Identification of a Novel Mutant pAMT Allele Responsible for Low-pungency and Capsinoid Production in Chili Pepper: Accession ‘No. 4034’ (Capsicum chinense)

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Capsinoids are low-pungent capsaicinoid analogues in chili pepper fruits. They exhibit various bioactivities in humans similar to capsaicinoids, but do not produce a nasty burning sensation, encouraging their application in foods and supplements. Previous reports demonstrated that loss-of-function of putative aminotransferase (pAMT) leads to low-pungency and capsinoid accumulation. Therefore, the pant allele is a useful gene in chili pepper breeding programs to enhance health-promoting properties. Eight loss-of-function alleles have been identified in the Capsicum genus, but the variation in pant alleles remains to be fully elucidated. In this study, we identified one novel loss-of-function allele from the analysis of low-pungent chili pepper ‘No. 4034’ (C. chinense). ‘No. 4034’ contained mainly capsinoid with an undetectable level of capsaicinoid. A genetic complementation test was conducted by crossing ‘No. 4034’ with other accessions. The results indicated that ‘No. 4034’ possessed a loss-of-function pant allele. Sequence analysis showed that the novel mutant allele contained a 7-bp insertion (TCGGTAC) in the 16th exon region, which we designated as pant9. The insertion caused a frameshift mutation and resulted in a truncated protein. Gene expression analysis showed that the expression level of pAMT specifically decreased among biosynthetic genes tested here in ‘No. 4034’, compared with that of pungent accession. pant9 will be useful for low-pungency and capsinoid breeding, and will provide additional information for variations in pAMT mutants.

Key Words: allelic variation, capsaicinoid, frameshift mutation, health functional ingredient.

Introduction

Pungency is one of the most important commercial traits of chili pepper fruit, and is caused by unique alkaloids called capsaicinoids (Aza-Gonzalez et al., 2011; Nelson and Dawson, 1923). The basic chemical structure of capsaicinoid is a fatty acid amide of vanillylamine, and the dominant capsaicinoids in nature are capsaicin and dihydrocapsaicin, which are produced mainly in the placental tissue of chili pepper fruit (Stewart et al., 2007; Suzuki et al., 1980). The capsaicinoid biosynthesis pathway consists of two pathways: the phenylpropanoid pathway produces vanillylamine derived from phenylalanine, and the other pathway produces a branched fatty acid derived from valine or leucine (Aza-Gonzalez et al., 2011; Mazourek et al., 2009; Suzuki et al., 1981). In the final step, an acyltransferase, which is encoded in the Pun1 gene, promotes the condensation of vanillylamine and a branched fatty acid to form capsaicinoid (Stewart et al., 2005). Capsaicinoid has various beneficial functions in humans such as anti-obesity, cancer prevention, and antioxidant activity (Srinivasan, 2016). However, capsaicinoids cause a strong burning sensation, which prevents their application in foods and supplements.

Capsinoids are low-pungent capsaicinoid analogues, which were originally isolated from a low pungent C. annuum mutant ‘CH-19 Sweet’ (Kobata et al., 1998; Yazawa et al., 1989). The fundamental structure of capsinoid is a fatty acid ester of vanillyl alcohol, and major capsinoids in nature are capsiate and dihydrocapsiate. Capsinoids have various biological properties similar to...
Capsaicinoid, but they are less pungent and therefore favorable for consumption (Haramizu et al., 2011; Luo et al., 2011; Sasahara et al., 2010). Capsaicinoids have attracted attention due to their capsaicinoid-like biological properties and lack of burning sensation. Thus, capsinoid is an attractive target in chili pepper breeding programs.

Molecular genetic studies on “CH-19 Sweet” and low-pungent accessions have revealed that capsinoid production is caused by loss-of-function of the putative aminotransferase (pAMT) gene (Lang et al., 2009; Tanaka et al., 2010a, b). pAMT encodes the enzyme that catalyzes the conversion of vanillin to vanillylamine (Curry et al., 1999). The loss-of-function in pAMT suppresses production of vanillylamine from vanillin, and vanillyl alcohol is produced instead, which results in a significant increase in capsinoid content (Han et al., 2013; Kobata et al., 2013; Sutoh et al., 2006). Therefore, the pamt mutation is the key factor in genetic improvements in terms of pungency and capsinoid content in Capsicum breeding programs. Previous research identified eight loss-of-function alleles in the Capsicum genus (Koeda et al., 2014; Lang et al., 2009; Park et al., 2015; Tanaka et al., 2010a, b, 2015). Each allele is defined by a specific mutation feature such as a single nucleotide substitution, short nucleotide insertion, and transposon insertion. However, the variation in pamt alleles remains to be fully elucidated. Our previous study suggested that pamt is the most frequent genetic factor contributing to low-pungency in C. chinense accessions, and they have different pamt alleles (Tanaka et al., 2015). This led us to believe that some undiscovered pamt alleles still exist in low-pungent C. chinense accessions. The objective of this study was to characterize pAMT gene and capsinoid production in the low-pungent chili pepper accession ‘No. 4034’ (C. chinense).

Materials and Methods

Plant materials

Three chili pepper accessions were used in the current study: two low-pungent accessions (‘No. 4034’, ‘Aji Dulce strain 2’) and one pungent accession (‘Red Habanero’). All accessions belonged to Capsicum chinense. In addition, cross tests were conducted by crossing ‘No. 4034’ with ‘Red Habanero’ (pAMT/ pAMT) or ‘Aji Dulce strain 2’ (pamt/pamt) in order to check whether pamt was responsible for low-pungency in ‘No. 4034’. ‘No. 4034’ was derived from Peru, and the accession was collected in a Latin America scientific exploration project in the 1970s (Yamamoto, 1978). Plant materials were grown in the open field of Okayama university farm in 2015 and 2016. The fruits from each strain and cross were investigated for their capsaicinoid and capsinoid contents by high-performance liquid chromatography (HPLC) as described below.

HPLC analysis of capsaicinoid and capsinoid contents

Three mature green fruits (approximately 30 days after anthesis) from individual plants were harvested from all C. chinense accessions and F1 hybrid plants. All samples were lyophilized by a freeze drier, and ground in a blender. Capsaicinoids and capsinoids were extracted from 200 mg of dry fruit powder in 4 mL acetone. After filtering the extracts with a filter (DISMIC 13HP; ADVANTEC, Tokyo, Japan), HPLC analysis was performed to determine capsaicinoid and capsinoid contents as presented by Tanaka et al. (2015). HPLC analysis was conducted with three biological replicates.

RNA extraction and cDNA synthesis

Mature green fruits of ‘Red Habanero’ and ‘No. 4034’ were harvested, and the placental septum was separated for RNA extraction. The total RNA was extracted with Sepasol®-RNA I Super G (Nacalai, Kyoto, Japan) and purified with an RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). To remove contamination of genomic DNA, RNA was treated with DNase I (QIAGEN). First-strand cDNA was synthesized using ReverTra Ace (TOYOBO, Osaka, Japan) for cDNA sequence analysis, or PrimeScript™ RT Master Mix (Takara, Shiga, Japan) for expression analysis.

Genomic and cDNA sequence analysis of novel pamt allele

The full-length pAMT cDNA sequence of ‘No. 4034’ was amplified using primer sets (pAMT F1 and pAMT R1481). The primer set was designed from the pAMT cDNA sequence in the database (GenBank accession no. AF085149). The RT-PCR was performed in a 20 μL reaction mixture containing 0.3 μL of KOD FX neo (TOYOBO), 10 μL of buffer (provided with the polymerase), 4 μL of dNTPs (2 mM), 0.5 μL of forward and reverse primers (10 μM), and a 0.5 μL aliquot of genomic DNA. The PCR procedure 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 2 min at 68°C; and a final extension of 5 min at 68°C.

The genomic sequences covering the full-length open reading frame (ORF) were amplified using four sets of primers (pAMT F1 and pAMT R282, pAMT 3rd_intron F and pAMT 9th_intron R, pamt3-fwd and pAMT 16th_intron R, and pAMT F1270 and pAMT 3_of_stop R). Genomic DNA was isolated from leaf tissues using Nucleon PhytoPure (GE Healthcare Japan, Tokyo, Japan). The genomic PCR was performed using KOD FX neo (TOYOBO) as presented by Tanaka et al. (2015). Primer sequences for genomic and RT-PCR are shown in Table 1.

PCR products were separated on a 1.0% agarose gel and stained with Gel Red (Biotium, Hayward, CA, USA) to confirm their amplifications. The PCR products were then purified with the Exo Star kit (GE Healthcare Japan) and nucleotide sequencing was per-
formed by the Eurofins sequencing service (Eurofins Genomics, Tokyo, Japan). ATGC (GENETYX, Tokyo, Japan) was applied to analyze nucleotide sequences. After sequencing analysis, the sites of introns were determined by comparison with a cDNA sequence. In addition, deduced amino acid sequence alignment of pAMTs from ‘No. 4034’ and pungent cultivars was carried out using the ClustalW program.

**Development of a DNA marker to distinguish novel pamt derived from ‘No. 4034’**

A DNA marker was developed on the basis of a 7-bp insertion in the 16th exon that led to a frameshift mutation. The primers (pAMT 4034 F and pAMT 4034 R) were designed on both sides of the insertion. The PCR was performed in a 21 μL reaction mixture containing 10 μL of GoTaq Green Master Mix (Promega KK, Tokyo, Japan), 1.0 μL of forward and reverse primers (10 μM), and a 1.0 μL aliquot of genomic DNA. The PCR procedure was as follows: 1 cycle of 2 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C; and a final extension of 5 min at 72°C. PCR products were separated on a 6.0% polyacrylamide gel and stained with Gel Red (Biotium) to check the product size. Wild-type pAMT produces a 121-bp fragment, while pAMT<sub>4034</sub> produces a 128-bp fragment.

**Gene expression analysis**

Quantitative RT-PCR (qRT-PCR) was performed using the KOD SYBR(R) qRT-PCR Mix (TOYOBO) on a Roche Light Cycler Nano Real-Time PCR System according to the manufacturer’s protocol. The amplification program consisted of 1 cycle of 2 min at 98°C, followed by 45 cycles of 10 s at 98°C, 10 s at 60°C, and 30 s at 68°C. A reference gene (Actin) was used to normalize the expression level. The comparative CT method (2<sup>−ΔΔCT</sup> method) was used to analyze expression levels (Livak and Schmittgen, 2001). Each reaction was repeated three times as biological replicates, and means and standard errors were calculated. Primer sequences for qRT-PCR are summarized in Table 1.

### Results

The low-pungent chili pepper accession ‘No. 4034’ produced capsinoid

‘Red Habanero’ had a high content of capsaicinoids and produced capsinoids in trace amounts. In contrast, ‘Aji Dulce strain 2’ harbouring the loss-of-function
The composition of capsaicinoid and capsainoid suggests that some mutation in pAMT is responsible for the low-pungency in ‘No. 4034’. To test this hypothesis, cross tests were carried out by crossing ‘No. 4034’ with other accessions. The crossing of ‘No. 4034’ with the pungent cultivar ‘Red Habanero’ (pAMT/pAMT) resulted in pungent plants, which mainly produced capsaicinoid (Table 2). This indicates that the low-pungency in ‘No. 4034’ is a recessive trait. On the other hand, the F1 hybrids derived from ‘No. 4034’ and ‘Aji Dulce strain 2’ (pamt/pamt) were low-pungent plants, which produced mainly capsainoid and little capsainoid. These results demonstrated that ‘No. 4034’ is homozygous for pamt.

The novel loss-of-function pamt allele derived from ‘No. 4034’ had the loss-of-function pamt allele

Complementation tests indicated that ‘No. 4034’ had an undetectable level of capsaicinoid, but produced trace amounts of capsinoid (Table 2). ‘No. 4034’ had a 7-bp insertion in the 16th exon region, which results in a frameshift mutation and a premature stop codon. Therefore, the 7-bp insertion could be the causal mutation responsible for inactivation of pAMT. From here on, the novel pamt derived from ‘No. 4034’ is designated as pamt9. We have deposited the complete genomic sequence of pamt9 in GenBank (accession no. LC273300). We also developed a PCR marker to detect the pamt9 allele based on its 7-bp insertion (Fig. 2A). Next, we tested the PCR marker for various C. chinense accessions. The result confirmed that the PCR marker designed here could distinguish pamt9 from wild-type pAMT or other loss-of-function pamt (Fig. 2B).

The expression of five capsaicinoid biosynthetic pathway genes (Pal, pAMT, BCAT, KAS, and Pun1) proximately 11 kb (Tanaka et al., 2010b). pAMT cDNA has a 1377-bp ORF and encodes a peptide of 459 amino acids. In order to reveal the molecular mechanism for low-pungency and capsainoid production, we determined the complete genomic and cDNA sequences of pAMT in ‘No. 4034’. The genomic pAMT sequence in ‘No. 4034’ was also composed of 17 exons, but it contained a 7-bp insertion (TCGGTAC) in the 16th exon (Fig. 1). The insertion led to a frameshift mutation and resulted in a truncated protein (409 amino acids), which is shorter than functional pAMT. The insertion has not been observed in any loss-of-function pamt reported to date. There was no critical amino acid substitution in the comparison between pungent accessions and ‘No. 4034’, except for the frameshift mutation. Therefore, the 7-bp insertion could be the causal mutation responsible for inactivation of pAMT. From here on, the novel pamt derived from ‘No. 4034’ is designated as pamt9. We have deposited the complete genomic sequence of pamt9 in GenBank (accession no. LC273300). We also developed a PCR marker to detect the pamt9 allele based on its 7-bp insertion (Fig. 2A). Next, we tested the PCR marker for various C. chinense accessions. The result confirmed that the PCR marker designed here could distinguish pamt9 from wild-type pAMT or other loss-of-function pamt (Fig. 2B).

The expression of five capsaicinoid biosynthetic pathway genes (Pal, pAMT, BCAT, KAS, and Pun1)

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**Table 2.** Capsaicinoid and capsainoid contents in F1 hybrids between ‘No. 4034’ and other accessions.

| Accession or F1 hybrid | Contents (μg·g−1DW) | Capsaicin | Dihydrocapsaicin | Total capsaicinoid | Capsiate | Dihydrocapsiate | Total capsainoid |
|------------------------|---------------------|-----------|------------------|--------------------|----------|----------------|-----------------|
| Red Habanero           |                     | 11021.5±862.29 | 3243.2±337.69   | 14264.7±1197.82   | 282.2±20.52 | 103.1±45.89   | 385.3±58.15    |
| Aji Dulce strain 2     |                     | 37.3±17.98   | 4.9±3.07         | 42.2±19.95        | 4064.2±1196.25 | 660.9±165.44  | 4725.1±1347.06  |
| No. 4034               |                     | nd          | nd               | 404.7±34.55       | 52.1±2.59  | 456.9±52.40   |                 |
| Red Habanero × No. 4034|                     | 10872.7±425.91 | 4479.1±89.69   | 15351.8±456.04  | 328.3±28.89 | 25.7±13.02   | 354.1±31.81    |
| Aji Dulce strain 2 × No. 4034 |     | 16.9±5.50   | 2.7±1.41         | 19.6±6.91        | 789.4±27.00  | 128.8±5.67   | 918.2±32.46    |
| No. 4034 × Aji Dulce strain 2 |     | 25.1±5.15   | 2.1±1.12         | 27.3±4.03        | 725.9±35.81  | 140.1±43.33  | 866.0±74.35    |

z: average± standard error (n = 3).
y: not detected.

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![Fig. 1](image-url)  
A schematic representation of the genomic structure and cDNA of pamt9. The pamt9 allele contained a 7-bp insertion (TCGGTAC) in the 16th exon region, which results in a frameshift mutation and a premature stop codon.
was investigated in ‘Red Habanero’ and ‘No. 4034’. The expression level of \( pAMT \) was significantly lower than that of ‘Red Habanero’, although similar expression levels of other biosynthetic genes were observed in both accessions (Fig. 3).

![Fig. 2. Development of a DNA marker to distinguish novel \( pant \) alleles in ‘No. 4034’. (A) Genomic sequence of the \( pAMT \) gene and primer design. The sequence in the box indicates the 16th exon, and other parts are intron sequences. The underlined sequence indicates the 7-bp insertion in ‘No. 4034’. The arrows indicate primer binding sites. (B) Genomic PCR analysis to determine novel \( pant \) alleles. 1. ‘Red Habanero’ (\( pAMT \)), 2. ‘No. 4034’ (a novel \( pant \) designated as \( pant \)), 3. ‘Belize Sweet’ (\( pant \)), 4. ‘Zavory Hot’ (\( pant \)), 5. ‘Aji Dulce strain 2’ (\( pant \)), 6. ‘No. 80’ (\( pant \)), 7. ‘LP6’ (\( pant \)). The size of the amplicon is indicated to the left. A novel \( pant \) derived from ‘No. 4034’ produced a 128-bp fragment, while wild-type \( pAMT \) and other \( pant \) produced a 121-bp fragment.]

![Fig. 3. Comparative expression analysis of capsaicinoid biosynthesis-related genes. A pungent accession ‘Red Habanero’ (RH) and a low-pungent accession ‘No. 4034’ were used, and total RNA was extracted from placental tissue in the mature green fruit stage. Gene expression levels were determined for five capsaicinoid biosynthesis-related genes (\( Pal \): phenylalanine ammonia-lyase, \( pAMT \): putative aminotransferase, \( BCAT \): branched-chain amino acid transferase, \( KAS \): \( \beta \)-ketoacyl ACP synthase, and \( Pun1 \): acyltransferase). The expression levels of genes in RH were set to 1. All data are presented as means of three biological replicates. Error bars indicate standard errors.]

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Table 3. Allelic variations in pamt and their structural features.

| pamt allele | Structural features | Representative accession harboring the allele | Reference |
|-------------|---------------------|---------------------------------------------|-----------|
| pamt<sup>1</sup> | one T insertion in 16th exon | CH-19 Sweet (<em>C. annuum</em>) | Lang et al. (2009) |
| pamt<sup>2</sup> | one SNP in 11th exon leading to amino acid substitution in PLP binding domain | Himo (<em>C. annuum</em>) | Tanaka et al. (2010a) |
| pamt<sup>3</sup> | 5 bp (TGGGC) insertion in 8th exon | Belize Sweet (<em>C. chinensis</em>) | Tanaka et al. (2010b) |
| pamt<sup>4</sup> | 2.3 kb transposon insertion in 5th intron | Zavorý Hot (<em>C. chinensis</em>) | Tanaka et al. (2010b) |
| pamt<sup>5</sup> | 2.3 kb transposon insertion in 3rd intron | Aji Dulce strain 2 (<em>C. chinensis</em>) | Tanaka et al. (2010b) |
| pamt<sup>6</sup> | 8 bp (GCCACGAC) insertion in 6th exon | No. 80 (<em>C. chinensis</em>) | Koeda et al. (2014) |
| pamt<sup>7</sup> | 7 bp (CTTTACT) insertion in 2nd exon | LP6 (<em>C. chinensis</em>) | Tanaka et al. (2015) |
| pamt<sup>8</sup> | 2.8 kb transposon insertion in 2nd exon | S3212 (<em>C. frutescens</em>) | Park et al. (2015) |
| pamt<sup>9</sup> | 7 bp (TCCGGTAC) deletion in 16th exon | No. 4034 (<em>C. chinensis</em>) | In this study |

Discussion

The mutation in pAMT is an effective genetic factor to control pungency and enhance capsinoid accumulation in chili pepper fruits. One novel loss-of-function allele responsible for low-pungency and capsinoid production was identified in this study. The allele named pamt<sup>2</sup> was characterized by a 7-bp insertion in the 16th exon, which led to frameshift mutation and created a premature stop codon (Fig. 1). qRT-PCR analysis showed that the expression level of pamt<sup>2</sup> decreased compared with functional pAMT (Fig. 3). The reduction in pamt<sup>2</sup> expression level in ‘No. 4034’ could be due to a nonsense-mediated mRNA decay (NMD) mechanism. NMD is a surveillance pathway that eliminates mRNAs containing premature stop codons to prevent toxic effects by truncated proteins (Conti and Izaurralde, 2005). The mechanism exists in all eukaryotes, and the involvement of NMD in the reduction of gene expression in plants has also reported in inactive alleles of anthocyanin biosynthetic genes. The expression level of inactive anthocyanin biosynthetic gene alleles containing premature stop codons was significantly reduced in the morning glory and onion (Hoshino et al., 2003; Kim et al., 2009).

The first loss-of-function allele was reported from ‘CH-19 Sweet’ (Lang et al., 2009). The pamt<sup>1</sup> contained one T insertion in the 16th exon, which resulted in a frameshift mutation. Since finding pamt<sup>1</sup>, 7 loss-of-function alleles have been found additionally in the Capsicum genus (Table 3). The 2nd pamt in <em>C. annuum</em> was reported in the Japanese low-pungent landrace ‘Himo’ (Tanaka et al., 2010a). pamt<sup>2</sup> has a unique single nucleotide substitution in the 11th exon, which results in one amino acid substitution. The substitution is located in the pyridoxal 5-phosphate binding domain, which plays an important role in aminotransferase activity. Recently, one pamt allele (pamt<sup>8</sup>) has been identified in a low-pungent <em>C. frutescens</em> accession (Park et al., 2015). The pamt allele contained a 12-bp deletion in the 7th exon, which is related to low-pungency. In <em>C. chinensis</em>, 5 pamt alleles have been reported prior to pamt<sup>1</sup> in this study. A common observation about <em>C. chinense</em> pamt alleles is that the mobilization of a hAT family transposon could be involved in their mutations. Three alleles (pamt<sup>4</sup>, pamt<sup>6</sup>, and pamt<sup>7</sup>) contain a hAT family transposon in the exon or intron region, which leads to abnormal splicing or a frameshift mutation (Jang et al., 2015; Tanaka et al., 2010b). pamt<sup>4</sup> and pamt<sup>6</sup> harbored an almost identical transposon, Tcc1, in different introns, while pamt<sup>7</sup> had a larger hAT family transposon in the 2nd exon, which shared high identity with Tcc1 (Tanaka et al., 2010b, 2015). The three (pamt<sup>4</sup>, pamt<sup>6</sup>, and pamt<sup>7</sup>) alleles have a short insertion in the exon region, which disrupts pAMT function (Koeda et al., 2014; Tanaka et al., 2010b). The short insertions in exon regions can be regarded as footprints after the transposon jumped (Tanaka et al., 2015). The comparison between pamt<sup>4</sup> and pamt<sup>7</sup> clearly demonstrated that the short insertion in pamt<sup>4</sup> is a footprint sequence left after excision of the Tcc2 transposon. In the current study, we found pamt<sup>9</sup> in ‘No. 4034’, and identified a 7-bp insertion in the 16th exon. The insertion in ‘No. 4034’ is also a transposon footprint. Therefore, the discovery of pamt<sup>9</sup> in this study provides further support for our hypothesis that a Tcc family transposon could drive the occurrence of various pamt alleles in <em>C. chinense</em>.

The collective findings in this report add novel information on diverse pamt alleles. This information will be useful for low-pungency and capsinoid production in chili pepper breeding programs.

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