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A *Comt*1 Loss of Function Mutation Is Insufficient for Loss of Pungency in *Capsicum*

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Abstract

The participation of *O*-methyltransferase (COMT) in phenylpropanoid-mediated capsaicinoid biosynthesis has long been proposed. Ferulic acid, a phenylpropanoid intermediate, is a precursor of capsaicinoid biosynthesis and is produced from caffeic acid by the action of COMT. As previously reported that silencing *Comt* expression caused a drastic decrease in capsaicinoid accumulation, it was presumed that a *Comt* loss-of-function mutation would cause loss of pungency in *Capsicum*. This hypothesis was tested by cloning *Comt*1 and *Comt*2 from the placenta tissue of the pungent cultivar Habanero. The phylogenetic analysis and comparison of critical amino-acid residues for enzyme function showed that the two COMTs had high similarity with the COMTs of other plant species. Moreover, as the two *Comt*s were both expressed in placenta tissue and expressed prior to the accumulation of capsaicinoids, the two genes could be candidates for capsaicinoid biosynthesis. Second, *Comt*1 loss-of-function mutants were screened from the germplasm. A truncated *Comt*1 transcript was expressed in non-pungent pepper No.3341 caused by deletion of the genomic region. The predicted No.3341 COMT1 lacked His-265, which was absolutely necessary for enzymatic activity. Contrary to our expectations, the *Comt*1 mutation was not related to non-pungency of No.3341, as the deletion of *Comt*1 did not co-segregate with non-pungency in the F_2 population obtained from crossing No.3341 with Habanero. This result was confirmed by screening several pungent accessions harboring the same *Comt*1 deletion mutation. Although the participation of COMT in phenylpropanoid-mediated capsaicinoid biosynthesis has long been proposed, our present study shows that *Comt*1 can not be a target for controlling fruit pungency.

Keywords

Capsaicinoids, *Capsicum*, *Comt*, Pepper, Phenylpropanoid Pathway, Pungency

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1. Introduction

Capsicum, a member of the family Solanaceae, originated from and was first domesticated in South and Central Americas [1]. Based on archeological evidence, *Capsicum* was already domesticated in 6000 B.P., making it one of the earliest domesticated plant genera [2]. *Capsicum* was introduced to Europe at the end of the 15th century, after the first voyage of Christopher Columbus, and its use spreads rapidly over the Old World continents. Because *Capsicum* accumulates pungent capsaicinoid analogs in its fruit, it is one of the most important spices used worldwide. Moreover, as non-pungent cultivars, such as bell pepper or paprika, contain a high amount of vitamins, *Capsicum* is also an important vegetable. Thus, to understand how capsaicinoids are biosynthesized is important, as both pungent and non-pungent peppers are valuable targets for spice and vegetable breeding.

Capsaicinoids are synthesized in placenta tissue from L-phenylalanine through the phenylpropanoid pathway to extract cinnamic, *p*-coumaric, caffeic and ferulic acid followed by vanillin and vanillylamine, which is ultimately linked to the branched fatty acid residues (8-methyl-6-nonenoic, 8-methylnonanoic, 7-methyloctanoic, 9-methyl-6-decenoic, or 9-methyldecanoic acid) that are synthesized from L-valine or L-leucine to give five capsaicinoid analogs: capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin, and homodihydrocapsaicin [3]-[6] (**Figure 1**). The general capsaicinoid biosynthetic pathway has been studied for almost half a century, and transcripts expressed in placenta tissue of pungent cultivars have been isolated [7]-[9].

A single genetic source for non-pungency has been suggested by the early identification in the 1500s of a widely distributed non-pungent *C. annuum* pepper [10], now known to carry a recessive *acyltransferase* (*Pun*1) allele [11]. Most of the non-pungent cultivars within *C. annuum* carry the same single *Pun*1 recessive allele [11]. Two recessive *putative aminotransferase* (*p-AMT*) alleles have been reported in the CH-19 Sweet and Himo non-pungent cultivars [12] [13]. In addition, a single recessive *Pun*1 allele has been reported for *C. chinense*, *C. frutescens*, and *C. chacoense* [14], and four recessive *p-AMT* alleles have been reported for *C. chinense* [15] [16]. Although the capsaicinoid biosynthetic pathway involving specific enzymes and genes has been proposed,

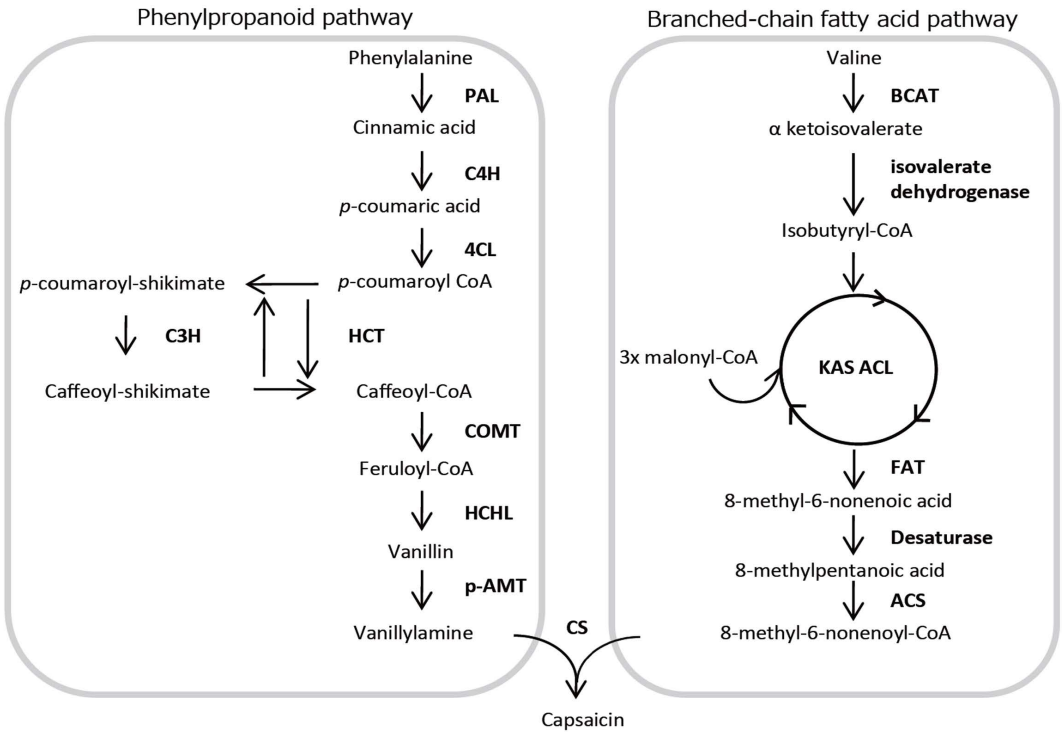

Figure 1. Capsaicinoid biosynthetic pathway (modified from Stewart *et al.* [11]). PAL, phenylalanine ammonia-lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; HCT, hydroxycinnamoyl transferase; C3H, coumaroyl shikimate/quinate 3-hydroxylase; COMT, caffeic acid *O*-methyltransferase; HCHL, hydroxycinnamoyl-CoA hydratase lyase; p-AMT, putative aminotransferase; BCAT, branched-chain amino acid transferase; KAS, β-ketoacyl-[acyl-carrier-protein] (ACP) synthase; ACL, acyl carrier protein; FAT, acyl-ACP thioesterase; ACS, acyl-CoA synthase; CS, capsaicin synthase.

and some transcripts for those genes specifically accumulate in placenta tissue, except for *Pun*1 and *p-AMT*, no direct evidence of their participation in capsaicinoid production has been reported.

Abraham-Juarez *et al*. [6] silenced *Comt*, 3-*keto-acyl-ACP synthase* (*Kas*), and *p-AMT* by virus induced gene silencing (VIGS) and showed that the accumulation of capsaicinoids decreased drastically. This result indicates that a loss-of-function mutation in *Comt* or *Kas* may induce loss of pungency in *Capsicum*. Ferulic acid is a precursor of capsaicinoid biosynthesis and is produced from caffeic acid by the action of COMT [5] [7] [17] [18]. COMT is one of the S-adenosyl-L-methionine (SAM)-dependent *O*-methyltransferases (OMTs) that methylate small molecules involved in the biosynthesis of lignin, flavonoids, alkaloids, and many other plant secondary products [19]. The *O*-methylation patterns of polyhydroxylated small molecules are crucial in plants to determine final product distribution via multiple branched biosynthetic pathways using the same or similar intermediates and substrates [20]. Two *OMT*s expressed in *Capsicum* placenta tissue, *CaOMT*1 (U83789.1) from *C. annuum* [21] and *Comt* (AF081214.1) from *C. chinense* [7] have been cloned. Although the participation of *Comt* in phenylpropanoid-mediated capsaicinoid biosynthesis has long been proposed, it is still unclear whether *Comt* could be a target for controlling fruit pungency.

The objectives of the present study were 1) to clarify whether the two previously cloned *OMT*s were allelic or different genes coded at different loci, 2) to investigate whether the *Comt* loss-of-function mutation caused loss of pungency in *Capsicum*. To this end, two *OMTs* were cloned from placenta tissue of Habanero (*C. chinense*). In addition, phylogenetic and expression analyses of the two *OMTs* were conducted. Moreover, natural deletion mutants of one of the OMTs that had high similarity to *Comt* (AF081214.1) were screened from the germplasm and the phenotype were analyzed for pungency.

2. Materials and Methods

2.1. Plant Materials

C. chinense cultivars Habanero, No.3341, No.3582, No.3614, No.3616, No.3628, No.3639, No.3641, No.3643, No.3645, and No.3646 were used. An inbred line of Habanero was prepared by self-fertilizing six times. F_1 and F_2 progeny were obtained by crossing Habanero with No.3341. All plants were grown on the Kyoto University Experimental Farm, from March to October 2012 and 2013.

2.2. Phenotyping Fruit Pungency

After the fruits were freeze-dried, the capsaicinoids were extracted and quantified according to the method described by Koeda *et al*. [16]. Capsaicinoid content was calculated as the sum of capsaicin and dihydrocapsaicin.

2.3. *Comt* cDNA Sequence Analysis

Pepper fruits were harvested at three stages, and the placenta was separated for RNA extraction. Total RNA was extracted and reverse transcribed according to the method described by Koeda *et al*. [22]. *CaActin* (AY572427) was used as a positive internal control for reverse transcription-polymerase chain reaction (RT-PCR). The partial *Comt*1 cDNA sequence was amplified using Comt1-F and Comt-R1 primer sets, and *Comt*2 was amplified using Comt2-F and Comt-R1 primer sets (**Table 1**). The full-length *Comt*1 cDNA sequence was amplified using Comt1-F and Comt1-R primer sets, and *Comt*2 was amplified using Comt2-F and Comt2-R primer sets (**Table 1**). A Gene Racer kit (Invitrogen, Carlsbad, CA, USA) was used to clone truncated *Comt*1 of No.3341. PCR was performed using KOD Plus Neo (Toyobo, Osaka, Japan). The reaction mixtures for all PCR reactions were initially denatured at 94˚C for 2 min, followed by 35 cycles at 94˚C for 30 s, 60˚C for 30 s, and 72˚C for 2 min, terminating with a 3 min extension at 72˚C. Electrophoresis using 1.0% (w/v) agarose gels was performed on the amplified PCR products. Three biological replicates of RT-PCR were performed for all treatments using independently prepared total RNA, and similar results were obtained. The full-length *Comt*1 and *Comt*2 sequences amplified by RT-PCR were cloned into the 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).

2.4. *Comt* Genomic Sequence Analysis

Genomic DNA was extracted from young leaves of pepper plants using Nucleon PhytoPure (GE Healthcare,

Table 1. Primers used in this study.

Primer name	Sequence (5'-3')	Tm (˚C)
Comt1-F	TTTCCGAATTCAGTTATTCAAACA	60
Comt1-R	TCAATAGAGATTCTGGTTTTCTCA	60
Comt2-F	TTTCTTCCCTTAGCTTGCCTAAT	60
Comt2-R	GAATCCTTCAAAGGAATTGGAT	60
Comt-R1	ACACTAACAAACATGTCACCAC	60
Comt-R2	GCTTCGTAGCAGTTCTTCAACA	60
Comt-R3	TACTCTTTGTGGCAGCTGATGT	60
Comt-R4	CTGCTAAACCAACCCCAGTTAG	60
del-F3	GGTGCTACTGTGAACATGATTGTCTCC	67
del-R4	CAGTCGCCCAGGTAGTGGTATAAGAG	67

Buckinghamshire, UK). The *Comt*1 genomic region was amplified using Comt1-F and Comt1-R primer sets, and *Comt*2 was amplified using Comt2-F and Comt2-R primer sets (**Table 1**). A Straight Walk kit (BEX Co., Ltd., Tokyo, Japan) was used for *Comt*1 genome walking. PCR, electrophoresis, and sequencing were performed as described above.

2.5. Sequence Analysis

The *Comt*1 and *Comt*2 nucleic acid sequences were translated to their corresponding peptide sequences using EMBOSS Transeq (The European Bioinformatics Institute) [23]. BLAST (National Center for Biotechnology Information) [24] was used to search for similar sequences in the GenBank database. The sequences were aligned using Clustal W and a phylogenetic tree was generated using a Molecular Evolutionary Genetic Analysis software ver. 6.0 with 1000 bootstrap replicates [25].

3. Results

3.1. Cloning of *Comt*1 and *Comt*2 from Habanero

Comt1-F and Comt1-R primer sets and Comt2-F and Comt2-R primer sets were used for cloning the two *OMT*s from Habanero (*C. chinense*) placenta tissue. Two *OMT* sequences were cloned from Habanero and named *Comt*1 and *Comt*2 in this study. Both *Comt*1 and *Comt*2 had 1080 bp coding regions (CDS), and had 92% similarity in nucleic acid sequence. *Comt*1 had 99% similarity with *Comt* (AF081214.1), and *Comt*2 had 99% similarity with *CaOMT*1 (U83789.1). Sequencing the genomic regions of *Comt*1 and *Comt*2 clarified that both genes had four exons, and the CDS comprised 407, 311, 65, and 300 bp. In contrast to the exons, the sequences and the length of the introns differed between the two genes. *Comt*1 consisted of three introns, each comprising 241, 105, and 423 bp, whereas *Comt*2 comprised 1164, 251, and 440 bp. The predicted protein sequences were 359 amino acids for COMT1 and COMT2. The two protein sequences showed 88% residue identity (315/359), and showed high similarity with other OMTs, particularly with COMTs reported from other plants species (**Figure 2**).

3.2. Phylogenetic Analysis of COMT1 and COMT2

A phylogenetic tree was constructed with COMT1, COMT2, and SAM methyltransferases (SAM-Mtases) of other plant species (**Figure 3**). Twenty-nine SAM-Mtases were clustered into three groups: group 1 comprised COMT, chalcone OMT (CHOMT), and catechol OMT (CTOMT); group 2 comprised various SAM-Mtases including myricetin OMT (MOMT), flavonoid OMT (FOMT), and isoflavone OMT (IOMT); group 3 comprised jasmonic acid carboxyl methyltransferase (JAMT), salicylic acid carboxyl methyltransferase (SAMT), and indole-3-acetate OMT (IAOMT). COMT1 and COMT2 were located close to group 1.

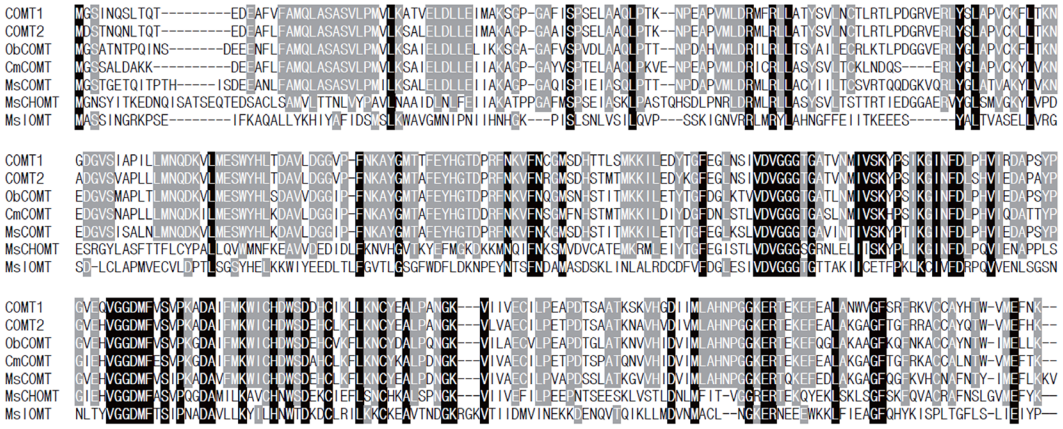

Figure 2. Alignment of the deduced amino acid sequences of Habanero caffeic acid *O*-methyltransferases (COMTs) with similar plant sequences. COMT1 and COMT2 of Habanero were aligned to ObCOMT (*Ocimum basilicum*, AAD38189.1), CmCOMT (*Chrysanthemum* × *morifolium*, BAK42963.1), MsCOMT (*Medicago sativa*, AAB46623.1), MsCHOMT (*M. sativa*, AAB4334159.1), and MsIOMT (*M. sativa*, AAC 49927.1). Shaded black indicates the nucleic acids conserved in all OMTs. Shaded gray indicates the nucleic acids conserved in all COMTs.

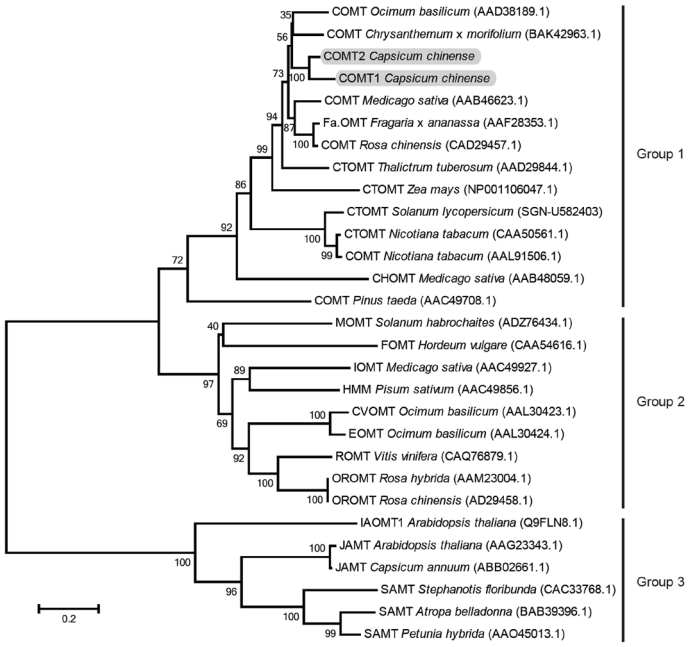

Figure 3. Phylogenetic relationships of Habanero caffeic acid *O*-methyltransferases (COMTs) with S-adenosyl-L-methionine methyltransferases (SAM-Mtases) of other plant species. A neighbor-joining tree comparing Habanero COMTs to the 27 SAM-Mtases. Three groups were present in the resulting tree: group 1 comprised COMT, chalcone OMT (CHOMT), and catechol OMT (CTOMT); group 2 comprised various SAM-Mtases including myricetin OMT (MOMT), flavonoid OMT (FOMT), and isoflavone OMT (IOMT); and group 3 comprised jasmonic acid carboxyl methyltransferase (JAMT), salicylic acid carboxyl methyltransferase (SAMT), and indole-3-acetate OMT (IAOMT). Branch labels include enzyme name, source plant, and GenBank protein ID. Bootstrap values are shown along the branches (from 1000 replicates).

3.3. Sequence Comparison of Critical Amino-Acid Residues of the OMTs

In the present study, 18 critical amino-acid residues of the SAM-binding domain, the catalytic domain, and the substrate binding domain were compared between 16 OMTs of groups 1 and 2 (**Figure 4**). Five of eight amino-acid residues in the SAM-binding domain were conserved in all OMTs, and all amino-acid residues were con-

		SAM binding domain								Catalytic domain			Substrate binding domain						
		202	204	227	228	247	248	261	267	265	293	325	126	172	176	310	315	316	319
Group 1	COMT1	D	G	D	L	D	M	K	W	H	E	E	M	F	M	V	I	M	H
	COMT2	D	G	D	L	D	M	K	W	H	E	E	M	F	M	V	V	M	H
	COMT (BAK42963.1)	D	G	D	L	D	M	K	W	H	E	E	M	F	M	V	I	M	H
	COMT (AAD38189.1)	D	G	D	L	D	M	K	W	H	E	E	M	F	M	V	I	M	H
	COMT (CAD29457.1)	D	G	D	L	D	M	K	W	H	E	E	M	F	M	V	I	M	H
	CTOMT (AAD29844.1)	D	G	D	L	D	M	K	W	H	E	E	M	F	M	F	V	M	H
	CTOMT (NP001106047.1)	D	G	D	L	D	M	K	W	H	E	E	M	F	M	F	I	M	H
	CHOMT (AAB48059.1)	D	G	D	L	D	M	K	W	H	E	E	F	F	M	S	L	M	T
Group 2	CVOMT (AAL30423.1)	D	G	D	L	D	M	K	W	H	D	E	V	F	M	F	M	A	S
	EOMT (AAL30424.1)	D	G	D	L	D	M	K	W	H	D	E	V	F	M	F	M	A	C
	OROMT (AAM23004.1)	D	G	D	L	D	M	K	W	H	D	E	A	F	M	F	M	L	A
	ROMT (CAQ76879.1)	D	G	D	L	D	M	K	W	H	D	E	A	F	M	F	M	T	I
	IOMT (AAC49927.1)	D	G	D	L	D	M	K	W	H	D	E	C	F	M	M	V	N	C
	HMM (AAC49856.1)	D	A	D	L	D	M	K	W	H	D	E	G	F	M	Y	V	V	T
	MOMT (ADZ76434.1)	D	G	D	R	S	M	K	W	H	D	E	F	F	M	M	L	I	I
	FOMT (CAA54616.1)	D	A	D	Q	D	M	K	W	H	D	V	L	F	M	M	L	S	M

Figure 4. Comparison of the critical amino acid residues for *O*-methyltransferases (OMTs). Critical amino acid residues for the S-adenosyl-L-methionine (SAM) binding domain, the catalytic domain, and the substrate binding domain were chosen according to Zubieta *et al.* [20]. The shaded box indicates the residues conserved in at least two different proteins.

served in group 1. The catalytic domain was also highly conserved in all OMTs, and all amino-acid residues were conserved in group 1. In contrast, only two (Phe-172 and Met-176) of seven amino-acid residues were conserved in the substrate binding domain of all OMTs, and the other five residues varied. In particular, group 2 OMT amino-acid residues were highly variable, suggesting that the difference in these amino-acid residues is critical for substrate specificity. The amino-acid residues were highly conserved in group 1 compared to group 2, and all residues in the COMTs were conserved except for Val-315 of COMT2.

3.4. *Comt*1 and *Comt*2 Expression Analysis

The *Comt*1 and *Comt*2 expression analysis was conducted with RNA samples derived from the placenta tissue (stage 1: immature green, stage 2: mature green and stage 3: mature red) (**Figure 5(a)**), leaf, stem, and root by RT-PCR. The accumulation of capsaicinoids in Habanero increased drastically beginning at stage 2 (**Figure 5(b)**). *Comt*1 and *Comt*2 showed the same expression pattern in placenta tissue. Both genes were highly expressed during stages 1 and 2 and then decreased during stage 3 (**Figure 5(c)**). Although *Comt*1 expression was not detected in leaf, stem, or root, *Comt*2 was highly expressed in these tissues (**Figure 5(c)**).

3.5. Analysis of *Comt*1 Deletion Mutants

Abraham-Juarez *et al*. [6] silenced *Comt* (AF081214.1) by VIGS, which has 99% similarity with *Comt*1, and showed that the accumulation of capsaicinoids decreased drastically. This result indicates that *Comt*1 loss-of-function mutation could induce loss of pungency in *Capsicum*. No.3341 (*C. chinense*) is a non-pungent cultivar found in our previous study that *Pun*1 and *p-AMT* could not account for its non-pungency [26]. No expression was detected when Comt1-F was designed to anneal the 5' untranslated region (UTR) and Comt1-R was designed to anneal the 3'UTR primer set for detecting *Comt*1 in RNA samples derived from the placenta of No.3341 (**Figure 6**). As it was possible that primers could not anneal to the cDNA of No.3341 because of single nucleotide polymorphisms (SNPs) located at the annealing position, an additional four reverse primers were designed and used for PCR. *Comt*1 was detected by all the primer sets in Habanero (**Figure 6**). In contrast, only Comt-R1, which was designed at almost the middle of the coding region, could detect the *Comt*1 transcript in No.3341 (**Figure 6**). The same result was obtained when genomic PCR was conducted using the same primers (**Figure 6**).

The race-method was used to obtain the full sequence, because expression of a truncated *Comt*1 transcript was suggested in No.3341. Sequencing the full-length *Comt*1 cDNA of No.3341 clarified that the truncated transcript was expressed. The predicted protein sequences were 283 amino acids for COMT1 of No.3341 compared to 359 amino acids for Habanero (**Figure 7**). COMT1 of No.3341 differed from 262 amino acids residue compared to

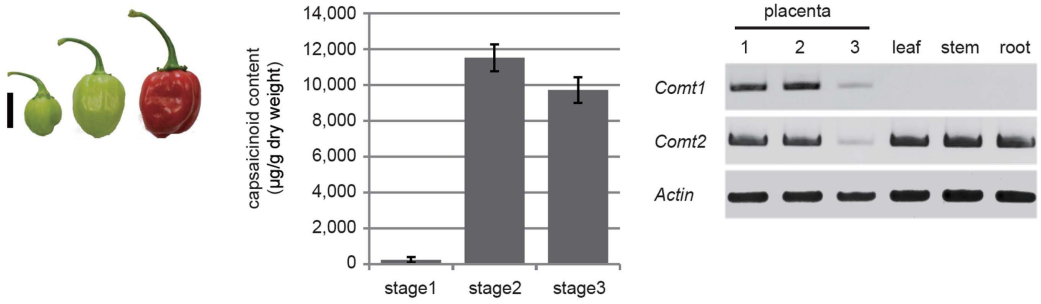

Figure 5. Assessment of capsaicinoid accumulation and expression analysis of caffeic acid *O*-methyltransferases (*Comt*s) in Habanero. (a) The Habanero fruit developmental stage used for the study (stage 1: immature green, stage 2: mature green and stage 3: mature red from left to right). Bar indicates 2 cm; (b) Capsaicinoid content at each Habanero fruit stage. Error bars: standard deviation for results of three plants. (c) *Comt*1 and *Comt*2 expression in placenta tissue (stage 1-3), leaf, stem, and root of Habanero. Actin was used as the positive internal control.

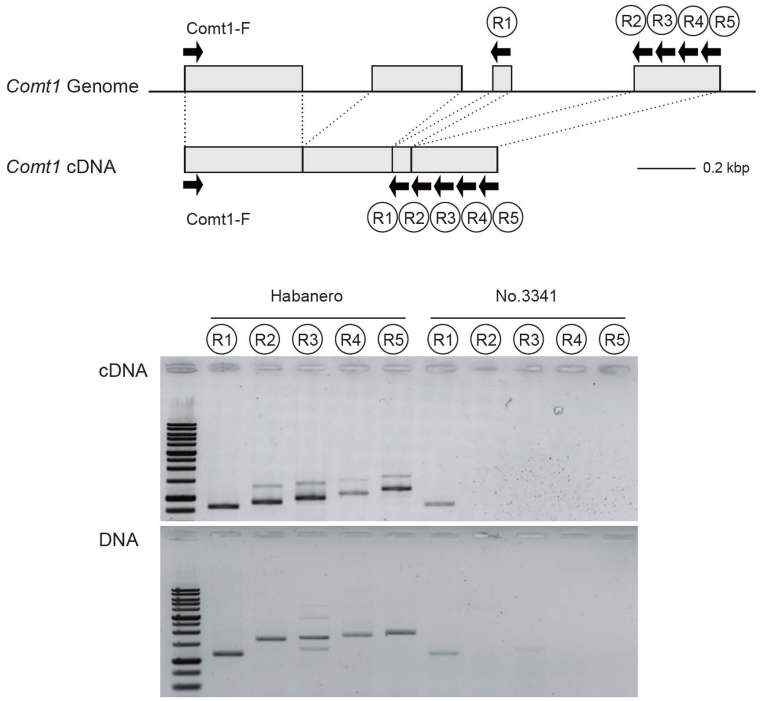

Figure 6. Genomic and cDNA structures of caffeic acid *O*-methyltransferase 1 (*Comt*1), and electrophoresis of polymerase chain reaction amplified fragments. Expression of the truncated *Comt*1 transcript was suggested in No.3341. R1, R2, R3, R4, and R5 indicate Comt-R1, Comt-R2, Comt-R3, Comt-R4, and Comt1-R, respectively.

Habanero, lacking almost half of the *O*-methyltransferase domain. The genomic region was sequenced by genome walk and clarified that *Comt*1 of No.3341 possessed a 2.3 kbp large deletion from the 5' region of the fourth exon to the intergenic region (**Figure 8**).

The co-dominant DNA markers del-F3 and del-R4 were designed to confirm the relationship between the *Comt*1 deletion mutation and non-pungency in No.3341 (**Table 1**). The pungent and non-pungent phenotype segregated 3:1 in the F_2 population obtained by crossing Habanero with No.3341 (n = 98, chi-square = 0.340, *P*-value = 0.560). Contrary to our expectations, the *Comt*1 mutation did not co-segregate with non-pungency of No.3341 (**Figure 9(a)**). Thus, the *Comt*1 mutation was not related to non-pungency of No.3341. This result was confirmed by screening several pungent accessions harboring the same *Comt*1 deletion mutation (**Figure 9(b)**).

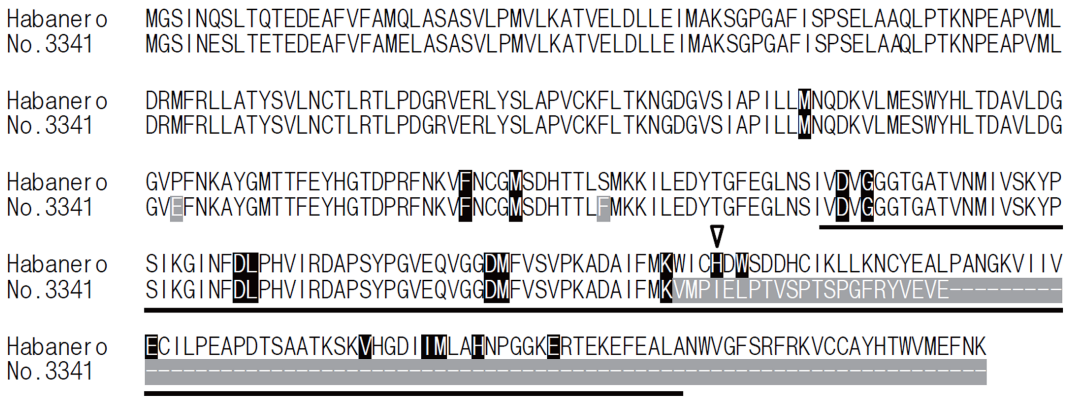

Figure 7. Alignment of the deduced amino acid sequence of caffeic acid *O*-methyltransferase 1 (COMT1) from Habanero and No.3341. COMT1 of Habanero and No.3341 were aligned. The underlined part indicates the *O*-methyltransferase domain. A truncated COMT1 was produced in No.3341 because of the 2.3 kbp genetic mutation. Shaded gray for No.3341 indicates the amino acid residues that differ from Habanero. Shaded black indicates the critical amino acid residues of the S-adenosyl-L-methionine (SAM) binding domain, catalytic domain, and substrate binding domain. Triangle indicates the His-265 necessary for catalytic activity.

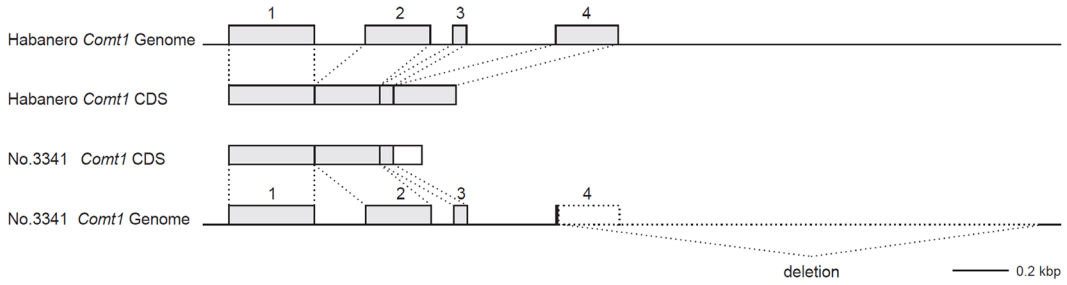

Figure 8. Genomic and coding region structures of caffeic acid *O*-methyltransferase 1 (*Comt*1) for Habanero and No.3341. Boxes indicate *Comt*1 exons. The location of the 2.3 kbp deletion is shown in the *Comt*1 No.3341 genomic structure.

4. Discussion

4.1. *Comt*1 and *Comt*2 Are Different Genes Coded in Different Loci

The *CaOMT*1 (U83789.1) from *C. annuum* [21] and *Comt* (AF081214.1) from *C. chinense* [7] are two *OMT*s expressed in placenta tissue that have been cloned previously. Because *CaOMT*1 (U83789.1) and *Comt* (AF081214.1) were cloned by different research teams and from different *Capsicum* species, it is unclear whether the two transcripts are allelic or genes coded by different loci. In the present study, two transcripts were cloned from the Habanero inbred line. *Comt*1, cloned from Habanero, had 99% similarity with *Comt* (AF081214.1) and *Comt*2 had 99% similarity with *CaOMT*1 (U83789.1). In addition, the length and sequences of the *Comt*1 and *Comt*2 introns differed. Moreover, whole genome sequencing of *Capsicum* clarified that *Comt* has undergone extensive gene duplication [27]. Searching the whole genome sequence of *Comt*1 and *Comt*2 revealed that the two genes are coded in chromosome 3 with a 13 kbp distance. Thus, it can be concluded that *Comt*1 and *Comt*2 are not allelic but different genes coded by different loci.

4.2. *Comt*1 and *Comt*2 are Candidate Genes for Capsaicinoid Biosynthesis

Plant SAM-Mtases are key enzymes in phenylpropanoid, flavonoid, and many other metabolic pathways [28]. OMT is one of the SAM-Mtases and can be classified into two groups, a group including COMT and another group that includes other OMTs with various substrate specificities [19] [28]-[30]. In the present study, a phylogenetic analysis of 29 SAM-Mtases was conducted and showed that two Habanero OMTs were located closely with COMTs in group 1 of other plant species (**Figure 3**). These results suggest that COMT1 and COMT2 have similar substrate preferences to COMTs of other plant species.

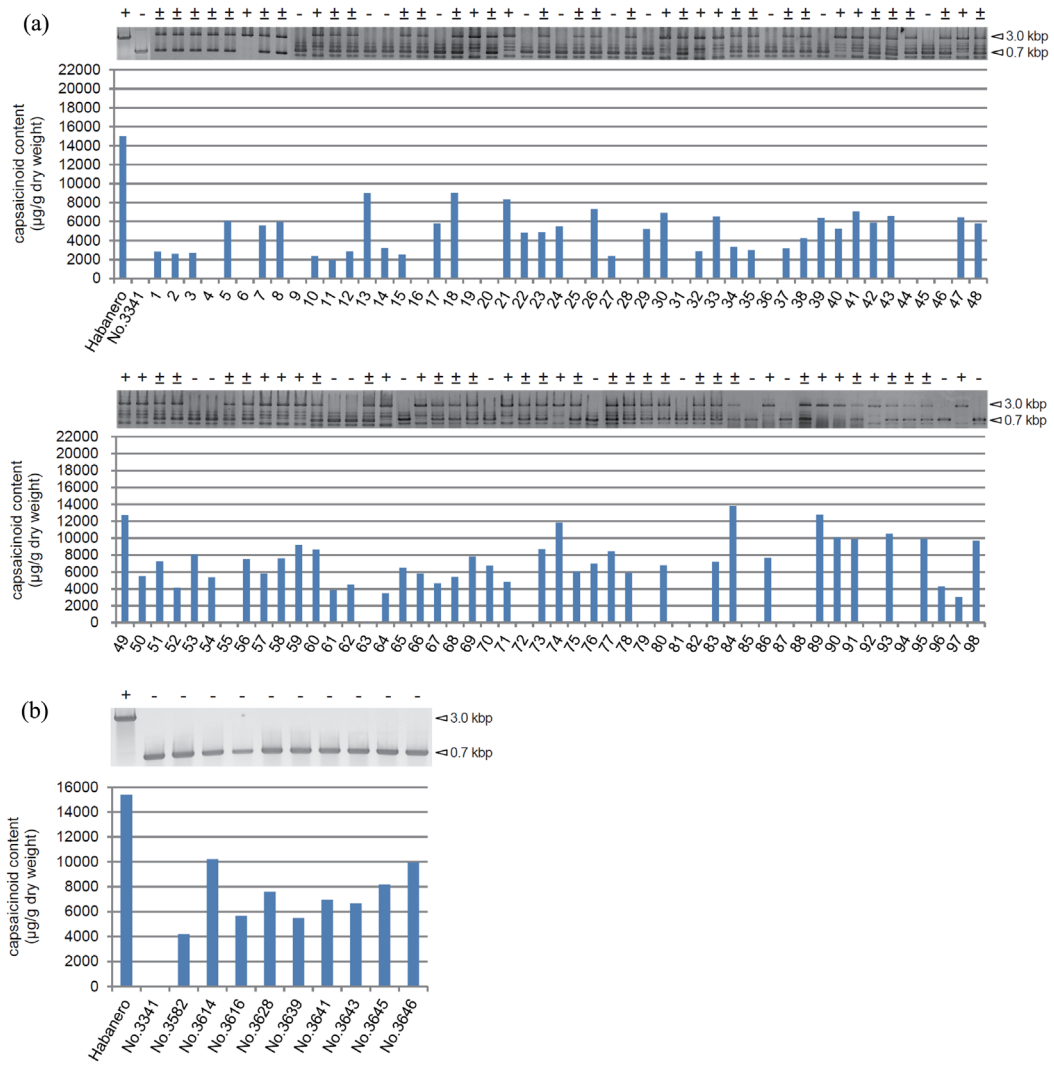

Figure 9. DNA marker analysis. (a) The DNA marker pattern of caffeic acid *O*-methyltransferase 1 (*Comt*1) did not co-segregate with the pungency trait in F_2 individuals. The capsaicinoid contents measured by high performance liquid chromatography are expressed in $\mu g \cdot g^{-1}$ dry weight. The gels show DNA marker patterns of the F_2 individuals. + and – indicates individuals homozygous in the Habanero type (3 kbp) and the No. 3341 type (0.7 kbp), respectively, and ± indicates a heterozygote. (b) Pungency phenotype in *Comt*1-deletion mutants.

Zubieta*et al*. [20] studied the precise structural basis of enzyme activity using CHOMT and IOMT of *Medicago sativa* (alfalfa). Plant OMTs use SAM as a methyl source, yielding S-adenosyl-L-homocysteine and the methyl ether derivatives as products. Critical residues for the SAM-binding domain and the catalytic domain were highly conserved in the 16 OMTs clustered in groups 1 and 2 of our phylogenetic tree (**Figure 3** and **Figure 4**). In contrast, substrate binding domain diversity was observed for critical residues (**Figure 4**). As sequential diversity was observed particularly in the OMTs of group 2, these differences seemed to reflect substrate specificity. Six out of seven residues among the five COMTs of group 1 were conserved, suggesting that these OMTs use the same substrate. However, as amino acid 315 was a valine only in COMT2, but an isoleucine in other COMTs, further enzymological study is needed to clarify how this difference affects substrate preference.

Capsaicinoids start to accumulate in *Capsicum* beginning 20 days post-anthesis [11], which coincides with the accumulation pattern observed in Habanero (**Figure 5(b)**). As *Comt*1 and *Comt*2 are expressed in placenta tissue where the capsaicinoids are synthesized and expressed prior to capsaicinoid accumulation, the two genes could be candidates for capsaicinoid biosynthesis (**Figure 5(c)**). The expression of the two genes differed in other or-

gans. Only *Comt*2 was expressed in the leaf, stem, and root (**Figure 5(c)**), suggesting that *Comt*2 has broad functions in addition to capsaicinoid synthesis. Considering gene duplication of *Comt* [27] and our phylogenetic analysis (**Figure 3**), *Comt*1 may have been duplicated from *Comt*2.

4.3. The *Comt*1 Loss-of-Function Mutation Does Not Affect Capsaicinoid Biosynthesis in *Capsicum*

Based on our phylogenetic and expression analyses, *Comt*1 and *Comt*2 could be candidates for capsaicinoid biosynthesis. Because Abraham-Juarez *et al*. [6] silenced *Comt* (AF081214.1) by VIGS, which had 99% similarity with *Comt*1, and showed that the accumulation of capsaicinoids decreases drastically, it was assumed that the *Comt* loss-of-function mutation would cause a loss of pungency in *Capsicum*. As transformation of *Capsicum* is technically difficult [31], a natural mutant of a specific gene is valuable for predicting gene function. In the present study, non-pungent No.3341 was screened for the expression of the truncated *Comt*1 transcript which was caused by the large deletion mutation in the genomic region (**Figure 8**), lacking almost half of the OMT domain. Zubieta *et al*., [20] have clearly shown that histidine in CHOMT (His-278) catalytic domain and IOMT (His-257) are absolutely necessary catalytic residues. The predicted COMT1 of No.3341 lacks His-265 due to a mutation, which would diminish enzyme activity. F_2 populations obtained from crossing No.3341 and Habanero were prepared to elucidate the relationship between mutated *Comt*1 and capsaicinoid synthesis. Contrary to our expectations, the *Comt*1 mutation did not co-segregate with the non-pungency trait (**Figure 9(a)**). With our previous study [26], it was shown that loss of pungency in No.3341 is controlled by a single recessive gene that is neither *Pun*1, *p-AMT* nor *Comt*1. For further confirmation, *Comt*1 mutants were screened from the *Capsicum*-germplasm and nine pungent accessions harboring the same *Comt*1 deletion mutation were screened (**Figure 9(b)**). Thus it was concluded that *Comt*1 loss-of-function mutation is insufficient for loss of pungency in *Capsicum*. Because *Comt*1 loss-of-function mutants were frequently found, those might have been selected artificially for other valuable traits caused by mutation of *Comt*1. Further study is needed to clarify this point.

In the present study, *Comt*1 and *Comt*2 were cloned from the Habanero pungent pepper (*C. chinense*). Moreover, an analysis of *Comt*1-mutated peppers clarified that *Comt*1 loss-of-function mutation was insufficient for loss of pungency. In contrast, Abraham-Juarez *et al*. [6] silenced *Comt* (AF081214.1) by VIGS, which had 99% similarity with *Comt*1, and showed that the accumulation of capsaicinoids decreased drastically. Expression of *Comt*2 in placenta tissue must be considered to explain these contradictory findings. In the study of Abraham Juarez *et al*. [6], *Comt*(*Comt*1)-silenced plants had undetectable levels of *Comt* mRNA or a reduction of an 8 - 24 folds by northern blotting and RT-PCR. Our results showed that the *Comt*1 and *Comt*2 sequence similarity was 92% in the coding regions. Thus, even *Comt*1 was specifically silenced by VIGS, *Comt*2 would still be detected by the DNA probes or primers designed to anneal coding regions of *Comt*1. Two possibilities should be discussed to explain these results. When two or more genes have high sequence similarity, a specific sequence of siRNA induces simultaneous silencing such as in petunia, *Trenia* plants, yellow beans (*Glycine max* L.), and dahlia [32]-[37]. Silencing of *Comt*1 by VIGS may have simultaneously silenced *Comt*2; thus, leading to decrease capsaicinoid accumulation. The second possibility is that serrano chili pepper (*C. annuum* cv. Tampiqueño 74) used in the study of Abraham Juarez *et al*. [6] had a *Comt*2 expression defect, such as the deletion of the promoter region. If *Comt*1 was silenced in such a pepper plant, both *Comt*1 and *Comt*2 would not function and capsaicinoid accumulation would decrease drastically. In the present study, 280 pepper germplasms were screened for a *Comt*2 loss-of-function mutant, which will be valuable for asking the relation of *Comt*s to capsaicinoid synthesis, using high-throughput sequencing technology, but no mutant was found (data not shown). It can be concluded that the *Comt*1 mutation is insufficient for the loss of pungency in *Capsicum*. Participation of COMT in phenylpropanoid-mediated capsaicinoid biosynthesis has long been proposed [5] [7] [18] [19]. Mozourek *et al*. [9] proposed the participation of caffeoyl-CoA *O*-methyltransferase (CCoAOMT) instead of COMT in the phenylpropanoid pathway of capsaicinoid biosynthesis. Thus, further study is needed to clarify which genes are critically important for capsaicinoid biosynthesis and could be a target for controlling fruit pungency in *Capsicum*.

GenBank Accession Numbers

CDS sequence of *Comt*1 (AB971124), *Comt*2 (AB971126), genomic sequence of *Comt*1 (AB971125), and *Comt*2 (AB971127) of Habanero. CDS sequence of *Comt*1 (LC050124) and genomic sequence of *Comt*1

(LC050125) of No. 3341.

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