun1*/*Pun1*) was selected because it is a pungent variety that is amenable to virus-induced gene silencing (VIGS). Baseline expression data prior to VIGS experiments via RNA blot analysis indicated that *AT3* transcripts were first detected in Thai Hot at 20 dpa (Figure 5a). *AT3* transcripts were not detectable by 40 dpa. *AT3* expression in Bell pepper (*pun1/pun1*) was conspicuously absent in all organs and at all stages of fruit development. Immunoblot analysis of AT3 protein accumulation was consistent with observations of *AT3* transcript expression. AT3 began to accumulate at approximately 20 dpa and was absent by 50 dpa in fruit of Thai Hot (Figure 5b). As predicted, AT3 was not detectable in Bell pepper. Capsaicinoid accumulation, monitored by HPLC, initiated with expression of *AT3*. Capsaicinoids were absent in Bell pepper, but in Thai Hot steadily increased through development starting at 20 dpa (Figure 5c). Consistent with observations made in Habanero, *AT3* expression in Thai Hot was examined in various fruit tissue. This transcript was expressed solely in the placental tissue and absent from pericarp and seeds (data not shown). The absence of this transcript in Bell pepper is consistent with the nature of the lesion observed at *Pun1*.

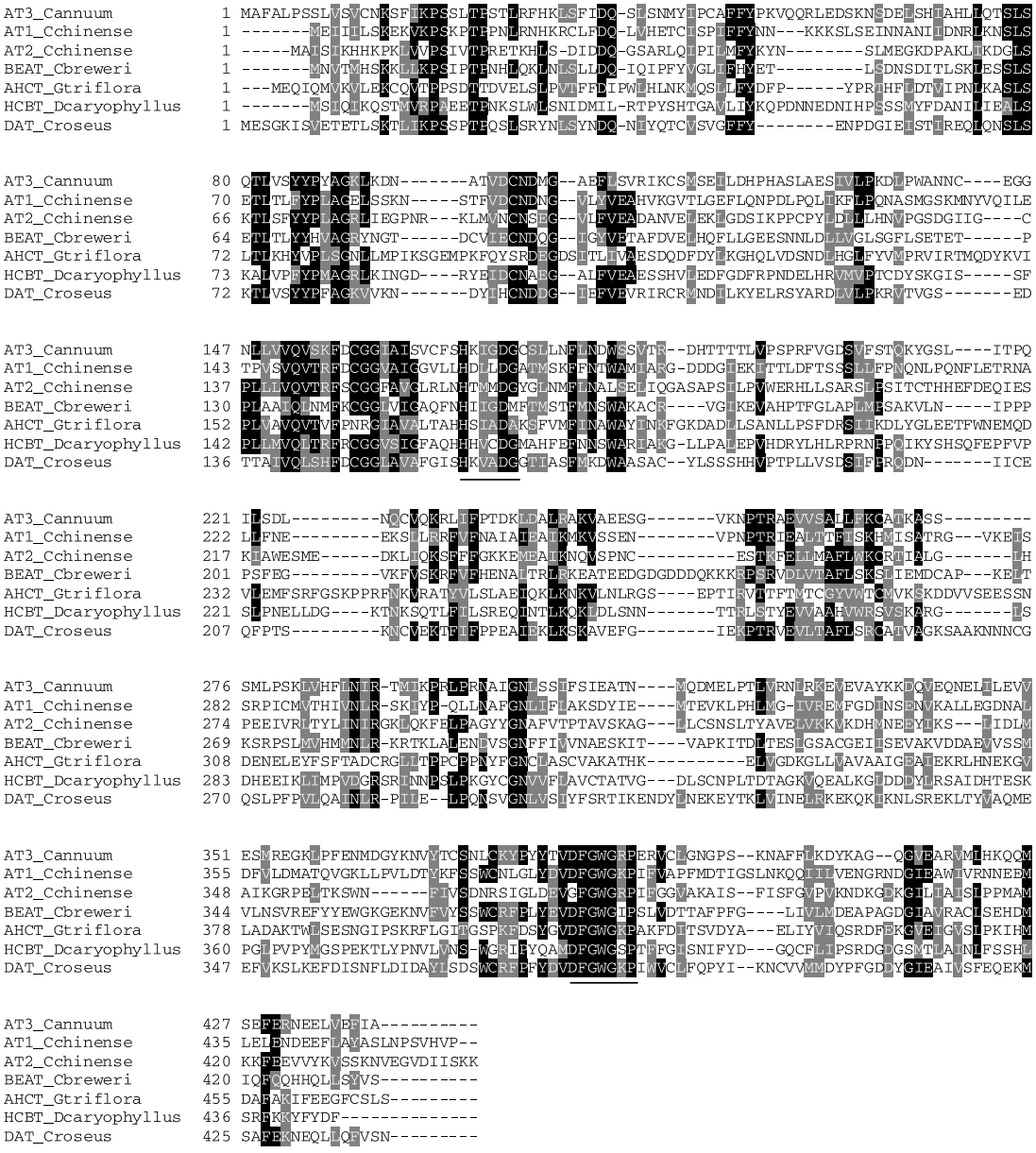

Figure 3. Alignment of the deduced amino acid sequence of AT3 from *Capsicum annuum* with similar sequences of plant origin.
AT3 was aligned to two other *Capsicum* acyltransferases, AT1 (*C. chinense*, AAN85435) and AT2 (*C. chinense*, AAN85436) and to benzylalcohol acetyltransferase (BEAT, *Clarkia breweri*, AAF04787), anthocyanin 5-aromatic acyltransferase (AHCT, *Gentiana triflora*, BAA74428), anthranilate *N*-hydroxycinnamoyl/benzoyltransferase (HCBT, *Dianthus caryophyllus*, CAB06427), deacetylvindoline 4-*O*-acetyltransferase (DAT, *Catharansus roseus*, AAC99311) acyltransferases of the BAHD superfamily. Gaps are indicated by dashes. Conserved amino acids are shaded black and similar amino acids are shaded gray. Conserved domains are underlined. Amino acid sequences were aligned using CLUSTALW (Chenna *et al.*, 2003; Thompson *et al.*, 1994).

Virus-induced gene silencing of AT3

Pepper is recalcitrant to transformation, consequently VIGS was used to examine the function of *AT3 in vivo*. For VIGS analysis of *AT3*, a tobacco rattle virus (TRV) construct was generated, TRV:AT3 (Liu *et al.*, 2002). This construct contained a 0.4-kb region from the first exon spanning the predicted active site (indicated in Figure 2a). Mean capsaicinoid accumulation in mature red fruit from TRV:AT3-treated plants was 2100 p.p.m. compared with 4500 p.p.m. in un-inoculated plants, a reduction of over 50% (Figure 6b). This reduction in capsaicinoid accumulation is a phenotype consistent with a proposed role for this gene in capsaicin biosynthesis. In contrast, capsaicin accumulation in empty TRV vector-treated plants, 7700 p.p.m., was higher than the un-inoculated control. This increase is likely a result of stress from the virus infection and wounding during infiltration and is consistent with an increase in pungency observed in response to other

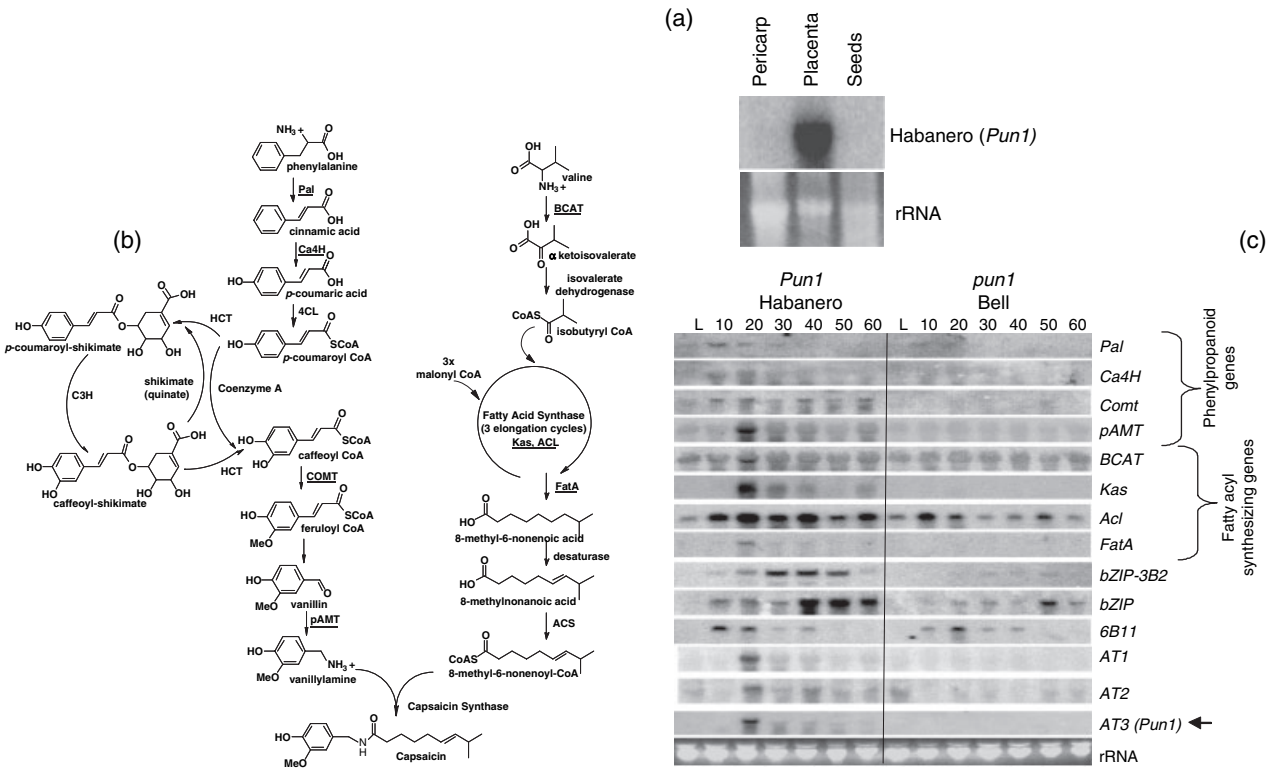

Figure 4. RNA gel blot analysis of expression of capsaicinoid biosynthetic candidate genes in *Pun1* and *pun1* genotypes from *Capsicum chinense* Habanero and *C. annuum* Bell pepper, respectively.

(a) *AT3* is expressed specifically in the placenta of *Pun1* fruit. Immature Habanero fruit was harvested then dissected into pericarp, placenta and seeds, and analyzed for *AT3* expression. The full-length cDNA of *AT3* was used as a probe. Ethidium bromide-stained ribosomal RNA was used as a control.

(b) Proposed model of the capsaicin biosynthetic pathway. Enzymes are shown adjacent to the reactions they catalyze. For those enzymes underlined, the gene encoding them has been cloned in *Capsicum*. *Kas* is the only enzyme functionally characterized in *Capsicum*; all others are based on differential gene expression. PAL, phenylalanine ammonia lyase; Ca4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate CoA ligase; HCT, hydroxycinnamoyl transferase; C3H, coumaroyl shikimate/quinate 3-hydroxylase; COMT, caffeic acid *O*-methyltransferase; pAMT, aminotransferase; BCAT, branched-chain amino acid transferase; KAS, β-ketoacyl ACP synthase; ACL, acyl carrier protein; FAT, acyl-ACP thioesterase; ACS, acyl-CoA synthetase; CS, capsaicin synthase.

(c) Northern survey of *AT3* and other candidate genes involved in capsaicinoid biosynthesis through fruit development in *Pun1* and *pun1* genotypes. Blots containing RNA from leaves and different developmental stages of *C. chinense* Habanero and *C. annuum* Bell fruit were probed with existing capsaicinoid candidate genes as described in Blum *et al.* (2003). Ethidium bromide-stained rRNA was used as a control.

environmental stresses (Estrada *et al.*, 1999). Given the observed increase in capsaicinoid accumulation as a result of viral infection, TRV:AT3-infected plants likely had a reduction in capsaicinoid accumulation of over 70% (2100 p.p.m. compared with 7700 p.p.m.). Immunoblot analysis revealed significantly reduced amounts of AT3 protein in TRV:AT3-treated plants relative to controls (Figure 6a). Given the correlation between transcript and protein accumulation observed in Figure 5, it can be concluded that endogenous production of *AT3* transcripts in the TRV:AT3-treated plants is significantly reduced.

Phylogenetic analysis of AT3

As of June 2004, a PsiBLAST search identified 35 AT3 plant homologs in GenBank that had been functionally characterized. All of these enzymes were members of the BAHD superfamily of acyltransferases based on the presence of characteristic conserved motifs (Figure 3). A phylogenetic analysis was performed to determine the relationship of AT3 to these enzymes of known function. Two other putative acyltransferases of unknown function, AT1 and AT2, isolated from *C. chinense* Habanero were also included. At least six clades were resolved that were supported by similar enzymatic roles, as recognized in previous studies (Figure 7) (Burhenne *et al.*, 2003; Hoffmann *et al.*, 2003; St. Pierre and De Luca, 2000). CER2 from *Arabidopsis thaliana* and Glossy2 from *Zea mays* were used to root the tree (clade 6) because St. Pierre and De Luca (2000) determined this clade to be basal in the BAHD superfamily. Unlike the specialized functions of the other acyltransferases, CER2 and Glossy2 function in a common reaction of fatty acid elongation to produce epicuticular waxes (Tacke *et al.*, 1995; Xia *et al.*, 1996). The topology of this tree was consistent regardless of algorithm or evolutionary model with three exceptions. The bootstrap support of clade 1 varied greatly for sequences below AT3,

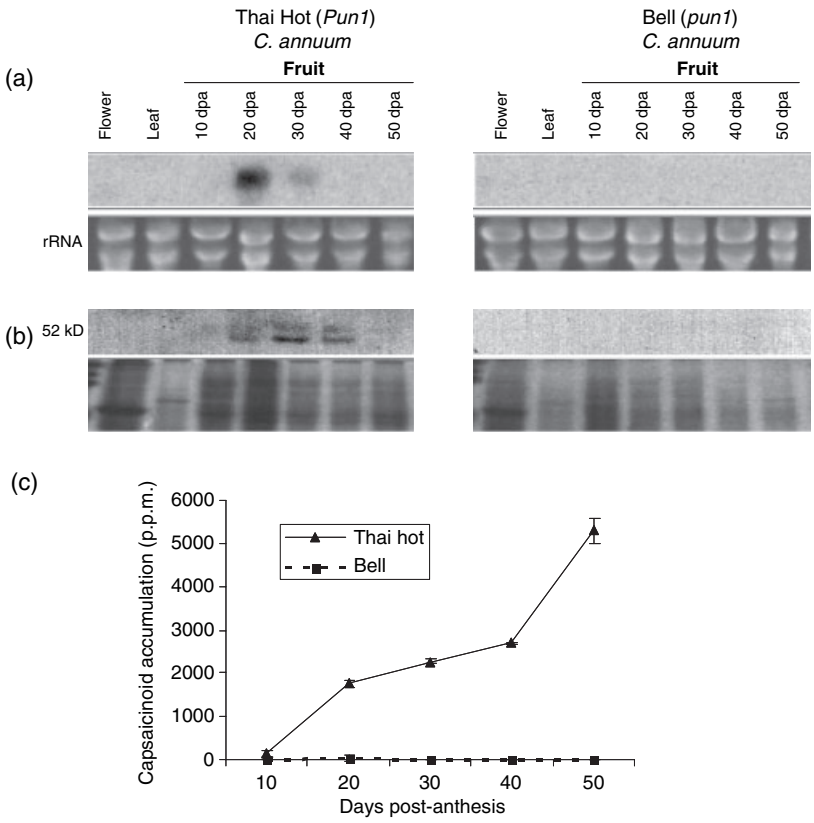

Figure 5. Analysis of *AT3* expression during fruit development in *Capsicum annuum Pun1* and *pun1* genotypes.
(a) *AT3* expression was analyzed in flower, leaf and fruit placenta through development. The full-length cDNA of *AT3* was hybridized to blots containing RNA from different developmental stages of *C. annuum* 'Thai Hot' and *C. annuum* Bell fruit placenta. Whole fruit was used at 10 days post-anthesis (dpa) due to the small size of the fruit.
(b) Immunoblot analysis of AT3 expression. Total protein was extracted from flowers, leaves and multiple stages of fruit placenta through development. A polyclonal antibody raised against the recombinant AT3 was used as the primary antibody and horseradish peroxidase-conjugated anti-rabbit IgG antibody as the secondary antibody at 1:500 dilution. Whole fruit was used for 10 dpa sample due to small size. Coomassie-stained protein loading controls from the same extractions are shown.
(c) HPLC analysis of capsaicinoid accumulation through fruit development is presented from fruits of *C. annuum* Thai Hot and Bell pepper. Due to fruit size, whole fruit samples were measured for Thai Hot and placental tissue samples for Bell pepper. Values represent the mean ± SD for at least three plants.

DAT and MAT depending on the methods employed. The placement of Gt5AT alternated from below to within the other anthocyanin acyltransferases of clade 2. ACT was also placed either basal to clades 3–5 or within clade 3. Burhenne *et al.* (2003) described ACT as a member of a distinct clade when uncharacterized close homologs of ACT from other genera were included.

Within the resulting tree, AT3 was placed immediately basal to DAT and MAT, both acyltransferases from *Catharanthus roseus* that catalyze *O*-acetylations in monoterpenoid indole alkaloid biosynthesis. This division was consistently well supported by a high bootstrap value. AT3 was closely related to AT1 in clade 1, which includes enzymes known to perform acylations of a variety of related phytochemicals. Within this clade was placed Ss5MaT2, an unusual anthocyanin acyltransferase because of its phylogenetic separation from other anthocyanin acyltransferases (Suzuki *et al.*, 2004). AT3 was not closely related to AT2, which grouped with ripening-related/wound-induced acyltransferases of clade 4. All three pepper acyltransferases are distinctly

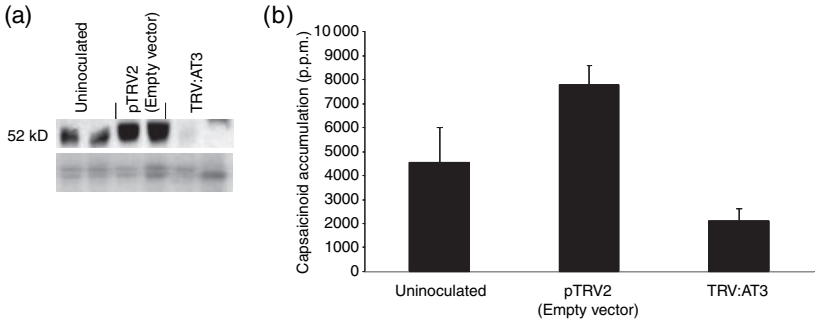

Figure 6. Effects of virus-induced gene silencing of *AT3* in *Capsicum* fruit.
(a) Immunoblot analysis of AT3 protein accumulation in silenced fruit (red ripe) from TRV:AT3-infected plants or equivalent tissue from un-inoculated and empty TRV vector (pTRV2)-infected plants. Replicate samples within each treatment are shown. Coomassie-stained protein loading controls from the same extractions are shown.
(b) HPLC analysis of capsaicinoid accumulation in un-inoculated, pTRV2 and TRV:AT3-infected plants. Values represent the mean ± SD for at least three plants.

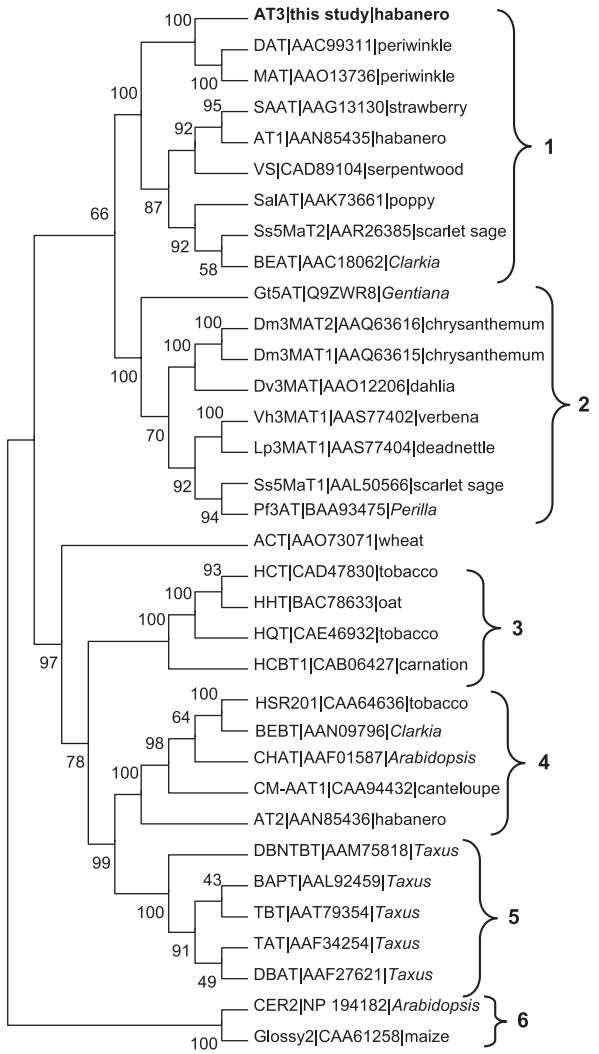

Figure 7. Phylogenetic relationship of *AT3* with other plant acyltransferases. A neighbor-joining tree comparing habanero acyltransferases, AT1, AT2 and AT3 to the 35 BAHD acyltransferases of known function. At least six clades were present in the resulting tree: (1) acyltransferases with diverse substrates, (2) anthocyanin acyltransferases, (3) hydroxycinnamoylransferases, (4) wound-inducible/ripening-related acytransferases, (5) acyltransferases of taxol biosynthesis, and (6) epicuticular wax enzymes. ACT constitutes a possible seventh clade. Branch labels include enzyme namelGenBank protein IDlsource plant. Numbers adjacent to nodes indicate bootstrap support as a percentage of 1000 replications.

separated from clades 2, 3 and 5 which catalyze anthocyanin, hydroxycinnamoyl, and taxol biosynthesis acylations, respectively. Currently, we are evaluating AT3 and the other acyltransferases to identify their potential roles in capsaicin biosynthesis.

Discussion

Capsicum originated in South America, most likely in the area now known as Bolivia (Andrews, 1984). The explorer Columbus played a key role in dispersing peppers throughout the world, returning to Spain with several pungent forms of *Capsicum*, most of which were members of the *C. annuum* species (Walsh and Hoot, 2001). From here, peppers spread throughout the world and eventually were reintroduced to the Americas. A single genetic source for non-pungency within *C. annuum* is suggested by the early identification of a non-pungent pepper in the 1500s that was widely distributed (Boswell, 1937), and documented and inferred pedigrees of non-pungent pepper varieties released by breeding programs in the United States, South America and Europe (Cook, 1984a,b; Homma, 1986; Millet and Jones, 1982; Prashar and Enevoldsen, 1984; Smith *et al.*, 1987; Villalon, 1986; Villalon *et al.*, 1986). This dispersal pattern and subsequent selective propagation of lines containing favorable mutations such as non-pungency likely resulted in a progressive narrowing of the genetic base of subsequent populations, in effect creating genetic bottlenecks. Similar effects on genetic diversity have been documented in soybean and wheat (Duvick, 1977; Harlan, 1987). An early genetic event in *C. annuum* domestication such as the deletion responsible for non-pungency at the *Pun1* locus, identified in this study, would be expected to be present broadly within the species. While it is probable that there is more than one mutation that results in non-pungency, the presence of the deletion in *Pun1* across many *C. annuum* genotypes tested indicates that it likely arose early in the domestication of *C. annuum*.

The recessive allele at the *Pun1* locus has been used in diverse breeding programs around the world for at least 300 years (Boswell, 1937). When the recessive allele now known as *pun1* is present in the homozygous condition capsaicinoids are not produced (Webber, 1911). Previous research mapped this locus to pepper chromosome 2 and identified several markers that cosegregated with *Pun1* (Blum *et al.*, 2002). *AT3*, an acyltransferase, was identified as a strong candidate for *Pun1* based on its map position and its hybridization pattern that correlated it with pungency. We utilized VIGS to demonstrate that silencing of the *AT3* gene caused a significant decrease in capsaicinoid accumulation and AT3 protein accumulation, a result consistent with the *pun1* phenotype. Some inconsistencies were observed in that immunoblot analysis showed no protein accumulation while HPLC showed limited production of capsaicinoids. This is likely due to the inherent instability of VIGS and differences in detection limits between the immunoblot and HPLC. Hoffmann *et al.* (2004) obtained similar but less dramatic silencing when VIGS was applied to silence HCT, a homologous acyltransferase also involved in the metabolism of phenylpropanoid compounds. Although we have not yet demonstrated an activity for this protein consistent with its phenotypes, the combination of gene mapping, allele sequencing, expression and silencing data provides strong evidence that *Pun1* encodes an acyltransferase critical to

capsaicinoid biosynthesis, and that the recessive allele, *pun1*, used for several hundred years in pepper breeding, is due to a large deletion that spans the promoter and first exon.

AT3 transcript and protein accumulation is highest during early fruit development and gradually decreases as the fruit matures. *Pal1*, *Ca4h* and *Comt*, phenylpropanoid genes involved in capsaicinoid biosynthesis, show a similar pattern of expression through fruit development (Curry *et al.*, 1999). *Acl*, *FatA* and *Kas*, subunits of the fatty acid synthase complex involved in capsaicinoid biosynthesis, also showed the same pattern of expression (Aluru *et al.*, 2003). In both studies, the transcriptional state of these biosynthetic candidate genes was positively correlated with pungency. In the present work, the transcription of these genes and several others was higher in, if not limited to, *Pun1* fruits at the onset of pungency. Concomitantly, the lack of a functional *Pun1* allele, due to the large deletion, is associated with reduction or absent transcription of the same biosynthetic candidate genes. The accumulation of AT3 protein closely followed that of *AT3* transcripts, suggesting that the regulation of capsaicinoid biosynthesis may occur primarily at the level of transcription. A faint second band appeared slightly higher on the gel and may result from modification of the native protein or an unknown cross-reacting protein (Figure 5b). The tissue-specific expression of *AT3* in the placenta of *Pun1* fruits is logical given the exclusive presence of capsaicinoids in the placenta. Altogether, the differential expression of capsaicinoid structural genes between pungent and non-pungent varieties and tissue-specific localization of expression in fruit presents a clear trend regarding expression of genes required for and involved in capsaicinoid biosynthesis (Aluru *et al.*, 2003; Curry *et al.*, 1999).

It is of interest to note that several transcripts of capsaicin biosynthetic genes appear to undergo coordinate regulation. For example, mRNA expression patterns of *pAMT*, *Kas*, *FatA* and *AT3* through fruit development were all indistinguishable in these analyses. Coordinate regulation at the level of transcription has been reported for other alkaloid biosynthetic genes consistent with a regulatory mechanism common to genes involved in capsaicin biosynthesis, despite their apparent disparate genomic positions (Blum *et al.*, 2003; Facchini, 2001). One possibility consistent with early hypotheses regarding the identity of *Pun1* is that this locus directly regulates the capsaicin biosynthetic pathway. Recent reports document the ability of metabolic enzymes to act as transcriptional regulators (Hall *et al.*, 2004; Zheng *et al.*, 2003). Perhaps *Pun1* is part of a larger complex of proteins that work together to channel intermediates through the capsaicinoid biosynthetic pathway. Descriptions of such metabolic channeling activity have been reported for *Pal* and *Ca4H*, enzymes involved in phenylpropanoid metabolism (Achnine *et al.*, 2004; Winkel-Shirley, 1999). Alternatively, *Pun1* itself maybe regulated by a yet to be identified factor or the substrate of AT3 could effect feedback regulation of the pathway.

While the functions of AT3 and the other habanero acyltransferases remain unknown, phylogenetic analysis may at least suggest hypotheses regarding the reactions they do not catalyze. All previous models of capsaicin biosynthesis predict the involvement of a single acyltransferase, namely capsaicin synthase (Blum *et al.*, 2003). In the model we present here, the pathway has been updated to reflect current understanding of phenylpropanoid metabolism by the addition of the acyltransferase, HCT, and phenylpropanoids are represented as esters rather than as free acids (Hoffmann *et al.*, 2003; Humphreys and Chapple, 2002). The phylogenetic analysis presented here indicates that AT3 and AT1 reside in a clade of *O*-acyltransferases, suggesting these enzymes may likely be ester-forming enzymes. Alternatively, they may define the first *N*-acyltransferase in this clade. Given their similarity in sequence and expression, AT3 and AT1 may overlap with respect to functions. In contrast, AT2 was most closely related to the enzymes that are fruit ripening/wounding-induced. These are also *O*-acyltransferases, but are more closely related to clades of *N*-acyltransferases. None of these pepper acyltransferases is a good candidate for a pepper HCT, given their sequence divergence and phylogenetic separation. Similarly, none of the habanero acyltransferases is likely to be an anthocyanin acyltransferase, although one unusual anthocyanin acyltransferase, Ss5MaT2, groups near AT3 and AT1.

Three pepper acyltransferases, AT1, AT2 and AT3, have been cloned. The increase of their transcripts in pungent tissues suggests that they have a role in capsaicin biosynthesis. While it may be possible that they function in the biosynthesis of other ripening-related metabolites, they may also have a function in the conversion of ferulate to vanillin. Despite the importance of vanillin as a spice, little is known about its biosynthesis in plants, it may be possible that AT3 or another acyltransferase functions in its biosynthesis (Walton *et al.*, 2003). Classical analysis of pathway mutants makes use of the accumulation of intermediates immediately upstream of the biochemical defect to determine the missing activity and its place in the pathway. Due to the observed coordinate regulation of pathway transcripts, it is likely that without a functional *AT3*, several intermediates will not be present. Even in pungent peppers these intermediates are not commonly detectable (Sukrasno and Yeoman, 1993).

While it is a tantalizing hypothesis to suggest that *AT3* encodes capsaicin synthase, the terminal enzyme in capsaicin biosynthesis, previous results from other groups suggest that *AT3* may not be capsaicin synthase because capsaicin synthase activity was detected in extracts of non-pungent peppers (Fujiwake *et al.*, 1980; Iwai *et al.*, 1977,

1978). While capsaicin synthase appears to be a coenzyme A-dependent acyltransferase, it may not be a member of the BAHD superfamily (Fujiwake *et al.*, 1980). Other coenzyme A-dependent acyltransferases, most notably the tyramine acyltransferase, THT, catalyze similar reactions especially considering the similarity of tyramine to vanillylamine (Farmer *et al.*, 1999; Yu and Facchini, 1999). Regardless, we are pursuing the possibility that AT3 may be capsaicin synthase. However, coenzyme A esters of substrates for BAHD acyltransferases are not normally commercially available, and thus require custom synthesis (Beuerle and Pichersky, 2002). Recombinant expression of AT3 has been difficult due to extreme insolubility of the protein. Native enzyme extracts assayed for capsaicin synthesis are plagued with contamination of endogenous capsaicinoids indicating radiolabeled substrates are critical for this assay.

The cloning and characterization of *Pun1* was based on the candidate gene approach. The method remains a powerful tool for gene discovery in non-model species such as *Capsicum*. Traditional techniques such as map-based cloning are difficult in *Capsicum* due to its large genome size and the limited availability of genetic resources such as mutants, large EST libraries and molecular markers. The candidate gene approach has been effectively used to isolate genes involved in capsaicinoid biosynthesis (Aluru *et al.*, 2003; Curry *et al.*, 1999) and other secondary metabolic pathways (Lange *et al.*, 2000; Thorup *et al.*, 2000). Because *Pun1* has been known and used in diverse cultures and industries for hundreds of years as the only mutation known to affect the presence/absence of capsaicin, isolation of *Pun1* will undoubtedly provide a valuable resource for marker-assisted selection and other manipulations of capsaicin biosynthesis for human use. The development of allele-specific markers is currently underway. Our work now focuses on a more detailed understanding of the role AT3 plays in capsaicinoid biosynthesis and on the elucidation of the genetic, biochemical and other events that underlie the acquisition, evolution and regulation of this unique biosynthetic capacity in *Capsicum*.

Experimental procedures

Plant material

All plant materials were grown and maintained in greenhouse conditions at 27°C day/night with supplemental lighting. The following pungent varieties were obtained from commercial sources: *C. chinense* Habanero, *C. frutescens* Tabasco, and *C. annuum* Chung-Yang, Hot 1493, NuMex RNaky, Santa Fe Grande, Thai Hot. A Mexican landrace collected by J. Labode, *C. frutescens* BG2814-6, previously reported as pungent (Blum *et al.*, 2002), was also included. An array of non-pungent varieties obtained from commercial sources included *C. annuum* Cal Wonder, Jupiter, King of the North, Lehava, Maor, Sweet 3575 and Yolo Y.

Mapping of SB2-66

Pun1 and EST clone SB2-66 (BF723664) were mapped and pungency was scored in an F_2 mapping population ($n = 242$) derived from *C. frutescens* BG2816 (a pungent wild pepper) × *C. annuum* cv. Maor (a non-pungent inbred variety), as described in Blum *et al.* (2002).

Isolation of AT3 and sequence analysis

The full-length cDNA of SB2-66 was obtained by 5′ RACE using total RNA from immature placental tissue of Habanero according to the manufacturer's instructions (Clontech, Palo Alto, CA, USA). DNA isolated from Habanero and Jupiter, using the method of Doyle and Doyle (1990), was used for genome walking to isolate genomic DNA as described by the provider (Clontech). Primer sets based on Habanero genomic DNA were used to isolate homologous sequences from various pungent and non-pungent genotypes using PCR. The pGEM-T Easy Vector System I was used for all subcloning and sequencing (Promega, Madison,WI, USA). Sequencing was performed by the Bioresources Center, Cornell University (http://www.brc.cornell.edu).

DNA gel blot hybridization

For DNA gel blot analysis, genomic DNA was extracted from young leaves of pungent and non-pungent *Capsicum* genotypes (Doyle and Doyle, 1990), and quantitated using UV absorbance. Ten micrograms of DNA was digested with *Bst*NI, electrophoretically separated, and transferred to Hybond N+ membrane (Amersham Biosciences, Piscataway, NJ, USA). Full-length *AT3* cDNA was radiolabeled using Prime-It RmT Random Labelling Kit (Stratagene, La Jolla, CA, USA) and hybridized to the membrane according to standard protocols (Sambrook and Russell, 2001). Filters were washed three times with 0.5X SSC for 15 min each at 65°C. Filters were exposed on a PhosphorImager storage screen (Molecular Dynamics, Sunnyvale, CA, USA).

RNA gel blot hybridization analysis

Capsicum chinense Habanero, *C. annuum* Thai Hot, *C. annuum* Gadir and *C. annuum* Maor pepper fruits were harvested at 10-day intervals beginning 10 dpa through 60 dpa. Fruits from multiple plants were pooled together. The placentas of the fruits were isolated, except when the fruits were too small (10 dpa), and ground to powder using liquid nitrogen. Total RNA was extracted from fruit placenta, leaf and open flowers, using Trizol reagent as described by the manufacturer (Invitrogen, Carlsbad, CA, USA). RNA levels were equilibrated by UV absorbance prior to loading, and visualized afterward by ethidium bromide staining of the gel. Northern blots were prepared using 10 μg of RNA transferred to Hybond N+ membrane (Amersham Biosciences). Full-length *AT3* cDNA was radiolabeled and hybridized as described above. Filters were exposed as described above or on Kodak XB-1 film with a BioMax MS intensifying screen (Eastman Kodak Company, Rochester, NY, USA). RNA gel bots were replicated at least once.

Capsicum cDNA clones for candidate capsaicinoid biosynthetic genes used for RNA gel blot analysis are described in Blum *et al.* (2003). Filters were probed as described above, then washed once in 2X SSC/0.1% SDS at 65°C for 10 min, then twice in 1X SSC/0.1% SDS at 65°C for 10 and 15 min, respectively. Filters were stripped using boiling 0.5% SDS, then incubated at 65°C for 20 min.

Expression of AT3 in Escherichia coli *and production of AT3 antibody*

The open-reading frame of the *AT3* gene was amplified with primers 5′-CATA-TGGCTTTTGCATTACCATCA-3′ and 5′-CTCGAGGTT-GACCGTAAACTTCCGTTG-3′ and cloned into *Nde*I and *Xho*I sites of the pET16b vector (Novagen, Madison, WI, USA). The N-terminus His-tagged AT3 recombinant protein was over-expressed in *E. coli* (DE3) and affinity purified using nickel-nitrilotriacetic agarose (Qiagen, Valencia, CA, USA). The purified AT3 protein gave a molecular mass of 52 kDa consistent with the predicted molecular mass of 50 kDa. After gel purification, the recombinant protein was used to immunize rabbits and antibody subsequently collected (Spring Valley Laboratories, Woodbine, MD, USA).

Immunoblotting of AT3

Capsicum annuum Thai Hot and Maor pepper fruits were harvested as described above for RNA gel blot hybridizations. Total protein from fruit, leaf and flower tissues was extracted into 200 μl loading buffer [0.5 M Tris-HCl pH 6.8, 10% glycerol, 10% SDS, 0.05% (w/v) bromophenol blue, 5% β-mercaptoethanol] (Martínez-Garcia *et al.*, 1999). Samples were boiled for 5 min and subsequently centrifuged at 12 000 × ***g*** for 15 min. The supernatant was then loaded onto a 12% SDS gel. Proteins were separated and transferred onto a PVDF membrane by wet electro-blotting. For detection of AT3, AT3 polyclonal antibodies and an anti-rabbit antibody conjugated to phosphatase (Amersham Biosciences) were used at 1:500 and 1:5000 dilutions, respectively. A Coomassie-stained gel from the same extractions was used as a protein loading control. Blots were developed using the ECL kit (Amersham Biosciences). Chemiluminescence emitted from the filter was detected using X-ray film.

Virus-induced gene silencing of AT3

pTRV1 and pTRV2 vectors were provided by G. Martin (Cornell University, NY, USA) and used for gene silencing (Liu *et al.*, 2002). Using the GATEWAY cloning system, a partial DNA fragment of *AT3* was amplified with primers attB1 sequence + GGATCCCGATAT-GGGAGCTGAGTT and attB2 sequence + AAGCTTACTTTCTGCC-CTTGTTGGA. This PCR product was recombined into pDONR207 vector containing the attP1 and attP2 recombination sites using the BP CLONASE enzyme (Invitrogen). The reaction mixture was transformed into *E. coli* DH10B cells. Plasmids were isolated from the transformed cells and pTRV2-attR1-attR2 destination vector and the LR CLONASE enzyme (Invitrogen) were added. This mixture was transformed into DH10B cells and selected on kanamycin-containing LB plates. The final construct was named TRV:AT3 and transformed into *Agrobacterium* strain GV2260. *Agrobacterium*-containing pTRV1, pTRV2 (empty vector) and TRV:AT3 constructs were grown overnight in a 28°C shaker in LB medium containing 50 μl ml^{-1} kanamycin and 50 μl ml^{-1} rifampicin. The next day, 50 ml of the culture was inoculated into 500 ml LB containing appropriate antibiotics, and incubated overnight in a 28°C shaker. The cells were harvested, washed once with cold 10 mM $MgCl_2$, resuspended in the induction media (10 mM $MgCl_2$, 10 mM MES and 150 μM acetosyringone), and held at room temperature for 2 h with gentle shaking. The *Agrobacterium* cells containing TRV:AT3 and pTRV2 were mixed with pTRV1 in order to get a final $OD_{600} = 0.4$ for pTRV1 and $OD_{600} = 0.1$ for pTRV2 constructs, the mixtures were then infiltrated into Thai Hot leaves using an artist's airbrush (Type H; Paasche Airbrush Co., Harwood Heights, IL, USA) connected to a portable air compressor (Model 919.152350; Sears, Roebuck and Co., Hoffman Estates, IL, USA) set at 75 psi. Un-inoculated and pTRV2 treatments were used as controls. The plants were kept at 25°C with artificial light on 12/12 h cycle. Mature red fruit were harvested and capsaicinoid content was extracted into acetonitrile. Total protein was extracted from mature red fruit and immunoblotted using AT3 polyclonal antibodies as described above.

Lu *et al.* (2003) suggested that a minimum window of 20 nucleotides perfectly matched to the VIGS insert are needed for silencing. Comparison of TRV:AT3 with the other acyltransferases described earlier shows that TRV:AT3 has a maximum window of 11 nucleotides, well below the suggested threshold strongly indicating that the silencing construct was specific to *AT3*.

HPLC analysis of capsaicinoids

One gram of dried, ground pepper samples were extracted into 10 ml of acetonitrile for 4 h at 80°C according to the method of Collins *et al.* (1995). The extract was syringe filtered and 20 μl of the filtered extract was injected for HPLC quantification using Beckman 126 pumps and a Beckman 166 detector set at 280 nm or 227 nm (Beckman Coulter Inc., Fullerton, CA, USA). An isocratic mobile phase consisting of 50% acetonitrile: 45% water: 5% tetrahydrofuran at 1.5 ml min^{-1} (Phenomenex method ALF-519) was used on 150 mm × 4.6 mm Phenomenex Synergi Hydro RP columns (Phenomenex, Torrance, CA, USA). External standards were prepared by dissolving commercial capsaicinoids (Sigma M3403; Sigma-Aldrich, St. Louis, MO, USA) in acetonitrile and making a dilution series.

Sequence and phylogeny analysis

The SMART protein prediction program predicted the presence of an acyltransferase domain in AT3 (Schultz *et al.*, 1998). Similarity searches using BLAST identified similar sequences in the GenBank database. Alignment of *AT3* with similar sequences of plant origin was performed using ClustalW (Chenna *et al.*, 2003; Thompson *et al.*, 1994). Phylogenetic analysis of the predicted protein sequence of *AT3* was performed using Psi-BLAST with a taxonomic restriction to green plants (Altschul *et al.*, 1997). Of the resulting matches, only those sequences that had been characterized at a level beyond genomic sequences were selected for further study. The selected protein sequences were aligned using DiAlign (Morgenstern *et al.*, 1998) and ClustalW. A phylogeny tree was constructed from this alignment using the MEGA2 phylogeny package (Kumar *et al.*, 2001). In the depicted tree, sequences were aligned using the BLUSOM weight matrix within ClustalW and analyzed using the neighbor-joining method, the *p*-distance model and pairwise deletion setting to handle gaps. Bootstrap replications ($n = 1000$) were performed as a test of the inferred phylogeny. Alternate alignment methods with maximum parsimony analysis and other neighbor-joining criteria gave similar results.

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References

Achnine, L., Blancaflor, E.B., Rasmussen, S. and Dixon, R.A. (2004) Colocalization of L-phenylalanine ammonia-lyase and cinnamate 4-hydroxylase for metabolic channeling in phenylpropanoid biosynthesis. *Plant Cell*, **16**, 3098–3109.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389–3402.

Aluru, M.R., Mazourek, M., Landry, L.G., Curry, J., Jahn, M.M. and O'Connell, M.A. (2003) Differential expression of fatty acid synthase genes, *Acl*, *Fat* and *Kas*, in *Capsicum* fruit. *J. Exp. Bot.* **54**, 1655–1664.

Andrews, J. (1984) *Peppers The Domesticated Capsicums*. Austin: University of Texas Press.

Appendino, G., Minnasi, A., Morello, A., De Petrocellis, L. and Di Marzo, V. (2002) *N*-acylvanillylamides: development of an expeditious synthesis and discovery of new acyl templates for powerful activation of the vanilloid receptor. *J. Med. Chem.* **45**, 3739–3745.

Basu, K.S. and Krishna De, A. (eds) (2003) *Capsicum*. Medicinal and Aromatic Plants – Industrial Profiles. London: Taylor & Francis.

Bennett, D.J. and Kirby, G.W. (1968) Constitution and biosynthesis of capsaicin. *J. Chem. Soc.* **C**, 442–446.

Beuerle, T. and Pichersky, E. (2002) Enzymatic synthesis and purification of aromatic coenzyme A esters. *Anal. Biochem.* **302**, 305–312.

Billing, J. and Sherman, P.W. (1998) Antimicrobial function of spices: why some like it hot. *Q. Rev. Biol.* **73**, 3–49.

Blum, E., Liu, K., Mazourek, M., Yoo, E.Y., Jahn, M.M. and Paran, I. (2002) Molecular mapping of the *C* locus for presence of pungency in *Capsicum*. *Genome*, **45**, 702–705.

Blum, E., Mazourek, M., O'Connell, M.A., Curry, J., Thorup, T., Liu, K., Jahn, M.M. and Paran, I. (2003) Molecular mapping of capsaicinoid biosynthesis genes and quantitative trait loci analysis for capsaicinoid content in *Capsicum*. *Theor. Appl. Genet.* **108**, 79–86.

Boswell, V.R. (1937) Improvement and genetics of tomatoes, peppers, and eggplant. In *Yearbook of Agriculture*. Washington: United States Government Printing Office, pp. 176–206.

Burhenne, K., Kristensen, B.K. and Rasmussen, S.K. (2003) A new class of *N*-hydroxycinnamoyltransferases. *J. Biol. Chem.* **278**, 13919–13927.

Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D. and Julius, D. (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature*, **389**, 816–824.

Caterina, M.J., Leffler, A., Malmberg, A.B., Martin, W.J., Trafton, J., Petersen-Zeitz, K.R., Koltzenburg, M., Blasbaum, A.I. and Julius, D. (2000) Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science*, **288**, 306–313.

Chancellor, M.B. and De Groat, W.C. (1999) Intravesical capsaicin and resiniferatoxin therapy: spicing up the ways to treat the overactive bladder. *J. Urol.* **162**, 3–11.

Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T.J., Higgins, D.G. and Thompson, J.D. (2003) Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res.* **31**, 3497–3500.

Chu, C., Huang, S., De Petrocellis, L. *et al.* (2003) *N*-oleoyldopamine, a novel endogenous capsaicin-like lipid that produces hyperalgesia. *J. Biol. Chem.* **278**, 13633–13639.

Collins, M.D., Wasmund, L.M. and Bosland, P.W. (1995) Improved method for quantifying capsaicinoid in *Capsicum* using high-performance liquid chromatography. *Hortscience*, **30**, 137–139.

Cook, A.A. (1984a) 'Florida VR2-34' bell pepper. *Hortscience*, **19**, 311.

Cook, A.A. (1984b) 'Florida VR4' bell pepper. *Hortscience*, **19**, 456.

Cruz, F. (2004) Mechanisms involved in new therapies for overactive bladder. *Urology*, **63**, 65–73.

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

Deal, C., Schnitzer, T., Lipstein, E., Seibold, J., Stevens, R., Levy, M., Albert, D. and Renold, F. (1991) Treatment of arthritis with topical capsaicin: a double blind trial. *Clin. Ther.* **13**, 383–395.

Duvick, D.N. (1977) Major United States crops in 1976. *Ann. N. Y. Acad. Sci.* **287**, 86–96.

Estrada, B., Pomar, F., Díaz, J., Merino, F. and Bernal, M.A. (1999) Pungency level in fruits of the Padrón pepper with different water supply. *Sci. Hortic.* **81**, 385–396.

Facchini, P.J. (2001) Alkaloid biosynthesis in plants: biochemistry, cell biology, molecular regulation, and metabolic engineering applications. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52**, 29–66.

Farmer, M.J., Czernic, P., Michael, A. and Negrel, J. (1999) Identification and characterization of cDNA clones encoding hydroxycinnamoyl-CoA:tyramine N-hydroxylcinnamoyltransferase from tobacco. *Eur. J. Biochem.* **263**, 686–694.

Fujiwake, H., Suzuki, T., Oka, S. and Iwai, K. (1980) Enzymatic formation of capsaicinoid from vanillylamine and iso-type fatty acids by cell-free extracts of *Capsicum annuum* var. *annuum* cv. Karayatsubusa. *Agric. Biol. Chem.* **44**, 2907–2912.

Hall, D.A., Zhu, H., Zhu, X., Royce, T., Gerstein, M. and Snyder, M. (2004) Regulation of gene expression by a metabolic enzyme. *Science*, **306**, 482–484.

Han, S., Keum, Y., Seo, H., Lee, S. and Surh, Y. (2001) Capsaicin suppresses phorbol ester-induced activation of NF-κB and AP-1 transcription factors in mouse epidermis. *Cancer Lett.* **164**, 119–126.

Harlan, J.R. (1987) Gene centers and gene utilization in American agriculture. In *Plant Genetic Resources: A Conservation Imperative* (Yeatman, C.W., Kafton, D. and Wilkes, G., eds). Boulder, CO: Westview, pp. 111–129.

Harvell, K.P. and Bosland, P.W. (1997) The environment produces a significant effect on pungency of chiles. *Hortscience*, **32**, 1292.

Hoffmann, L., Besseau, S., Geoffroy, P., Ritzenthaler, C., Meyer, D., Laplerre, C., Pollet, B. and Legrand, M. (2004) Silencing of hydroxycinnamoyl-coenzyme A Shikimate/quinate hydroxycinnamoyl transferase affects phenylpropanoid biosynthesis. *Plant Cell*, **16**, 1446–1465.

Hoffmann, L., Maury, S., Martz, F., Geoffroy, P. and Legrand, M. (2003) Purification, cloning, and properties of an acyltransferase controlling shikimate and quinate ester intermediates in phenylpropanoid metabolism. *J. Biol. Chem.* **278**, 95–103.

Homma, S. (1986) MI-221 and MI-600 pepper breeding populations. *Hortscience*, **21**, 1469.

Huang, S.M., Bisogno, T., Trevisani, M. *et al.* (2002) An endogenous capsaicin-like substance with high potency at recombinant and native vanilloid VR1 receptors. *Proc. Natl Acad. Sci. USA*, **99**, 8400–8405.

Humphreys, J.M. and Chapple, C. (2002) Rewriting the lignin roadmap. *Curr. Opin. Plant Biol.* **5**, 224–229.

Iwai, K., Suzuki, T., Lee, K.-R., Kobashi, M. and Oka, S. (1977) *In vivo* and *In vitro* formation of dihydrocaspaicin in sweet pepper fruits, *Capsicum annuum* L. var. *grossum*. *Agric. Biol. Chem.* **10**, 1877–1882.

Iwai, K., Lee, K.-R., Kobashi, M., Suzuki, T. and Oka, S. (1978) Intracellular localization of the capsaicinoid synthesizing enzyme in sweet pepper fruits. *Agric. Biol. Chem.* **42**, 201–202.

Iwai, K., Suzuki, T. and Fujiwake, H. (1979) Formation and accumulation of pungent principle of hot pepper fruits, capsaicin and its analogous, in *Capsicum annuum* var. *annuum* cv. Karayatsubusa at different growth stages after flowering. *Agric. Biol. Chem.* **43**, 2493–2498.

Jordt, S.-E. and Julius, D. (2002) Molecular basis for species-specific sensitivity to 'Hot' chili peppers. *Cell*, **108**, 421–430.

Kim, M., Kim, S., Kim, S. and Kim, B.D. (2001) Isolation of cDNA clones differentially accumulated in the placenta of pungent pepper by suppression subtractive hybridization. *Mol. Cell*, **11**, 213–219.

Kumar, S., Tamura, K., Jakobsen, I.B. and Nei, M. (2001) MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics*, **17**, 1244–1245.

Lange, B.M., Wildung, M.R., Stauber, E.J., Sanches, C., Pouchnik, D. and Croteau, R. (2000) Probing essential oil biosynthesis and secretion by functional evaluation of expressed sequence tags from mint glandular trichomes. *Proc. Natl Acad. Sci. USA*, **97**, 2934–2939.

Leete, E. and Louden, M.C.L. (1968) Biosynthesis of capsaicin and dihydrocaspaicin in *Capsicum frutescens*. *J. Am. Chem. Soc.* **90**, 6837–6841.

Liu, Y., Schiff, M. and Dinesh-Kumar, S.P. (2002) Virus-induced gene silencing in tomato. *Plant J.* **31**, 777–786.

Lu, R., Martin-Hernandez, A.M., Peart, J.R., Malcuit, I. and Baulcombe, D.C. (2003) Virus-induced gene silencing in plants. *Methods*, **30**, 296–303.

Martínez-Garcia, J., Monte, E. and Quail, P. (1999) A simple, rapid and quantitative method for preparing *Arabidopsis* protein extracts for immunoblot analysis. *Plant J.* **20**, 251–257.

Millet, A.H. and Jones, R.A. (1982) 'Garden Sunshine' bell pepper. *Hortscience*, **17**, 683.

Morgenstern, B., Frech, K., Dress, A. and Werner, T. (1998) DIALIGN: finding local similarities by multiple sequence alignment. *Bioinformatics*, **14**, 290–294.

Nam, K., Dudareva, N. and Pichersky, E. (1999) Characterization of benzylalcohol acetyltransferases in scented and non-scented *Clarkia* species. *Plant Cell Physiol.* **40**, 916–923.

Ohta, Y. (1962) Physiological and genetical studies on the pungency of *Capsicum*, IV. Secretory organs, receptacles and distribution of capsaicin in the *Capsicum* fruit. *Jpn. J. Breed.* **12**, 43–47.

Prashar, P. and Enevoldsen, M. (1984) 'Pick-Me-Quick' bell pepper. *Hortscience*, **19**, 683.

Sambrook, J. and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Schultz, J., Milpetz, F., Bork, P. and Ponting, C. (1998) SMART, a simple modular architecture research tool: identification of signaling domains. *Proc. Natl Acad. Sci. USA*, **95**, 5857–5864.

Smith, P.G., Villalon, B. and Villa, P.L. (1987) Horticultural classification of peppers grown in the United States. *Hortscience*, **22**, 11–13.

Srinivasan, K. (2005) Spices as influencers of body metabolism: an overview of three decades of research. *Food Res. Int.* **38**, 77–86.

St. Pierre, B. and De Luca, V. (2000) Evolution of acyltransferase genes: origin and diversification of the BAHD superfamily of acyltransferases involved in secondary metabolism. *Recent Adv. Phytochem.* **34**, 285–315.

St. Pierre, B., Laflamme, P., Alarco, A.-M. and De Luca, V. (1998) The terminal *O*-acetyltransferase involved in vindoline biosynthesis defines a new class of proteins responsible for coenzyme A-dependent acyl transfer. *Plant J.* **14**, 703–713.

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

Surh, Y.J. (2002) More than a spice: capsaicin in hot chili peppers makes tumor cells commit suicide. *J. Natl Cancer Inst.* **94**, 1263–1265.

Surh, Y.J. (2003) Cancer chemoprevention with dietary phytochemicals. *Nat. Rev. Cancer*, **3**, 768–780.

Suzuki, T., Fujiwake, H. and Iwai, K. (1980) Intracellular localization of capsaicin and its analogues, capsaicinoid, in *Capsicum* fruit 1. Microscopic investigation of the structure of the placenta of *Capsicum annum* var. *annum* cv. Karayatsubusa. *Plant Cell Physiol.* **21**, 839–853.

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

Suzuki, H., Sawada, S., Watanabe, K., Nagae, S., Yamaguchi, M., Nakayama, T. and Nishino, T. (2004) Identification and characterization of a novel anthocyanin malonyltransferase from scarlet sage (*Salvia splendens*) flowers: an enzyme that is phylogenetically separated from other anthocyanin acyltransferases. *Plant J.* **38**, 994–1003.

Tacke, E., Korfhage, C., Michel, D., Maddaloni, M., Motto, M., Lanzini, S., Salamini, F. and Döring, H.P. (1995) Transposon tagging of the maize *Glossy2* locus with the transposable element *En/Spm*. *Plant J.* **8**, 907–917.

Tewksbury, J. and Nabhan, G.P. (2001) Directed deterrence by capsaicin in chillies. *Nature*, **412**, 403–404.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighing, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673–4680.

Thorup, T.A., Tanyolac, B., Livingstone, K.D., Popovsky, S., Paran, I. and Jahn, M.M. (2000) Candidate gene analysis of organ pigmentation loci in the Solanaceae. *Proc. Natl Acad. Sci. USA*, **97**, 11192–11197.

Thresh, J.C. (1876) Capsaicin, the active principle of *Capsicum* fruits. *Pharm. J.* **7**, 21.

Villalon, B. (1986) 'Tambel-2' bell pepper. *Hortscience*, **21**, 328.

Villalon, B., Dainello, F.J., Lipe, W.N. and Taylor, R. (1986) 'Tam Mild Chile-2' chile pepper. *Hortscience*, **21**, 1468–1469.

Walsh, B.M. and Hoot, S.B. (2001) Phylogenetic relationships of *Capsicum* (Solanaceae) using DNA sequences from two noncoding regions: the chloroplast *atpB-rbcL* spacer region and nuclear *waxy* introns. *Int. J. Plant Sci.* **162**, 1409–1418.

Walton, N.J., Mayer, M.J. and Narbad, A. (2003) Molecules of interest: vanillin. *Phytochemistry*, **63**, 505–515.

Watson, C., Tyler, K., Bickers, D., Millikan, L., Smith, S. and Coleman, E. (1993) A randomized vehicle-controlled trial of topical capsaicin in the treatment of postherpetic neuralgia. *Clin. Ther.* **15**, 510–526.

Webber, H. (1911) Preliminary notes on pepper hybrids. *Am. Breed. Assoc. Annu. Rep.* **7**, 188–199.

Winkel-Shirley, B. (1999) Evidence for enzyme complexes in the phenylpropanoid and flavonoid pathways. *Physiol. Plant.* **107**, 142–149.

Xia, Y., Nikolau, B.J. and Schnable, P.S. (1996) Cloning and characterization of *CER2*, an *Arabidopsis* gene that affects cuticular wax accumulation. *Plant Cell*, **8**, 1291–1304.

Yu, M. and Facchini, P.J. (1999) Purification, characterization, and immunolocalization of hydroxycinnamoyl-CoA:tyramine *N*-(hydroxycinnamoyl)transferase from opium poppy. *Planta*, **209**, 33–44.

Zamski, E., Shoham, O., Palevitch, D. and Levy, A. (1987) Ultrastructure of capsaicinoid-secreting cells in pungent and non-pungent Red Pepper (*Capsicum annum* L.) cultivars. *Bot. Gaz.* **148**, 1–6.

Zewdie, Y. and Bosland, P.W. (2000) Evaluation of genotype, environment, and genotype-by-environment interaction for capsaicinoids in *Capsicum annum* L. *Euphytica*, **111**, 185–190.

Zewdie-Tarekegen, Y. (1999) Genetic study of capsaicinoids (pungency) in chile, *Capsicum*, spp. PhD Dissertation. New Mexico State University, Las Cruces, NM.

Zheng, L., Roeder, R.G. and Luo, Y. (2003) S phase activation of the histone H2B promoter by OCA-S, a coactivator complex that contains GAPDH as a key component. *Cell*, **114**, 255–266.

The sequences reported in this paper have been submitted to the Genbank database and have been assigned the following accession numbers: AY819026, AY819030, AY819027, AY819031, AY819028, AY819032, AY819029