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RESEARCH PAPER

Genetic control of pungency in *C. chinense* via the *Pun1* locus

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Abstract

Capsaicin, the pungent principle in hot peppers, acts to deter mammals from consuming pungent pepper pods. Capsaicinoid biosynthesis is restricted to the genus *Capsicum* and results from the acylation of the aromatic compound, vanillylamine, with a branched-chain fatty acid. The presence of capsaicinoids is controlled by the *Pun1* locus, which encodes a putative acyltransferase. In its homozygous recessive state, *pun1/pun1*, capsaicinoids are not produced by the pepper plant. HPLC analysis confirmed that capsaicinoids are only found in the interlocular septa of pungent pepper fruits. Immunolocalization studies showed that capsaicinoid biosynthesis is uniformly distributed across the epidermal cells of the interlocular septum. Capsaicinoids are secreted from glandular epidermal cells into subcuticular cavities that swell to form blisters along the epidermis. Blister development is positively associated with capsaicinoid accumulation and blisters are not present in non-pungent fruit. A genetic study was used to determine if the absence of blisters in non-pungent fruit acts independently of *Pun1* to control pungency. Screening of non-pungent germplasm and genetic complementation tests identified a previously unknown recessive allele of *Pun1*, named *pun1^2*. Sequence analysis of *pun1^2* revealed that a four base pair deletion results in a frameshift mutation and the predicted production of a truncated protein. Genetic analysis revealed that *pun1^2* co-segregated exactly with the absence of blisters, non-pungency, and a reduced transcript accumulation of several genes involved in capsaicinoid biosynthesis. Collectively, these results establish that blister formation requires the *Pun1* allele and that *pun1^2* is a recessive allele from *C. chinense* that results in non-pungency.

Key words: Blister, capsaicin, capsaicinoids, *Capsicum*, pepper, pungency, secretion.

Introduction

Capsaicin and its analogues, collectively called capsaicinoids, are the pungent principle of peppers (Thresh, 1876). Peppers (*Capsicum* spp.) are well known for their ability to cause an intense organoleptic sensation of heat when consumed. Capsaicinoids are produced only within the genus *Capsicum* and their presence has driven the domestication of several *Capsicum* species (Walsh and Hoot, 2001). Peppers probably originated in Bolivia, as this area contains many of the 20–27 recognized species of *Capsicum* (Andrews, 1984; Hunziker, 2001; Walsh and Hoot, 2001). Bell pepper seeds were first traded nearly 500 years ago, and archeological evidence indicates peppers have a history of human use dating back nearly 9000 years, making *Capsicum* one of the oldest plant genera to be domesticated (Boswell, 1937; Pickersgill, 1966; Heiser Jr 1969; Basu and Krishna De, 2003).

Ecological studies suggest that capsaicinoids evolved as a means of directed deterrence (Tewksbury and Nabhan, 2001). Birds, the favoured seed-dispersal agents of wild *Capsicum*, do not perceive capsaicinoids. After ingestion by avian frugivores, seeds are transported within the birds' gut, and then excreted, often in favourable habitats under trees that provide appropriate light levels for plant growth. Furthermore, passage through the avian digestive system appears to promote the germination of pepper seeds (Tewksbury and Nabhan, 2001). In contrast to birds,

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mammals are extremely sensitive to capsaicinoids, perceiving pain when capsaicinoids contact epidermal tissues. Furthermore, mammals possess crushing molars and harsh, acidic, digestive systems that harm pepper seeds (Tewksbury and Nabhan, 2001). The pain response to capsaicinoids is due to the activation of the TRPV1 (VR1) receptor in mammals by capsaicinoids; in birds, this receptor does not respond to capsaicinoids (Caterina *et al*., 1997, 2000; Jordt and Julius, 2002). In fact, the TRPV1 receptor is involved in several pain-sensing pathways, which has prompted much basic and clinical research on the use of capsaicinoids to treat a variety of human ailments, including arthritis, bladder and digestive problems, and cancer (Deal *et al*., 1991; Chancellor and De Groat, 1999; Han *et al*., 2001; Surh, 2002; Cruz, 2004; Srinivasan, 2005). Natural capsaicin analogues, which may function as neurotransmitters, have been found in mammalian brains, and endorphins have been shown to be released in the brain when capsaicin is consumed (Appendino *et al*., 2002; Huang *et al*., 2002; Chu *et al*., 2003).

It is generally accepted that capsaicinoids are produced solely in pepper fruits, although the location of the biosynthesis and accumulation of capsaicinoids within the fruits has been debated. A recent report describes the detection of capsaicinoids in vegetative organs, and others have reported small amounts of capsaicinoids in seeds (Ohta, 1962; Balbaa *et al*., 1968; Estrada *et al*., 2002). Within the pepper fruit, capsaicinoids chiefly accumulate along the epidermal cells of the interlocular septum which defines the fruit locules and is derived from the tissue connecting the placenta to the pericarp (Judd *et al*., 1999). The epidermal cells of the interlocular septum have been implicated in capsaicinoid biosynthesis, based on morphological changes during fruit development and the existence of osmiophilic granules in these cells (Furuya and Hashimoto, 1954; Ohta, 1962; Suzuki *et al*., 1980). In pungent varieties, epidermal protrusions or blisters arise from the lifting of the cuticle layer from the cell wall during the filling of subcuticular cavities with capsaicinoids (Rowland *et al*., 1983; Zamski *et al*., 1987).

Very little is known about the regulation of capsaicinoid biosynthesis. Capsaicinoids are produced by condensation of a branched-chain fatty acid, derived from either valine or leucine, with vanillylamine, derived from phenylalanine (Fig. 1a) (Bennett and Kirby, 1968; Leete and Louden, 1968; Suzuki *et al*., 1981; Sukrasno and Yeoman, 1993). In previous research, the identity of *Pun1*, a putative acyltransferase named *AT3*, was reported (Stewart *et al*.,

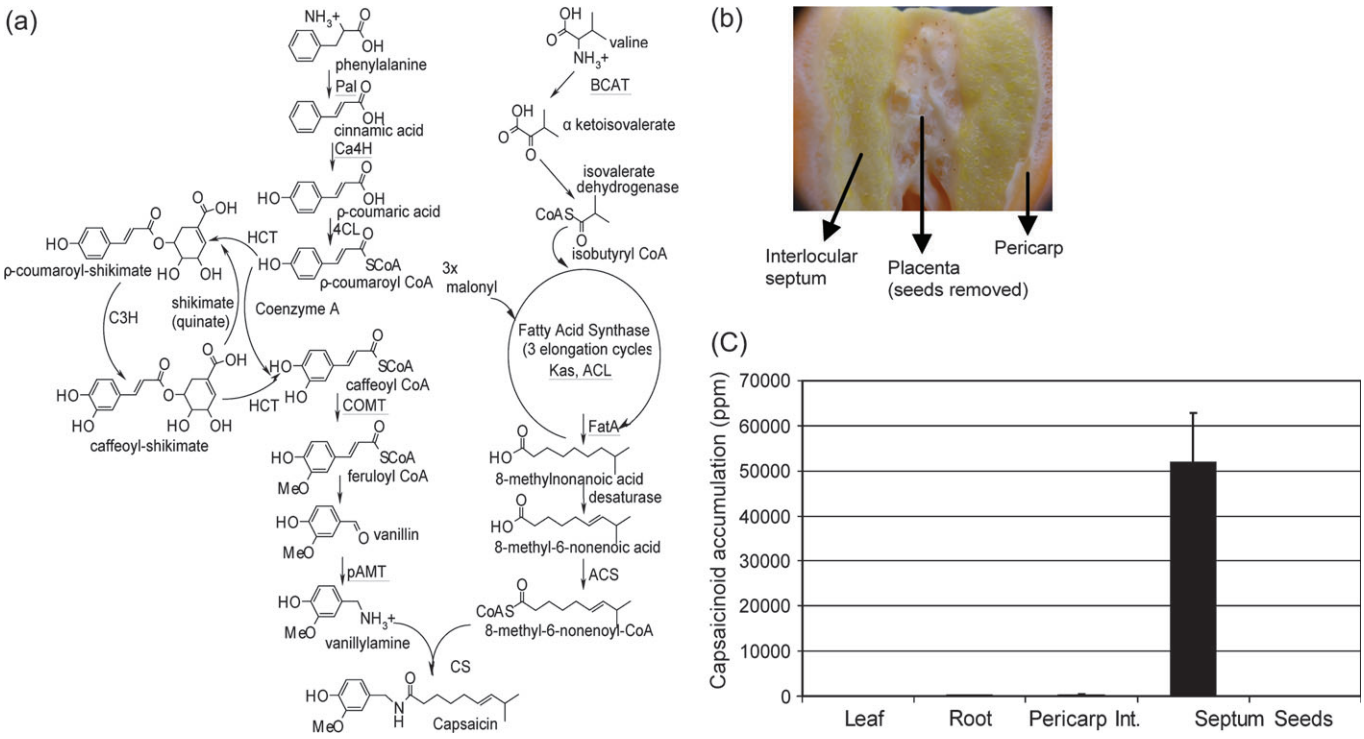

Fig. 1. Tissue specificity of capsaicinoid accumulation in pepper fruits. (a) Current model of the capsaicinoid biosynthetic pathway. Enzymes are shown adjacent to the reactions they catalyse. For those enzymes underlined, the gene encoding them has been cloned in *Capsicum*. Kas is the only enzyme functionally characterized in *Capsicum*; all other enzyme attributions are based on nucleotide similarity and 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, 3-keto-acyl ACP synthase; ACL, acyl carrier protein; Fat, acyl-ACP thioesterase; ACS, acyl-CoA synthetase; CS, capsaicin synthase. (b) *C. chinense* 'Habanero' fruit dissected and seeds removed to show interlocular septum, placenta, and pericarp locations. (c) HPLC analysis of capsaicinoid accumulation in leaf, root, pericarp, interlocular septum, and seed tissues of Habanero. Values represent the mean ±SD of at least three plants.

2005). The *Pun1* locus, first investigated nearly a century ago, is responsible for non-pungency throughout *C. annuum* based on inferred breeding pedigrees and sequencing results (Webber, 1911; Stewart *et al*., 2005). Non-pungent *C. annuum* genotypes were observed to have a 2.5 kb deletion spanning the putative promoter and first exon of *AT3*. The *pun1* allele defined by this large deletion is the only known mutation to date that has a qualitative effect on the presence/absence of capsaicinoids (Webber, 1911; Blum *et al*., 2002).

Studies of this locus have revealed that *AT3* transcript is expressed specifically in the placenta and begins to accumulate along with several other capsaicinoid biosynthetic genes at 20 days post-anthesis (dpa). It was noted that several capsaicinoid biosynthetic genes, including *AT3*, were either not expressed or reduced in expression when non-pungent and pungent genotypes were compared (Stewart *et al*., 2005). Similarly, Curry *et al.* (1999) and Aluru *et al*. (2003) observed that transcript accumulation of several capsaicinoid biosynthetic genes was correlated with the level of pungency.

In the book, *The Paprika*, Somos described the presence of blisters that contained capsaicin and suggested it may be possible to regulate capsaicinoid production by regulating the presence of blisters (Somos, 1984). A recent report termed these blister structures 'vesicles' and stated that the absence of these capsaicinoid-accumulating vesicles is inherited as a single recessive gene at a new locus designated 'loss-of-vesicles' (*lov*) (Votava and Bosland, 2002). This locus, *lov*, was proposed as a second locus, in addition to *Pun1*, that has a qualitative effect on pungency. The current study was undertaken critically to test the genetic relationship between blisters, capsaicinoid biosynthesis, *lov*, and *Pun1*.

The localization of capsaicinoid biosynthesis and accumulation to the epidermal cells of the interlocular septa in pungent fruits is reported here. The presence of blisters in pungent fruits represents sites of capsaicinoid secretion and storage and is precisely related to capsaicinoid accumulation. A recessive allele of the *Pun1* locus was identified and it is shown that in the allelic state at *Pun1*, transcript accumulation of capsaicinoid biosynthetic genes and capsaicinoid accumulation are highly correlated. Based on these results, it is concluded that mutations at a single locus are responsible for non-pungency within *C. annuum* and *C. chinense* and that this locus is also responsible for the presence/absence of the blistered structures that contain capsaicinoids.

Materials and methods

Plant material

Seeds of *C. chinense* 'Habanero' and *C. annuum* 'Maor' were obtained from commercial sources (Tomato Growers Supply Co., Ft. Myers, FL). *C. chinense* NMCA 30036, previously described by Votava and Bosland (2002), was obtained from the NMSU Chile Pepper Institute.

For genetic studies in the field, plants were sown in the greenhouse and then transplanted to fields located in Varna, NY during the summer of 2004. For gene expression studies, all plant materials were grown and maintained in greenhouse conditions at 27 °C day/night with supplemental lighting unless otherwise noted.

Scanning-electron and light microscopy

Interlocular septum tissue from fresh, unfixed, immature green Habanero and NMCA 30036 fruits were removed and dissected cross-sectionally by hand into 5 mm^2 pieces using a razor blade. The samples were mounted on aluminium stubs and coated twice under vacuum with a film of fine-grain gold using a Denton Vacuum Sputter Coater. The surface of the sections was examined under a Hitachi S-3200N Variable Pressure Scanning Electron Microscope (SEM).

For light microscopic analysis, the interlocular septa of ripe Habanero fruits were fixed and embedded into low viscosity resin (Electron Microscopy Sciences, Hatfield, PA) as described in Rowland *et al*. (1983) with the following modifications. After fixation, samples were washed three times with cold 50 mM PIPES buffer, pH 5.6, dehydrated in a graded ethanol series, 30 min at room temperature for each step, and incubated overnight in 100% ethanol at 4 °C. Ethanol was removed through a graded acetone/ethanol series, 30 min at 4 °C for each step, and soaked in acetone for 2–3 h. Samples were infiltrated with resin as described in the manufacturer's protocol. Sections 1 μm thick were cut using a glass knife and stained using a 1% (w/v) crystal violet solution. An Olympus BX60 compound microscope attached to a digital camera and computer for video-capture was used to view samples.

Blisters on the epidermis of the interlocular septum of Habanero and NMCA 30036 were photographed at 10 d intervals through fruit development using a dissecting microscope attached to a digital camera mounted with the ScopeTronix MaxView Plus System (Scopetronix, Cape Coral, FL). Images were taken of multiple fruit from multiple plants.

Immunolocalization of capsaicinoids

Thick sections, approximately 8–10 mm wide, were dissected from the interlocular septa of immature green Habanero and NMCA 30036 fruits (approximately 30 dpa) using a double-edge razor blade. Samples were immediately placed in fixative consisting of 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.2, and incubated overnight at 4 °C. Samples were washed five times with 0.1 M sodium phosphate buffer and dehydrated through a graded ethanol series, 30 min at room temperature for each step. Samples were incubated overnight at 4 °C in 100% ethanol, then moved through a graded Histoclear (National Diagnostics, Atlanta, GA) series, 1.5 h at each step, and held overnight in 100% Histoclear at ambient temperature. Samples were infiltrated with Paraplast (Kendall, Mansfield, MA) as per the manufacturer's instructions. Sections, approximately 8 μm thick, were cut, paraffin removed, and rehydrated in a graded ethanol series. A rabbit capsaicin antibody (Beacon Analytical Systems Inc., Portland, ME) was used for immunolocalization at a 1:250 dilution using the ImmPRESS Anti-Rabbit Ig Peroxidase Kit (Vector Laboratories, Burlingame, CA) following the manufacturer's instructions. According to the manufacturer, the capsaicin antibody has reactivity against capsaicin and dihydrocapsaicin, the dominant capsaicinoids in commercial peppers. Sections were viewed using an Olympus BX50 microscope attached to a digital camera.

HPLC and ELISA analysis of pungency

Extracts of Habanero, Maor, NMCA 30036, and F_2 progeny from the cross of Habanero and NMCA 30036 were prepared and analysed for capsaicinoid accumulation via HPLC as previously described in Stewart *et al.* (2005).

Interlocular septa from ripe Habanero fruits were removed and either directly dried or gently brushed with a small spatula to remove blisters and then dried. Capsaicinoids were extracted and analysed via HPLC as previously described in Stewart *et al.* (2005).

The presence/absence of pungency in fruit produced for the genetic complementation tests was assessed with ELISA due to the greater sensitivity of this method for capsaicinoid detection. Multiple ripe fruits from Maor, NMCA 30036, and the F_1 progeny of the complementation cross were analysed for capsaicinoid accumulation using the Capsaicin High Sensitivity Plate Kit (Beacon Analytical Sys. Inc, Portland, ME) that utilizes an antibody that specifically recognizes the capsaicinoid small molecule as the epitope. The manufacturer's protocol was followed with the followng modification. Methanol extracts of the dried pepper samples were not diluted prior to analysis.

Inheritance and co-segregation of blisters, pungency, and Pun1

Ripe fruit from F_1 and F_2 populations (n=495), derived from *C. chinense* Habanero and *C. chinense* NMCA 30036 and its reciprocal cross, were analysed for pungency and presence/absence of blisters, and then genotyped to determine which *Pun1* allele(s) were present. Plants were scored twice independently as pungent or non-pungent by a panel of three trained individuals. Non-pungent phenotypes were confirmed using HPLC as described above. The presence/absence of blisters along the interlocular septum of the fruits was visually scored and the absence of blisters was independently verified by two observers. A PCR-based, co-dominant marker based on the following primers 5′-ATGTCAACCGGCCAGCAGCAT-3′, 5′-CTGATTCTTCTGCCACCTTCAATCCC-3′, 5′-CCCACAATCAAACTTACTTGAAC-3′ was used to determine the allelic state at *Pun1* for all individuals. PCR was performed using Advantage 2 Polymerase (Clontech, Palo Alto, CA) on DNA isolated from the interlocular septa according to the method of Doyle and Doyle (1990) with one modification. Microfuge tubes were treated with polyvinylpolypyrrolidone (Sigma P6755), prior to DNA extraction to minimize interference of phenolic compounds during PCR. RNA and total protein from interlocular septa of mature green F_2 plants, confirmed homozygous dominant or recessive at *Pun1*, were isolated as described below to analyse gene expression. Capsaicinoid accumulation in the same samples was analysed via HPLC as described above.

Genetic complementation tests to determine whether the absence of pungency in NMCA 30036 and Maor is due to defects at the same or different loci were conducted by crossing NMCA 30036×Maor. The interspecific F_1 progeny were confirmed to be hybrids visually and by genotyping. Reciprocal crosses in the opposite direction did not pollinate. Ripe F_1 fruits from each cross were assayed for capsaicinoid accumulation via ELISA as described above.

RNA gel blot analysis

RNA was extracted from pepper fruits at time points through fruit development for gel blot analysis as described in Stewart *et al.* (2005). Fruits from multiple plants were pooled together to minimize variation due to environmental effects. Due to small fruit size, whole fruits with seeds removed were used for all 10 dpa time points. Ten μg of RNA was transferred to Hybond N^+ membrane (Amersham Bioscience, Piscataway, NJ). Full-length *AT3* cDNA was radiolabelled using the Prime-It RmT Random Labeling Kit (Stratagene, La Jolla, CA) and hybridized to the membrane according to standard protocols (Sambrook and Russell, 2001). *Pal* and *Kas*, known capsaicinoid biosynthetic genes, were radiolabelled and used as probes as described previously (Curry *et al.*, 1999; Aluru *et al.*, 2003; Blum *et al.*, 2003). Filters were washed twice at 65 °C in 2× SSC/0.1% SDS for 10 min and twice in 1× SSC/0.1% SDS for 10 min. Filters were exposed on a PhosphorImager storage screen (Molecular Dynamics). RNA gel bots were replicated at least twice from independent extractions.

Immunoblotting of AT3

Habanero, Maor, and NMCA 30036 fruits were harvested at 10 d intervals. Total protein was extracted and blotted as described in Stewart *et al.* (2005). For detection of the AT3 protein, a rabbit polyclonal antibody against AT3 and an anti-rabbit secondary antibody conjugated to phosphatase (Amersham Bioscience, Piscataway, NJ) were used at 1:2000 and 1:5000 dilutions, respectively. The production, use, and specificity of the AT3 antibody were described previously in Stewart *et al.* (2005). Coomassie staining of replicate gels from the same extractions or of the probed membrane was used to verify equal protein loading. Blots were developed using the ECL kit (Amersham Biosciences, Piscataway, NJ). Chemiluminescence emitted from the filter was detected using X-ray film. Immunoblots were replicated at least twice.

Cloning and sequencing

Primer sets based on Habanero genomic DNA were used to isolate homologous sequence from NMCA 30036 using PCR. The pGEM-T Easy Vector System I was used for all subcloning and sequencing (Promega, Madison, WI). Sequencing was performed by the Bioresource Center, Cornell University (www.brc.cornell.edu).

Results

Localization of capsaicinoid accumulation to the epidermis of the Capsicum *interlocular septum*

A cross-section of a ripe *C. chinense* Habanero fruit with seeds removed, with the pericarp, placenta, and interlocular septum labelled, is shown in Fig. 1b. Capsaicinoids were extracted from Habanero leaf, root, pericarp, interlocular septum, and seed tissues. Capsaicinoid accumulation, monitored by HPLC, was present in the interlocular septum, 52 000±11 000 parts per million (ppm), and absent from all other tissues sampled (Fig. 1c). Residual capsaicinoids were detected in the pericarp, probably due to imperfect separation of the interlocular septum from the pericarp tissues. Close inspection revealed that when the fruit is cut open or handled roughly, capsaicinoids from the blisters (see below) can contaminate nearby seeds. These results are consistent with early radioisotope analysis localizing capsaicinoids to the interlocular septum of pepper fruits (Iwai *et al.*, 1979).

Immunolocalization studies using a capsaicinoid antibody were conducted to determine the cellular location of capsaicinoid accumulation within the interlocular septum. Capsaicinoids were not detected in *C. chinense* NMCA 30036,

a non-pungent variety, or negative controls (Fig. 2a–c). Figure 2d shows the immunolocalization of 3-keto-acyl ACP synthase (KAS), a capsaicinoid biosynthetic enzyme, to the epidermal cells consistent with previous reports (Aluru *et al*., 2003). Similarly, capsaicinoids were localized to the epidermal cells of the interlocular septum (Fig. 2e). Signal was also detected in the cell layer immediately subtending the epidermal cells, but it was not possible to determine whether this was actually due to the presence of capsaicinoids or an artefact of sample preparation. Capsaicinoids and KAS were detected in all the epidermal cells; their expression was not spatially restricted and did not occur in a noticeable pattern in the epidermal cells. Staining of the specimens with crystal violet, a general cytological stain, revealed cellular differentiation between cells under the blisters compared with cells not under the blisters (Fig. 2g). This suggests their involvement in secretion of capsaicinoids into the subcuticular cavity. The results of the organ-specificity of capsaicinoid biosynthesis and immunolocalization studies taken together establish that capsaicinoid accumulation and biosynthesis are localized to the epidermal cells of the interlocular septum in the pepper fruit.

Association of blisters and presence of capsaicinoids

Examination of the blistered structures observed on the interlocular septa of pepper fruit showed that they were only present in the pungent genotype, Habanero, and were entirely absent from the non-pungent genotype, NMCA 30036 (Fig. 3a). To examine the relationship between blisters and pungency more closely, the capsaicinoid accumulation along the interlocular septum was measured before and after brushing the tissue to remove blisters along the surface (Fig. 3b). Similar to studies where exudates are produced in glandular trichomes, it was not possible to remove all blisters from the interlocular septum by brushing (Fridman *et al*., 2005). Capsaicinoid accumulation in brushed interlocular septa was 20 600±3700 ppm compared with 52 000±6800 ppm in unbrushed samples, a reduction of 60% (Fig. 3).

The distribution of blisters was examined to understand the relationship between blister development and capsaicinoid accumulation. Blisters were not observed on interlocular septa of ripe non-pungent NMCA 30036 or 10 dpa Habanero fruits (Fig. 4a, b). Blisters were first observed at 20 dpa in Habanero fruit and persisted throughout fruit development (Fig. 4c–f). Generally, the size of the

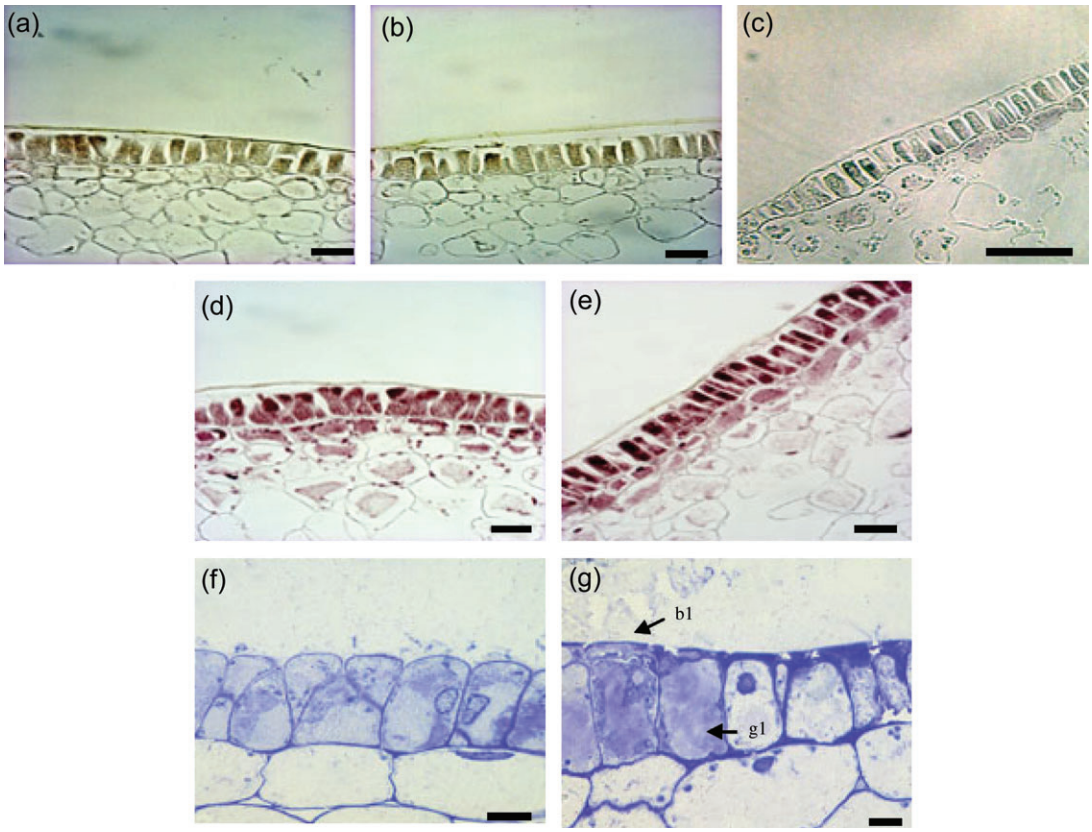

Fig. 2. Immunolocalization of capsaicinoid biosynthesis to the interlocular septum epidermis in pungent and non-pungent genotypes. (a) Habanero (pungent), rabbit preimmune serum; (b) Habanero, secondary antibody, diluted 1:1500; (c) *C. chinense* NMCA 30036 (non-pungent), capsaicinoid antibody diluted 1:250; (d) Habanero, KAS antibody, diluted 1:250; (e) Habanero, capsaicinoid antibody, diluted 1:250; (f, g) Habanero interlocular septum sections stained with Crystal Violet. Bar=50 μm (a–e) or 10 μm (f, g). bl, blister; gl, glandular cells.

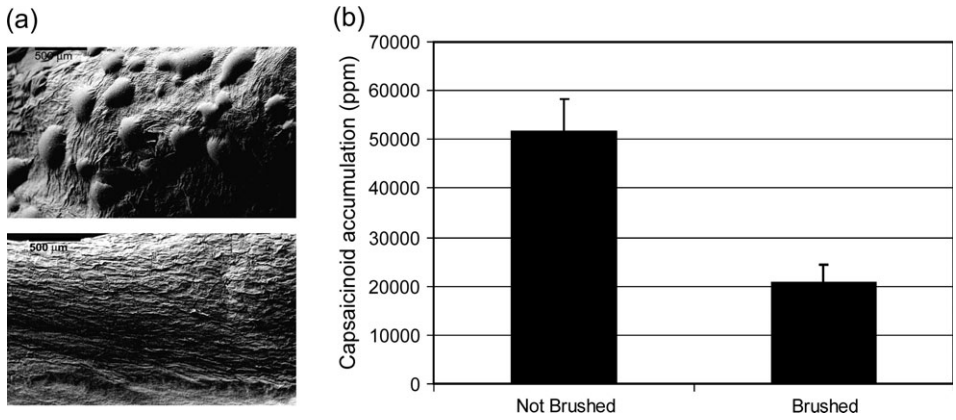

Fig. 3. Localization of capsaicinoid accumulation to blisters along the epidermis of the interlocular septum in Habanero. (a) The epidermal surface of the interlocular septum in Habanero (top) and NMCA 30036 (bottom), pungent and non-pungent, respectively, were viewed using a scanning electron microscope. (b) Comparison of the absolute levels of capsaicinoids in Habanero interlocular septa tissue with or without a brushing treatment to remove blisters. Septum tissue was extracted directly with acetonitrile (not brushed) after drying and weighing or gently brushed (brushed) before drying, weighing, and extraction. Values represent the mean ±SD of at least three plants.

individual blisters increased markedly during fruit development although definitive measurements were hard to make. Consistent with this trend, capsaicinoids did not begin to accumulate in Habanero until 20 dpa, gradually increasing throughout fruit development (Fig. 4g). These results and the previous finding that capsaicinoid biosynthesis is not restricted to cells under the blisters suggest that the blisters act primarily as a site of capsaicinoid storage for materials synthesized in the epidermal cells.

Genetic analysis of pungency, blisters, and Pun1

An F_2 population (n=495) derived from reciprocal crosses of Habanero×NMCA 30036 was generated to confirm an earlier report that the presence/absence of blisters along the interlocular septum is controlled by the single recessive gene, *lov*, at a second locus controlling pungency (Votava and Bosland, 2002). Habanero is blistered and pungent while NMCA 30036 is non-blistered and non-pungent. The resulting F_1 plants were blistered and pungent, confirming the dominance of blisters and pungency. Results for the F_2 population, shown in Table 1, indicate that absence of blisters precisely co-segregated with non-pungency. Blisters were never observed in non-pungent plants. In a testcross population, blisters co-segregated exactly with pungency consistent with a 1:1 ratio, confirming monogenic inheritance and perfect association of these traits in *C. chinense* (Table 1).

The genomic sequence at the *Pun1* locus was determined in NMCA 30036 to develop PCR-based markers for testing whether *Pun1* segregated independently from the blister phenotype. During the development of these primers, the *Pun1* locus from *C. chinense* was sequenced. A deletion of four base pairs (bp) located centrally in the first exon was identified in *C. chinense* NMCA 30036 at the *Pun1* locus (Fig. 5). This deletion was confirmed by sequencing cDNA from 30 dpa NMCA 30036 fruit. Sequence analysis predicted that this 4 bp deletion causes a frameshift mutation which results in a truncated AT3 protein. The truncated AT3 is not predicted to include the putative active site, HXXXDG (St Pierre and De Luca, 2000).

Based on this deletion, a co-dominant marker system (Fig. 5) for *Pun1* in *C. chinense* was developed. Three primers were used for genotyping, with primer 3 spanning the deletion found in NMCA 30036. When all the primers are mixed together during PCR, the reaction favours primer 3 as the reverse primer in the NMCA 30036 genotypes, generating a 0.5 kb amplicon (the shorter of two possible amplicons). Alternatively, in Habanero genotypes, primer 2 is the preferred reverse primer and generates a 1.1 kb amplicon. Primer 3 does not generate a significant amplification product in Habanero genotypes due to base pair mismatching between the primer and template. Genotyping of F_2 individuals from the segregating population described above revealed that this 4 bp deletion, tentatively named *pun1*^{2}, co-segregated precisely with the absence of blisters and non-pungency in the F_2 population shown in Table 1. These results suggest that non-pungency in NMCA 30036 previously attributed to a second locus, *lov*, is actually perfectly associated with a 4 bp deletion at the *Pun1* locus. To confirm this result, genetic complementation analysis was performed to assess whether *lov* in *C. chinense* and *Pun1* in *C. annuum* are allelic.

Complementation tests to determine allelic diversity of the Pun1 *locus*

To test the hypothesis that the 4 bp deletion identified in the *Pun1* locus of NMCA 30036 is the gene previously designated *lov*, a genetic complementation test was

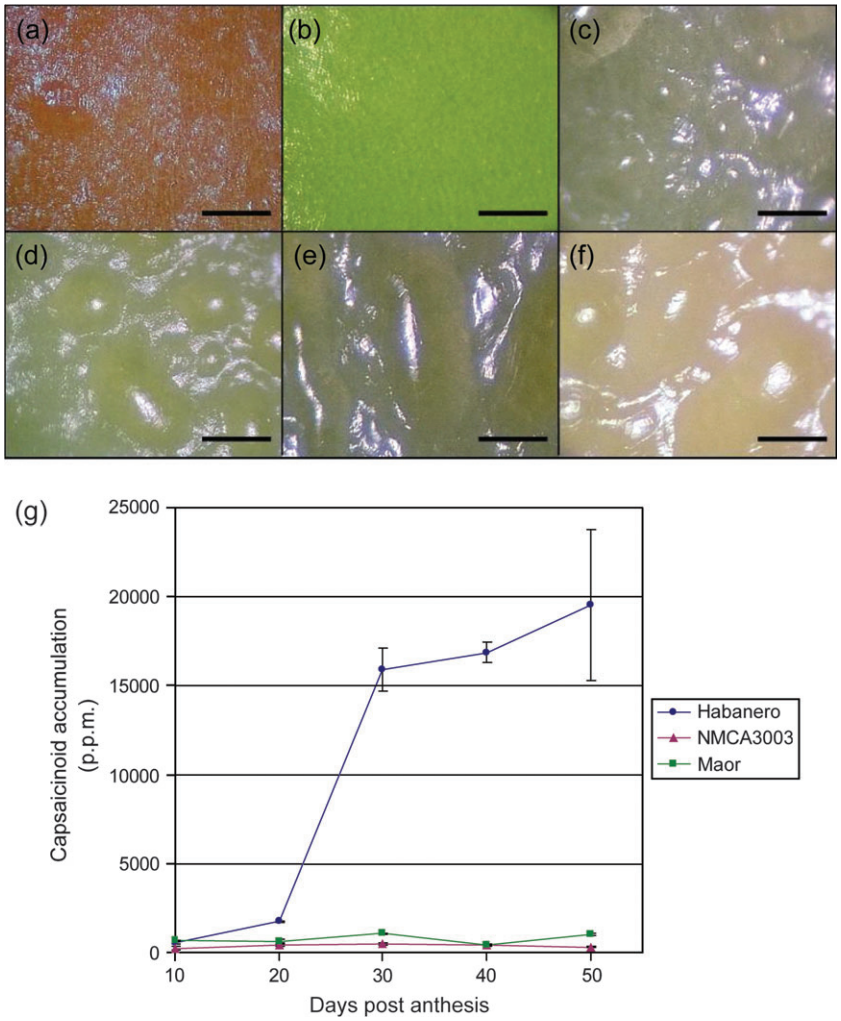

Fig. 4. Association of blister development and capsaicinoid accumulation. (a) Interlocular septum of a ripe NMCA 30036 fruit. Bar=250 μm. (b–f) Habanero fruits were harvested at 10 d intervals, and the interlocular septa photographed. All images are representative of at least three fruits. Bar=250 μm. (g) HPLC analysis of capsaicinoid accumulation through fruit development in Habanero, NMCA 30036, and *C. annuum* Maor. Capsaicinoids were extracted from interlocular septa tissue except for 10 d post-anthesis samples, in which whole fruits with seeds removed, were used due to small fruit size. Values represent the mean ±SD for at least three plants.

performed by crossing *C. chinense* NMCA 30036×*C. annuum* Maor (non-pungent, *pun1/pun1*). Capsaicinoid accumulation was not detectable in either parent, NMCA 30036 or Maor (Table 2). Capsaicinoid accumulation in all F_1 plants was below the lower detection limit of the ELISA assay system (0.50 ppm) indicating that the loss-of-pungency mutations in Maor and NMCA 30036 fail to complement. These data conclusively establish that the locus previously designated *lov* is actually a second recessive allele of the *Pun1* locus, defined by a 4 bp deletion. According to conventions in *Capsicum* gene nomenclature, it is proposed that this allele be designated *pun1*^{2}, and listed as synonymous with *lov*. The F_1 plants that were recovered from this cross did produce fruit, but did not produce viable seeds and were deformed and exhibited virus-like symptoms, which is often observed in the progeny of such crosses. The reciprocal cross could not be obtained.

Regulation of AT3, Pal, *and* Kas *gene expression in peppers containing the alleles,* Pun1, pun1, *and* pun1^2

Transcript accumulation of capsaicinoid biosynthetic genes is hypothesized to correlate with the level and presence of pungency (Curry *et al*., 1999; Aluru *et al*., 2003). The expression patterns of *AT3*, a putative acyltransferase encoded by the *Pun1* locus, and phenylalanine ammonia lyase (*Pal*) and *Kas*, two structural genes implicated in capsaicinoid biosynthesis, were examined in Habanero (*Pun1/Pun1*), Maor (*pun1/pun1*), and NMCA 30036 (*pun1*^{2}*/pun1*^{2}). In Habanero, *Pal* and *Kas* expression was similar; both were strongly expressed by 20 dpa before decreasing gradually throughout later fruit development (Fig. 6a). *Pal* and *Kas* were specific to the interlocular septum, although a small amount of signal was detected in flowers and seeds. This background probably resulted from the fact that both *Pal* and *Kas* are

Table 1. *Genetic analysis and co-segregation of blisters, pungency, and* Pun1 *genotype*

A F_2 population (n=495) derived from reciprocal crosses Habanero (*Pun1*/*Pun1*, pungent, blistered)×NMCA 30036 (*pun1*^{2}/*pun1*^{2}, non-pungent, not blistered) was analysed for the inheritance and co-segregation of blisters, pungency, and *Pun1*. Bl=blistered; NB=not blistered.

	n	Pungent		Non-pungent		Expected ratio	Chi-square
	Pop. size	Bl *Pun1*	Bl *pun1*^{2}	NB *Pun1*	NB *pun1*^{2}	(Bl:NB)	(*P*-value)
P_1 Habanero	20	20	0	0	0		
P_2 NMCA 30036	22	0	0	0	22		
F_1 (P_1×P_2)	1	1	0	0	0		
F_1 (P_2×P_1)	8	8	0	0	0		
F_2 [(P_1×P_2)×(P_1×P_2)]	214	169	0	0	45	3:1	1.80 (0.180)
F_2 [(P_2×P_1)×(P_2×P_1)]	281	202	0	0	79	3:1	1.45 (0.228)
Pooled F_2	495	371	0	0	124	3:1	0.0007 (0.979)
BC_1P_1 [(P_2×P_1)×P_2)]	85	48	0	0	37	1:1	1.42

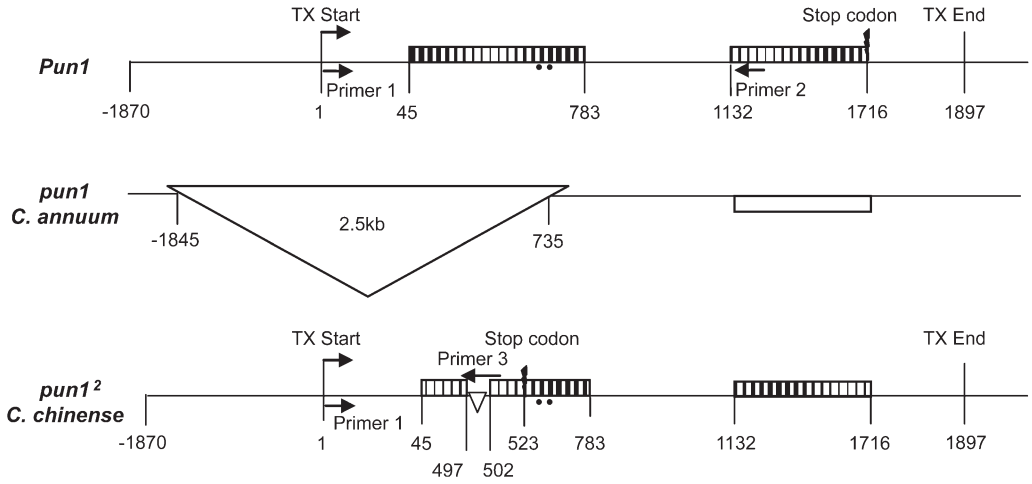

Fig. 5. Genomic DNA structure of the *Pun1* locus from pungent (*Pun1*/*Pun1*) and non-pungent (*pun1*/*pun1*; *pun1*^{2}/*pun1*^{2}) genotypes. Schematic diagram of the *Pun1*, *pun1*, and *pun1*^{2} alleles. Exons (closed boxes) were deduced from cDNA sequence. Open boxes depict exons predicted not to be transcribed due to a deletion in the gene. The deletions in the *pun1* and *pun1*^{2} alleles are represented with an inverted triangle. Transcription start/end sites and translation stop codons are also indicated. The putative active site of AT3 is identified by two dots. Primers used for genotyping are indicated with arrows.

Table 2. *Genetic complementation tests to determine allelism at the* Pun1 *locus in non-pungent genotypes*

Parents and F_1 hybrids were assayed for capsaicinoid accumulation using an ELISA system.

	Genotype	*n*	Capsaicinoid accumulation (ppm)
Maor	*pun1*	8	nd[a]
NMCA 30036	*pun1*^{2}	8	nd
NMCA 30036×Maor	*pun1*^{2}×*pun1*	2	nd*(<0.50±0.10)[b]

[a] nd, Not detectable.
[b] Asterisk denotes capsaicinoid accumulation was below limits of assay detection.

members of gene families and are essential genes in phenylpropanoid and fatty acid metabolism, respectively (Blum *et al*., 2003).

AT3 expression was also specific to the interlocular septum and was first detected at 20 dpa. *AT3* expression persisted throughout much of fruit development but was not detectable by 50 dpa. The expression patterns of *Pal*, *Kas*, and *AT3* closely followed that of capsaicinoid accumulation throughout fruit development (Fig. 4g). Capsaicinoids were first detected at 20 dpa and increased through fruit development. At 50 dpa capsaicinoids still accumulated, even though expression of *Pal*, *Kas*, and *AT3* was not detected. It is plausible that metabolic turnover, for example, a reduction in the degradation of capsaicin, could account for the continued capsaicinoid accumulation at 50 dpa (Bernal *et al*., 1993; Estrada *et al*., 2000).

In Maor, both *Pal* and *Kas* had significantly reduced transcript accumulation throughout much of fruit development relative to Habanero (Fig. 6a). Similar to Habanero, however, there was background expression of both *Pal* and *Kas* in leaf, flower, seed, and pericarp tissues. In addition, there was strong expression of *Pal* and *Kas* in the seeds, probably due to lignins and fatty acids known to accumulate in seeds (Buchanan *et al*., 2000). *AT3* expression in Maor was not detectable during fruit development.

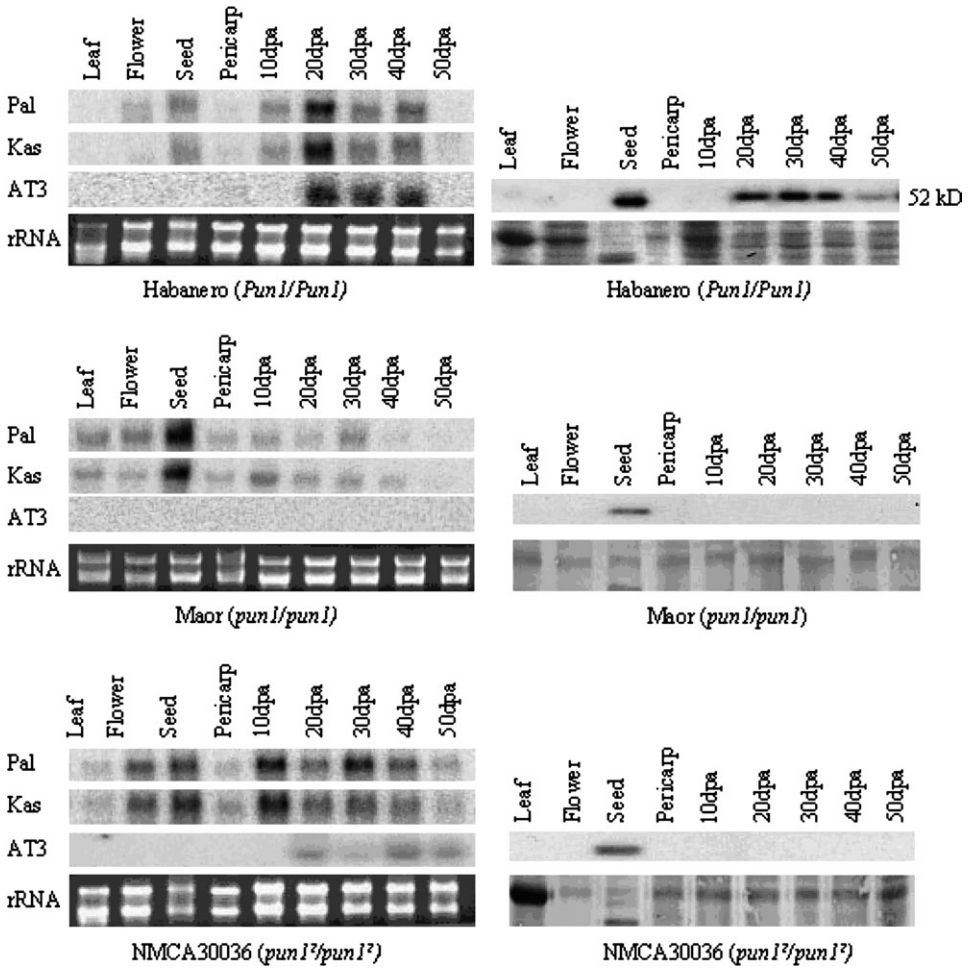

Fig. 6. Gene expression patterns of capsaicinoid biosynthetic genes *Pal*, *Kas*, and *AT3* during fruit development in *Pun1*, *pun1*, and *pun1*^{2} genotypes. (a) *Pal*, *Kas*, and *AT3* expression was analysed in leaf, flower, seed, pericarp, and interlocular septum tissues through development at 10 d intervals. The full-length cDNA of *AT3* was hybridized to blots containing RNA from Habanero, Maor, and NMCA 30036. Whole fruit was used for samples taken at 10 dpa due to their small size. (b) Immunoblot analysis of AT3 expression in the same tissues as described above. Total protein was probed with an AT3 polyclonal antibody (1:2000 dilution) as the primary antibody and horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:5000 dilution). Whole fruits were used for samples taken at 10 dpa due to their small size. Membranes were Coomassie stained after probing to show equal loading of protein.

This observation was consistent with the lack of capsaicinoid accumulation and was also consistent with previous results showing that the deletion in *AT3* disrupts transcription and translation (Fig. 4g) (Stewart *et al*., 2005).

Pal and *Kas* had similar expression profiles in NMCA 30036 (Fig. 6a). Both *Pal* and *Kas* were strongly expressed early in fruit development, 10 dpa versus 20 dpa in Habanero. At 20 dpa, however, both *Pal* and *Kas* were less abundant in NMCA 30036 than in Habanero. Expression levels of *Pal* and *Kas* were higher in NMCA 30036 than in Maor, perhaps due to genotype- or species-specific differences. In NMCA 30036, *AT3* transcripts were expressed at significantly lower levels when compared with Habanero from 20 dpa through 50 dpa (Fig. 6a).

AT3 protein accumulation in Habanero followed the same pattern as transcript accumulation, first detected at 20 dpa, and reduced by 50 dpa (Fig. 6b). AT3 protein was not detectable in Maor or NMCA 30036 genotypes, except in seeds. The absence of AT3 protein accumulation in these genotypes is consistent with their mutations, a 2.5 kb deletion in *pun1* and a 4 bp deletion in *pun1*^{2}, which disrupt translation. A cross-hybridizing band was detected in the seed tissues of all genotypes tested. This band persisted despite variations in protein extraction procedures and was consistently found in pungent and non-pungent genotypes. There are probably several acyltransferases involved in the production and storage of fatty acids and oils operating in seed tissues (Buchanan *et al*., 2000). A similar cross-hybridizing band was reported in the isolation and characterization of DAT, an acyltransferase involved in vindoline biosynthesis (St Pierre *et al*., 1998).

Segregation of gene expression and Pun1 *genotype*

RNA gel blots were made from representative samples of mature green (approximately 40 dpa) fruit from the F_2 homozygous progeny classes resulting from the cross of Habanero×NMCA 30036. *Pal* gene expression did not segregate between the different F_2 classes (Fig. 7b). This is consistent with the gene expression studies shown in Fig. 6 which show that *Pal* is similarly expressed in both Habanero and NMCA 30036 at 40 dpa. *Kas* appeared to accumulate more in *Pun1*/*Pun1* F_2 progeny even though *Kas* expression at 40 dpa between the two parental genotypes is not significantly different. It is plausible that this difference in *Kas* gene expression is a result of environmental variation between the parental genotypes shown in (Fig. 6) and F_2 progeny (Fig. 7b). *AT3* was clearly more abundant in the *Pun1*/*Pun1* progeny than in the *pun1*^{2}/*pun1*^{2} progeny. Within the *Pun1*/*Pun1* class there were not major differences in *AT3* expression even though total capsaicinoid accumulation varied significantly (Fig. 7d). AT3 protein was not detectable in *pun1*^{2}/*pun1*^{2} progeny, consistent with the genetic lesion that produces the truncated protein (Fig. 7c). Taken together, the results clearly establish a strong relationship between the allelic state of *Pun1*, pungency, and *AT3* and *Kas* expression.

Discussion

Due to centuries of human manipulation, taxonomists disagree on the criteria necessary to identify some of the species and varieties of *Capsicum*. In particular, *C. annuum*, *C. frutescens*, and *C. chinense* are referred to as the *C. annuum* complex due to the difficulty of demarcating the individual species. Members of this complex include pungent and non-pungent, wild and domesticated varieties which interbreed and are morphologically similar (Walsh and Hoot, 2001). In previous research, it was established that the *pun1* allele is responsible for non-pungency within *C. annuum* as a result of a large deletion at *Pun1* that has been conserved and propagated for several centuries (Stewart *et al*., 2005). In this report, another recessive allele of *Pun1*, namely *pun1*^{2}, is identified. DNA sequencing of *pun1*^{2} revealed a 4 bp deletion in the centre of the first exon of *AT3*. Inheritance studies revealed that *pun1*^{2} co-segregated with the absence of blisters, non-pungency, and decreased expression of two capsaicinoid biosynthetic genes, *Kas* and *AT3*. Genetic complementation tests confirmed that *pun1*^{2} is allelic to *pun1*. We have tentatively identified another allele of *pun1* in *C. frutescens* (GM Stellari and M Mazourek, unpublished data). This study establishes that, to date, only mutations in *Pun1* define known genetic sources of non-pungency in domesticated *Capsicum* spp.

Results from this study support the long-held assertion that capsaicinoids are synthesized primarily or exclusively in the interlocular septa of pungent fruits. This tissue has independently been referred to as the septum (Ohta, 1962; Rowland *et al*., 1983), dissepiment (Furuya and Hashimoto, 1954; Balbaa *et al*., 1968), cross wall (Huffman *et al*., 1978), or placenta (Iwai *et al*., 1977*a*, *b*, 1979; Fujiwake

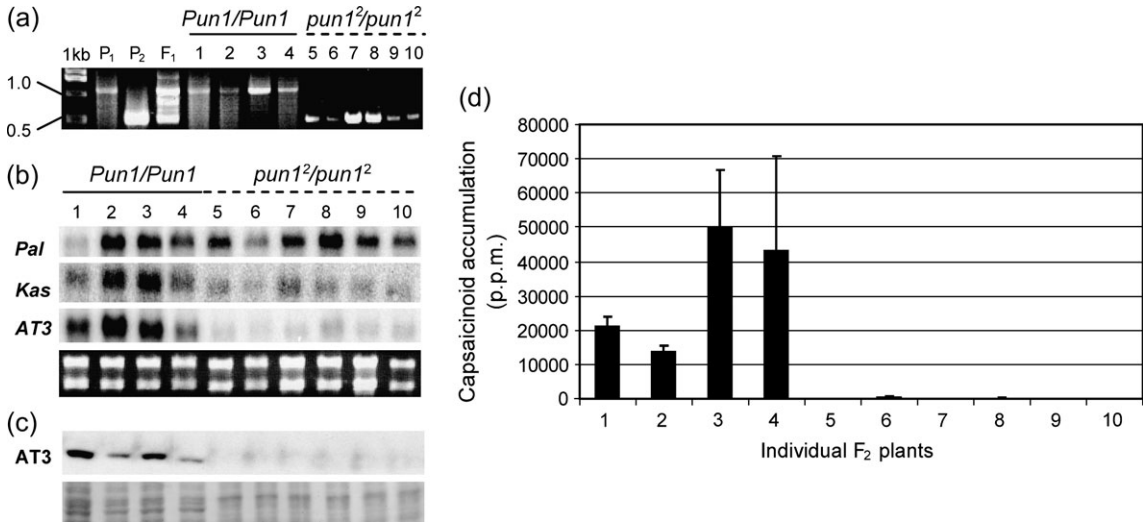

Fig. 7. Segregation of *Pal*, *Kas*, and *AT3* gene expression with the allelic state of *Pun1* in a *C. chinense* Habanero (*Pun1*/*Pun1*)×*C. chinense* NMCA 30036 (*pun1*^{2}/*pun1*^{2}) F_2 population. (a) Agarose gel showing PCR bands from *Pun1* genotyping of the F_2 population. Numbers represent individual plants. 1 kb, P_1, and P_2 represent the 1 kb DNA ladder, Habanero parent, and NMCA 30036 parent, respectively. (b) RNA gel-blots of *Pal*, *Kas*, and *AT3* expression in mature green F_2 interlocular septum tissue. EtBr staining of rRNA was used as a loading control. Numbers represent individual plants. (c) Immunoblot analysis of AT3 expression in mature green interlocular septum tissue. Total protein was probed with an AT3 polyclonal antibody (1:2000 dilution) as the primary antibody and horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:5000 dilution). Equal protein loading was confirmed by Coomassie staining the membrane after probing. (d) HPLC analysis of capsaicinoid accumulation in the interlocular septa of mature green *Pun1*/*Pun1* and *pun1*^{2}/*pun1*^{2} F_2 progeny. Values represent the mean ±SD of duplicate extractions.

et al., 1980; Suzuki *et al*., 1980), generating confusion in the literature. In small-fruited varieties, the distinction between the placenta and interlocular septum is difficult to discern and in other varieties this tissue breaks down during ripening (Rao and Paran, 2003). Huffman *et al*. (1978) attributed the small amounts of capsaicinoids detected in the seeds to surface contamination during dissection. There has been persistent debate in the literature regarding the existence of glands that accumulate capsaicinoids. At least two reports concluded that the existence of specialized glands containing capsaicinoids could not be verified (Erwin, 1932; Huffman *et al*., 1978). Conversely, other reports have documented the presence of capsaicin-secreting glands, sometimes called receptacles, vesicles, or blisters, along the interlocular septum of pungent genotypes (Furuya and Hashimoto, 1954; Ohta, 1962; Suzuki *et al*., 1980; Rowland *et al*., 1983; Zamski *et al*., 1987; Votava and Bosland, 2002). In botany, the term 'gland' is used broadly, often having the connotation of specialized secretory structures (e.g. glandular trichomes of tomato or mint). However, any cells secreting specialized metabolites can be referred to as glandular even if they lack glandular trichomes (Fahn, 1979). For example, the papillae cells of the stigma surface are called glandular based on their secretion of carbohydrates and lipids to aid pollen tube growth, yet they lack formal secretory structures (Buchanan *et al*., 2000).

The term blister has been used to describe the epidermal swellings produced by the filling of subcuticular cavities along the interlocular septum of pungent peppers (Rowland *et al*., 1983; Zamski *et al*., 1987). In an analogous system, the resin exudate of epidermal cells along young leaves and stipules in *Populus* spp. (Salicaceae) is secreted into the space between the outer wall of the cells and the cuticle, forming a bulge or blister (Langehheim, 2003). In this article, the term blister was used in reference to such epidermal swellings in pungent pepper fruits and the term glandular cells in broad reference to any cells that secrete capsaicin.

Blisters in Habanero begin forming at 20 dpa concomitant with capsaicinoid accumulation. This link between specialized morphological features, blisters, and their specialized metabolites, capsaicinoids, is consistent with studies in sage and thyme (Lamiaceae), showing that the development of the glandular trichomes is correlated with essential oil production (Croteau *et al*., 1981; Yamaura *et al*., 1989). Immunolocalization of capsaicinoids and *Kas*, a subunit of the fatty acid synthase complex involved in capsaicinoid biosynthesis, showed that the epidermal cells are the site of capsaicinoid biosynthesis. Capsaicinoid biosynthesis is not restricted to the cells immediately subtending the blisters but occurs along the entire epidermis. Based on differential staining observed during microscopic analysis, the cells immediately under the blister are glandular cells, or idioblasts, that secrete capsaicinoids. Capsaicinoids are secreted into spaces between the cell wall and the cuticle and, as the pressure builds, the cuticle is separated and bulges to form a blister with a subcuticular cavity (Furuya and Hashimoto, 1954; Ohta, 1962; Rowland *et al*., 1983; Zamski *et al*., 1987). Similar to the blisters on resin-secreting tissues in *Populus* spp., the pepper blisters are formed from the exudate secreted from multiple cells; however, it is not known whether capsaicinoids are transported intercellularly from nearby cells not immediately under a blister (Langehheim, 2003). These results are consistent with the conclusion of Rowland *et al*. (1983), who reported that capsaicinoids were only found in the glandular areas along the interlocular septum of Jalapeño peppers.

Several reports in the literature describe blisters in non-pungent *Capsicum* varieties (Rowland *et al*., 1983; Zamski *et al*., 1987). Such a phenomenon was not observed in this analysis. A possible source of these observations may be that intense yellow-pigmented areas occur in both pungent and non-pungent varieties; however, careful examination reveals no association with pungency. This is consistent with previous work that concluded that the presence of the yellow pigmentation does not necessarily indicate the presence of capsaicinoids (Huffman *et al*., 1978).

A previous report stated that the presence/absence of blisters constituted a second locus controlling pungency, *lov*, in the non-pungent *C. chinense* genotype NMCA 30036 (Votava and Bosland, 2002). Analysis of F_2 and testcross populations indicated that the absence of blisters and non-pungency perfectly co-segregate in every population examined, consistent with the possibility that a single defect has pleiotropic effects on capsaicinoid biosynthesis and blisters. This implies that capsaicinoid biosynthesis is required for blister formation. Genetic complementation tests reported here definitively prove that non-pungency in NMCA 30036 is due to a recessive allele at the *Pun1* locus herein designated *pun1*^{2}.

Our results also support the hypothesis that the transcriptional state of some genes in the capsaicinoid biosynthetic pathway is positively correlated with pungency (Curry *et al*., 1999; Aluru *et al*., 2003). The complete absence of AT3 protein accumulation in *pun1* and *pun1*^{2} genotypes is consistent with a lack of capsaicinoid accumulation. The *pun1* allele is characterized by the absence of *AT3* expression, owing to a 2.5 kb deletion of *AT3*, and residual levels of *Pal* and *Kas* expression (Stewart *et al*., 2005). In *pun1*^{2} genotypes, a small 4 bp frameshift mutation results in the absence of AT3 protein accumulation. Also within *pun1*^{2} both *Pal* and *Kas* are expressed at levels greater than *pun1* but less than *Pun1*. A strong up-regulation of several capsaicinoid biosynthetic genes (*pAMT*, *Pal*, *Kas*, *BCAT*, *FatA*) occurs 20 dpa after flowering coinciding with capsaicinoid accumulation in pungent varieties of both *C. annuum* and *C. chinense* (Stewart *et al*., 2005). In addition, the transcript

accumulation of *AT3* and *Kas* at 20 dpa was higher in pungent genotypes than in non-pungent genotypes from both *C. annuum* and *C. chinense*. This pattern of expression was confirmed in an F_2 population. This suggests that the association of elevated transcript levels of genes involved in capsaicinoid biosynthesis with capsaicinoid biosynthesis, itself, is consistent across multiple *Capsicum* species. While there were small differences in *Pal* expression between pungent and non-pungent varieties, its expression did not segregate in an F_2 population. This indicates that coordinated transcriptional regulation of capsaicinoid biosynthesis occurs downstream of *Pal* with genes that are specific to this metabolic pathway. Current research efforts aim to characterize relevant gene expression in non-pungent *Capsicum* accessions drawn from diverse species and to analyse the genetic basis for variation in the expression of genes implicated in this biosynthetic pathway.

The identification of a new recessive allele of *Pun1* and the development of co-dominant markers will be useful for marker-assisted selection in breeding programmes and studies of allelic diversity at loci relevant for agriculture. Growing evidence suggests that dramatic changes in phenotypes during plant domestication can result from perturbations in single dominant genes, such as *Pun1*. The gain and loss of strawberry flavour, the architecture of maize inflorescence, and the liberating of maize kernels from the hardened fruitcases of the maize progenitor teosinte are all traits controlled by single dominant genes (Aharoni *et al*., 2004; Gallavotti *et al*., 2004; Wang *et al*., 2005). Manipulation of such genes will be useful in the metabolic engineering of natural products for the benefit of humanity.

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