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Novel Loss-of-Function *putative aminotransferase* Alleles Cause Biosynthesis of Capsinoids, Nonpungent Capsaicinoid Analogues, in Mildly Pungent Chili Peppers (*Capsicum chinense*)

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Capsinoids are a group of nonpungent capsaicinoid analogues produced in *Capsicum* fruits. They have similar bioactivities to capsaicinoids such as suppression of fat accumulation and antioxidant activity. They are more palatable ingredients in dietary supplements than capsaicinoids because of their low pungency. Previous studies on nonpungent *Capsicum annuum* cultivars showed that capsinoid biosynthesis is caused by loss-of-function *putative aminotransferase* (*p-amt*) alleles. This study showed that three mildly pungent cultivars of *Capsicum chinense* (Zavory Hot, Aji Dulce strain 2, and Belize Sweet) contain high levels of capsinoid. It was shown that these cultivars have novel *p-amt* alleles, which contain mutations that differ from those of *C. annuum*. Sequence analysis of *p-amt* in Belize Sweet revealed that a 5 bp insertion (TGGGC) results in a frameshift mutation. A transposable element (*Tcc*) was found in the *p-amt* of Zavory Hot and Aji Dulce strain 2. *Tcc* has features similar to those of the *hAT* transposon family. This was inserted in the fifth intron of Zavory Hot and in third intron of Aji Dulce strain 2. The *p-amt* alleles harboring *Tcc* cannot produce an active p-AMT. These mildly pungent cultivars will provide a new natural source of capsinoids.

KEYWORDS: *Capsicum chinense*; capsinoid; nonpungent capsaicinoid analogue; putative aminotransferase; frameshift mutation; transposon

INTRODUCTION

Capsaicin and its analogues, collectively called capsaicinoids, are the pungent components of *Capsicum* fruits (*1*). The fundamental chemical structure of capsaicinoids comprises an acid amide of vanillylamine combined with a fatty acid. Previous studies have shown that capsaicinoids have a wide range of bioactivities, such as the enhancement of thermogenesis and suppression of fat accumulation (*2*, *3*). However, their strong pungency prevents their use as ingredients in foods and supplements.

Capsinoids are nonpungent capsaicinoid analogues, which were first isolated from a nonpungent *Capsicum annuum* cultivar, CH-19 Sweet (*4*, *5*). They are similar in structure to capsaicinoids, but have an ester group instead of the amide moiety (**Figure 1**). Compared to capsaicinoids, capsinoids have similar bioactivity but considerably lower pungency (*6*, *7*). Several studies confirm that capsiate, a member of the capsinoids, promotes thermogenesis or endurance capacity through activation of the capsaicin receptor, transient receptor potential vanilloid type 1 (*8*, *9*). Moreover, various effects of capsinoids, such as suppression of fat accumulation, apoptosis induction, and anticancer and antioxidant properties, have been reported (*10*−*15*). Capsinoids are more palatable than capsaicinoids because of their low pungency. From this perspective, capsinoids are attractive ingredients for dietary supplements.

Our research on two *C. annuum* cultivars, CH-19 Sweet and Himo, showed that capsinoid biosynthesis was caused by the loss-of-function alleles of the *putative aminotransferase* (*p-AMT*) gene (*16*, *17*). p-AMT is thought to catalyze the formation of vanillylamine from vanillin in the capsaicinoid biosynthetic pathway (*18*, *19*). In the $p\text{-}amt^{CH\text{-}19\ Sweet}$ allele, a T nucleotide insertion forms a new stop codon (TGA), which prevents the translation of the *p-AMT* gene. In the $p\text{-}amt^{Himo}$ allele, a single nucleotide substitution results in one amino acid substitution in the pyridoxal 5-phosphate (PLP) binding domain. These mutations suppress the formation of vanillylamine from vanillin, instead of which vanillyl alcohol is produced, which in turn leads to the production of capsinoids (**Figure 1**). Nonpungent cultivars containing capsinoids, other than these two cultivars, have not been reported.

Capsicum chinense is a different species from CH-19 Sweet and Himo (*C. annuum*). Most *C. chinense* cultivars are pungent. Of the domesticated species of the genus *Capsicum*, *C. chinense* is recognized as having the most pungent fruit, because the extremely pungent *Capsicum* cultivars belong to *C. chinense*, including

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Habanero and Scotch Bonnet (*20*). However, there are some mildly pungent cultivars in *C. chinense*, called Aji Dulce (which means "sweet chili" in Spanish). The genetic mechanism underlying the mild pungency of *C. chinense* is unknown. Germplasm screening revealed that these mildly pungent cultivars contain high levels of capsinoids. The aim of this study is to identify the genetic mechanism of capsinoid biosynthesis in mildly pungent *C. chinense* cultivars.

MATERIALS AND METHODS

Plant Material. Four *C. chinense* cultivars were used in this study: three mildly pungent cultivars (Zavory Hot, Aji Dulce strain 2, and Belize Sweet) and one pungent cultivar (Red Habanero). Immature fruits of the mildly pungent cultivars are shown in **Figure 2**. All plants were grown at the experimental farm of Kyoto University, from March to October 2009. The capsinoid and capsaicinoid contents of pepper fruits were determined using HPLC as described below.

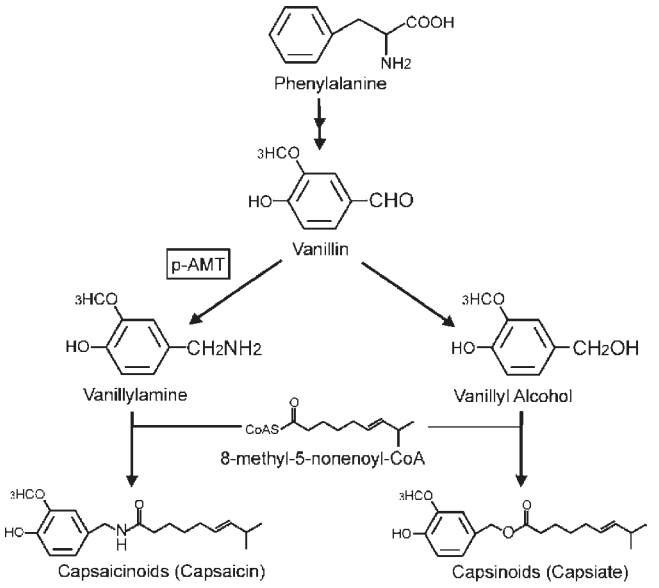

Figure 1. Proposed biosynthesis pathway of capsaicinoid and capsinoid in *Capsicum*. Putative aminotransferase (p-AMT) is supposed to catalyze the formation of vanillylamine from vanillin.

Extraction of Capsinoids and Capsaicinoids. To determine the capsinoid and capsaicinoid contents, three immature fruits at approximately 30 days after flowering or mature fruits at approximately 45 days after flowering were used. These fruits become red at the mature fruit stage. Their contents were determined as described in our previous study (*17*). After the pericarps were cut with a knife, whole fruits were dried completely in a freeze-dryer (FDU-540, EYELA) for 3 days. Dried fruits were ground in a blender (MK-61M, National) at room temperature. A 4 mL volume of acetone (Wako) was added to 200 mg of dry fruit powder. After vortexing, the sample tube was allowed to settle for 15 min at room temperature. The supernatant was collected, and 1 mL of acetone was added to the residue. Then the supernatant was collected again. After this process had been repeated, 1 mL of ethyl acetate was added to the residue, and the supernatant was collected. The combined supernatant volume was completely evaporated in a rotary evaporator (VC-960, Taitec) at 36 °C under vacuum. The residue was dissolved in 2 mL of ethyl acetate, and the resulting solution was filtered into a 2 mL glass vial using a Sep-Pak cartridge C18 (Waters) and then used for HPLC (Hitachi L-7420 system) analysis.

HPLC Analysis Conditions. The separation was performed on a μ-Bondapak C18 column (10 μm, 3.9 mm × 150 mm, Waters) coupled with a guard column (μ-Bondapak Guard-Pak, Waters). The eluent, which was detected at 280 nm with a UV detector, was a mixture of MeOH/H_2O (70:30 v/v) with a flow rate of 1.0 mL/min. The capsinoid content was calculated as the sum of capsiate and dihydrocapsiate, and the capsaicinoid content was calculated as that of capsaicin and dihydrocapsaicin.

Genomic Sequence Analysis of *p-AMT*. The genomic sequence covering the full length of *p-AMT* was determined for pungent and mildly pungent cultivars. Genomic DNA was extracted from young leaves of pepper plants using Nucleon PhytoPure (GE Healthcare, U.K.). The *p-AMT* genomic sequence covering the full-length open reading frame (ORF) was amplified using primer sets: F1 and 7th intron R, F443 and R788, 10th intron F and R1313, F747 and R1055, 14th intron F and R1481 (**Figure 3**). The sequences of primers are shown in **Table 1**. The genomic region harboring the insertion in Zavory Hot was amplified using F304 and 7th intron R, and that in Aji Dulce strain 2 was amplified using 3rd intron F and R282. The genomic PCR reaction mixture consisted of 0.5 μL of LA Taq polymerase (TAKARA, Japan), 5 μL of buffer (provided with the polymerase), 8 μL of dNTP mixture (2.5 mM), 5 μL of $MgCl_2$ (25 mM), and 1.0 μL of forward and reverse primer (20 μM) and

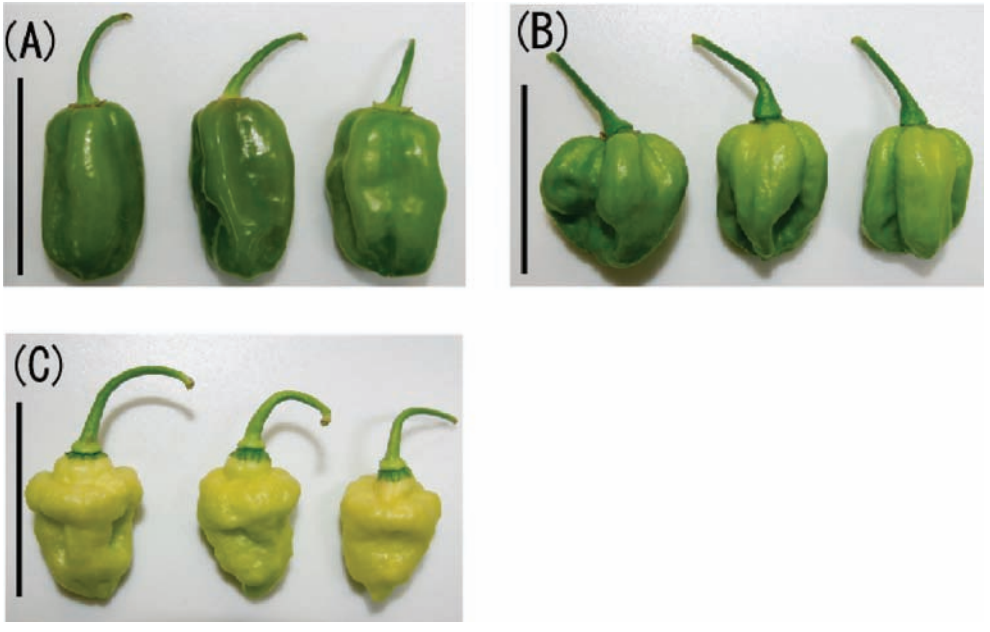

Figure 2. Mildly pungent *C. chinense* cultivars (immature fruit stage, approximately 30 days after flowering): (**A**) Zavory Hot; (**B**) Aji Dulce strain 2; (**C**) Belize Sweet. Bar indicates 5 cm.

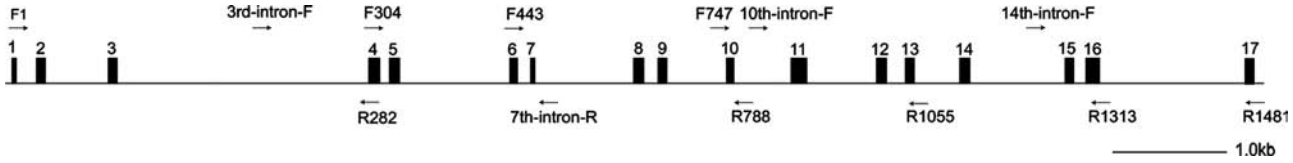

Figure 3. Design and location of primers for genomic PCR of *p-AMT* gene: schematic representation of the genomic organization of the *p-AMT* gene in a pungent cultivar, Red Habanero. Black boxes indicate exons, and lines indicate introns. Arrows indicate location and direction of primers. The sequence of each primer is given in **Table 1**.

Table 1. Primers Used for Genomic Sequence Analysis of *p-AMT*

primer name	primer sequence (5′−3′)
F1	TCTTTCTCTTTCCTTAGCAAT
3rd intron F	CCCCCTCTTATGGGTGAAAC
R282	GCAGCTTCAACAAGTCGAGTC
F304	GCCATTTTATCATTCATTTTGGA
F443	GGTGAAGATGGTGTGGTATT
7th intron R	AAATGATCATGTTATGTTCAAAAA
F747	TCCTAGGAGCAGCAGGTGTAAT
R788	AATATGTTGCGGGAGGAAGT
10th intron F	CCCGCTTTGGTCCTCTCTCTG
R1055	CAGGGTGTCCGGAATAAGTAAA
14th intron F	AATATGCTTCGCCCCTAAAT
R1313	CCAACATCCCGTACTTAGCACA
R1481	ATAAACAAGCTTTCGCCGTGA

was adjusted to 50 μL with superdistilled water. Approximately 30 ng of genomic DNA was used as a template. The genomic PCR procedure was as follows: 1 cycle of 2 min at 94 °C; 35 cycles of 10 s at 98 °C, 30 s at 55 °C, and 15 min at 68 °C, with a final extension of 15 min at 68 °C. For cloning purposes, the *p-AMT* sequence amplified by genomic PCR was cloned to a pCR-XL-TOPO vector using a TOPO XL PCR Cloning Kit (Invitrogen, USA). Nucleotide sequencing was carried out in an ABI PRISM 3100 genetic analyzer with an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA).

cDNA Sequence Analysis of *p-AMT*. The full-length cDNA sequences of *p-AMT* were determined in pungent and mildly pungent cultivars. Pepper fruits were harvested at 30 days after flowering, and the placenta was separated for RNA extraction, because placental dissepiment is the site of capsaicinoid and capsinoid biosynthesis (*21*−*23*). *p-AMT* is expressed specifically in the placenta (*16*, *18*). Total RNA was extracted from the placenta using a QuickGene RNA Cultured Cell Kit S (Fuji Film, Japan) with some modifications. All RNA used for RT-PCR was treated with DNase I prior to cDNA synthesis to remove DNA contamination. The RT reaction was performed using 0.5 μL of ReverTra Ace (TOYOBO, Japan) with a reaction mixture composed of 2 μL of RT buffer, 1 μL of dNTPs (10 mM), 0.5 μL of RNase inhibitor, 0.5 μL of oligo (dT) primer (20 μM), and 4.5 μL of superdistilled water. Total RNA was adjusted to approximately 500 ng/μL, and 1 μL was used as a template. This mixture was incubated at 42 °C for 30 min and at 99 °C for 5 min. The full-length cDNA sequence of *p-AMT* was amplified using primer sets, F1 and R1481. The primer sequences were designed on the basis of the studied nucleotide sequence for *p-AMT* (GenBank accession no. AF085149); they were designed to amplify a 1481 bp fragment of the *p-AMT* cDNA sequence. The RT-PCR reaction mixture consisted of 0.4 μL of KOD FX polymerase (TOYOBO, Japan), 20 μL of buffer (provided with the polymerase), 8 μL of dNTPs (2 mM), and 8 μL of forward and reverse primer (20 μM) and was adjusted to 40 μL with superdistilled water. A 1 μL aliquot of cDNA was used as a template. The PCR procedure to amplify *p-AMT* was as follows: 1 cycle of 2 min at 96 °C; 35 cycles of 10 s at 98 °C, 30 s at 55 °C, and 3 min at 68 °C; and a final extension of 10 min at 68 °C. RT-PCR products were separated on a 1% agarose gel, stained with ethidium bromide, and visualized using a UV transilluminator. The full-length sequence of *p-AMT* amplified by RT-PCR was cloned to pUC118 by using a Mighty Cloning Kit (TAKARA, Japan). Nucleotide sequencing was carried out as described above. BLAST (http://blast.ncbi.nlm.nih.gov/) was applied to analyze nucleotide and derived amino acid sequences as well as multiple alignments.

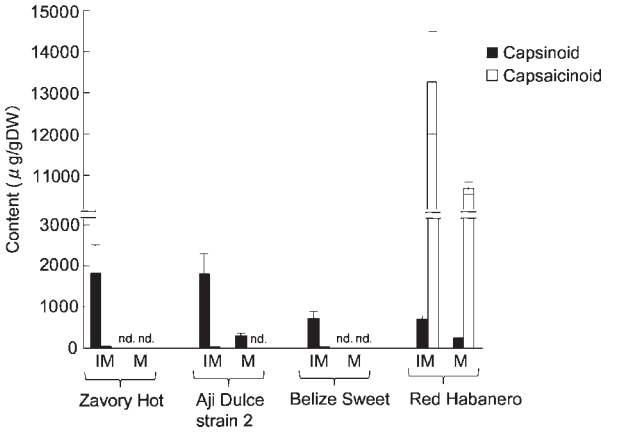

Figure 4. Capsinoid and capsaicinoid contents in *C. chinense* cultivars: IM, immature fruit stage; M, mature fruit stage. Bar indicates standard error (n = 3). nd indicates not detected.

RESULTS AND DISCUSSION

Mildly Pungent *C. chinense* Cultivars Contain Capsinoid. The pungent cultivar, Red Habanero, mainly contained capsaicinoids and produced capsinoids in trace amounts. On the contrary, the three mildly pungent cultivars contained high levels of capsinoid and produced capsaicinoid in trace amounts (**Figure 4**). The capsinoid contents in Zavory Hot, Aji Dulce strain 2, and Belize Sweet were 1812, 1797, and 732 μg/g DW, respectively. Previous studies have shown that the capsinoid content decreased more drastically than capsaicinoid content as fruits matured (*4*, *24*). In mildly pungent cultivars, the capsinoid content also decreased drastically during fruit maturation (**Figure 4**). Immature fruit should be collected to obtain a high level of capsinoid from these cultivars. Capsinoids, which are esters of a fatty acid and vanillyl alcohol, decompose easily in polar solvents such as water and methanol (*25*). This instability in water could be responsible for its rapid decrease during fruit maturation.

Genomic Sequence Analysis of *p-amt* Alleles. Our previous studies showed that capsinoid biosynthesis in *C. annuum* is determined by loss-of-function *p-amt* alleles (*16*, *17*). We investigated the *p-AMT* gene to identify the genetic mechanism of capsinoid biosynthesis in mildly pungent *C. chinense* cultivars. The *p-AMT* genomic sequence corresponding to the ORF was amplified from the pungent cultivar, Red Habanero, and the resulting 11 kb sequence was determined. The *p-AMT* of Red Habanero consists of 17 exons separated by 16 introns, and its sequence is very similar to that of the *C. annuum* cultivars (**Figure 5A**). The *p-AMT* of Belize Sweet also consists of 17 exons, but a 5 bp insertion (TGGGC) was found in the eighth exon (**Figure 5B**). In Zavory Hot and Aji Dulce strain 2, insertions of transposable elements were found in introns of *p-AMT* (**Figure 5C**,**D**). When a genomic region between the fourth exon and seventh intron was amplified, a 1.5 kb fragment was obtained from the *C. chinense* cultivars except for Zavory Hot (**Figure 6A**). In contrast, a 3.8 kb fragment from Zavory Hot was amplified,

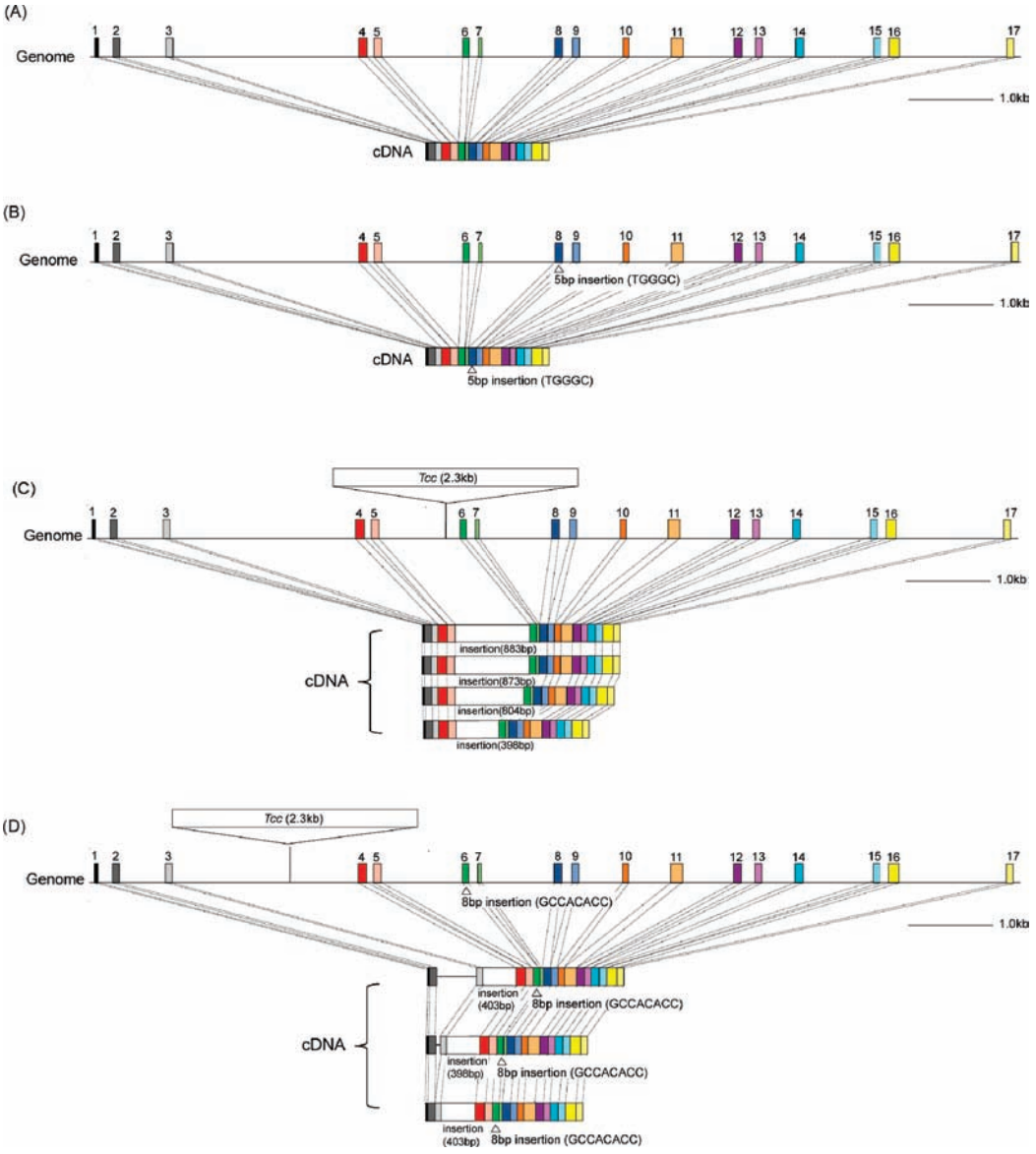

Figure 5. Schematic diagrams of the *p-AMT* allele and RT-PCR-derived cDNAs in *C. chinense* cultivars. (**A**) Schematic representation of the genomic organization of the *p-AMT* gene in a pungent cultivar, Red Habanero. Colored boxes indicate exons and lines indicate introns. The cDNA encodes a full-length open reading frame of *p-AMT*. The three mildly pungent cultivars have loss-of-function *p-amt* alleles, which contain a transposable element of *C. chinense* (*Tcc*) or a short nucleotide insertion. (**B**) Schematic representation of the $p\text{-}amt^{Belize\ Sweet}$ allele and cDNA sequence. The $p\text{-}amt^{Belize\ Sweet}$ allele contains a 5 bp insertion (TGGGC) in the eighth exon, which leads to a frameshift mutation. (**C**) Schematic representation of the $p\text{-}amt^{Zavory\ Hot}$ allele and cDNA sequence. The $p\text{-}amt^{Zavory\ Hot}$ allele contains *Tcc* in the fifth intron region. *Tcc* is a member of the *hAT* transposon superfamily and is 2.3 kb long. The cDNAs in Zavory Hot have insertions of the *Tcc* sequence between the fifth and sixth exons. Splice variants were identified by sequencing cDNA fragments, none of which encode the full-length *p-AMT* open reading frame. (**D**) Schematic representation of the $p\text{-}amt^{Aji\ Dulce\ strain\ 2}$ allele and cDNA sequences. The $p\text{-}amt^{Aji\ Dulce\ strain\ 2}$ allele contains *Tcc* in the third intron region. The cDNAs in Aji Dulce strain 2 also have insertions of the *Tcc* sequence between the third and fourth exons. In addition, some cDNAs contain second intron sequences and an 8 bp insertion (GCCACACC) in the sixth exon.

cloned, and sequenced. A distinct long insertion sequence was found in this fragment. The insertion sequence was 2299 bp long and was located in the 3′ part of the fifth intron (**Figure 5C**). A short ORF (104 amino acids in length) was found in this insertion sequence and showed similarity in its amino acid sequence to the *hAT* dimerization domain. This insertion sequence contained 23 bp terminal inverted repeats with an 8 bp target site duplication (CCCTTACA). These structure characteristics indicated that this insertion is a member of the *hAT* transposon superfamily. We named this element a "transposon of *C. chinense*" (*Tcc*) (see the Supporting Information). *Tcc* appears to be nonautonomous because it is not long enough for its amino acid sequence to encode the entire transposase. When a genomic region between the third intron and the fourth exon was amplified, a 0.8 kb fragment was obtained from the *C. chinense* cultivars except Aji Dulce strain 2 (**Figure 6B**). In contrast, a 3.1 kb fragment from Aji Dulce strain 2 was amplified, cloned, and sequenced. This insertion is identical to *Tcc* with an 8 bp target site duplication (CTATGACC). In addition, an 8 bp insertion (GCCACACC) was found in the sixth exon of Aji Dulce strain 2 (**Figure 5D**).

Aberrant *p-AMT* mRNAs Are Expressed in Mildly Pungent Cultivars. Analysis of the *p-AMT* expression by RT-PCR was conducted with a primer set of F1 and R1481. The 1.5 kb fragments were amplified in Red Habanero and Belize Sweet, but larger cDNA fragments were amplified in Zavory Hot and Aji Dulce strain 2 (**Figure 7**). cDNA sequences of *p-AMT* were

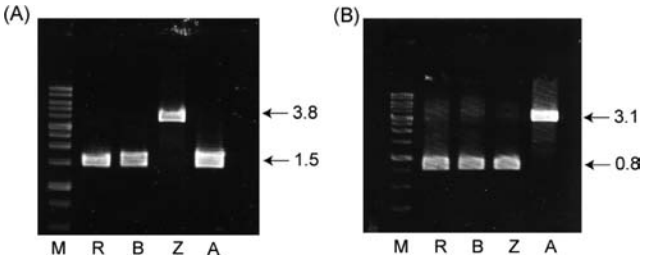

Figure 6. Genomic PCR analysis of *p-AMT*. (**A**) Amplification of the region between the fourth exon and seventh intron. Primer F304 and seventh intron R were used. (**B**) Amplification of the region between the third intron and fourth exon. Primer third intron F and R282 were used. The size of the amplified fragment is indicated to the right (kb). Lanes: M, DNA ladder marker; R, Red Habanero; B, Belize Sweet; Z, Zavory Hot; A, Aji Dulce strain 2.

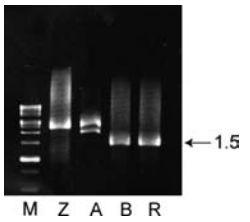

Figure 7. RT-PCR for full length of *p-AMT* in *C. chinense* cultivars. Lanes: Z, Zavory Hot; A, Aji Dulce strain 2; B, Belize Sweet; R, Red Habanero. Zavory Hot, Aji Dulce strain 2, and Belize Sweet are mildly pungent cultivars containing capsinoid. Red Habanero is a pungent cultivar. Primers for RT-PCR were designed to amplify the full-length coding sequence of *p-AMT* (1481 bp). The size of the amplified fragment is indicated to the right (kb).

determined in these cultivars. **Figure 5** shows a schematic representation of the genomic sequence of *p-AMT* and a graphic summary of distinct RT-PCR cDNA clones derived from the placenta. In Red Habanero, all cDNA sequences contained a complete ORF of 1377 bp encoding a putative aminotransferase of 459 amino acids (**Figure 5A**). p-AMT of Red Habanero is completely identical to that of a pungent cultivar (GenBank accession no. AF085149) in the amino acid sequence. This result indicates that mRNAs encoding functional p-AMT are synthesized in the placenta of Red Habanero.

In contrast, cDNA sequences encoding p-AMT were not detected in mildly pungent cultivars. The *p-AMT* cDNA of Belize Sweet contains a 5 bp insertion at the eighth exon, which results in a frameshift mutation (**Figure 5B**). This would lead to a truncated protein of 217 amino acids lacking the PLP-binding domain, which is an important domain for aminotransferase activity and is present in the p-AMT of pungent cultivars. Therefore, the insertion at the eighth exon could prevent the translation of the active p-AMT in Belize Sweet. The *p-AMT* cDNAs of Zavory Hot contained a 400−900 bp insertion between the fifth and sixth exons (**Figure 5C**). The sequence of the insertion is identical to a part of *Tcc*. The insertion position corresponds to that of *Tcc* in the genomic sequence. The *p-AMT* cDNAs of Aji Dulce strain 2 also contain a part of the *Tcc* sequence between the third and fourth exons (**Figure 5D**). In addition, an 8 bp nucleotide (GCCACACC) and a second intron sequence were inserted into the cDNA of Aji Dulce strain 2. *Tcc* insertions in the *p-AMT* cDNA sequence could lead to a frameshift mutation, resulting in a truncated p-AMT. It has been shown that the insertion of a transposable element changes the splicing pattern of a gene, resulting in an mRNA containing a sequence of the element (*26*). The *Tcc* insertion at the intron region could change the splicing pattern of the *p-AMT* gene, and the mRNA containing the *Tcc* sequence and some splice variants could be synthesized.

p-AMT cDNA sequence analysis suggests that functional *p-AMT* mRNAs were not produced in mildly pungent cultivars. Taken together with the genomic sequences, mildly pungent cultivars have loss-of-function alleles of *p-amt*, designated $p\text{-}amt^{Belize\ Sweet}$, $p\text{-}amt^{Zavory\ Hot}$, and $p\text{-}amt^{Aji\ Dulce\ strain\ 2}$. A loss-of-function *p-amt* allele could suppress the formation of vanillylamine from vanillin, producing vanillyl alcohol instead, which in turn results in capsinoid formation in mildly pungent cultivars, such as in CH-19 Sweet and Himo (**Figure 1**).

Multiple Loss-of-Function Alleles of *p-amt* Contribute to the Generation of Mild Pungency and Capsinoid Biosynthesis in *C. chinense*. At least two genotypes of nonpungent cultivars in *C. annuum* have been reported so far. The first are the *pun1*/*pun1* cultivars, which contain neither capsaicinoid nor capsinoid. The *Pun1* gene was cloned and characterized as a *putative acyltransferase* gene, which can produce capsaicinoids from vanillylamine and a fatty acid (*27*, *28*). The single loss-of-function allele of *pun1* was found to be responsible for nonpungency throughout many *C. annuum* cultivars. *pun1* has a 2.5 kb deletion spanning the promoter and the first exon, which prevents the translation of acyltransferase (*27*). The second are the *p-amt*/*p-amt* cultivars, which do not contain capsaicinoid but produce capsinoid. It has been found that there are two types of loss-of-function *p-amt* alleles in *C. annuum*. In the $p\text{-}amt^{CH\text{-}19\ Sweet}$ allele, a T nucleotide insertion at 1291 bp forms a new stop codon and prevents the translation of normal p-AMT, whereas in the $p\text{-}amt^{Himo}$ allele, one nucleotide substitution results in one amino acid substitution in the PLP binding domain (*16*, *17*). Each *p-amt* allele is specific to CH-19 Sweet and Himo, respectively.

In *C. chinense*, another recessive allele of $pun1^2$ was found in nonpungent accession NMCA30036, which contains a 4 bp deletion, resulting in a frameshift mutation of *Pun1* (*23*). Here we revealed that the three mildly pungent cultivars have novel loss-of-function alleles of *p-amt*. These *p-amt* alleles have a transposon (*Tcc*) or short inserted nucleotides (**Figure 5**). The inserted positions are different among the three cultivars, suggesting that these mutations occurred independently in the breeding process of the *C. chinense* cultivars. Therefore, mildly pungent *C. chinense* cultivars were generated not by single mutation but multiple loss-of-function mutations in *p-amt*.

Two of three *p-amt* alleles are generated by *Tcc* insertion. *Tcc* insertion was not found in $p\text{-}amt^{Belize\ Sweet}$, but this allele has a 5 bp insertion (TGGGC) in the eighth exon. It has been found that the excision of the *hAT* family transposon from a given site can generate 5−8 bp nucleotides, called footprints (*29*). These extra sequences can cause a frameshift mutation (*30*). The 5 bp insertion (TGGGC) in the $p\text{-}amt^{Belize\ Sweet}$ allele could be a footprint generated by insertion and the subsequent excision of a transposon, such as *Tcc*. It was assumed that *Tcc* could widely contribute to the generation of mild pungency in *C. chinense*. The other mildly pungent *C. chinense* cultivars were reported (*31*). Further analysis of mildly pungent *C. chinense* cultivars will reveal the relationship between *Tcc* and *p-amt*, and it remains unclear whether these three *p-amt* alleles are specific to *C. chinense* or also exist in other *Capsicum* species. To understand the domestication and breeding process concerning nonpungency, further analysis of *p-AMT* should be conducted using not only *C. chinense* but also other *Capsicum* species.

In this study, we found that three mildly pungent cultivars of *C. chinense* contain high levels of capsinoid, a nonpungent capsaicinoid analogue. Our previous study showed that capsinoid biosynthesis is caused by a loss-of-function *p-amt* gene in nonpungent *C. annuum* cultivars CH-19 Sweet and Himo. It was revealed that

mildly pungent *C. chinense* cultivars have novel loss-of-function *p-amt* alleles. The *p-amt* alleles of the three cultivars contain different mutations. Sequence analysis of $p\text{-}amt^{Belize\ Sweet}$ revealed that a 5 bp insertion (TGGGC) results in a frameshift mutation. $p\text{-}amt^{Zavory\ Hot}$ and $p\text{-}amt^{Aji\ Dulce\ strain\ 2}$ contain a transposable element (*Tcc*) in the intron regions. *Tcc* shares features with the *hAT* transposon family. This was inserted in the fifth intron of $p\text{-}amt^{Zavory\ Hot}$ and in the third intron of $p\text{-}amt^{Aji\ Dulce\ strain\ 2}$. The *p-amt* alleles harboring *Tcc* could not produce functional *p-AMT* mRNAs. We conclude that multiple loss-of-function mutations of *p-amt* could contribute to the generation of mildly pungent *C. chinense* cultivars with capsinoid biosynthesis. These mildly pungent *C. chinense* cultivars will be a useful natural source of capsinoid.

Supporting Information Available: Sequence of *Tcc* inserted in *p-amt* alleles. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Nelson, E. K.; Dawson, L. E. The constitution of capsaicin, the pungent principle of *Capsicum*. III. *J. Am. Chem. Soc.* **1923**, *45*, 2179−2181.

(2) Kawada, T.; Hagihara, K.; Iwai, K. Effects of capsaicin on lipid metabolism in rats fed a high fat diet. *J. Nutr.* **1986**, *116*, 1272−1278.

(3) Kawada, T.; Sakabe, S.; Aoki, N.; Watanabe, T.; Higeta, K.; Iwai, K.; Sugimoto, E. Intake of sweeteners and pungent ingredients increases the thermogenin content in brown adipose tissue of rat. *J. Agric. Food Chem.* **1991**, *39*, 651−654.

(4) Yazawa, S.; Suetome, N; Okamoto, K; Namiki, T. Content of capsaicinoids and capsaicinoid-like substances in fruit of pepper (*Capsicum annuum* L.) hybrid made with 'CH-19 Sweet' as a parent. *J. Jpn. Soc. Hortic. Sci.* **1989**, *58*, 601−607.

(5) Kobata, K.; Todo, T.; Yazawa, S.; Iwai, K.; Watanabe, T. Novel capsaicinoid-like substances, capsiate and dihydrocapsiate, from the fruits of a nonpungent cultivar, CH-19 Sweet, of pepper (*Capsicum annuum* L.). *J. Agric. Food Chem.* **1998**, *46*, 1695−1697.

(6) Iida, T.; Moriyama, T.; Kobata, K.; Morita, A.; Murayama, N.; Hashizume, S.; Fushiki, T.; Yazawa, S.; Watanabe, T.; Tominaga, M. TRPV1 activation and induction of nociceptive response by a non-pungent capsaicin-like compound, capsiate. *Neuropharmacology* **2003**, *44*, 958−967.

(7) Sasahara, I.; Furuhata, Y.; Iwasaki, Y.; Inoue, N.; Sato, H.; Watanabe, T.; Takahashi, M. Assessment of the biological similarity of three capsaicin analogs (capsinoids) found in non-pungent chili pepper (CH-19 Sweet) fruits. *Biosci., Biotechnol., Biochem.* **2010**, *74*, 274−278.

(8) Ohnuki, K.; Niwa, S.; Maeda, S.; Inoue, N.; Yazawa, S.; Fushiki, T. CH-19 Sweet, a non-pungent cultivar of red pepper, increased body temperature and oxygen consumption in humans. *Biosci., Biotechnol., Biochem.* **2001**, *65*, 2033−2036.

(9) Haramizu, S.; Mizunoya, W.; Masuda, Y.; Ohnuki, K.; Watanabe, T.; Yazawa, S.; Fushiki, T. Capsiate, a nonpungent capsaicin analog, increases endurance swimming capacity of mice by stimulation of vanilloid receptors. *Biosci., Biotechnol., Biochem.* **2006**, *70*, 774−781.

(10) Kawabata, F.; Inoue, N.; Yazawa, S.; Kawada, T.; Inoue, K.; Fushiki, T. Effects of CH-19 Sweet, a non-pungent cultivar of red pepper, in decreasing the body weight and suppressing body fat accumulation by sympathetic nerve activation in humans. *Biosci., Biotechnol., Biochem.* **2006**, *70*, 2824−2835.

(11) Ohnuki, K.; Haramizu, S.; Oki, K.; Watanabe, T.; Yazawa, S.; Fushiki, T. Administration of capsiate, a non-pungent capsaicin analog, promotes energy metabolism and suppresses body fat accumulation in mice. *Biosci., Biotechnol., Biochem.* **2001**, *65*, 2735−2740.

(12) Macho, A.; Sancho, R.; Minassi, A.; Appendino, G.; Lawen, A.; Muñoz, E. Involvement of reactive oxygen species in capsaicinoid-induced apoptosis in transformed cells. *Free Radical Res.* **2003**, *37*, 611−619.

(13) Macho, A.; Lucena, C.; Sancho, R.; Daddario, N.; Minassi, A.; Muñoz, E.; Appendino, G. Non-pungent capsaicinoids from sweet pepper, synthesis and evaluation of the chemopreventive and anticancer potential. *Eur. J. Nutr.* **2003**, *42*, 2−9.

(14) Masuda, Y.; Haramizu, S.; Oki, K; Ohnuki, K.; Watanabe, T.; Yazawa, S.; Kawada, T.; Hashizume, S.; Fushiki, T. Upregulation of uncoupling proteins by oral administration of capsiate, a non-pungent capsaicin analog. *J. Appl. Physiol.* **2003**, *95*, 2408−2415.

(15) Rosa, A.; Deiana, M.; Casu, V.; Paccagnini, S.; Appendino, G.; Ballero, M.; Dessi, M. A. Antioxidant activity of capsinoids. *J. Agric. Food Chem.* **2002**, *50*, 7396−7401.

(16) Lang, Y; Kisaka, H.; Sugiyama, R.; Nomura, K.; Morita, A.; Watanabe, T.; Tanaka, Y.; Yazawa, S.; Miwa, T. Functional loss of pAMT results in biosynthesis of capsinoids, capsaicinoid analogs, in *Capsicum annuum* cv. CH-19 Sweet. *Plant J.* **2009**, *59*, 953−961.

(17) Tanaka, Y.; Hosokawa, M.; Miwa, T.; Watanabe, T.; Yazawa, S. Newly mutated *putative-aminotransferase* in nonpungent pepper (*Capsicum annuum*) results in biosynthesis of capsinoids, capsaicinoid analogues. *J. Agric. Food Chem.* **2010**, *58*, 1761−1767.

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

(19) Del Rosario Abraham-Juárez, M.; Del Carmen Rocha-Granados, M.; López, M. G.; Rivera-Bustamante, R. F.; Ochoa-Alejo, N. Virus-induced silencing of *Comt*, *pAmt* and *Kas* genes results in a reduction of capsaicinoid accumulation in chili pepper fruits. *Planta* **2008**, *227*, 681−695.

(20) Canto-Flick, A.; Balam-Uc, E.; Bello-Bello, J. J.; Lecona-Guzmán, C.; Solís-Marroquín, D.; Avilés-Viñas, S.; Gómez-Uc, E.; López-Puc, G.; Santana-Buzzy, N.; Iglesias-Andreu, L.G. Capsaicinoids content in Habanero pepper (*Capsicum chinense* Jacq.): hottest known cultivars. *HortScience* **2008**, *43*, 1344−1349.

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

(22) Sutoh, K.; Kobata, K.; Yazawa, S.; Watanabe, T. Capsinoid is biosynthesized from phenylalanine and valine in a non-pungent pepper, *Capsicum annuum* L. cv. CH-19 Sweet. *Biosci., Biotechnol., Biochem.* **2006**, *70*, 1513−1516.

(23) Stewart, C., Jr.; Mazourek, M.; Stellari, G. M.; O'Connell, M.; Jahn, M. Genetic control of pungency in *C. chinense* via the *Pun1* locus. *J. Exp. Bot.* **2007**, *58*, 979−991.

(24) Tanaka, Y.; Hosokawa, M.; Otsu, K.; Watanabe, T.; Yazawa, S. Assessment of capsiconinoid composition, nonpungent capsaicinoid analogues, in *Capsicum* cultivars. *J. Agric. Food Chem.* **2009**, *57*, 5407−5412.

(25) Sutoh, K.; Kobata, K.; Watanabe, T. Stability of capsinoid in various solvents. *J. Agric. Food Chem.* **2001**, *49*, 4026−4030.

(26) Hoshino, A.; Morita, Y.; Choi, J.-D.; Saito, N.; Toki, K.; Tanaka, Y.; Iida, S. Spontaneous mutations of the flavonoid 3′-hydroxylase gene conferring reddish flowers in the three morning glory species. *Plant Cell Physiol.* **2003**, *44*, 990−1001.

(27) Stewart, C., Jr.; Kang, B.-C.; Liu, K.; Mazourek, M.; Moore, S. L.; Yoo, E. Y.; Kim, B.-D.; Paran, I.; Jahn, M. M. The *Pun1* gene for pungency in pepper encodes a putative acyltransferase. *Plant J.* **2005**, *42*, 675−688.

(28) Lee, C.-J.; Yoo, E. Y.; Shin, J. H.; Lee, J.; Hwang, H.-S.; Kim, B.-D. Non-pungent *Capsicum* contains a deletion in the capsaicinoid synthetase gene, which allows early detection of pungency with SCAR markers. *Mol. Cells* **2005**, *19*, 262−267.

(29) Moon, S.; Jung, K.-H.; Lee, D.; Jiang, W.-Z.; Koh, H. J.; Heu, M.-H.; Lee, D. S.; Suh, H. S.; An, G. Identification of active transposon *dTok*, a member of the *hAT* family, in rice. *Plant Cell Physiol.* **2006**, *47*, 1473−1483.

(30) Quattrocchio, F.; Wing, J.; Woude, K.; Souer, E.; Vetten, N.; Mol, J.; Koes, R. Molecular analysis of the *antocyanin2* gene of petunia and its role in the evolution of flower color. *Plant Cell.* **1999**, *11*, 1433−1444.

(31) Votava, E. J.; Bosland, P. W. 'NuMex Suave Red' and 'NuMex Suave Orange' mild *Capsicum chinense* cultivars. *HortScience* **2004**, *39*, 627−628.

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