Non-pungency in a Japanese Chili Pepper Landrace (Capsicum annuum) is Caused by a Novel Loss-of-function Pun1 Allele

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Pungency in peppers is due to the presence of the alkaloid capsaicin and its analogues, collectively known as capsaicinoids. These compounds are only produced in the Capsicum genus and function as deterrents to mammals from consuming the pepper fruits. Pungency in pepper is qualitatively controlled by the Pun1 locus, which encodes a putative acyltransferase enzyme. Mutations in the Pun1 gene result in a loss of pungency, and several Pun1 loss-of-function alleles have been identified in sweet peppers to date (pun11–3). However, variations in pun1 alleles have not been completely elucidated. In the present study, we report a new type of loss-of-function pun1 allele, named pun14, in a Japanese sweet pepper cultivar, ‘Nara Murasaki’ (C. annuum). Sequence analysis at the Pun1 locus revealed that this type of Pun1 allele is caused by a single adenine nucleotide insertion in the second exon region. This insertion is unique to ‘Nara Murasaki’ and is not present in wild-type Pun1. This insertion causes a frameshift mutation and a change in the amino acid sequence, resulting in a truncated protein. The results of gene expression analysis indicated that the expression of Pun1 in ‘Nara Murasaki’ was hardly detectable, while the transcripts of this gene were strongly expressed in a pungent cultivar. In a co-segregation test, the pun14 genotype perfectly co-segregated with non-pungency in 103 F2 population plants of a cross between ‘Nara Murasaki’ and a pungent cultivar. ‘Nara Murasaki’ and a DNA marker to distinguish the pun14 allele will be informative for understanding the domestication process of sweet peppers.

Key Words: capsaicinoid, dCAPS marker, domestication, putative acyltransferase.

Introduction

Capsaicin and its analogues, collectively known as capsaicinoids, are the primary source of pungency in Capsicum spp., which belongs to the family Solanaceae (Bennett and Kirby, 1968; Suzuki et al., 1980). These alkaloids confer pungency, or ‘heat’, to pepper fruits and are detected by taste receptor cells in mammals. The perception of pain on contact with this receptor cell during ingestion indicates that capsaicinoids act as deterrents (Jordt and Julius, 2002; Tewksbury and Nabhan, 2001). The absence or presence of a small amount of capsaicinoids is desirable when using peppers as a vegetable, while the presence of a larger amount is preferred for their use as a spice or hot food.

The biosynthesis of these compounds occurs in epidermal cells in the interlocular septa of the placental tissues in pepper fruits (Stewart et al., 2007). Capsaicinoids are produced by the condensation of vanillylamine with a branched fatty acid, derived from either valine or leucine, with vanillylamine being derived from phenylalanine (Suzuki et al., 1981). Variations in capsaicinoid contents in peppers have been attributed to environmental influences during plant growth and also to the fruit position on the plant (Harvell and Bosland, 1997). The accumulation of capsaicinoids in pepper fruit placental tissues occurs between 20–30 days after flowering in pungent cultivars (Iwai et al., 1986).

Although capsaicinoid contents and the intensity of pungency are quantitative traits, the presence of capsaicinoids is controlled by the single Pun1 locus. Pun1 encodes a putative acyltransferase, which acylates vanillylamine with a fatty acid to produce capsaicinoids (Stewart et al., 2005). In the homozygous recessive state, capsaicinoids are not produced by the pepper plant. Three loss-of-function alleles of Pun1 have been reported to date, pun11–3. The allele pun11 is widely
distributed in sweet *C. annuum* cultivars. This type of *pun1* has a 2.5-kb deletion spanning from the putative promotor region to most of the first exon region (Stewart et al., 2005). The protein is not transcribed or translated. The second type of *Pun1* allele, *pun1* has a 4-bp deletion in the first exon region that creates an early stop codon. In this allele, there is transcription, but no protein product is produced (Stewart et al., 2007). In the third allele, *pun1* has a large deletion in the 2nd exon region leading to the loss of 70 amino acids in the Pun1 protein (Stellari et al., 2010). This allele is neither transcribed nor translated. Mutations in this locus have, for a long period of time, been preferred and utilized in the breeding of non-pungent peppers (Deshpande, 1935). However, variations in the loss-of-function allele have not been fully understood.

Therefore, the objective of the present study was to elucidate the mechanisms responsible for the loss of pungency in the Japanese landrace sweet pepper cultivar, ‘Nara Murasaki’. This cultivar differs from other sweet peppers in that it has elongated purple fruits, while most sweet pepper cultivars share common characteristics of bell-type fruit and the *pun1* allele. This motivated our research into the genetic mechanism of non-pungency in ‘Nara Murasaki’. We successfully identified a novel loss-of-function *pun1* allele, dubbed *pun1*. ‘Nara Murasaki’ and a DNA marker to distinguish the *pun1* allele will be informative for understanding the domestication process of sweet peppers.

**Materials and Methods**

**Plant materials**

Four chili pepper cultivars were used in the present study: three non-pungent cultivars (‘Nara Murasaki’, ‘Wonder Bell’ (*pun1*/*pun1*), and ‘Chocolate’ (*pun1*/*pun1*)) and one pungent cultivar (‘Demon Red’). ‘Nara Murasaki’ was purchased from a seed company (Fukase, Japan). This cultivar is a Japanese landrace with purple fruits. The cultivar is called ‘Murasaki (“purple” in Japanese)’ in Nara prefecture in Japan. In order to distinguish it from other purple cultivars, we describe the cultivar as ‘Nara Murasaki’ in the present study. All cultivars belonged to *Capsicum annuum*. Plant materials were grown in a greenhouse in Okayama university farm between 2014 and 2015. The seeds were germinated in black polythene pots and later transferred to larger plastic pots in a greenhouse. Regular watering and fertilization was performed. The capsaicinoid contents of the pepper fruits were determined using high-performance liquid chromatography (HPLC) as described below.

**HPLC analysis of capsaicinoid contents**

Three fruits were harvested at 30 days post anthesis, lyophilized, and freeze-dried for 3 days. They were later ground in a blender and the contents extracted in 4 mL acetone from 200 mg dry fruit powder. The supernatant was filtered (DISMIC 13HP; ADVANTEC TOYO, Japan) and used in the HPLC analysis. The analysis was conducted using a JASCO PU-2080 Plus pump equipped with a UV-Vis detector SPD-10A (Shimadzu, Japan) and a Shimadzu CR-6A integrator. Separation was performed using an Inertsil ODS-3 column (250 × 4.6 mm) (GL Science Inc., Japan). The eluent was a mixture of methanol and water (75:25) at a flow rate of 1 mL·min⁻¹. The detector was set at a wavelength of 280 nm. The run time was set at 17 min. The total capsaicinoid content was calculated from the area indices of capsaicin and dihydrocapsaicin.

**Crossing test for non-pungency**

F1 and F2 populations (n = 103), derived from a cross between ‘Nara Murasaki’ and pungent ‘Demon Red’ were grown to test the inheritance patterns of non-pungency. This population was also used for a cosegregation analysis with *pun1*. Genetic complementation tests were conducted by crossing ‘Nara Murasaki’ with *pun1*-defective cultivars in order to determine whether non-pungency in ‘Nara Murasaki’ is due to defects at the *Pun1* or different loci. F1 hybrids were constructed by crossing ‘Nara Murasaki’ with ‘Wonder Bell’ or ‘Chocolate’. The fruits from each cross were investigated for their capsaicinoid contents by HPLC.

**DNA extraction**

Young fresh leaves were harvested from all experimental plant materials and used for DNA extraction using a Nucleon Phytopure kit (GE Healthcare, UK).

**Genomic PCR and sequence analysis of the Pun1 gene in ‘Nara Murasaki’**

Genomic PCR and sequence analyses were conducted to investigate the *Pun1* locus in ‘Nara Murasaki’. The PCR mixture contained 0.4 μL of KOD FX neo (Toyobo co., Ltd., Japan), 10 μL of buffer, 4 μL of dNTPs (2 mM), and 0.5 μL each of the forward and reverse primers (10 μM) and template DNA. The volume was topped up to 20 μL using super distilled water. PCR conditions were as follows: 1 cycle at 94°C for 2 min, followed by 35 cycles at 98°C for 10 s, 55°C for 30 s, and 68°C for 2 min. This was followed by a final extension at 68°C for 4 min. The list of primers used is shown in Table 1. The exon region was amplified by CSF1 and Pun1 stop 3′ R. The genomic region spanning from promoter to the 1st exon was obtained with BF6 and BR8. Gel red-stained PCR products were separated on a 1% agarose gel in order to confirm their amplification. The PCR products were cleaned using the Exo Star Kit (GE Healthcare) and nucleotide sequencing was performed by the Eurofins sequencing service (Eurofins, Japan). The ATGC program (GENETYX Co., Japan) was used to align the nucleotide sequences. The entire genomic *Pun1* locus in ‘Nara Murasaki’ and
‘Demon Red’ was sequenced and aligned. The deduced amino acid sequence alignment was carried out using the Clustal W program.

Development of the derived Cleaved Amplified Polymorphic Sequence (dCAPS) Marker

In order to distinguish the Nara-Murasaki (NM)-type pun1, a dCAPS marker was developed using a one-nucleotide insertion at the Pun1 gene in ‘Nara Murasaki’. We designed one primer set to create a MseI site in the PCR product; Pun1MseI F (5'-AGAAGGGAAACTGCATTGAAAATTGGATGGCTATAAGAATGTTTTA-3') and Pun1 MseI R (5'-GCCTTGCCAGCTTTGTAAAT-3'). The genomic PCR mixture consisted of 0.3 μL of KOD FX neo polymerase (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 template DNA was adjusted to 20 μL with super distilled water. PCR conditions were as follows: 1 cycle at 94°C for 2 min, followed by 35 cycles at 98°C for 10 s, 55°C for 30 s, and 68°C for 30 s. This was followed by a final extension at 68°C for 4 min. The reaction mixture contained 3 μL of the PCR product, 2 μL 10X buffer, and 1 μL MseI (Takara Bio Inc., Japan). The volume was topped up to 20 μL with super distilled water and kept at 37°C for 18–24 hours. The restriction enzyme product was stained with gel red and visualized on a 2% agarose gel using a UV transilluminator.

Co-segregation of the Pun1 genotype and non-pungency

F1 and F2 populations, derived from a cross between ‘Nara Murasaki’ and ‘Demon Red’, were used for this experiment. The dCAPS marker was used to genotype Pun1 for all plants. The fruits of all plants were assayed for capsaicinoid contents by HPLC.

qRT-PCR analysis

qRT-PCR was conducted in order to investigate the expression levels of Pun1 genes. Total RNA was extracted from the placental tissues of pepper fruits harvested at 30 days post anthesis. Total RNA was extracted using Sepasol-RNA I Super G (Nacalai, Japan) and an RNeasy Mini spin column (Qiagen, Germany). All RNA used for RT-PCR were treated with DNase I prior to cDNA synthesis in order to remove DNA contamination. Five hundred nanograms of DNA-free RNA were converted into cDNA using the PrimeScriptTM RT Master Mix (Takara). Quantitative PCR was performed using the KOD SYBR(R) qPCR Mix (Toyobo) on the Light Cycler Nano Real-Time PCR System (Roche Diagnostics Inc., Switzerland) according to the manufacturer’s instructions. The thermal cycle used was 98°C for 2 min followed by 45 cycles at 98°C for 10 s, 60°C for 10 s, and 68°C for 30 s. Primer sequences for qRT-PCR were as follows: Actin (F 5’-AGCAACTTGGGACATATTGGAAG-3’, R 5’-AAGAGCAACACCCGCTGAATAGC-3’), Pun1 (F 5’-CTCAACGAGTGGCGTGACAGAAAGACT-3’, R 5’-GTGAACACCTTTGATGGTGATG-3’). The reference gene (Actin) was used for normalization. The comparative CT method (2-ΔΔCT method) was used to analyze expression levels. Data represent the mean relative expression of three biological replicates with standard error bars. Reaction specificities were verified by a melting curve analysis (60°C to 97°C).

Results

Crossing test

The results of the HPLC analysis showed that the pungent cultivar ‘Demon Red’ contained a large amount of capsaicin and dihydrocapsaicin, whereas the three non-pungent cultivars ‘Nara Murasaki’, ‘Wonder Bell’, and ‘Chocolate’ did not contain any detectable amounts of capsaicinoids (Table 2). In order to test the genetic pattern of non-pungency in ‘Nara Murasaki’, we made several crosses with pungent and non-pungent cultivars. The crossing of ‘Nara Murasaki’ with a pungent cultivar resulted in a pungent hybrid. This result showed that non-pungency in ‘Nara Murasaki’ is a recessive trait. Previous studies attributed non-pungency to a loss-of-function mutation in the Pun1 locus (Stewart et al., 2005). Crosses of ‘Nara Murasaki’ with non-pungent cultivars (pun1/pun1) resulted in non-pungent hybrids (Table 2). This result suggests that the loss of pungency in ‘Nara Murasaki’ is controlled by the Pun1 locus and the type of allele responsible is a recessive allele.

DNA marker for the known Pun1 allele

Pun1 encodes an acyltransferase that acylates vanil-lylamine with a fatty acid to produce capsaicinoids. The recessive allele pun1 has a 2.5-kb deletion between the

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Oligonucleotide</th>
<th>Size (nt)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF1</td>
<td>5’-ATGGCTTTTGCAATTACCATCA-3’</td>
<td>21</td>
<td>Lee et al. (2005)</td>
</tr>
<tr>
<td>CSR4</td>
<td>5’-TCAACACCAAAAAAGCTGTG-3’</td>
<td>22</td>
<td>Lee et al. (2005)</td>
</tr>
<tr>
<td>BF6</td>
<td>5’-GAAAGATCCGACCTCGTCAA-3’</td>
<td>20</td>
<td>Lee et al. (2005)</td>
</tr>
<tr>
<td>BR8</td>
<td>5’-TGACACACCAATTGAGTGCT-3’</td>
<td>22</td>
<td>Lee et al. (2005)</td>
</tr>
<tr>
<td>Pun1 stop 3’ R</td>
<td>5’-TTTGTAATAATCCCTCTCTTC-3’</td>
<td>23</td>
<td>In this study</td>
</tr>
</tbody>
</table>
promoter region and first exon, which prevents transcription and translation of the gene and leads to reduced capsaicinoid synthesis (Stewart et al., 2005). Previous studies on various pepper varieties reported that pun1 was widely distributed among non-pungent C. annuum varieties. In order to further test the pun1 allele in ‘Nara Murasaki’, we used a DNA marker to distinguish pun1 (Lee et al., 2005). If plants possess the wild-type Pun1 allele, the 1.6-kb PCR fragment is amplified with CSF1/CSR4 primers. On the other hand, when plants are homozygous for pun1 alleles, the PCR fragment is not amplified. The pungent cultivar ‘Demon Red’ produced the 1.6-kb fragment, whereas ‘Wonder Bell’ (pun1/pun1) did not. This was consistent with previous findings. However, non-pungent ‘Nara Murasaki’ produced a PCR fragment similar to ‘Demon Red’ (Fig. 1). These results indicate that the recessive pun1 alleles in ‘Nara Murasaki’ were not distinguishable with the known pun1 marker, and that ‘Nara Murasaki’ may contain a novel pun1 allele.

Sequence analysis of Pun1 in ‘Nara Murasaki’

We sequenced the entire genomic region of the Pun1 locus in ‘Nara Murasaki’, and compared it with the wild-type sequence. No significant mutation was found in the promoter region or 1st exon of Pun1, whereas the reported pun1 has a 2.5-kb deletion region spanning from the promoter to exon 1. However, the sequence analysis revealed a single nucleotide insertion in the 2nd exon region in Pun1 of ‘Nara Murasaki’, which was absent in the pungent cultivar (Fig. 2). The insertion contained an adenine nucleotide leading to a frameshift mutation.

Deduced amino acid sequence alignment

We performed an amino acid sequence alignment between NM-type and wild-type Pun1 using Clustal W to

![Fig. 1. DNA marker to distinguish the known pun1 allele. 1: ‘Demon Red’ (pungent), 2: ‘Nara Murasaki’ (non-pungent), 3: ‘Wonder Bell’ (non-pungent: pun1/pun1). In ‘Wonder Bell’ (pun1/pun1), the 1670-bp DNA fragment was not amplified because pun1 does not have the 2.5-kb region to which the forward primer binds.](image)

![Fig. 2. Structures at the Pun1 locus from pungent (Pun1/Pun1) and non-pungent (pun1/pun1, pun1/pun1) cultivars. Schematic illustration of the Pun1 locus. Pun1 consists of two exons. pun1 possesses a 2.5-kb deletion in the putative promoter region and exon 1, which is represented by the inverted triangle. pun1 does not have a structural mutation in the promoter region or exon 1, but possesses a single nucleotide insertion in the 2nd exon, which causes a frameshift mutation.](image)

<table>
<thead>
<tr>
<th>Cultivar or F1 hybrid</th>
<th>Content (μg·g−1 of fruit DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Capsaicin</td>
</tr>
<tr>
<td>Nara Murasaki</td>
<td>nd</td>
</tr>
<tr>
<td>Demon Red</td>
<td>2854 ± 231</td>
</tr>
<tr>
<td>Chocolate</td>
<td>nd</td>
</tr>
<tr>
<td>Wonder Bell</td>
<td>nd</td>
</tr>
<tr>
<td>(Nara Murasaki × Demon Red) F1</td>
<td>1359 ± 140</td>
</tr>
<tr>
<td>(Nara Murasaki × Chocolate) F1</td>
<td>nd</td>
</tr>
<tr>
<td>(Nara Murasaki × Wonder Bell) F1</td>
<td>nd</td>
</tr>
</tbody>
</table>

* Not detected.

* Mean ± standard error (n = 3).

Table 2. Primers of genomic PCR for the Pun1 locus.
confirm the effects of the single nucleotide insertion on the amino acid sequence (Fig. 3). The deduced amino acid alignment revealed the effects of the insertion present in ‘Nara Murasaki’ on the amino acid sequence. From the point of the A insertion, a subsequent change was observed in the amino acid sequence owing to a frameshift and there was also a premature stop codon. This resulted in a shorter amino acid sequence, with a loss of 50 amino acids. This mutation may lead to a loss of function in the Pun1 enzyme. In contrast, wild-type Pun1 had a complete amino acid sequence.

Development of a dCAPS marker to distinguish the ‘Nara Murasaki’ type Pun1 allele

A dCAPS marker was developed to distinguish the pun1 allele present in ‘Nara Murasaki’. Using a single nucleotide substitution in the primer Pun1 MseI F, we created an MseI restriction enzyme site in the NM-type Pun1 sequence (Fig. 4A). The resulting amplicon was digested by the MseI restriction enzyme. Only the NM-type Pun1 allele was digested and a shorter band of 133 bp was generated when the restriction enzyme product was separated on a 2% agarose gel using electrophoresis (Fig. 4B). ‘Demon Red’ produced a 181-bp band. This marker was used in co-segregation of the pun1 genotype and non-pungency in the F2 population between ‘Nara Murasaki’ and ‘Demon Red’.

dCAPS marker patterns co-segregated with non-pungency

A co-segregation test was conducted using the F2 population from the cross between ‘Nara Murasaki’ and ‘Demon Red’ with the dCAPS marker described above to confirm that the NM-type pun1 allele is the recessive gene determining non-pungency (Table 3). A total of 103 F2 population plants were investigated for genotyping with the dCAPS marker. The NM-type genotype formed a 133-bp band, the wild-type formed a 181-bp band, and the heterozygous genotype formed 133- and 181-bp bands (Fig. 5). In all plants tested, the pun1 genotypes perfectly co-segregated with non-pungency. Among the 103 F2 plants tested, 24 F2 plants contained only the 181-bp fragment, while 44 plants formed the 133-bp and 181-bp bands. The two groups may correspond to wild-type homozygotes for this locus (Pun1/Pun1) and those that were heterozygous at this locus (Pun1/pun1), respectively. On the other hand, the remaining 35 plants had non-pungent fruits and contained a 133-bp fragment, which indicated that they were homozygous for the NM-type allele (pun1/pun1) (Table 3). These results suggest that pun1 is the recessive gene determining non-pungency in ‘Nara Murasaki’. The segregation ratio was also consistent with the expectation that non-pungency is controlled by a single recessive gene, pun1 (X2 = 3.12; P = 0.21). Taken together...
with nucleotide sequence data, the co-segregation analysis strongly suggests that non-pungency in ‘Nara Murasaki’ is caused by a new loss-of-function pun1 allele, which is different from the widely distributed pun1 allele among C. annuum.

Expression level of Pun1

Total RNA was extracted from the placental tissues of ‘Demon Red’, ‘Nara Murasaki’, and ‘Wonder Bell’ (pun1/pun1), cDNAs were synthesized, and qPCR was conducted for an expression analysis of Pun1. The results of the gene expression analysis showed that Pun1 gene transcripts were strongly expressed in ‘Demon Red’, but were not detected in ‘Wonder Bell’. Pun1 expression in ‘Nara Murasaki’ was detected, but the expression level was $1.3 \times 10^4$ times lower than that in ‘Demon Red’ (Fig. 6).

Discussion

The domestication and human use of chili pepper (Capsicum) occurred at least 7000–9000 years ago based on archaeological evidence, making it one of the earliest domesticated plant species (Basu and De Krishna, 2003). The origin of Capsicum may be traced to Bolivia in Central South America, with this area being a habitat to most of the 20–27 recognized species of Capsicum. (Andrews, 1984). Columbus, from his explorations, returned to Spain with different pungent forms of the C. annuum species. Consequently, he is credited with having played a major role in the dispersal of peppers throughout the world (Walsh and Hoot, 2001). Peppers spread from Europe to all over the world with an ultimate reintroduction to the Americas from where they had

Table 3.  Co-segregation of pun1$^4$ and non-pungency.

<table>
<thead>
<tr>
<th>Population size</th>
<th>n</th>
<th>Pun1 genotype</th>
<th>Phenotype</th>
<th>Expected ratio</th>
<th>Chi-squared (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Nara Murasaki × Demon Red)</td>
<td>24</td>
<td>Pun1/Pun1</td>
<td>Capsaicin Dihydrocapsaicin Total</td>
<td>1</td>
<td>44</td>
</tr>
<tr>
<td>F2</td>
<td>103</td>
<td>Pun1/pun1$^4$</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>pun1$^4$/pun1$^4$</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

* The genotype was distinguished by the developed dCAPS marker.

* Value represents the capsaicin level (μg·g$^{-1}$ of fruit dry weight).

* Not detected.

Fig. 4. The dCAPS marker developed to distinguish the NM-type of the Pun1 allele. (A) Genomic sequences of the Pun1 gene and primer design. The figure shows the 2nd exon sequence of Pun1. The asterisk indicates an A residue that was replaced by a T to construct an Msel site in the forward primer Pun1-Mse1 F. The box indicates an Msel site in the PCR product derived from NM-type pun1. (B) The amplicon resulting from PCR was treated with the Msel restriction enzyme, and only the NM sequence was digested. ‘Nara Murasaki’ formed a shorter band of 133 bp than the wild-type (‘Demon Red’), which formed a 181-bp band, on 2% agarose gel electrophoresis. 1: ‘Demon Red’, 2: ‘Nara Murasaki’.
originated. Sweet bell peppers were first characterized approximately 500 years ago, and their seeds were traded around the same time. The first description referring to a bell variety distinctively identified for cultivation occurred in 1774. Early non-pungent pepper forms were from a single genetic source (*Capsicum annuum*) identified in the 16th century. This was the origin of the non-pungent pepper cultivars released in Europe, North and South America (Boswell, 1937; Cook, 1984a, b; Homma, 1986; Millet and Jones, 1982).

In genetic studies, Webber (1911) reported that the absence of pungency was controlled by a single recessive gene, *pun1* (formerly known as *C*), which has a qualitative effect on pungency. Blum et al. (2002) then mapped the *Pun1* locus to chromosome 2 in a crossing population between a pungent *C. frutescens* accession and non-pungent *C. annuum* bell pepper. Stewart et al. (2005) successfully cloned and characterized *Pun1* encoding acyltransferase to form capsaicinoids from vanillylamine and fatty acids. Recessive *pun1* was found to have a large deletion spanning from the promoter to the exon. The abundantly distributed *pun1* allele utility in different breeding programs involving non-pungent peppers has occurred for approximately 3 centuries (Boswell, 1937; Lee et al., 2005).

Even though two loss-of-function *pun1* alleles have been reported in other *Capsicum* species, including *pun1* in *C. chinense* and *pun1* in *C. frutescens*, no additional *pun1* allele has been identified among *C. annuum* (Stellari et al., 2010; Stewart et al., 2007; Wyatt et al., 2012). This finding indicates that breeding for non-pungent peppers is relying on the *pun1* allele worldwide.

We herein report a novel *pun1* allele, named *pun1*4, in the Japanese non-pungent cultivar ‘Nara Murasaki’ (*C. annuum*). The sequence analysis of *pun1*4 revealed that one A insertion in the 2nd exon causes a frameshift mutation. Pun1 is a member of the plant BAHD acyltransferase family. The BAHD family shares two highly conserved domains such as the HXXXDG domain near the center portion of the enzyme, and the DFGWG motif near the C-terminus (D’Auria, 2006). The frameshift mutation in *pun1*4 changes the DFGWG motif,
which could lead to loss of function (Fig. 3). This mutation is not found in \textit{pun1}, and is unique to \textit{pun1}$. In contrast to \textit{pun1}, \textit{pun1} has a complete promoter and exon 1. This indicates that \textit{pun1} may have originated independently of \textit{pun1} from pungent peppers. However, since the origin of this allele is unknown, when the mutation occurred and how it was introduced into ‘Nara Murasaki’ remains an open question. There is one possibility that the mutation resulting in \textit{pun1} occurred in Japan, and two possible theories have been proposed for the introduction of peppers to Japan (Yazawa, 2008). The first theory suggests that peppers were introduced to Japan in the 16th century from the Korean peninsula. The second theory suggests that peppers were introduced to Japan by Lusitanians. In both theories, pungent forms were introduced to Japan. By the time sweet peppers were introduced from western countries to Japan in the 19th century, several low-pungent landraces had been developed domestically. As an example, a previous study reported that the low-pungent Japanese landrace ‘Himo’ has a unique loss-of-function allele of \textit{putative aminotransferase}, which is a structural gene in the capsaicinoid biosynthesis pathway (Tanaka et al., 2010). Further studies are needed in order to clarify the origin of ‘Nara Murasaki’ and \textit{pun1} and reveal the phylogenetic relationship among \textit{Capsicum} accession.

In the present study, the expression levels of the \textit{Pun1} gene in ‘Nara Murasaki’ and ‘Wonder Bell’ were similar because the mRNA transcripts were negligibly low or undetected in qPCR in both cultivars. In comparison, the pungent cultivar ‘Demon Red’ exhibited abundant levels of \textit{Pun1} mRNA. This is consistent with the hypothesis that the level of transcripts accumulating for capsaicinoid-associated genes positively correlates with the level of pungent compounds (Aluru et al., 2003; Curry et al., 1999). The loss of transcription in ‘Wonder Bell’ (\textit{pun1}) has been attributed to a 2.5-kb deletion in the putative promoter region and exon 1. On the other hand, the transcripts of \textit{Pun1} were rarely detected in ‘Nara Murasaki’, even though there is a complete promoter region and exon 1, as in the case of wild-type \textit{Pun1} (Fig. 2). A similar pattern was reported in \textit{pun1}$^2$ and \textit{pun1}$^3$, in which there was reduced transcription even though the 2.5-kb deletion was absent (Stellari et al., 2010; Stewart et al., 2007). The inhibition of transcription is in contrast to our expectation that some \textit{Pun1} transcription may have occurred due to possession of the full promoter region and exon 1. The transcription inhibitory mechanism in the mutated \textit{pun1} alleles may be due to an unknown factor. Even if the \textit{pun1}$^4$ allele were transcribed, the truncated protein lacking a conserved domain would be non-functional.

In conclusion, we herein found a novel allele of \textit{Pun1}, \textit{pun1} in \textit{Capsicum annuum}, which is responsible for non-pungency in the Japanese sweet pepper ‘Nara Murasaki’. This allele is the second allele in \textit{C. annuum} after \textit{pun1} with a distinct mode of loss of function. It opens the door for further research on allelic variations present in all domesticated \textit{Capsicum} species. The co-dominant dCAPS marker will be useful in marker-assisted selection to distinguish this new type allele of \textit{Pun1}. ‘Nara Murasaki’ and \textit{pun1}$^4$ will be a new genetic source of non-pungency.

\textbf{Literature Cited}


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