JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 1761–1767 1761
DOI:10.1021/jf903282r

Newly Mutated *putative-aminotransferase* in Nonpungent Pepper (*Capsicum annuum*) Results in Biosynthesis of Capsinoids, Capsaicinoid Analogues

Yoshiyuki Tanaka,*,† Munetaka Hosokawa,† Tetsuya Miwa,‡ Tatsuo Watanabe,§ and Susumu Yazawa†

†Graduate School of Agriculture, Kyoto University, Oiwake-cho, Kitashirakawa, Kyoto 606-8502, Japan, ‡Research Institute for Health Fundamentals, Ajinomoto Company Inc., 1-1 Suzuki-cho, Kawasaki-ku, Kawasaki 210-8681, Japan, and §School of Food and Nutritional Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan

Capsinoids make up a group of nonpungent capsaicinoid analogues produced in *Capsicum* fruits. They have bioactivities similar to those of capsaicinoids such as suppression of fat accumulation and antioxidant activity. Because of their low pungency, they are more palatable ingredients in dietary supplements than capsaicinoids. We recently reported that capsinoid biosynthesis is caused by nonsense mutation in a putative aminotransferase gene (*p-AMT*) in a nonpungent cultivar CH-19 Sweet. Here we report on the screening of nonpungent germplasm that revealed a nonpungent cultivar Himo, which contains high levels of capsinoids. We have shown that Himo has a recessive allele of *p-amt*, which contains a mutation different from that of CH-19 Sweet. Sequence analysis of *p-amt* in Himo revealed that a single-nucleotide substitution results in one amino acid substitution from cysteine to arginine in the pyridoxal 5-phosphate binding domain. Genetic analysis using a cleaved amplified polymorphic sequence marker confirmed that the *p-AMT* genotype was precisely cosegregated with capsinoid biosynthesis and nonpungency. Himo will provide a new natural source of capsinoids.

KEYWORDS: ***Capsicum*; nonpungent capsaicinoid analogue; capsinoid; putative aminotransferase; one-amino acid substitution**

INTRODUCTION

The pungent components in *Capsicum* fruits are capsaicin and its analogues, capsaicinoids (*1*). The fundamental chemical structure of capsaicinoids comprises an acid amide of vanillylamine with a fatty acid. Capsaicinoids are reported to have many bioactivities such as the enhancement of thermogenesis and suppression of fat accumulation (*2*, *3*). However, their use as ingredients in foods and supplements has been limited by their pungency.

Capsinoids are nonpungent capsaicinoid analogues, which were first isolated from a nonpungent 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*). Several reports confirm that capsiate, a member of the family of capsinoids, promotes thermogenesis or endurance capacity through activation of the capsaicin receptor, transient receptor potential vanilloid type 1 (TRPV1) (*7*, *8*). Moreover, various effects of capsinoids such as suppression of fat accumulation, apoptosis induction, and anticancer and antioxidant properties have been reported (*9*−*14*). Because of their low pungency, capsinoids are more palatable than capsaicinoids. From this perspective, capsinoids are attractive ingredients for dietary supplements.

CH-19 Sweet contains a large amount of capsinoids, but most pungent cultivars produce trace amounts of capsinoids (*15*). Our recent research on CH-19 Sweet has shown that capsinoid biosynthesis was caused by the functional loss of the *putative-aminotransferase* (*p-AMT*) gene (*16*). *p-AMT* is thought to catalyze the formation of vanillylamine from vanillin in the capsaicinoid biosynthetic pathway (*17*, *18*). A single-nucleotide polymorphism analysis of cDNA sequence revealed a T nucleotide insertion at base pair 1291 in the *p-AMT* gene of CH-19 Sweet. This insertion formed a new stop codon, TGA, which prevents the translation of the *p-AMT* gene. This suppressed the formation of vanillylamine from vanillin, and vanillyl alcohol was produced instead, which in turn led to the production of capsinoids (*16*, *19*) (**Figure 1**).

Nonpungent cultivars containing high levels of capsinoid, other than CH-19 Sweet, have not been reported. Recently, our screening of germplasm revealed that a nonpungent cultivar Himo (*Capsicum annuum*) also contains high levels of capsinoids.

*To whom correspondence should be addressed. Telephone: +81-75-753-6048. Fax: +81-75-753-6068. E-mail: kapipara1014@yahoo.co.jp.

© 2009 American Chemical Society

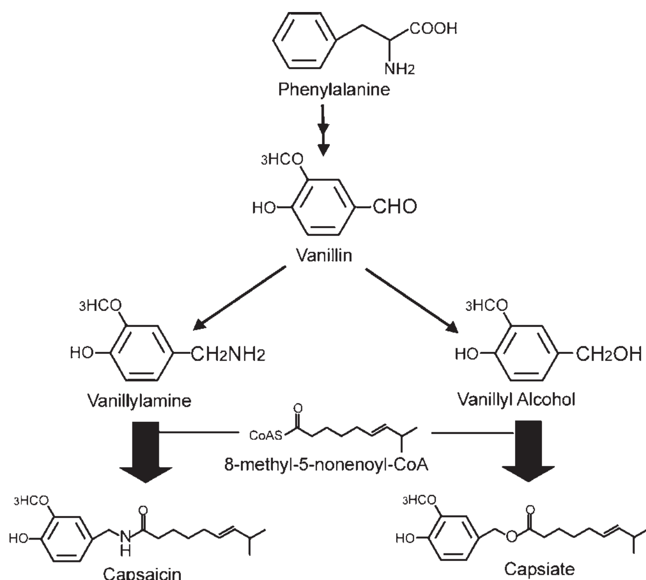

Figure 1. Proposed biosynthesis pathway of capsaicinoid and capsinoid in *Capsicum*.

Table 1. Capsinoid and Capsaicinoid Contents in the F_1 Hybrids between Himo and Other Cultivars[a]

	content (μg/g of fruit dry weight)	
cultivar or F_1 hybrid	capsinoid	capsaicinoid
Himo	1240 ± 105	nd[b]
CH-19 Sweet	5825 ± 286	110 ± 3
No. 3446	113 ± 17	5264 ± 360
Yatsufusa	145 ± 28	3707 ± 994
California Wonder	nd[b]	nd[b]
Wonder Bell	nd[b]	nd[b]
Himo × CH-19 Sweet	951 ± 203	24 ± 12
Himo × No. 3446	113 ± 17	6649 ± 116
Himo × Yatsufusa	nd[b]	800 ± 189
Himo × California Wonder	nd[b]	777 ± 389
Himo × Wonder Bell	nd[b]	1098 ± 521

[a] Mean ± the standard error (n = 3). [b] Not detected.

Himo will provide a new natural source of capsinoids. The aim of this study is to identify the genetic mechanism of capsinoid biosynthesis in Himo.

MATERIALS AND METHODS

Plant Material. Ten *Capsicum* cultivars were used in this study: two nonpungent cultivars containing capsinoids (Himo and CH-19 Sweet), five pungent cultivars (No. 3446, Yatsufusa, Hungarian Yellow Wax, Tabasco, and Habanero), and three nonpungent cultivars (California Wonder, Wonder Bell, and Sweet Banana). All plants were grown at the experimental farm of Kyoto University, from March to October 2007. The typical capsinoid and capsaicinoid contents of *Capsicum* cultivars are listed in **Table 1**. The capsinoid and capsaicinoid contents of pepper fruit were determined using high-performance liquid chromatography (HPLC) as described below.

Extraction of Capsinoids and Capsaicinoids. To determine the capsinoid and capsaicinoid contents, 3−10 fruits approximately 30 days after flowering were used. The content was determined as described in our previous report (*20*). After the pericarps were cut with a knife, whole fruits were dried completely in a freeze-drier (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 0.2 mg of dry fruit powder. After the samples had been vortexed, 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 was 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 this solution was filtered into a 2 mL glass vial using 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 was a mixture of MeOH and H_2O (70:30, v/v) at a flow rate of 1.0 mL/min. Detection was performed at 280 nm with a UV detector. Capsinoid and capsaicinoid contents were calculated as the sum of capsiate and dihydrocapsiate, and capsaicin and dihydrocapsaicin, respectively.

Inheritance of Capsinoid Content in Himo. To test inheritance patterns of capsinoid content, F_1 and F_2 populations (n = 80), derived from a cross between Himo and a wild pungent accession, No. 3446, were grown for segregation analysis. An F_1 generation derived from a cross between Himo and a pungent cultivar, Yatsufusa, was also used for this experiment. Genetic complementation tests were conducted by crossing Himo with CH-19 Sweet to determine whether capsinoid biosynthesis in CH-19 Sweet and Himo is due to defects at the same or different loci.

Early genetic studies reported that a single dominant gene (*Pun1*) controlled pungency and capsaicinoid biosynthesis. Recently, the *Pun1* gene was cloned and characterized as a putative acyltransferase gene, which can produce capsaicinoids from vanillylamine and a fatty acid (*21*, *22*). It was reported that nonpungent *C. annuum* cultivars have homozygous recessive allele *pun1* (*23*). To investigate the relationship of *pun1* to nonpungency in Himo, F_1 hybrids derived from a cross between Himo and two nonpungent cultivars, California Wonder and Wonder Bell, were used. California Wonder and Wonder Bell are *pun1*/*pun1*. F_1 fruits from each cross were assayed for their capsinoid and capsaicinoid contents by HPLC as described above.

Semiquantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). To compare expression levels of *p-AMT*, semiquantitative RT-PCR was conducted. Himo and two pungent cultivars, Yatsufusa and Hungarian Yellow Wax, were used for this experiment. Pepper fruits were harvested 30 days after flowering, and the placenta was separated for total RNA extraction using QuickGene RNA cultured cell kit S (Fuji Film) 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) 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 RT-PCR mixture consisted of 0.1 μL of Blend Taq polymerase (TOYOBO), 1 μL of buffer (provided with the polymerase), 1 μL of dNTPs (2 mM), and 0.2 μL of forward and reverse primer (20 μM) and was adjusted to 9 μL with superdistilled water. A 1 μL aliquot of cDNA was used as the template. The PCR procedure to amplify *p-AMT* was as follows: one cycle of 2 min at 94 °C; 38 cycles of 30 s at 94 °C, 30 s at 52 °C, and 2 min at 72 °C; and a final extension of 10 min at 72 °C. The primer sequences for *p-AMT* were 5′-TCTTTCTCTTTCCTTAGCAAT-3′ (forward primer) and 5′-ATAAACAAGCTTTCGCCGTGA-3′ (reverse primer). The primer sequences were designed on the basis of the reported nucleotide sequence for *p-AMT* (GenBank accession number AF085149); they were designed to amplify a 1481 bp fragment of the *p-AMT* cDNA sequence. The housekeeping gene *Actin* was used as a constitutively expressed gene. The PCR procedure for amplifying *Actin* was follows: one cycle of 2 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C; and a final extension of 10 min at 72 °C. The primer sequences for *Actin* were 5′-CTTGTCTGTGATAATGGAACAG-3′ (forward primer) and 5′-GGGATACTTCAAGGTGAGAATA-3′ (reverse primer). The primer sequences were designed on the basis of the reported nucleotide sequence for *Actin* (GenBank accession number AY572427); they were designed to amplify a 189 bp fragment of the *Actin* cDNA sequence. RT-PCR products were separated in a 1% agarose gel, stained with ethidium bromide, and visualized using a UV transilluminator.

Cloning and Sequence of the *p-AMT* Gene. The full-length sequence of *p-AMT* amplified by RT-PCR was cloned to p-TAC1 by using the DynaExpress TA PCR Cloning Kit (BioDynamics Laboratory Inc.). Nucleotide sequencing was conducted in an ABI PRISM 3100 genetic analyzer and the ABI PRISM BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems). Blast (http://blast.ncbi.nlm.nih.gov/) and GeneDoc (http://www.nrbsc.org/gfx/genedoc/) were applied for the analysis of nucleotide and derived amino acid sequences as well as multiple alignments.

Development of the Cleaved Amplified Polymorphic Sequence Marker. A cleaved amplified polymorphic sequence (CAPS) DNA marker was developed using one-nucletide substitution at base pair 755 of the *p-AMT* gene in Himo. This one-nucleotide substitution produced an *Rsa*I site. For CAPS analysis, genomic DNA was extracted from young leaves of pepper plants using Nucleon PhytoPure (GE Healthcare). The genomic PCR mixture consisted of 0.1 μL of KOD FX polymerase (TOYOBO), 5 μL of buffer (provided with the polymerase), 2 μL of dNTPs (2 mM), and 0.2 μL of forward and reverse primer (20 μM) and was adjusted to 10 μL with superdistilled water. Approximately 10 ng of genomic DNA was used as template. The primer sequences for the genomic PCR were Intron F 5′-GAAAATCCTAACTCCCGCTTTG-3′ (forward primer) and R852 5′-CTTTGCTACAGAGACAAGAT-CAGG-3′ (reverse primer). The primer sequences were designed on the basis of genomic sequence for *p-AMT* and were designed to amplify a 320 bp fragment. The genomic PCR procedure is as follows: one cycle of 2 min at 94 °C; 35 cycles of 10 s at 98 °C, 30 s at 60 °C, and 1 min at 68 °C; and a final extension of 10 min at 68 °C. A volume of 5 μL of PCR amplicon was mixed with 1 μL of buffer (provided with the enzyme) and 0.1 μL of *Rsa*I restriction enzyme (TOYOBO), adjusted to 10 μL with superdistilled water, vortexed, and incubated at 37 °C for 60 min. The digestion products were separated by electrophoresis on a 1% agarose gel, stained with ethidium bromide, and visualized using a UV transilluminator. Plants that are homozygous for the wild-type allele produce only 320 bp fragments. Plants that are homozygous for the Himo-type allele produce 241 and 79 bp fragments, because *Rsa*I cuts the 320 bp fragment. Heterozygous plants produce three fragments: 320, 241, and 79 bp.

CAPS analysis was conducted to check whether the single-nucleotide substitution at base pair 775 in *p-AMT* is specific to Himo. Ten cultivars, including Himo, were used for CAPS analysis.

Cosegregation of *p-AMT* Genotype and Capsinoid Biosynthesis. F_1 and F_2 populations, derived from a cross between Himo and No. 3446, were used for this experiment. The CAPS marker was used to determine the allelic state at *p-AMT* for all plants. Fruits of all plants were assayed for capsinoid and capsaicinoid content by HPLC as described above.

RESULTS AND DISCUSSION

Genetic Analysis of Capsinoid Content. To investigate the hereditary pattern of capsinoid content, genetic analysis was conducted using F_1 and F_2 populations derived from crossing Himo and a pungent cultivar. Himo contained high levels of capsinoid, but capsaicinoid was not detectable (**Table 1**). Pungent cultivars, No. 3446 and Yatsufusa, mainly contained capsaicinoids and produced capsinoids in trace amounts. Both Himo × No. 3446 F_1 and Himo × Yatsufusa F_1 plants mainly produce capsaicinoids, indicating the dominance of pungency and capsaicinoid biosynthesis (**Table 1**). Of a total of 80 Himo × No. 3446 F_2 plants, 59 plants mainly produced capsaicinoids and 21 produced capsinoids. This segregation ratio is consistent with the expected ratio of three capsaicinoids per capsinoid plant (χ^2 = 0.07; P = 0.79). These results indicate that capsinoid biosynthesis in Himo is controlled by a single recessive gene. Our previous report showed that capsinoid biosynthesis in CH-19 Sweet is determined by a single recessive allele of *p-amt* (*16*). To test the hypothesis that the recessive gene in Himo is *p-amt*, a genetic complementation test was conducted by crossing Himo and CH-19 Sweet. CH-19 Sweet mainly produces capsinoid and contains a small amount of capsaicinoid. Himo × CH-19 Sweet F_1 plants also mainly produced capsinoids and contained a small amount of capsaicinoid (**Table 1**). These data conclusively indicate that capsinoid synthesis in Himo is the result of *p-amt*, as in CH-19 Sweet.

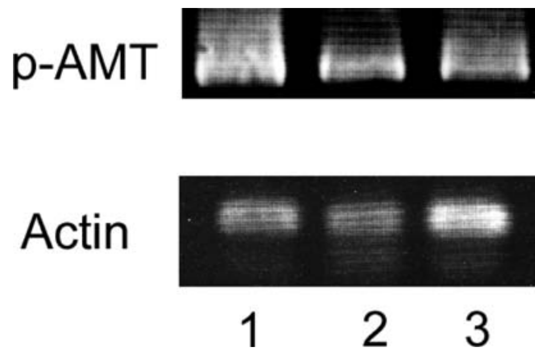

Figure 2. Semiquantitative RT-PCR of *p-AMT* in placenta tissue: (1) Himo, (2) Yatsufusa, and (3) Hungarian Yellow Wax. *Actin* was used as constitutively expressed gene.

pun1 is a recessive allele that results in nonpungency (*21*, *22*). Most nonpungent cultivars, including California Wonder and Wonder Bell, have *pun1* in a homozygous state (*23*). *Pun1* encodes a putative acyltransferase, which conducts acylation of vanillylamine with a fatty acid to form capsaicinoids. *pun1* contains a deletion in the open reading frame (ORF) region, which prevents the translation of acyltransferase (*21*). To investigate how *pun1* relates to the nonpungency of Himo, capsaicinoid and capsinoid contents were assayed in F_1 hybrids derived from crossing Himo and California Wonder, and Himo and Wonder Bell. Both F_1 hybrids were pungent plants, which produced mainly capsaicinoids (**Table 1**). These results demonstrated that there are at least two types of nonpungent cultivars in *Capsicum*. The first type includes cultivars that are homozygous for *pun1*. It was confirmed that many nonpungent cultivars such as California Wonder and Wonder Bell are *pun1*/*pun1* (*23*). *pun1*/*pun1* cultivars do not produce capsaicinoids, because of the functional loss of acyltransferase. Recently, a new recessive allele of *pun1* was reported in *Capsicum chinense*, which contains a 4 bp deletion, resulting in a frameshift mutation of *Pun1* (*24*). The second type includes cultivars that are homozygous for *p-amt*. As far as we know, only two cultivars, CH-19 Sweet and Himo, have been confirmed as *p-amt*/*p-amt* cultivars. It is of note that *p-amt*/*p-amt* cultivars contain nonpungent capsaicinoid analogues, capsinoids. In this and our previous report, F_1 hybrids derived from crosses between two nonpungent cultivars were pungent plants (*25*). The generation of pungent plants by crossing between nonpungent cultivars often causes problems in breeding programs for nonpungent peppers. A genetic classification of nonpungent cultivars is therefore needed by breeders. Classifications based on *pun1*/*pun1* or *p-amt*/*p-amt* will be helpful for the earlier and more accurate identification of nonpungent peppers in breeding programs.

The *p-AMT* Gene Is Transcribed Normally in Himo. To identify the genetic mechanism of capsinoid biosynthesis in Himo, we investigated the *p-AMT* gene. Transcription levels of *p-AMT* in Himo and pungent cultivars were compared by using semiquantitative RT-PCR. There was no significant difference in transcript level detected between Himo and pungent cultivars, suggesting that capsinoid biosynthesis is not caused by the absence of *p-AMT* expression (**Figure 2**).

Unique Single-Amino Acid Substitution in the p-AMT of Himo. cDNA sequences of *p-AMT* were determined in Himo, CH-19 Sweet, and pungent cultivars. All cDNA sequences contained a complete ORF of 1377 bp encoding a putative aminotransferase of 459 amino acids, except CH-19 Sweet (**Figure 3**). Our previous report showed that the *p-AMT* gene of CH-19 Sweet contains a T

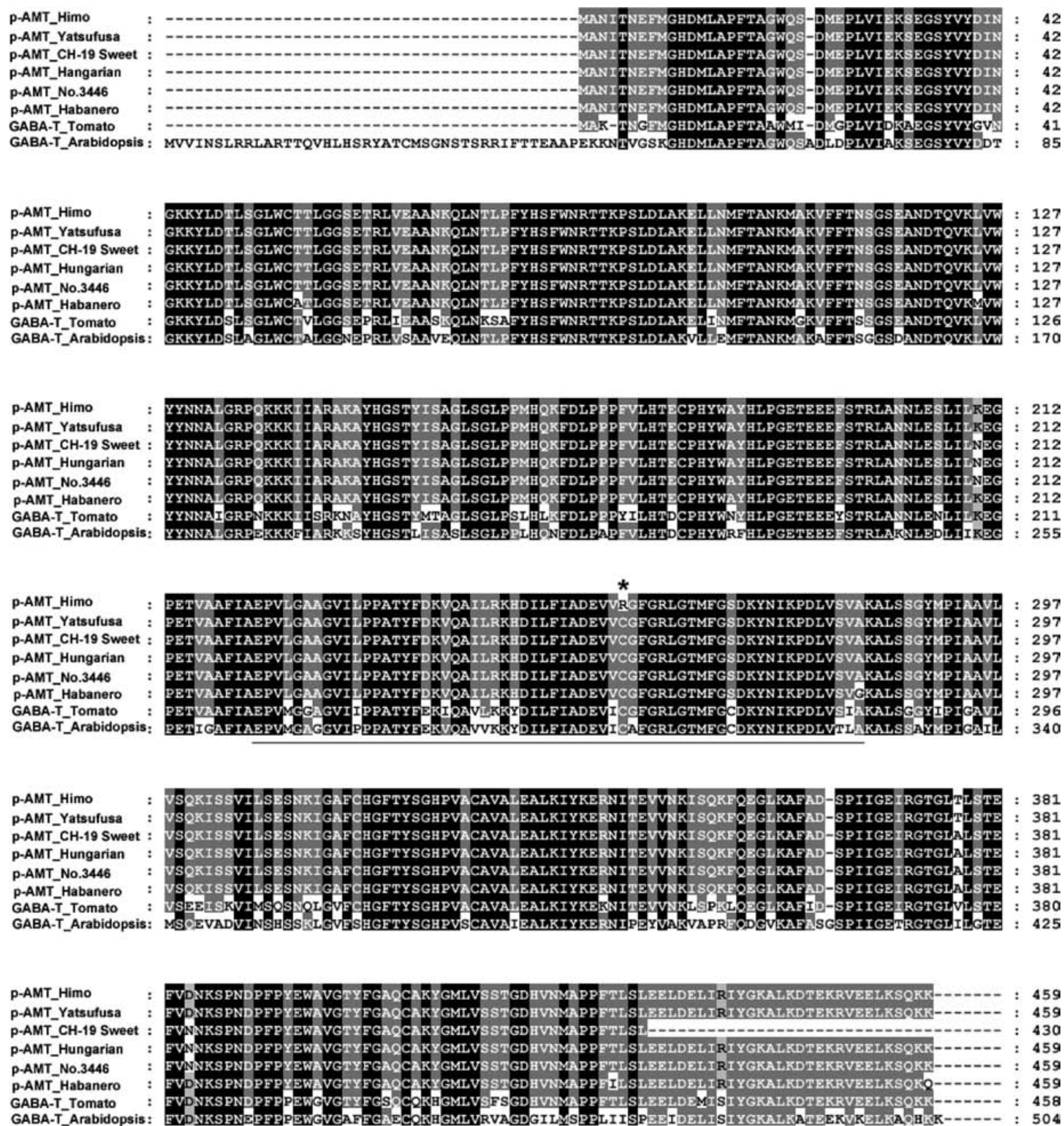

Figure 3. Alignment of the deduced amino acid sequence of p-AMT from *Capsicum* cultivars. Amino acid sequences were aligned using Gene Doc. Consensus amino acids are highlighted in black, and major amino acids are highlighted in gray. p-AMT derived from five *Capsicum annuum* cultivars (Himo, Yatsufusa, CH-19 Sweet, Hungarian Yellow Wax, and No. 3446) were aligned with the Habanero putative aminotransferase (*C. chinense*, GenBank entry AF085149), γ-aminobutyrate transaminase (GABA-T) (*Solanum lycopersicum*, GenBank entry AA092256), and GABA-T (*Arabidopsis thaliana*, GenBank entry AAK52899). The underlined part indicates the PLP binding domain. The asterisk indicates one-amino acid substitution (C → R) in Himo. In CH-19 Sweet, a truncated p-AMT could be produced because of a T nucleotide insertion at base pair 1291, which forms a stop codon, TGA.

nucleotide insertion at base pair 1291, which forms a new stop codon, TGA, which prevents the translation of p-AMT (*16*). The nucleotide insertion at base pair 1291 was not confirmed in the *p-AMT* sequence derived from Himo.

Sequence analysis by protein BLAST indicated that p-AMT has a putative cofactor pyridoxal 5-phosphate (PLP) binding domain, so the enzyme activity of p-AMT can be PLP-dependent. p-AMT has a high degree of amino acid identity to γ-aminobutyrate transaminase (GABA-T) found in other plants, for example, 83% identical to *Solanum* GABA-T and 75% identical to *Arabidopsis* GABA-T. It is reported that the GABA-T activity was strongly inhibited by aminooxyacetate and gabaculine, compounds that interfere with the binding of PLP (*26*). These facts imply that the PLP binding domain is important for p-AMT enzyme activity. The comparison of nucleotide sequences between Himo and a pungent cultivar Yatsufusa showed that the *p-AMT* sequence of Himo contained a single T → C change at base pair 775 of the protein-coding region. This nucleotide change results in the conversion of cysteine 259 into an arginine (**Figure 3**). This change in amino acid residue is located within the putative PLP binding domain. This residue is well conserved not only among p-AMT types in pungent cultivars but also among aminotransferases in other plants, such as *Solanum lycopersicum* (GenBank accession number AA092256) and *Arabidopsis thaliana* (GenBank accession number AAK52899), implying that it has an important role in aminotransferase activity (**Figure 3**). The conversion from cysteine to arginine in Himo is a drastic change from a neutral amino acid residue to a basic one.

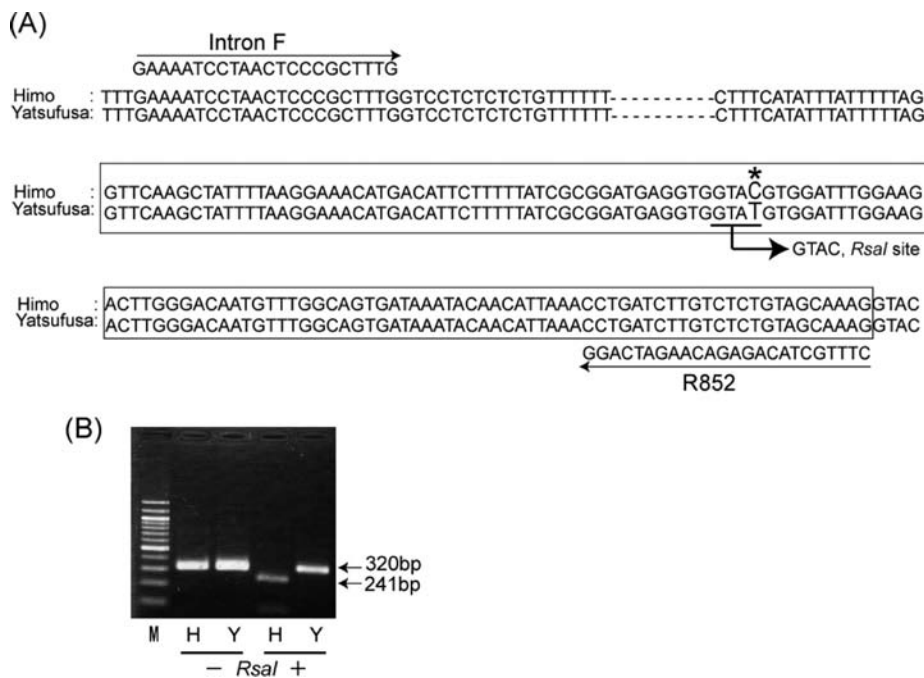

Figure 4. Development of the CAPS marker for the Himo-type allele of the *p-AMT* gene. (A) Genomic sequences of the *p-AMT* gene and primer design. Sequences in the box make up the exon sequence (base pairs 721−852 of cDNA sequences), and other sequences indicate intron sequences of the *p-AMT* gene. The asterisk indicates a single-nucleotide polymorphism (SNP) between Yatsufusa and Himo (T → C). This SNP produces an *Rsa*I site (GTAC) in the Himo-type allele. Primers (Inton F and R852) were designed to amplify the region including the *Rsa*I site. (B) Genomic PCR products amplified by primers (Intron F and R852) were digested with *Rsa*I to judge allelic states: M, DNA ladder marker; H, Himo; Y, Yatsufusa. The two left lanes were not digested with *Rsa*I, and the two right lanes were digested with *Rsa*I.

This suggests that this mutation has a crucial effect on p-AMT function, resulting in a decrease in enzyme activity. This one-amino acid substitution in p-AMT could suppress the formation of vanillylamine from vanillin, and vanillyl alcohol was produced instead, which in turn leads to capsinoid in Himo, as in CH-19 Sweet.

On the basis of a single-nucleotide substitution at base pair 755 in Himo, a codominant CAPS DNA marker was developed (**Figure 4A**). Genomic PCR was conducted using primers (Intron F and R852) for the *p-AMT* gene. Primers for genomic PCR were designed to amplify the region including a one-nucleotide substitution (T → C) at base pair 775. DNA from Himo and DNA from Yatsufusa were used for the genomic PCR. This genomic PCR generated a 320 bp amplicon in both cultivars. The PCR products were digested using *Rsa*I, and the digested fragments were separated by agarose electrophoresis. A 241 bp fragment was found in Himo, whereas a 320 bp fragment was found in Yatsufusa, a pungent cultivar (**Figure 4B**). These results confirmed a single-nucleotide substitution at base pair 755 in the *p-amt* gene of Himo and demonstrated that the CAPS marker can distinguish the *p-amt* allele of Himo. This CAPS analysis was conducted for 10 cultivars, including Himo. The results show that only Himo has the single-nucleotide substitution (T → C) at base pair 755, suggesting that this mutation is unique to Himo (**Figure 5**).

The wild-type *p-AMT* allele of pungent cultivars encodes normal p-AMT, which can produce vanillylamine from vanillin. Taken together with our previous study of CH-19 Sweet, there are two types of loss-of-function *p-amt* alleles in *C. annuum*. In the *p-amt* allele of CH-19 Sweet, a T nucleotide insertion at base pair 1291 forms a new stop codon and prevents the translation of normal p-AMT, while in the *p-amt* allele of Himo, a one-nucleotide substitution at base pair 755 results in a one-amino acid substitution in the PLP binding domain (*16*). In this report, we named these types of alleles CH-19 Sweet-type and Himo-type alleles, respectively.

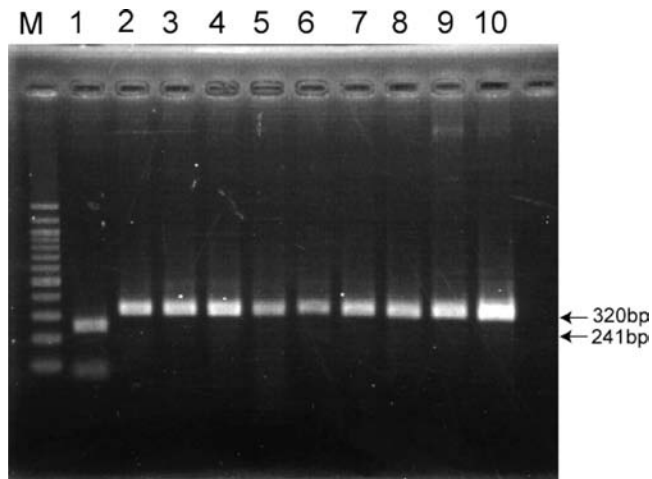

Figure 5. CAPS pattern in various *Capsicum* cultivars: M, DNA ladder marker; lane 1, Himo (*C. annuum*, nonpungent with capsinoid); lane 2, CH-19 Sweet (*C. annuum*, nonpungent with capsinoid); lane 3, No. 3446 (*C. annuum*, pungent); lane 4, Yatsufusa (*C. annuum*, pungent); lane 5, Hungarian Yellow Wax (*C. annuum*, pungent); lane 6, Wonder Bell (*C. annuum*, nonpungent); lane 7, California Wonder (*C. annuum*, nonpungent); lane 8, Sweet Banana (*C. annuum*, nonpungent); lane 9, Tabasco (*Capsicum frutescens*, pungent); lane 10, Habanero (*C. chinense*, pungent). In the Himo-type allele, a single-nucleotide substitution at base pair 755 of *p-AMT* produces an *Rsa*I site. Genomic PCR products amplified by primers (Intron F and R852) were digested with *Rsa*I to judge allelic states in each cultivar. A cultivar that has a Himo-type allele produces a 241 bp fragment.

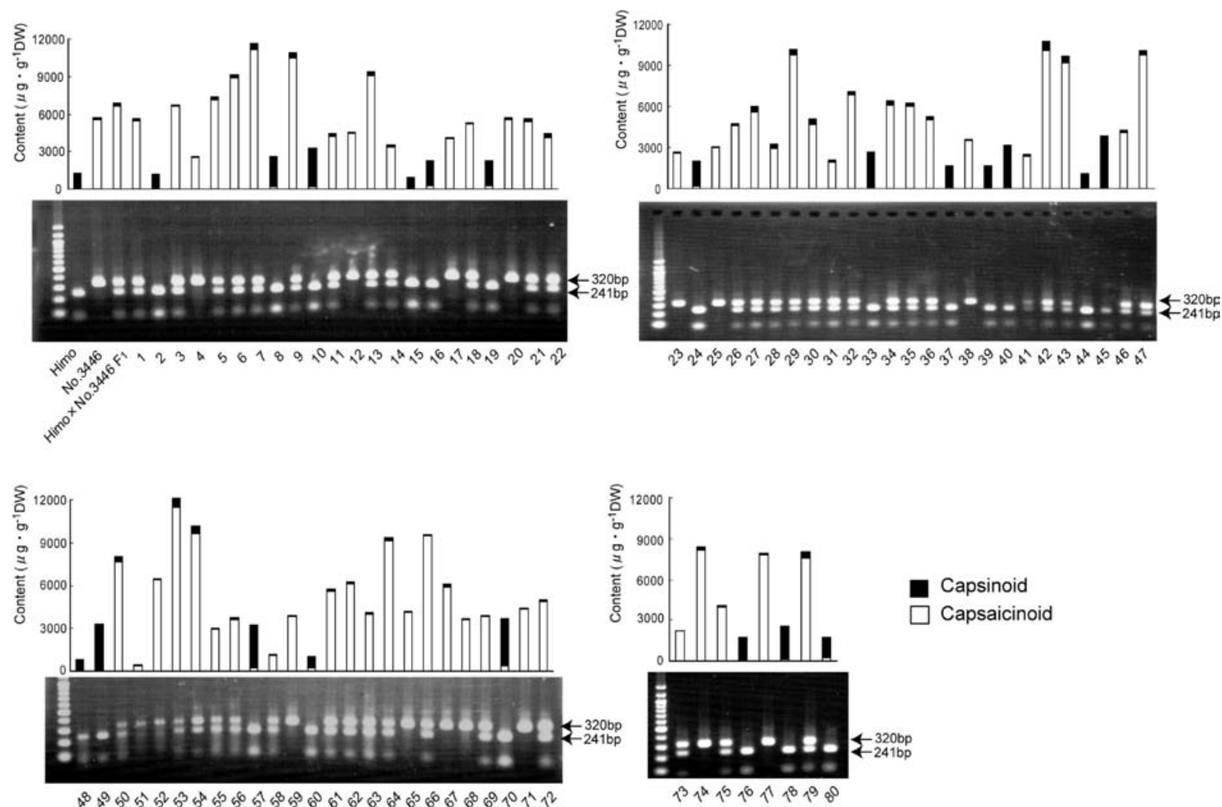

Figure 6. Cosegregation of the *p-AMT* genotype and capsinoid biosynthesis in an F_2 population derived from Himo and No. 3446. The bar charts show the contents of capsinoid and capsaicinoid in parents, F_1 and F_2 plants. Capsinoid and capsaicinod contents were determined by the HPLC method. The *p-AMT* genotypes were determined with the CAPS marker. The homozygotes of Himo-type (*p-amt*/*p-amt*) and wild-type (*p-AMT*/*p-AMT*) forms generate 241 and 320 bp fragments, respectively, and the heterozygotes (*p-AMT*/*p-amt*) generate both 320 and 241 bp fragments when digested with *Rsa*I.

CAPS Marker Patterns Cosegregated with Capsinoid Biosynthesis. To confirm that the Himo-type *p-amt* allele is the recessive gene determining capsinoid biosynthesis, a cosegregation test was conducted using the F_2 population from the cross between Himo and No. 3446, a pungent accession that produces capsaicinoid, with the CAPS marker described above. CAPS analysis in the F_2 population identified three patterns of PCR product fragments (**Figure 6**). A total of 21 F_2 plants with capsinoid fruit contained a 241 bp fragment, which indicated that they were homozygous for the Himo-type allele (*p-amt*/*p-amt*). A further 59 F_2 plants with capsaicinoid fruit could be divided into two groups, one consisting of 16 plants with a 320 bp fragment and the other consisting 43 plants with both 241 and 320 bp fragments. The two groups could correspond to wild-type homozygotes for this locus (*p-AMT*/*p-AMT*) and those that were heterozygous at this locus (*p-AMT*/*p-amt*), respectively. Plants that were homozygous for the Himo-type allele (*p-amt*/*p-amt*) contained mainly capsinoid, indicating that *p-amt* is the recessive gene determining capsinoid biosynthesis. Taken together with the results of nucleotide sequence analysis, the results of CAPS analysis strongly suggest that capsinoid biosynthesis in Himo is caused by a new loss-of-function *p-amt* allele, which is different from the CH-19 Sweet-type *p-amt* allele.

This report found that a nonpungent cultivar Himo contains high levels of capsinoids, nonpungent capsaicinoid analogues. Our previous report showed that capsinoid biosynthesis is caused by the mutated *p-amt* gene in a nonpungent cultivar CH-19 Sweet (*16*). In CH-19 Sweet, a T nucleotide insertion at base pair 1291 forms new stop codon, TGA. In this report, it was revealed that Himo has a new loss-of-function *p-amt* allele, which has a single-nucleotide substitution (T → C) at base pair 775 in the protein-coding region. This mutation results in a one-amino acid substitution in the PLP binding domain, which could lead to a functional loss of p-AMT. Himo will provide a new natural source of capsinoid. In this report, a CAPS-based codominant marker was developed to distinguish the Himo-type allele. This CAPS marker can be used for the early detection of plants containing capsinoid in breeding programs.

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) 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.

(5) Kobata, K.; Sutoh, K.; Todo, T.; Yazawa, S.; Iwai, K.; Watanabe, T. Nordihydrocapsiate, a new capsinoid from the fruits of a non-pungent pepper, *C. annuum*. *J. Nat. Prod.* **1999**, *62*, 335–336.

(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) Onuki, 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.

(8) 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.

(9) 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.

(10) Onuki, 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.

(11) 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.

(12) 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.

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

(14) 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.

(15) 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.

(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) 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.

(18) 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.

(19) 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.

(20) 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.

(21) 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.

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

(23) 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.

(24) 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.

(25) Yazawa, S.; Ueda, M.; Suetome, N.; Namiki, T. Capsaicinoids content in the fruit of interspecific hybrids in *Capsicum*. *J. Jpn. Soc. Hortic. Sci.* **1989**, *58*, 353–360.

(26) Van Cauwenberghe, O. R.; Shelp, B. J. Biochemical characterization of partially purified GABA:pyruvate transaminase from *Nicotiana tabacum*. *Phytochemistry* **1999**, *52*, 575–581.

Received for review September 17, 2009. Accepted November 27, 2009.