Analysis of Non-pungency, Aroma, and Origin of a *Capsicum chinense* Cultivar from a Caribbean Island

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‘No.80’ (*Capsicum chinense*) from the Caribbean is a valuable genetic source from the aspect of its non-pungent and highly aromatic traits. In the present study, the non-pungency, volatile components, and phylogenetic origin of ‘No.80’ were analyzed with another *C. chinense* cultivar, ‘No.2’ from Brazil, which is also non-pungent but less aromatic. Expressions and deduced amino acid sequences of acyltransferase (*Pun1*) of ‘No.80’ and ‘No.2’ were normal compared with a pungent cultivar, ‘Habanero’. Insertions of 7-bp and 8-bp resulting in frameshift mutations were found in the coding regions of putative aminotransferase (*p-AMT*) of ‘No.80’ and ‘No.2’, respectively. Co-segregation of these insertions with the non-pungent phenotypes in F1 and F2 populations obtained from crossing ‘No.80’ or ‘No.2’ with ‘Habanero’ suggested that non-pungency in these cultivars arose from genetic mutations of *p-AMT* that occurred independently. Moreover, molecular phylogenetic analysis suggested that ‘No.80’, a close relative of ‘No.2’, originates from capsicums migrated from the South American mainland. In addition to pungency, we assessed the volatile components of the highly aromatic ‘No.80’, the less aromatic ‘No.2’, and their F1 hybrid using gas chromatography. ‘No.80’ contained higher levels of aroma-contributing volatiles than ‘No.2’, which correlated with the stronger and weaker aromas of two cultivars. Further, the fruit of F1 progenies emitted a number of volatile compounds between or higher than their corresponding parents. Based on these results, the approaches for breeding highly aromatic non-pungent cultivars are discussed.

**Key Words:** aromatic flavor, F1 hybrid, non-pungency, volatiles.

Introduction

The pungency of chili pepper fruits is caused by a group of analogs known as capsaicinoids (Bennett and Kirby, 1968). These unique compounds are exclusively produced by the fruits of *Capsicum* (Andrews, 1984). Based on the range of capsaicinoid levels, cultivars can be categorized into non-pungent, mildly, moderately, highly, and very highly pungent (Howard and Wildman, 2007). In Japan, consumption of non-pungent cultivars (eg., bell peppers, paprika, and ‘Shishitou’) is higher than that of pungent cultivars.

*Capsicum* consists of several wild species and five domesticated species, *C. annuum*, *C. chinense*, *C. frutescens*, *C. pubescens*, and *C. baccatum* (Bosland and Votava, 2000). Of the five domesticated species, *C. annuum*, which originates from southern Mexico, is the most widely cultivated worldwide, including Japan. *C. chinense* is of Amazonian origin and is indigenous to South America and the Caribbean (Eshbaugh, 1993). *C. chinense* cultivars such as ‘Habanero’ and ‘Scotch Bonnet’ are highly pungent and have highly aromatic flavors, which *C. annuum* cultivars lack (Moreno et al., 2012). In addition, ‘NMCA30036’, which accumulates no capsaicinoids, or ‘Zavory Hot’, ‘Aji Dulce strain 2’, and ‘Belize Sweet’, which accumulate capsaicinoids in a trace amount, have been reported (Stewart et al., 2007; Tanaka et al., 2010b). Based on the classification of Howard and Wildman (2007), these cultivars can be categorized as non-pungent cultivars. Although *C. annuum* is economically the most important worldwide, *C. chinense*, especially its non-pungent cultivars, have potential as vegetables with an aromatic flavor.
In our previous study, we conducted a field and market survey of capsicums in the Caribbean (Koeda, 2012). *Capsicum chinense* is widely used in Trinidad, a Caribbean island in the southern-most part of the Lesser Antilles, where more than 10 pungent and highly aromatic *Capsicum chinense* cultivars can be found; however, only one highly aromatic non-pungent *C. chinense* cultivar, locally known as ‘Pimento’, could be found in the local markets or supermarkets. The green immature fruits or red mature fruits are used for seasoning food. In addition, although precise records are not available, ‘Pimento’ appears to be an old, traditional cultivar in Trinidad, the genetic background of its non-pungency remains incompletely understood.

‘Pimento’ is a valuable genetic source from the aspect of its non-pungency and highly aromatic trait. In the present study, ‘Pimento’ was analyzed with another *C. chinense* cultivar ‘Pimentinha’ from Brazil, which is also non-pungent but less aromatic. Since mutations in acyltransferase (*Pun1*) or putative aminotransferase (*p-AMT*) are reported as the genetic causalities of loss of pungency in Capsicum (Stewart et al., 2005, 2007; Lang et al., 2009; Tanaka et al., 2010a, b), we investigated the *Pun1* and *p-AMT* genes to elucidate the genetic basis of the non-pungent phenotypes of ‘Pimento’ and ‘Pimentinha’ in the present study. Moreover, the composition of aromatic volatiles and the relationship between the two cultivars were investigated. Based on the results, the approaches for breeding highly aromatic non-pungent cultivars are discussed.

**Materials and Methods**

**Plant materials, crossing combinations, and growth conditions**

Four *C. chinense* cultivars ‘No.80’, ‘No.2’, ‘NMCA30036’, and ‘Habanero’ were used in this study. ‘Pimento’ and ‘Pimentinha’ were collected from Port of Spain (Trinidad) and Tome-acu (Brazil) and named ‘No.80’ and ‘No.2’, respectively. *C. chinense* accessions Tr-1 and Tr-17, previously reported in Koeda et al. (2013), are both ‘No.80’. ‘NMCA30036’ is a non-pungent cultivar carrying the recessive allele of *Tr-1* and *Tr-17*, previously reported in Koeda et al. (2007), and ‘Habanero’ is a pungent cultivar. *F₁* and *F₂* populations were obtained by crossing ‘Habanero’ with ‘No.80’, or ‘Habanero’ with ‘No.2’ to determine the inheritance pattern of the fruit pungency. In addition, other *F₁* populations were prepared by crossing ‘NMCA30036’ with ‘No.80’, ‘NMCA30036’ with ‘No.2’, and ‘No.80’ with ‘No.2’. All plants were grown at Kyoto University experimental farm from March to October in 2012 and 2013. Segregation data were evaluated by the chi-square test.

**Phenotyping of fruit pungency**

Organoleptic testing of mature fruits (*F₁* and *F₂* populations) was performed using two randomly sampled fruits of each plant by a minimum of two trained persons. If both fruits were pungent, the plant was considered pungently pungent. From plants that were considered as non-pungent by the organoleptic test, capsaicinoids were extracted and quantified by high-performance liquid chromatography (HPLC) as described in the section given below. The capsaicinoid contents of ‘No.80’, ‘No.2’, ‘NMCA30036’, and ‘Habanero’ were also confirmed by HPLC. After the fruits were freeze-dried, capsaicinoids were extracted and quantified according to the method described by Tanaka et al. (2010b). The capsaicinoid content was calculated as the sum of capsaicin and dihydrocapsaicin.

**cDNA sequence analysis of *Pun1* and *p-AMT***

The full-length cDNA sequences of *Pun1* and *p-AMT* were determined for ‘No.80’, ‘No.2’, and ‘Habanero’. Pepper fruits were harvested 20 days after flowering, and the placenta was separated for RNA extraction. Total RNA was extracted and reverse transcribed according to the method described by Koeda et al. (2013). In RT-PCR analysis, *CaActin* (AY572427) was used as a positive internal control. The full-length cDNA sequence of *Pun1* was amplified using F1 and R1481 primer sets (Tanaka et al., 2010b). PCR was performed using Blend Taq (Toyobo, Osaka, Japan). For all PCR reactions, the reaction mixtures were initially denatured at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min, terminating with 3 min of extension at 72°C. Electrophoresis using 1.0% (w/v) agarose gel was performed on the amplified PCR products. For all treatments, three biological replicates of RT-PCR analysis were performed using independently prepared total RNA and similar results were obtained. The full-length sequences of *Pun1* and *p-AMT* amplified by RT-PCR were cloned into pTaq1 cloning vector (BioDynamics Laboratory, Tokyo, Japan). Nucleotide sequencing was performed in an ABI PRISM 3100 genetic analyzer with an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

**Genomic sequence analysis of *p-AMT***

Genomic DNA was extracted from young leaves of pepper plants using Nucleon PhytoPure (GE Healthcare, Buckinghamshire, UK). The genomic region of *p-AMT* harboring the insertion in ‘No.80’ was amplified using F1 and R282 primers, and that in ‘No.2’ was amplified using F304 and 7th intron R primers (Tanaka et al., 2010b). PCR, electrophoresis, and sequencing were performed as described above.
Molecular phylogenetic analysis of C. chinense

Forty-six C. chinense accessions, which include ‘No.80’ and ‘No.2’, a C. annuum accession and a C. frutescens accession were used for phylogenetic analysis (Fig. 4). All the accessions were categorized as very highly pungent cultivars (DW) and ‘No.2’. The capsaicinoid contents of ‘Habanero’ (15353 ± 2485 μg·g⁻¹ DW), ‘No.80’ (25 ± 14 μg·g⁻¹ DW), ‘No.2’ (not detected), and ‘NMCA30036’ (not detected) were assessed by HPLC. According to Howard and Wildman (2007), ‘Habanero’ was categorized as a very highly pungent cultivar (> 5333 μg·g⁻¹ DW) and ‘No.80’, ‘No.2’, and ‘NMCA30036’ were categorized as non-pungent cultivars (< 47 μg·g⁻¹ DW) (Table 1). To determine the genetic basis of the non-pungent phenotype, we crossed ‘No.80’ and ‘No.2’ with another non-pungent cultivar ‘NMCA30036’ and assessed the capsaicinoid contents of the progeny. The progeny were grown in a greenhouse and analyzed using HPLC. The capsaicinoid contents of the progeny were compared with those of the parents to determine the genetic basis of the non-pungent phenotype.

Analysis of volatiles

Fruits of ‘No.80’, ‘No.2’, F₁ (‘No.80’ × ‘No.2’), and F₂ (‘No.2’ × ‘No.80’) were used for analysis. Volatiles were extracted by headspace solid-phase microextraction (HS-SPME). Four to five fruits (31.5–34.9 g) were cut into halves and placed in a headspace vial [sample weight (g) × 25 mL], and sealed with plastic wrap. Equilibration was achieved by heating the vial for 20 min in a water bath (30°C). Prior to the first analysis, an SPME fiber (DVB/CAR/PDMS; Supelco, Bellefonte, PA, USA) was conditioned for 1 h at 250°C in the injector port of a gas chromatograph (GC). To adsorb volatiles, the fiber was exposed to the headspace of sample vials for 20 min at 30°C. For thermal desorption, the needle was inserted into the injection port (250°C) of the GC (GC-2014; Shimadzu, Kyoto, Japan). Prior to the next analysis, the fiber was reconditioned at 250°C to ensure that no compounds from the previous sample were carriedover.

Volatile components in Capsicum fruits were analyzed with GC2014 and GCMS2010 systems (Shimadzu). The GC2014 was equipped with a flame ionization detector and used for qualitative determination. The GCMS2010 was equipped with a quadrupole MS detector and used for quantitative determination. These GC systems were equipped with DB-WAX (60 m × 0.25 mm i.d.; 0.25 μm film thickness) fused silica capillary columns (Agilent Technologies, Santa Clara, CA, USA). Injector and detector temperatures were set at 250°C. The column oven temperature was programmed as follows: after being held at 70°C for 5 min, the temperature was increased from 70°C to 220°C at a rate of 3°C·min⁻¹ and held at 220°C for 10 min. Helium was used as the carrier gas at 150 kPa, pressure control mode. The GC-MS system was operated in EI mode at 70 eV. The chromatograms were analyzed in scan mode of 35–500 m/z. The volatile compounds were identified by comparing their retention times and mass spectra with the NIST08/08s and FFNSC library data.

Results and Discussion

Genetic mechanisms of loss of pungency in ‘No.80’ and ‘No.2’

‘No.80’ is a valuable genetic source from the aspect of its non-pungency and the highly aromatic trait. In the present study, ‘No.80’ was analyzed with another non-pungent cultivar ‘No.2’. The capsaicinoid contents of ‘Habanero’ (15353 ± 2485 μg·g⁻¹ DW), ‘No.80’ (25 ± 14 μg·g⁻¹ DW), ‘No.2’ (not detected), and ‘NMCA30036’ (not detected) were assessed by HPLC. According to Howard and Wildman (2007), ‘Habanero’ was categorized as a very highly pungent cultivar (> 5333 μg·g⁻¹ DW) and ‘No.80’, ‘No.2’, and ‘NMCA30036’ were categorized as non-pungent cultivars (< 47 μg·g⁻¹ DW) (Table 1). To determine the genetic basis of the non-pungent phenotype, we crossed ‘No.80’ and ‘No.2’ with another non-pungent cultivar ‘NMCA30036’ and assessed the capsaicinoid contents of the progeny. The capsaicinoid contents of the progeny were compared with those of the parents to determine the genetic basis of the non-pungent phenotype.

| Parental cultivar and cross combination | Population size (n) | Number of plants | Expected ratio (Pungent:Non-pungent) | Chi-square (P-value) |
|----------------------------------------|---------------------|------------------|------------------------------------|---------------------|
| P, Habanero                            | 10                  | 10 Non-pungent   | 1:0                                | —                   |
| P, No.80                               | 10                  | 0 Pungent        | 1:0                                | —                   |
| P, No.2                                | 10                  | 0 Non-pungent    | 1:0                                | —                   |
| P, NMCA30036                           | 10                  | 0 Pungent        | 1:0                                | —                   |
| F₁ (P × P₂)                            | 15                  | 15 Non-pungent   | 1:0                                | —                   |
| F₁ (P × P₂)                            | 15                  | 15 Pungent       | 1:0                                | —                   |
| F₁ (P × P₂)                            | 11                  | 11 Non-pungent   | 1:0                                | —                   |
| F₁ (P × P₂)                            | 15                  | 15 Pungent       | 1:0                                | —                   |
| F₁ (P × P₂)                            | 10                  | 10 Non-pungent   | 1:0                                | —                   |
| F₁ (P × P₂)                            | 10                  | 10 Pungent       | 1:0                                | —                   |
| F₂ (P × P₂)                            | 48                  | 36 Non-pungent   | 3:1                                | 0.133 (0.715)       |
| F₂ (P × P₂)                            | 40                  | 31 Pungent       | 3:1                                | 0.000 (1.000)       |

* Non-pungency was determined by organoleptic test and HPLC analysis.
‘Habanero’. The pungent and non-pungent phenotypes were segregated as 1:0 in F1 populations and 3:1 in F2 populations (Table 1), suggesting that the non-pungent phenotypes of ‘No.80’ and ‘No.2’ were controlled by a single recessive gene. Moreover, because the F1 populations obtained by crossing ‘No.80’ with ‘No.2’ were non-pungent (Table 1), the non-pungency of ‘No.80’ and ‘No.2’ appeared to be controlled by the same locus.

We investigated the *Pun1* and *p-AMT* genes to elucidate the genetic basis of the non-pungent phenotype of ‘No.80’ and ‘No.2’. First, the expression of the *Pun1* gene was analyzed by RT-PCR. Fragments of 1.3 kbp of *Pun1* were amplified in ‘Habanero’, ‘No.80’, and ‘No.2’ (Fig. 1). cDNA sequences of *Pun1* were determined in these three cultivars. No genetic mutations affecting the deduced amino acid sequence were observed in ‘No.80’ and ‘No.2’ compared with ‘Habanero’ (data not shown). Because the F1 populations obtained by crossing ‘No.80’ or ‘No.2’ with ‘NMCA30036’ were all pungent (Table 1), *Pun1* could not account for the non-pungency of ‘No.80’ and ‘No.2’. Second, the expression of *p-AMT* was analyzed by RT-PCR. Fragments of 1.4 kbp of *p-AMT* were amplified in ‘Habanero’, ‘No.80’, and ‘No.2’ (Fig. 1). cDNA sequence analysis revealed that the *p-AMT* cDNA of ‘No.80’ contained a 7-bp (GTCTTTA) insertion in the second exon, and that of ‘No.2’ contained an 8-bp (GCCACACC) insertion in the sixth exon, resulting in frameshift mutations in both. These insertions led to truncated proteins of 25 and 218 amino acids, respectively, lacking the PLP domain (Fig. 2), which is essential for aminotransferase activity and mutations in this domain result in loss of pungency (Lang et al., 2009; Tanaka et al., 2010a, b).

Based on the insertions in ‘No.80’ and ‘No.2’, co-dominant markers for *p-AMT* were developed. When primers for detecting *p-AMT* were used for PCR, a 114-bp amplicon was generated in ‘Habanero’, whereas a 122-bp amplicon was generated in ‘No.2’ (Fig. 3). Genotyping of F2 progeny from the above segregating populations revealed that the 7-bp and 8-bp insertions of ‘No.80’ and ‘No.2’ co-segregated precisely with non-pungency in the F2 populations. These results indicate that the non-pungent phenotypes of ‘No.80’ and ‘No.2’ are controlled by 7-bp and 8-bp insertions in the *p-AMT*, respectively.

### Table 1

| Cultivar | GTTCTTTA | GCCACACC |
|----------|----------|----------|
| ‘Habanero’ | No insertion | No insertion |
| ‘No.80’ | GTCTTTA | GCCACACC |
| ‘No.2’ | No insertion | No insertion |

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**Fig. 1.** RT-PCR for full-length *Pun1* and *p-AMT* in ‘Habanero’, ‘No.80’, and ‘No.2’. Actin was used as a positive internal control.
pungency is suggested by the early identification in the 1500s of a widely distributed non-pungent pepper (Boswell, 1937), now known to carry a recessive allele of Pun1 (Stewart et al., 2005). Within C. annuum, most of the non-pungent cultivars carry the same single recessive allele of Pun1 (Stewart et al., 2005). Recently, two recessive alleles of p-AMT have been reported in two non-pungent cultivars, ‘CH-19 Sweet’ and ‘Himo’ (Lang et al., 2009; Tanaka et al., 2010a). In C. chinense, a single recessive allele of Pun1 in ‘NMCA30036’ and three recessive alleles of p-AMT in ‘Zavory Hot’, ‘Aji Dulce strain 2’, and ‘Belize Sweet’ are reported to be the genetic bases of non-pungency (Stewart et al., 2007; Tanaka et al. (2010b) have reported an 8-bp insertion in the sixth exon of ‘Aji Dulce strain 2’. ‘No.2’ carried the same 8-bp insertion in the same position as ‘Aji Dulce strain 2’, although we could not detect the large insertions (396 bp or 403 bp) reported for ‘Aji Dulce strain 2’ in p-AMT cDNA by Tanaka et al. (2010b). By genomic PCR using the 3rd-intron F and R282 primer set, an approximately 2.3-kbp insertion in the third intron similar to that in ‘Aji Dulce strain 2’ could also be detected in ‘No.2’ (data not shown). These results indicate that ‘No.2’ and ‘Aji Dulce strain 2’ carry the same recessive allele of p-AMT. The reason why partial sequences of 2.3-kbp were inserted in cDNA of ‘Aji Dulce strain 2’ but not in ‘No.2’ is unexplained. In ‘No.80’, the insertion sequence and insertion position were different from any other recessive alleles of p-AMT reported. This result suggests that the recessive allele of ‘No.80’ is a newly found allele. Although ‘No.80’ and ‘No.2’ possessed mutations in p-AMT, the former accumulated capsaicinoids in a trace amount (25 ± 14 μg·g⁻¹ DW), whereas the latter accumulated no capsaicinoids. ‘Zavory Hot’, ‘Aji Dulce strain 2’, and ‘Belize Sweet’ also accumulated capsaicinoids in a trace amount (Tanaka et al., 2010b). There is no doubt that the mutations in p-AMT largely affect the loss of pungency in Capsicum, but other genetic factors might be related to this small difference between the cultivars. Further study is needed to clarify this point.

In C. annuum, a single genetic source for non-

![Fig. 3](image-url)

**Fig. 3.** DNA polymorphism of ‘Habanero’, ‘No.80’, ‘No.2’, F₁, and F₂. Genomic DNA was isolated from leaves and PCR was conducted with (A) primer No80Ex2F2 and No80Ex2R2, (B) primer No2Ex6F1 and No2Ex6R1. Amplified fragments were electrophoresed on 8% polyacrylamide gels. M: DNA ladder marker, P₁: ‘Habanero’ (p-AMT/p-AMT), P₂: ‘No.80’ (p-amtNo.80/p-amtNo.80), P₃: ‘No.2’ (p-amtNo.2/p-amtNo.2), F₁ (A): (p-AMT/p-amtNo.80), F₁ (B): (p-AMT/p-amtNo.2) and 1–9 indicate F₂ of (A) ‘Habanero’ x ‘No.80’, (B) ‘Habanero’ x ‘No.2’. The sizes of the amplified fragments are indicated on the right (bp).

![Fig. 4](image-url)

**Fig. 4.** Neighbor-joining analysis of the Capsicum accessions based on modified Cavalli-Sforza distance (Dₛ). Bootstrap values higher than 70% are shown along the branches (from 1000 replicates). ‘No.80’ and ‘No.2’ are shaded grey. * indicate accessions from the Caribbean.
Tanaka et al., 2010b). Combined with our results for ‘No.80’ and ‘No.2’, recessive alleles of \( p\text{-AMT} \) appear to be the major genetic mechanism for the loss of pungency in \( C. \text{chinense} \) to date.

**Phylogenetic analysis of ‘No.80’ and ‘No.2’**

‘No.80’ is an important non-pungent cultivar widely cultivated in Trinidad, but its origin is unknown (Koeda, 2012). To infer the origin of ‘No.80’, molecular phylogenetic analysis of ‘No.80’, ‘No.2’, and other \( C. \text{chinense} \) accessions from the Caribbean and South America was performed using several \( Capsicum \) SSR markers. Forty-six accessions of \( C. \text{chinense} \) formed a different cluster from ‘No.72’ (\( C. \text{annuum} \)) and ‘No.81’ (\( C. \text{frutescens} \)) in the phylogenetic tree (Fig. 4). \( C. \text{chinense} \) accessions from the Caribbean formed a different cluster from the accessions from South America with strong support (87% bootstrap support; Fig. 4). Interestingly, ‘No.80’ fell into the cluster of South American accessions and was located near ‘No.2’ (Fig. 4). These results suggest two notable genetic backgrounds of ‘No.80’. Firstly, the origin of ‘No.80’ differs substantially from other \( C. \text{chinense} \) accessions of the Caribbean. Trinidad is only 10–15 km from Venezuela on the South American mainland. Combining the geographic location with our phylogenetic data, it is presumed that ‘No.80’ originates from a \( Capsicum \) accession transmitted from the South American mainland, probably via cultural exchange. Secondly, ‘No.80’ and ‘No.2’ are close relatives, although carrying different recessive alleles of \( p\text{-AMT} \). The mutations of \( p\text{-AMT} \) that contributed to the loss of pungency might have occurred independently in a small group of \( C. \text{chinense} \) including ‘No.80’ and ‘No.2’. Further studies are needed to test this hypothesis.

**Volatiles analysis of ‘No.80’, ‘No.2’, and \( F_1 \) progenies**

‘No.80’ is widely used in Trinidad because of its highly aromatic character (Koeda, 2012). In contrast, ‘No.2’, which is also a non-pungent cultivar, has less aromatic flavor. To investigate the high and low aromas of ‘No.80’ and ‘No.2’, and the inheritance pattern in \( F_1 \) hybrids, their volatile components were assayed by GC. Twenty volatiles were putatively identified on the basis of their mass spectra (Table 2). Of them, 16 volatiles were present in higher amounts in the aromatic cultivar ‘No.80’ than in the low-aromatic cultivar ‘No.2’ (Table 2). Rodríguez-Burruezo et al. (2010) determined the volatile composition in ripe fruits of 16 \( Capsicum \) accessions and combined their metabolite analysis with the taste panel data and sniffing port analyses. They concluded that the diversity in aromas found in their accessions was due to variation in the levels of at least one of the identified volatiles.

| Peak No. | Component | Odor description | \( \text{GC-FID peak area (×10}^4 \) | \( \text{No.80} \) | \( \text{No.2} \) | \( \text{No.80} \times \text{No.2} \) | \( \text{No.2} \times \text{No.80} \) |
|---------|-----------|-----------------|----------------------------------|----------|----------|---------------------|---------------------|
| 1       | hexyl 2-methylpropanoate | 243.3 | 324.8 | 873.1 | 820.9 |
| 2       | 4-methyl pentanol | 10.0 | 18.8 | 23.3 | 24.8 |
| 3       | penta 3-methylbutanoate | pungent, fruity, chemical | 106.5 | 60.6 | 126.4 | 145.9 |
| 4       | 4-methylpentyl 2-methylbutanoate | 465.9 | 239.8 | 785.2 | 1140.6 |
| 5       | 4-methylpentyl 3-methylbutanoate | fruity, peach | 2212.1 | 9.7 | 1877.8 | 2447.4 |
| 6       | hexyl 2-methylbutanoate | fruity | 180.7 | 41.9 | 81.1 | 128.7 |
| 7       | hexyl 3-methylbutanoate | fruity | 537.0 | 115.7 | 226.3 | 308.6 |
| 8       | \( \alpha \)-cubebene | 53.9 | 33.2 | 97.4 | 210.7 |
| 9       | cis-3-hexenyl-2-methylbutanoate | herbal, sweet | 115.8 | 17.7 | 39.2 | 57.1 |
| 10      | cis-3-hexenyl pentanoate | 396.3 | 10.3 | 39.3 | 51.2 |
| 11      | trans-2-hexenyl pentanoate | 59.4 | 11.2 | 24.7 | 32.3 |
| 12      | octanol | yuzu-like, sweet | 1.6 | 1.5 | 2.4 | 4.3 |
| 13      | hexyl hexanoate | peachy, plum | 29.9 | 51.2 | 162.0 | 309.8 |
| 14      | hexyl tiglate | 112.2 | 6.2 | 3.6 | 10.7 |
| 15      | octyl 2-methylbutanoate | 22.6 | 4.0 | 14.8 | 24.9 |
| 16      | \( \delta \)-cadinene | weak fruity-woody, spicy | 10.5 | 4.0 | 4.2 | 6.8 |
| 17      | \( \gamma \)-cadinene | thyme, herbal, woody | 2.6 | 1.9 | 1.3 | 1.9 |
| 18      | cadina-l(2),4-diene | 7.2 | 4.6 | 5.3 | 6.3 |
| 19      | \( \alpha \)-ionone | floral | 1.3 | nd. | nd. | nd. |
| 20      | \( \beta \)-ionone | fruity, floral | 1.9 | nd. | 2.1 | 2.3 |

* Eyres et al. (2007).
* Rodríguez-Burruezo et al. (2010).
* Jirovetz et al. (2002).
+ Jordán et al. (2002).
* Song et al. (2000).
* Jirovetz et al. (2006).
* Van Opstaele et al. (2012).
23 odor-contributing volatiles. In agreement with these authors, we found that ‘No.80’ contained high levels of aroma-contributing volatiles, such as 4-methylpentyl 3-methylbutanoate, hexyl 2-methylbutanoate, hexyl 3-methylbutanoate, α-ionone, and β-ionone, compared with ‘No.2’ (Table 2). Moreover, those having fruity and sweet flavors, such as pentyl 3-methylbutanoate (Eyres et al., 2007), cis-3-hexenyl-2-methylbutanoate (Jirovetz et al., 2002), and 6-cadinene (Jirovetz et al., 2006), were more abundant in ‘No.80’. These compounds appeared to accountable, at least in part, for the highly aromatic flavor of ‘No.80’. Moreover, because both ‘No.80’ and ‘No.2’ harbor mutations in p-AMT, it seems possible to breed non-pungency and aroma independently.

In the present study, volatile compounds of the non-pungent F1 hybrid obtained by crossing ‘No.80’ with ‘No.2’ were also assessed. Out of 20 identified volatiles, 8 volatiles were present in higher amounts in the F1 hybrid than in their corresponding parents, and 11 were present at intermediate levels (Table 2). Moreno et al. (2012) reported the transgressive and intermediate inheritance of individual volatiles in an F1 hybrid of Capsicum. Combined with our volatile data, hybridization may be useful for improving the aroma of cultivars.

**Future prospects for breeding aromatic non-pungent cultivars**

To breed highly aromatic non-pungent cultivars, pungency and aroma need to be controlled. Since pungency is controlled by a single gene mutation, it is a relatively simple trait, whereas aroma is a complex mixture of various volatile components. Based on our study, two approaches for breeding highly aromatic non-pungent cultivars can be suggested. One approach is hybridization between non-pungent lines harboring mutations in p-AMT. Considering our phylogenetic data, there is potential to find other non-pungent cultivars harboring mutations in p-AMT in lowlands east of South America, such as in Brazil, Guyana, and Venezuela. Those cultivars will be important genetic sources for breeding various types of aromatic non-pungent F1 hybrid cultivars. The second approach is to cross non-pungent cultivars with highly aromatic pungent cultivars. Since many highly aromatic pungent cultivars exist in C. chinense, various crossing combinations can be attempted. In such a breeding program, the genetic markers developed in this study will be useful for effective selection of non-pungency.

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**Literature Cited**

Andrews, J. 1984. Peppers: the domesticated Capsicums. University of Texas Press, Austin.

Bennett, D. J. and G. W. Kirby. 1968. Constitution and biosynthesis of capsaicin. J. Chem. Soc. C: 442–446.

Bosland, P. W. and E. J. Votava. 2000. Peppers: vegetable and spice capsicums. CABI Publishing, New York.

Bowell, V. R. 1937. Improvement and genetics of tomatoes, peppers, and eggplant. p. 176–206. In: H. A. Wallace (ed.). Yearbook of Agriculture. United States Government Printing Office, Washington.

Eshbaugh, W. H. 1993. Peppers: history and exploitation of a serendipitous new crop discovery. p. 132–139. In: J. Janick and J. E. Simon (eds.). New crops. Wiley, New York.

Eyres, G. T., P. J. Marriott and J. P. Dufour. 2007. Comparison of odor-active compounds in the spicy fraction of hop (Humulus lupulus L.) essential oil from four different varieties. J. Agric. Food Chem. 55: 6252–6261.

Howard, L. R. and R. E. C. Wildman. 2007. Antioxidant vitamin and phytochemical content of fresh and processed pepper fruit (Capsicum annuum). p. 165–191. In: R. E. C. Wildman (ed.). Handbook of nutraceuticals and functional foods, 2nd ed., CRC Press, Boca Raton.

Ince, A. G., M. Karaca and A. N. Onus. 2010. Polymorphic microsatellite markers transferable across Capsicum species. Plant Mol. Biol. Rep. 28: 285–291.

Jordan, M. J., K. L. Goodner and P. E. Shaw. 2002. Characterization of the aromatic profile in aqueous essence and fruit juice of yellow passion fruit (Passiflora edulis Sims. f. Flavicarpa degener) by GC-MS and GC/O. J. Agric. Food Chem. 50: 1523–1528.

Jirovetz, L., G. Buchbauer, Z. Denkova, A. Slavchev, A. Stoyanova and E. Schmidt. 2006. Chemical composition, antimicrobial activities and odor descriptions of various Salvia sp. and Thujia sp. essential oils. Nutrition 30: 152–159.

Jirovetz, L., D. Smith and G. Buchbauer. 2002. Aroma compound analysis of Eraca sativa (Brassicaceae) SPME headspace leaf samples using GC, GC-MS, and olfactometry. J. Agric. Food Chem. 50: 4643–4646.

Koeda, S. 2012. Cultivation and consumption of capsicums in tropical regions—C. chinense and C. frutescens—. Agric. Hortic. (Nogyo-ooyobi-Engei) 87: 29–33 (In Japanese).

Koeda, S., M. Hosokawa, H. Saito and M. Doi. 2013. Temperature-sensitive phenotype caused by natural mutation in Capsicum latescens in two tropical regions. J. Plant Res. 126: 675–684.

Lang, Y., H. Kisaka, R. Sugiyama, K. Nomura, A. Morita, T. Watanabe, Y. Tanaka, S. Yazawa and T. Miwa. 2009. Functional loss of pAMT results in biosynthesis of capsainoids, capsainoid analogs, in Capsicum annuum cv. CH-19 sweet. Plant J. 59: 953–961.

Lee, J. M., S. H. Nahm, Y. M. Kim and B. D. Kim. 2004. Characterization and molecular genetic mapping of microsatellite loci in pepper. Theor. Appl. Genet. 108: 619–627.

Moreno, E., A. Fita, M. C. González-Mas and A. Rodríguez-Burruezo. 2012. HS-SPME study of the volatile fraction of Capsicum accessions and hybrids in different parts of the fruit. Sci. Hortic. 135: 87–97.

Rodriguez-Burruezo, A., H. Kollmannsberger, M. C. González-Mas, S. Nitz and N. Fernando. 2010. HS-SPME comparative analysis of genotypic diversity in the volatile fraction and aroma-contributing compounds of Capsicum fruits from the annuum—chinense—frutescens complex. J. Agric. Food Chem. 58: 4388–4400.

Shirasawa, K., K. Ishii, C. Kim, T. Ban, M. Suzuki, T. Ito, T.
Tanaka, Y., M. Hosokawa, T. Miwa, T. Watanabe and S. Yazawa. 2010a. Newly mutated putative-aminotransferase in nonpungent pepper (*Capsicum annuum*) results in biosynthesis of capsinoids, capsaicinoid analogues. J. Agric. Food Chem. 58: 1761–1767.

Tanaka, Y., M. Hosokawa, T. Miwa, T. Watanabe and S. Yazawa. 2010b. Novel loss-of-function putative aminotransferase alleles cause biosynthesis of capsinoids, nonpungent capsaicinoid analogues, in mildly pungent chili peppers (*Capsicum chinense*). J. Agric. Food Chem. 58: 11762–11767.

Van Opstael, F., B. De Causmaecker, G. Aerts and L. De Cooman. 2012. Characterization of novel varietal floral hop aromas by headspace solid phase microextraction and gas chromatography-mass spectrometry/olfactometry. J. Agric. Food Chem. 60: 12270–12281.

Stewart, C. Jr., B. C. Kang, K. Liu, M. Mazourek, S. L. Moore, Y. Y. Eun, B. D. Kim, I. Paran and M. M. Jahn. 2005. The *Pun1* gene for pungency in pepper encodes a putative acyltransferase. Plant J. 42: 675–688.

Stewart, C. Jr., M. Mazourek, G. M. Stellari, M. O’Connell and M. Jahn. 2007. Genetic control of pungency in *C. chinense* via the *Pun1* locus. J. Exp. Bot. 58: 979–991.

Takezaki, N., M. Nei and K. Tamura. 2010. POPTREE2: Software for constructing population trees from allele frequency data and computing other population statistics with windows interface. Mol. Biol. Evol. 27: 747–752.

Tanaka, Y., M. Kobayashi, N. Nagata, S. Isobe and S. Tabata. 2012. Development of *Capsicum* EST-SSR markers for species identification and *in silico* mapping onto the tomato genome sequence. Mol. Breed. 31: 101–110.

Song, H. S., M. Sawamura, T. Ito, K. Kawashimo and H. Ukeda. 2000. Quantitative determination and characteristic flavour of *Citrus junos* (yuzu) peel oil. Flavour Fragr. J. 15: 245–250.

Stewart, C. Jr., M. Mazourek, N. Nagata, S. Isobe and S. Tabata. 2012. Development of *Capsicum* EST-SSR markers for species identification and *in silico* mapping onto the tomato genome sequence. Mol. Breed. 31: 101–110.

Song, H. S., M. Sawamura, T. Ito, K. Kawashimo and H. Ukeda. 2000. Quantitative determination and characteristic flavour of *Citrus junos* (yuzu) peel oil. Flavour Fragr. J. 15: 245–250.

Takezaki, N., M. Nei and K. Tamura. 2010. POPTREE2: Software for constructing population trees from allele frequency data and computing other population statistics with windows interface. Mol. Biol. Evol. 27: 747–752.

Stewart, C. Jr., B. C. Kang, K. Liu, M. Mazourek, S. L. Moore, Y. Y. Eun, B. D. Kim, I. Paran and M. M. Jahn. 2005. The *Pun1* gene for pungency in pepper encodes a putative acyltransferase. Plant J. 42: 675–688.

Takezaki, N., M. Nei and K. Tamura. 2010. POPTREE2: Software for constructing population trees from allele frequency data and computing other population statistics with windows interface. Mol. Biol. Evol. 27: 747–752.

Stewart, C. Jr., M. Mazourek, N. Nagata, S. Isobe and S. Tabata. 2012. Development of *Capsicum* EST-SSR markers for species identification and *in silico* mapping onto the tomato genome sequence. Mol. Breed. 31: 101–110.