A DISSERTATION FOR THE DEGREE OF MASTER OF SCIENCE

Investigation of Genetic Factors Controlling Capsiate Biosynthesis in Pepper

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SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

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ABSTRACT

Capsinoid which were found recently in non-pungent pepper show the same biological effects as capsaicinoid including anticancer and anti-obesity. A precursor of capsinoid, vanillyl alcohol, is known to be produced by mutations in the putative-aminotransferase (*pAMT*) gene. In the previous study, it was reported that capsinoid production is also controlled by the capsaicin synthase (*CS*) gene. However the relation between the *CS* activity and capsinoid contents has not been fully understood. This study was conducted to elucidate the role of *CS* in quantitative control of capsinoid contents. In the previous study, *C. chinense*, SNU11-001, which contains

capsinoid higher than *C. annuum* 'CH-19 Sweet' was identified. Non-functional mRNA of SNU11-001 had stop codon resulted from 403bp insertion and 45bp deletion. I analyzed the *CS* and *pAMT* activity using the five *Capsicum* accessions containing different levels of pungency including SNU11-001, the transcription levels of *CS* were higher in pungent *Capsicum* accessions. Molecular markers which can distinguish *CS* and *pAMT* genotype between SNU11-001 and pungent pepper cultivar 'Habanero' were developed. To investigate SNU11-001 x Habanero F_2 population was constructed and analyzed correlation between capsaicinoid and capsinoid concentration and *CS* genotypes of SNU11-001 and Habanero. Therefore, *CS* genotype of SNU11-001 and Habanero did not have influence on quantitative trait of capsinoid. Genetic factor which is not related in capsaicinoid biosynthesis might exist quantitative control of capsinoid synthesis. Transcriptome analysis between *Capsicum* pepper with capsinoid and pungent cultivar will identify effect on regulation of capsinoid contents in future studies.

Keywords: Capsaicinoid analogue, capsinoid, capsiate, *pAMT*, *CS,* SNU11-001

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Introduction

The unique characteristic of pepper is pungency, which is caused by capsaicinoid in fruits (Nelson and Dawson. 1923). Capsaicinoid is an alkaloid derived from pepper's placenta and have many biomedical functions such as cancer prevention, weight reduction, and cardiovascular (Thiele et al., 2008; Xiu-Ju et al., 2011). Capsaicin, one of the capsaicinoid analogs, is synthesized by condensation of vanillyl amine derived from phenylpropanoids pathway and 8-methyl-6-nonenoic acid from valine pathway. Vanillin is changed to vanillylamine by putative aminotransferase (*pAMT*). Capsaicin is a final product resulted from condensation reaction between branched-fatty acids and vanillylamine. Capsaicin synthase (*CS*) synthesizes capsaicin from 8-methyl-6-nonanoic acid and vanillylamine.

Capsinoid, capsaicinioid-like substance was first reported by Yazawa in 1989. Capsiate, one of the capsinoid analogs, has the same structure as capsaicin except for replacement of a peptide (NH) by an ester bond (O). The replacement of peptide with ester causes nonpungency of capsinoid. Non-pungency capsiate makes it more palatable and less toxic than capsaicin. Capsinoid are unstable and easily degraded in the aqueous phase. Therefore, capsinoid have advantageous characteristics compared to

capsaicinoid in biomedical uses.

Several genetic studies have been conducted on biosynthesis of capsinoid. Biosynthesis of capsiate is caused by mutations in the *pAMT* gene resulting in *pAMT* suppression of the formation vanillylamine from vanillin (Lang et al., 2009; Tanaka et al., 2010a). Dysfunction of *pAMT* shunts synthesis vanillylamine into vanillyl alcohol. Tanaka et al.(2010) identified several loss-of-function *pAMT* alleles that rendered production of capsinoid in pepper. Pepper can produce both capsaiciniods and capsinoid when pepper have functional *Pun1*. Otherwise *Pun1* mutation could not synthesize neither capsaicinoid nor capsinoid (Han et al., 2013).

Quatitative control of capsinoid synthesis may be affected by other factors besides *pAMT* and *CS*. Capsaicinoid accumulation is affected by environmental conditions and genetic constitations. Genetic studies on capsaicinoid contents have been conducted using QTL analysis and molecular mapping. Six QTLs in capsaicinoid accumulation were identified explaining 31% of the phenotypic variation. Nonetheless, studies have been performed to identifiey genetic factors affecting capsaicinoid accumulation. The Same genetic factors controlling capsaicinoid accumulation may be involved in capsinoid accumulation.

Genetic study of capsinoid biosynthesis has been performed since

capsiate was found in 1980th. However quantitative control of capsinoid in pepper was not elucidated. The purpose of this research is to investigate the genetic causes of capsinoid accumulation. To achieve the objective, analysis of capsaicinoid and capsinoid contents and genotype analysis of *pAMT* and *CS* was performed in F_2 population derived from *C. chinense* 'SNU11-001' x *C. chinense* 'Habanero'.

LITERATURE REVIEW

Biochemistry of capsaicinoid and capsinoid

Capsaicinoid

Pepper have been used as a food ingredient widely around the world. Main characteristic of pepper is pungency caused by capsaicin. Capsaicin is alkaloid unique in pepper and biosynthesized from placenta in pepper. Capsaicin biosynthesis is correlated with presence of blisters distributed along the epidermis in placenta. (Stewart et al., 2007) Blister was detected in specifically pungent pepper. Bilsters size and number were correlated with accumulation of capsaicinoid during fruit development. (Steward et al., 2007)

Capsinoid

Capsaicinoid-like compound, capsinoid was first found by Yazawa in 1989. The biggest difference between capsinoid and capsaicinoid is pungency. Capsinoid are not pungent. Capsinoid include capsiate, dihydrocapsiate, nordihydrocapsiate. Structure of capsiate is like capsaicin, but a peptide bond is replaced by an ester bond. (Kobata et al., 1998, 1999). Capsiate is less stable than capsaicin and decomposed easily in aqueous

phase while capsaicinoid are stable in nonpolar as well as polar solvent condition. (Tanaka et al., 2009)

Biomedical effect of capsaicionids and capsinoid

Capsaicinoid

Capsaicinoid have many pharmacological effects. Capsaicin has an effect on pain relief by blocking the painful endogenous substances that activate transient receptor potential vanilloid subfamily member 1 (TRPV1). TRPV1 is a nonselective cation channel and also known as capsaicin receptor. TRPV1 antagonists are considered to stave off pain perception. Biomedical effect of capsaicinoid to prevent cancer is ability to reduce the growth of cancer cell lines by induction of cycle arrest, apoptosis, autophagy or cellular metabolic activation. Capsaicinoid also affect weight reduction and regulation of obesity. Capsaicin along with dihydrocapsaicin affect thermogenesis and finally raise energy expenditure and energy metabolism. (Xiu-Ju et al., 2011) Although capsaicinoid have ability to weight reduction, capsaicinoid is not acceptable for all people because of pungency.

Capsinoid

Clinical benefit of capsinoid has not been reported as much as capsaicinoid. However, capsinoid attract attention owing to nonpungency and selectivity. Iida et al., (2003) reported that capsiate had no irritant response on skin surface in contrast to capsaicin. It was reported that capsiate relives pain while it must be investigated more due to instability on aqueous conditions. Capsinoid also have anticancer activity like capsaicinoid by blocking vascular endothelial growth factor (VEGF)-induced proliferation without irritating response. Capsinoid is effective to reduce weight by activating sympathetic nervous system and raising body temperature.

Biosynthesis of capsaicinoid and capsinoid

Capsaicinoid and capsinoid biosynthesis pathway

Capsaicin is synthesized by condensation of vanillylamine and 8-methyl-6-nonenoic acid. *pAMT* converts vanillin into vanillylaine in phenylpropanoid pathway and *CS* produces capsaicin using vanillylamine and branched fatty acid, 8-methyl-6-nonenoic acid, in the final step of capsaicin biosynthesis. On the other hand, *pAMT* mutation causes formation

of vanillyl alcohol instead of vanillylamine from vanillin. Capsiate is synthesized from vanillyl alcohol and branched chain fatty acid-CoA.

Role of *Pun1*

Functional *CS* is required for synthesis of capsaicinoid. *C* locus have been mapped to determine genomic region controlling pungency (Blum et al., 2002.2003). An EST clone 'SB2-66' was a candidate gene for *Pun1* since this clone was co-segregated with pungency and matched with *Pun1* region (Kim et al., 2002). cDNA and genomic sequence of *Pun1* was identified and it was reveal that *Pun1* contained two exons and an intron. Besides, *Pun1* in Habanero has acyltransferase domain not involved in AT1 and AT2 of Habanero. Consequently, *Pun1* was refered to AT3. (Stewart et al., 2005). The *Pun1* genotype in *C. annuum* have loss-of-function due to 2.5 kb deletion in promoter and first exon. Another recessive allele, $Pun1^{2,}$ was elucidated nonpungency in pepper due to frameshift mutation and cosegregation with absence of blisters in Placenta.

Role of *pAMT* mutation and *Pun1* in presence of pungency

It is only a short time since *pAMT* mutation was reported to have influence on capsinoid biosynthesis. CH-19 Sweet containing high level of

capsinoid have frameshift mutation owing to T insertion on *pAMT* while there was no significant difference on transcriptional level compared with pungent cultivar. (Lang et al., 2009). *C. annuum* cultivar, Himo also produce capsinoid. An amino acid substitution from cystein to arginine resulted in dysfunctional *pAMT*. Single amino acid substitution was involved in pyridoxal 5-phosphate (PLP) binding domain that influences on *pAMT* enzyme activity (Tanaka et al., 2010a). A transposable element was found in *C. chinense* cultivars (Zavory hot, Aji Dulce stain 2) with high contents of capsinoid (Tanaka et al., 2010b). "Transposable element of *C. chinense*" (refered to *Tcc*) is consisted of approximately 2.3 kb nucleotide sequence. *Tcc* was inserted in the fifth intron of Zavory hot and the third intron of Aji Dulce stain 2. *Tcc* affected synthesis of functional mRNA of *pAMT*. *Pun1* was also elucidated to control biosynthesis of capsinoid (Han et al., 2012). An F_6 recombinant inbred line population derived from SR211, pungent pepper with capsiniods and SR213, nonpungent pepper was used to determine the role of *Pun1* in capsinoid production. Plants with *Pun1*/*Pun1* genotype could synthesize both capsaicinoid and capsinoid but plants with *pun1*/*pun1* could not produce capsaicinoid as well as capsinoid. Therefore functional *Pun1* is necessary to produce capsinoid and nonfunctional *pAMT* is necessary condition for biosynthesis of capsinoid.

Regulation of capsaicinoid contents

Many researches have made efforts to elucidate relationship between capsaicinoid contents and enzymes on the capsaicinoid pathway. Relationship between transcript accumulation of genes in capsaicin biosynthesis pathway and degree of pungency was investigated. (Curry et al., 1999) Transcript level of *Pal*, *Ca4h*, *Comt*, *pAMT* and *Kas* were correlated with capsaicinoid contents. *pAMT* and *Kas* are expressed in placental tissue specifically. The *Kas*, *Acl* and *Fat* genes are involved in fatty acid synthase (FA) complex. (Aluru et al., 2003) These genes were found to be related to pungency level. mRNA of *Acl* and *Fat* in placenta was expressed in abundance remarkably. Transcripts of these genes were plentiful in immature green fruit before fruit became muture. Expression of other genes in phenylpropanoid pathway and valine pathway including *Acl*, *FatA*, *Kas* and *Pun1* was analyzed by RNA gel blot through fruit development in pungent pepper and nonpungent pepper. Almost all genes were detected highly in pungent peppers before peppers produced capsaicinoid maximally. On the other hand, gene expression was not detected in nonpungent peppers except *BCAT* and *Acl*.

Quantitative trait loci (QTL) analysis for capsaicinoid was conducted.

QTL *cap* in pungent cultivar BG2816 contributed to the increased level of pungency. (Blum et al., 2003) In later research, two QTL controlling capsaicinoid contents were found. QTL *cap7.1* with maker on chromosome 2 exerted an influence on the trait. QTL *cap7.2* might correspond to QTL *cap*. (Ben-chaim et al., 2006). However capsinoid contents with respect to genes in capsinoid pathway have never been investigated yet.

Cultivar containing high level of capsiate

Capsinoid are more palatable than capsaicinoid and have more advantage to intake. “Capsiate Natura” was developed for dietary supplement, easy-to-swallow vegetarian soft gel. New *Capsicum* cultivar ‘Maru Salad’ containing high level of capsinoid was also developed using CH-19 Sweet and Murasaki which is nonpungent pepper with nonfunctional *CS*. (Tanaka et al., 2014)

MATERIALS AND METHODS

Plant materials

A total of six *Capsicum* cultivars containing different levels of capsaicin and capsiate were used. SNU11-001 (*C. chinense*) contains the highest level of capsinoid and the lowest level of capsaicinoid. ECW (*C. annuum*) produces no capsaicinoid and capsinoid. Yuwol-cho (*C. annuum*) and Takanotsume (*C. annuum*), which are Korean landrace and Japan landrace respectively, have mild pungency. Habanero (*C. chinense*) and Jolokia (*C. chinense*) are the most pungent cultivars.

SNU11-001 and Habanero were used to construct F_1 and F_2 populations. Nine F_1 and 215 F_2 plants were grown in Seoul National University farm (Suwon, Korea).

pAMT and *CS* genotype analysis

For genotyping of *pAMT* of SNU11-001, we developed two types of molecular markers. To design SCAR markers, *pAMT* sequence was obtained from *C. annuum* genome database (http://cab.pepper.snu.ac.kr). The first primer set, third intron F and R (SNU11-001-ECW), was designed to detect insertion of repeat sequence on the third intron of the *pAMT* gene which is

specific to *C. Chinense*. The second marker, the third intron *Tcc*-R3 and third intron(R), was designed to detect the transposable element on the third intron in the *pAMT* gene which is specific to SNU11-001.

To distinguish the *CS* genotype between SNU11-001 and Habanero, two of CAPS markers were developed. First primer set was designed in first exon using *Alu1* site and another primer set was designed in second exon using *Rsa1* site. The latter marker set was used for the *CS* genotyping since whose band pattern is clearer than the former set.

For determination the *pAMT* genotype of SNU11-001 PCR screening was conducted in a 25μl reaction volume containing of 50 ng of template DNA. 10pmol of each primer, dNTP, 10x Hipi buffer and 1 unit of Taq polymerase. PCR was performed by following the conditions : 94℃ for 5 minutes followed by 35 cycles of 94℃ for 30 seconds, 60℃ for 30 seconds and 72℃ for 1 minutes and a final extension of 10 minutes at 72℃. PCR condition for determination of the *CS* genotype was similar to that of the *pAMT* genotype analysis. Annealing temperature of this marker was 57℃.

DNA extraction

Total genomic DNA was extracted from young leaves by CTAB

method of Han et al., (2013). To determinate concentration of genomic DNA, Nanodrop machine (Nanodrop Technology, Inc., Wilmington, DE, USA) was used. DNA samples were dissolved in the final volume 30μl in TE buffer (pH7.0).

Isolation RNA and cDNA synthesis

Total RNA was isolated from the placenta at 20 days after fruit setting by TRIzol reagent (Invitrogen company, Korea) method of Han et al., (2013). RNA samples were diluted in RNAse-free water (Hybrid-R, GeneAll Biotechnology, Seoul, Korea). To measure RNA concentration, Nanodrop machine was used. To synthesize cDNA, reverse-transcriptional PCR was performed in a 20μl PCR volume containing M-MLV 5x reaction buffer, dNTP, M-MLV RT 200 units and mixture of mRNA and oligo dT for 1 hour at 42℃.

HPLC analysis of capsaicinoid and capsinoid

Three fruits per a plant were harvested from all *Capsicum* accessions, SNU11-001 x Habanero F_1 and F_2 plants. Whole fruits including seeds were chopped and stored at -20℃. HPLC analysis was performed in the

Foundation of Agri. Tech. Commercialization and Transfer (FACT, Suwon, Korea) according to the method described by Han et al., (2013).

RESULTS

Capsaicinoid and capsinoid contents of the five cultivars

HPLC analysis was conducted to measure capsaicinoid and capsinoid concentration of five cultivars, SNU11-001, ECW, Yuwol-cho, Takanotsume and Habanero (Table 1.; Fig. 1.). We assumed that *CS* transcriptional level might be correlated with capsaicin contents. Capsaicinoid and capsinoid contents were measured at different four stages. Habanero contained the highest capsaicinoid concents (9195.3±591.29 μg/gDW) among five cultivars at stage 2. Yuwol-cho and Takanotsume had similar capsaicinoid levels, 3433.52±588.23 μg/gDW and 3153.73±518.04 μg/gDW, respectively. However capsaicinoid level of Takanotsume was higher than Yuwol-cho at stage 3 and 4. SNU11-001 contained almost no capsaicinoid (16.13±7.15 μg/gDW). ECW known as a nonpungent cultivar did not produce capsaicinoid. By contrast, SNU11-001 contained the highest capsinoid (6855.98±1795.53 μg/gDW). Habanero was followed by SNU11-001. ECW contained no capsinoid as well.

Table 1. Comparison of capsaicinoid and capsinoid contents in five cultivars by HPLC analysis.

Capsicum cultivars	species	Stage[b]	Capsaicinoid (μg/gDW)			Capsinoid (μg/gDW)		
			capsaicin	Dihydrocapsaicin	Total	capsiate	Dihydrocapsiate	Total
SNU11-001	***C. chinense***	**1**	17.9±5.26	15.5±4.79	33.4±10.04	2910.43±974.2	647.85±138.33	3558.28±1112.54
		2	16.13±4.28	0	16.13±7.15	6106.47±1609.48	749.52±186.96	6855.98±1795.53
		3	25.72±6.6	24.36±11.4	50.09±17.94	6669.92±613.8	771.88±90.47	7441.81±693.84
		4	17.72±5.08	17.31±2.99	35.03±7.94	4352.9±925.64	487.96±110.79	4840.86±1032.86
ECW	***C. annuum***	**1**	-	-	-	-	-	-
		2	nd[a]	nd	nd	nd	nd	nd
		3	nd	nd	nd	nd	nd	nd
		4	nd	nd	nd	nd	nd	nd
Yuwol-cho	***C. annuum***	**1**	116.52±10.29	125.45±4.09	241.97±6.19	87.59±3.58	22.9±3	110.48±0.59
		2	1572.32±325.99	1861.19±317.44	3433.52±588.23	273.17±145.78	79.21±15.7	352.37±159.43
		3	717.43±84.11	1063.48±152.96	1780.9±235.44	101.74±29.01	18.81±2.89	120.55±31.74
		4	516.92±204.75	841.51±186.46	1358.44±391.21	30.25±8.13	12.53±0.27	42.78±7.86
Takanotsume	***C. annuum***	**1**	-	-	-	-	-	-
		2	1632.78±203.98	1520.95±322.53	3153.73±518.04	364.53±21.62	85.46±8.32	449.98±29.49
		3	1362.76±92.97	1563.18±161.88	2925.93±324.81	275.4±22.97	82.58±7.09	357.98±29.96
		4	1780.93±174.57	2172.31±359.59	3953.24±399.23	187.81±36.12	40±6.76	227.81±42.84
Habanero	***C. chinense***	**1**	4771.1±677.47	4211.51±474.46	8982.62±1130.04	470.63±83.72	118.47±16.52	589.1±99.95
		2	4655.94±566.67	4539.36±53.88	9195.3±591.29	488.82±102.01	114.84±23.02	603.66±128.81
		3	2113.27±0*	2227.2±0*	4340.47±0*	169.34±0*	45.75±0*	215.1±0*
		4	2456.59±143.4	2651.17±140.63	5107.75±277.74	255.44±59.45	58.28±6.37	313.71±65.54

a nd=not detected

b stage, 1: 23 days after fruit set, 2: 30 days after fruit set, 3: 37 days after fruit set, 4: 45 days after fruit set

* indicates that this experiment was not repeated.

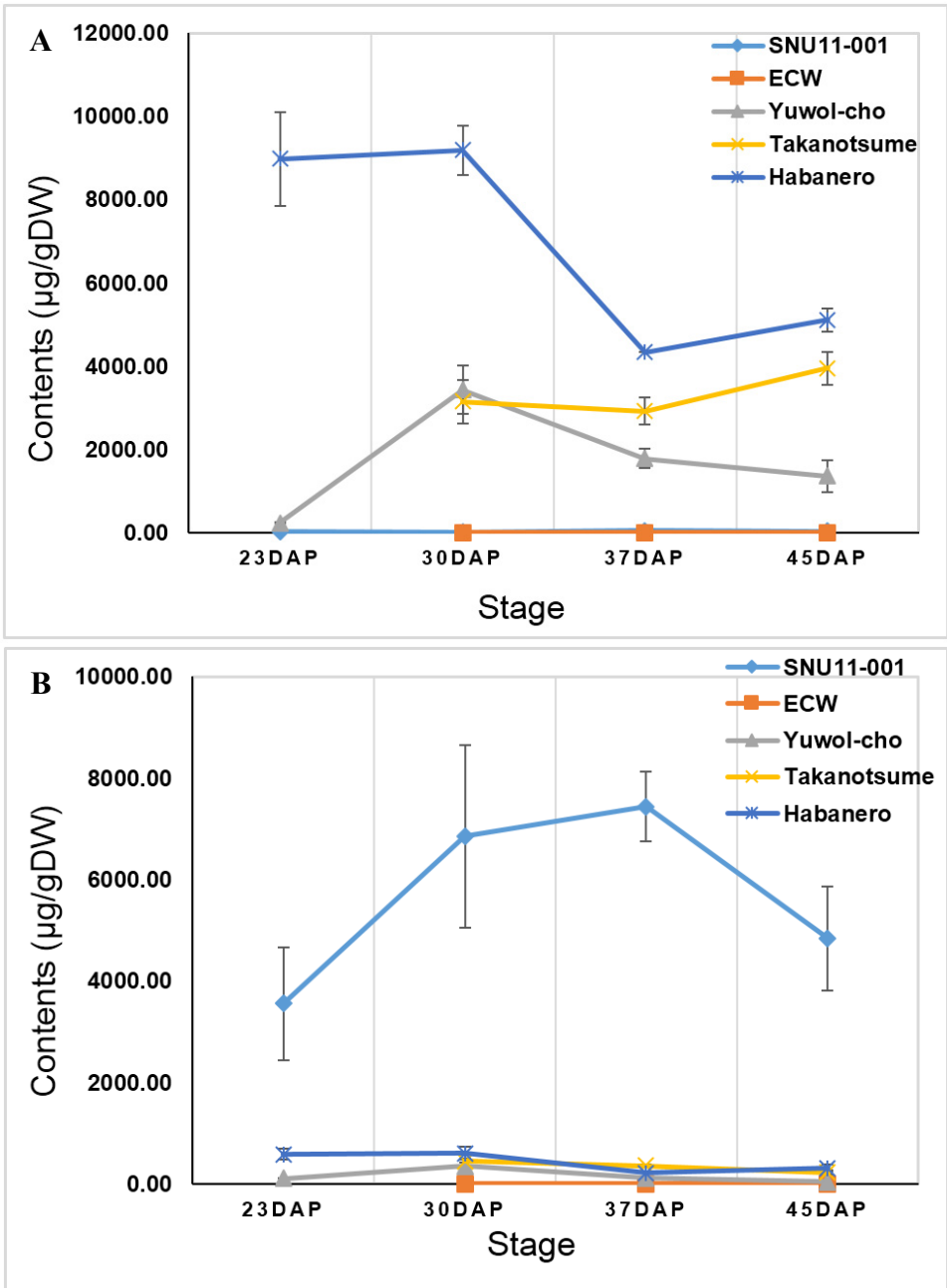

Figure 1. Comparison of (A) capsaicinoid and (B) capsinoid contents according to fruit developmental stages in five cultivars.

Construction of populations segregating capsinoid

To investigate relationship between capsiate production and *CS* activity, we constructed three F_1 populations using SNU11-001 and four cultivars showing various levels of pungency (ECW, Yuwolcho, Takanotsume, Habanero). In the interspecific crosses, *C. annuum* lines were used as maternal line and others as paternal lines to reduce the cross incompatibility. SNU11-001 was crossed with four cultivars to introgress the *pamt* allele. Only one F_2 population could be developed derived from a cross between SNU11-001 and Habanero.

Identification of *pAMT* and *CS* expression pattern

We tested *pAMT* and *CS* expression pattern in five cultivars. The primers for *pAMT* and *CS* were designed using an allele-specific copies based on *Capsicum* genome database (cab.pepper.snu.ac.kr). The cDNA of *pAMT* and *CS* were amplified as 1455 bp and 1206 bp in size, respectively. cDNAs at corresponds to 20 days after fruit set and 45 days after fruit set were used (Fig. 2.).

pAMT transcripts were amplified at immature stage in all cultivars.

pAMT transcription was detected in SNU11-001 but the two transcripts with different sizes were detected. The nonpungent cultivar ECW also expressed the *pAMT* gene. However, at mature stage, no *pAMT* expression was detected except Habanero. ECW express the *CS* gene as expected. *CS* expression was detected in the other cultivars including low pungent SNU11-001 at this stage. Habanero expressed highest *CS* transcript among the tested cultivars. By contrast, almost no *CS* transcript was detected at mature stage in all cultivars.

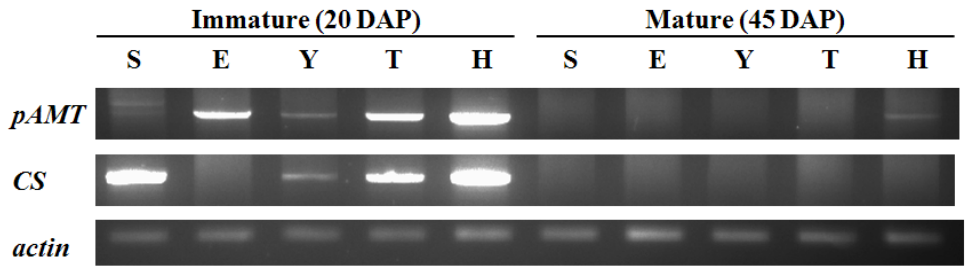

Figure 2. *pAMT* and *CS* expression patterns in five cultivars by RT-PCR. Immature and mature stages correspond to 20 and 45 days after fruit set respectively. *Actin* was used as control. *S* SNU11-001, *E* ECW, *Y* Yuwol-cho, *T* Takanotsume, *H* Habanero

cDNA sequence analysis of *pAMT* and *CS*

Two *pAMT* transcipts were detected in SNU11-001. Partial cDNA sequences of these transcripts from SNU11-001 were obtained (Fig. 3). Two *pAMT* transcripts of SNU11-001 were different from that of CM334. The longer transcript 1118bp in size contained a 403 bp and 8 bp insertions in the third and sixth exons. The longer transctipt was similar to one of the *pAMT* copies in Aji Dulce strain 2 (Tanaka et al., 2010b). The smaller transctipt had 45 bp deletion and 8 bp insertions. Two transcripts contained early stop codon.

To identify sequence differences of *CS* between SNU11-001 and Habanero, full sequences of the coding region in both cultivars was obtained (Fig. 4) and 4 SNPs were found. Three of them had amino-acid changes but one was synonymous mutation. First two non-synonymous mutations were located in the first exon. The other mutations were in the second exon.

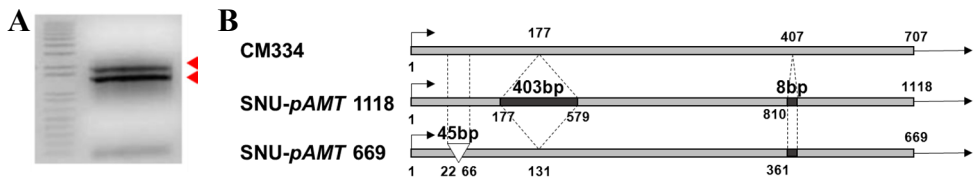

Figure 3. Two types of loss-of-function *pAMT* alleles in SNU11-001. (A) Two types of *pAMT* transcript were detected in SNU11-001. (B) The longer transcript contains a 403bp insertion between the third and the fourth exons and another 8 bp insertion but smaller transcript has 45 bp deletion and 8 bp insertion.

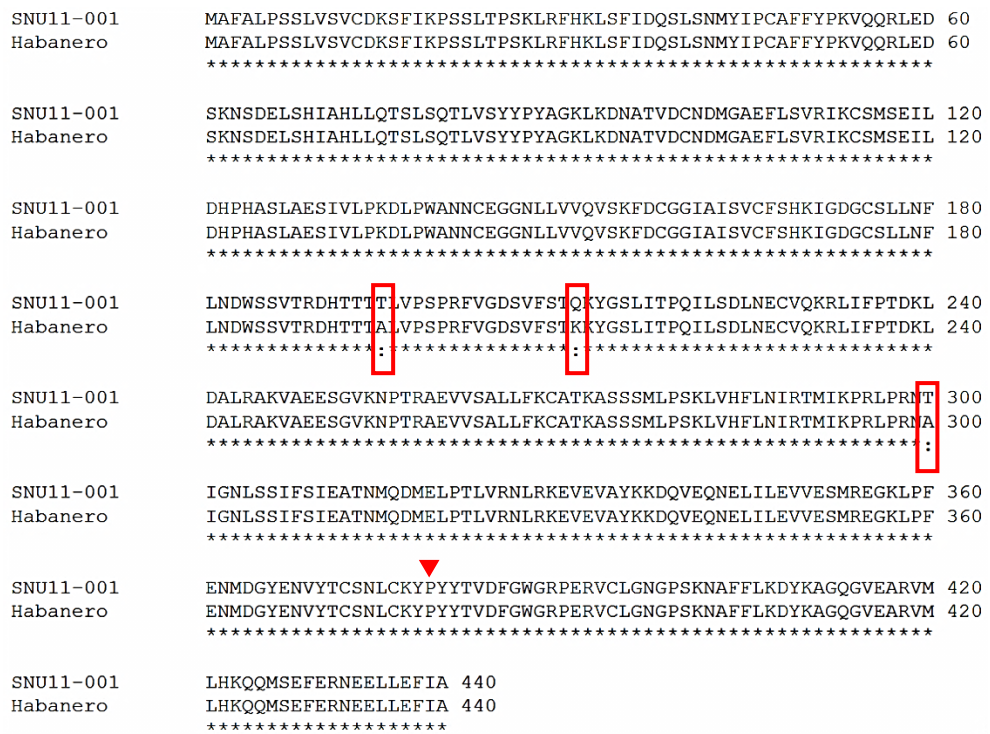

Figure 4. Amino acid sequence alignment of the *CS* gene in *C. chinense* SNU11-001 and Habanero. Four mutations were detected. Three of them in the box are non-synonymous mutation and another marked with triangle is synonymous mutation.

Molecular marker development for *pAMT* and *CS*

Two molecular marker sets were designed. One set was developed for the *pAMT* gene to select *pAMT* mutant and the other set was based on the *CS* gene to distinguish *CS* of SNU11-001 and Habanero. *pAMT* marker was designated in SNU-*pAMT*669. The insertion of transposable element (*Tcc*) on the third intron of the *pAMT* gene was specific to SNU11-001. This SCAR marker was developed from the sequence of *Tcc* in the third intron of SNU11-011 (Fig. 5.). Therefore, the primer set differentiated *pAMT* mutant cultivars which contain *Tcc* element. On the other hand, *CS* marker was developed to discriminate between normal *CS* in two cultivars using a SNP (Fig. 6.). This marker set was based on the synonymous mutation in second exon which can be detected by *Rsa1* site. This CAPS marker was used to genotype *CS* alleles in SNU11-001 x Habanero F_2 population.

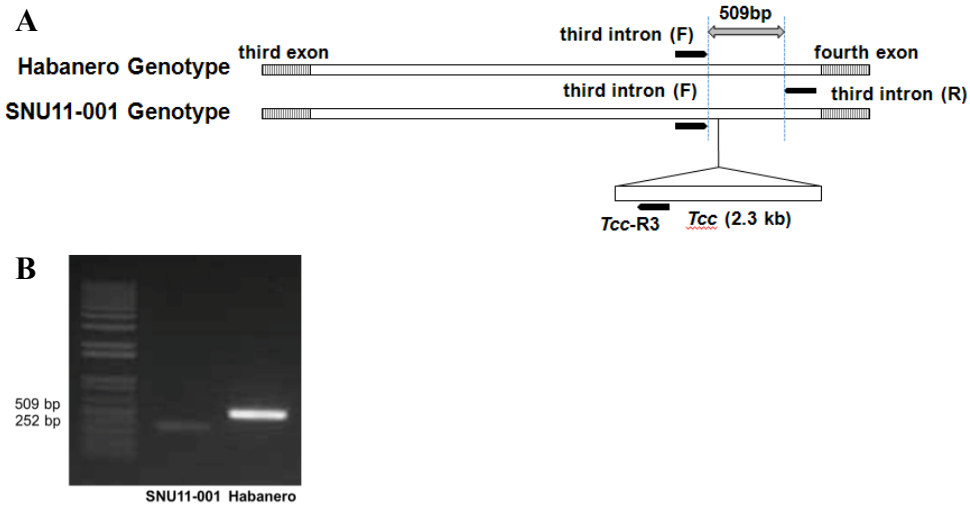

Figure 5. Development of molecular markers to select *pAMT* mutant. The SCAR marker set was designed from the sequence of *Tcc* in the third intron of SNU11-001 to select *pAMT* mutant plant. Striped box corresponds to exon and black bar indicates marker.

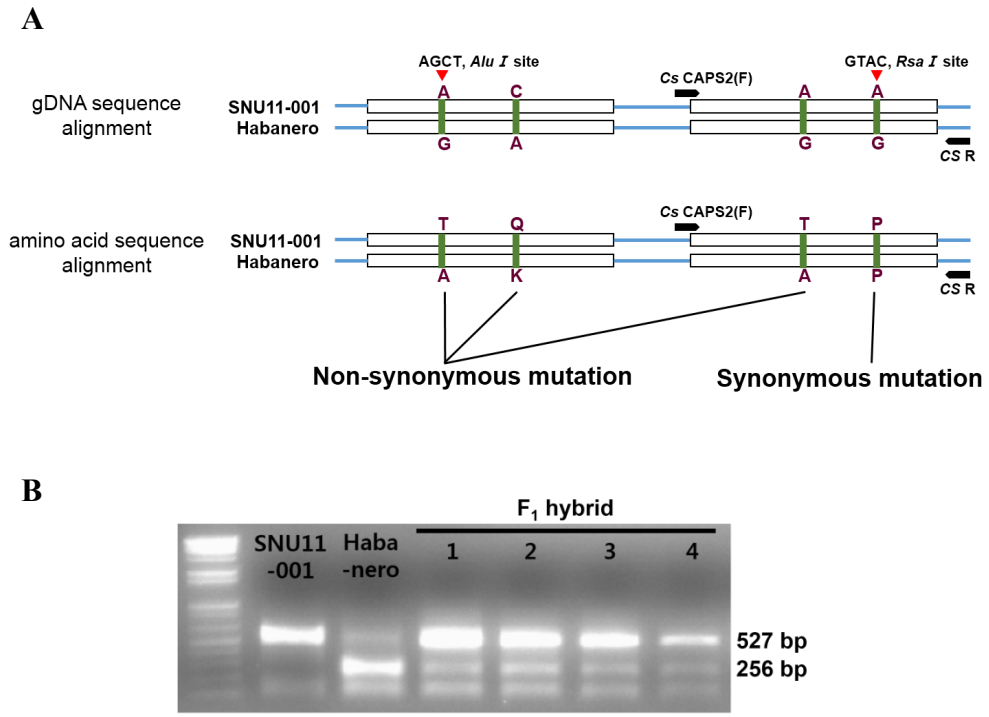

Figure 6. Development of molecular markers to distinguish the *CS* genotypes of SNU11-001 and Habanero. The CAPS marker set was developed in the second exon using *Rsa 1* site to distinguish *CS* genotypes of SNU11-001 and Habanero.

Segregation of *pAMT* and *CS* genotype in F_2 population

pAMT and *CS* genotype analysis was performed in SNU11-001 x Habanero F_2 population using SCAR and CAPS marker. We found that 49 plants were *pamt*/*pamt* homozygote (Table 2.). Heterozygous and homozygous plants were 160 in a total of 215 individuals. This segregation ratio fit an expected ratio 3:1 (x^2=0.27; p=0.6033). However, the number of *pAMT*/*pamt* heterozygote was 84, which was less than expected. In this case, segregation ratio did not match with the expected frequency.

CS of SNU11-001 type and CS^S/CS^s, CS^H/CS^H and CS^S/CS^H were 50, 150 and 108, respectively. This segregation ratio was consistent with 1:2:1 as expected (x^2=0.308; p=0.8524) indicating that *pAMT* and *CS* inherited independently.

Table 2. Genetic analysis of *pAMT* and *CS*

F_2 (SNU11-001 x Habanero)	Pop. size	Expected ratio	*pAMT* genotype			χ^2 (*p value*)	*CS* genotype			Undetermined	χ^2 (*p value*)
			pamt/ pamt	*pAMT/ pamt*	*pAMT/ pAMT*		CS^S/CS^S	CS^S/CS^H	CS^H/CS^H		
	215	1:2:1	49	84	76	14.7674 (0.00062130)				6	
		1:2:1					50	108	50	7	0.308(0.8574)

CS^S indicates *CS* of SNU11-001 type and CS^H correspond to *CS* of Habanero type.

Table 3. Investigation of inheritance pattern of *pAMT* and *CS* in SNU11-001 x Habanero F_2 population.

***pAMT* genotype**	**Number of individuals**	***CS* genotype**	**Number of individuals**
pamt/*pamt*	49	CS^S/CS^S	14
		CS^S/CS^H	25
		CS^H/CS^H	10
pAMT/*pAMT* *pAMT*/*pamt*	160	CS^S/CS^S	36
		CS^S/CS^H	83
		CS^H/CS^H	40

Capsinoid and capsaicinoid contents in an F_2 population

HPLC analysis for capsaicinoid and capsinoid contents was conducted in 49 *pamt*/*pamt* plants (Table 4.). Among 49 plants, seven plants were not analyzed due to problem in fruit sampling. The lowest concentration of capsinoid was 1485.61±115.58 μg/gDW of No. 170 and the highest concentration of capsinoid was 6050.75±698.74 μg/gDW of No. 76. Capsinoid contents of No. 76 was approximately 4.07 times higher than that of No. 39. Capsinoid content of No. 76 was similar to but less than that of SNU11-001.

Using 42 *pamt*/*pamt* plants, correlation between *CS* genotype and capsinoid contents were investigated. Individauls with CS^S/CS^S, CS^H/CS^H and CS^S/CS^H contained 3033.95±383.82 μg/gDW, 2664.02±198.43 μg/gDW and 2933.66±309.53 μg/gDW of capsinoid, respectively.

Table 4. Capsaicinoid and Capsinoid contents in SNU11-001 x Habanero F_2 population.

pamt mutant individual	*CS* type	Capsaicinoid (μg/gDW)			Capsinoid (μg/gDW)		
		capsaicin	Dihydrocapsaicin	Capsaicinoid	capsiate	Dihydrocapsiate	Capsinoid
6	CS^S/CS^H	50.15±7.37	56.64±5.48	106.79±12.85	2461.66±150.09	628.4±3.2	3090.06±153.29
15	CS^H/CS^H	31.48±6.91	38.63±7.38	70.11±14.15	1639.35±180.97	536.25±50.65	2175.61±227.96
18	CS^S/CS^H	39.55±9.66	46.97±13.46	86.52±23.07	2602.74±310.73	380.78±22.37	2983.52±328.45
23	CS^S/CS^H	30.93±1.7	28.37±8.32	59.3±10.02	2132.12±284.9	608.63±74.6	2740.75±359.49
26	CS^S/CS^S	15.34±3.97	13.88±5.06	29.22±8.87	1968.62±242.16	357.52±42.92	2326.15±284.86
38	CS^S/CS^H	36.13±9.44	50.99±14.34	87.12±23.71	2400.28±332.65	417.54±45.37	2817.82±377.72
39	CS^S/CS^H	17.34±11.57	20.64±14.1	56.97±30.67	1660.43±85.57	222.2±91.61	1993.72±104.73
42	CS^S/CS^H	23.1±9.23	30.45±7.65	48.93±14.63	2584.32±512.21	530.28±104.49	3114.6±615.35
48	CS^S/CS^S	23.05±0	14.08±0	37.13±0	2679.75±0	363.84±0	3043.59±0
64	CS^S/CS^S	6.25±2.05	6.46±2.84	12.71±1.87	2233.2±177.1	335.33±36.74	2568.52±211.91
66	CS^S/CS^S	41.46±2.86	38.06±10.56	79.52±11.32	1821.85±192.88	476.29±61.51	2298.14±226.68

69	CS^S/CS^S	37.8±0	45.57±0	83.37±0	2023.71±0	368.56±0	2392.27±0
76	CS^S/CS^H	48.04±3.84	46.16±1.38	94.2±5.05	4965.44±612.41	1085.31±96.63	6050.75±698.74
83	CS^S/CS^S	38.75±5.3	37.72±5.55	76.47±8.45	4126.7±543.42	609.02±52.33	4735.72±590.59
91	CS^H/CS^H	16.8±3.6	4.87±1.51	21.67±4.87	3955.06±651.07	679.28±84.92	4634.34±726.22
93	CS^S/CS^H	2.73±3.45	4.31±0.42	7.04±3.1	2025.29±205.33	316.07±18.71	2341.36±221.31
96	CS^H/CS^H	18.49±0.3	14.19±0.68	32.67±0.38	1444.06±34.14	396.94±7.57	1841±41.71
99	CS^S/CS^H	19.3±3.54	21.76±6.71	41.06±10.09	3352.42±504.51	620.18±88.21	3972.6±587.72
102	CS^S/CS^H	27.9±1.78	32.32±2.86	60.22±4.54	1358.05±396.12	185.67±51.42	1543.71±446.88
105	CS^S/CS^H	24.92±5.1	20.59±7.8	45.51±12.13	2807.51±295.68	351.31±31.35	3158.81±324.37
112	CS^S/CS^H	31.93±3.3	42.16±6.43	74.09±9.7	2935.99±174.97	364.32±23.69	3300.31±198.39
113	CS^H/CS^H	37.14±2.67	38.27±6.24	75.41±8.22	3280.82±316.12	455.38±45.02	3736.2±347.49
116	CS^S/CS^H	25.65±4.47	15.38±0.57	41.02±5.03	1504.93±527.96	396.94±93.4	1901.87±621.36
124	CS^S/CS^S	36.94±4.06	28.22±6.49	65.16±7.95	4521.08±1030.62	416.35±81.57	4937.43±1110.6
137	CS^S/CS^H	13.07±0.62	15.78±2.58	28.84±3.15	2075.9±227.96	437.74±32.47	2513.64±259.6

138	CS^S/CS^H	50.84±4.52	54.82±2.72	105.66±5.99	2436.68±302.49	511.15±88.74	2947.84±383.76
143	CS^S/CS^H	42.03±9.97	54.48±32.51	96.5±42.19	1313.25±120.88	477.32±45.04	1790.57±150.04
144	CS^S/CS^H	41.53±4.19	54.67±0.62	96.2±3.57	2147.34±144.62	416.6±3.83	2563.94±148.45
158	CS^H/CS^H	70.35±7.64	41.3±2.95	111.65±10.46	4570.33±596.52	625.23±76.64	5195.55±670.59
162	CS^S/CS^H	35.62±1.93	48.57±1.39	84.18±1.89	2381.47±213.64	313.65±19.2	2695.13±232.53
164	CS^S/CS^H	18.02±0	12.22±0	30.24±0	1295.06±0	518.11±0	1813.17±0
169	CS^H/CS^H	37.62±1.22	53.09±2.55	90.71±2.89	2488.37±303.34	310.02±21.96	2798.4±324.39
170	CS^S/CS^H	104.6±71.59	28.87±6.98	133.47±75.97	1234.92±120.15	250.7±10.06	1485.61±115.58
172	CS^S/CS^H	14.75±7.74	38.59±11.13	53.33±18.8	1490.46±446.02	291.66±74.74	1782.12±519.38
176	CS^H/CS^H	33.79±4.96	33.58±8.65	67.37±12.94	1967.44±360.63	347.38±55.76	2314.81±413.39
187	CS^H/CS^H	11.74±5.15	12.19±8.48	23.94±13.62	1607.09±35.09	244.92±13.91	1852.02±48.97
189	CS^S/CS^H	111.74±51.28	46.1±5	157.83±48.04	2026±239.97	445.22±37.51	2471.22±268.03
190	CS^S/CS^S	39.82±2.28	71.5±8.62	111.32±10.88	1841.77±286.08	339.51±20.32	2181.28±283.12
195	CS^S/CS^S	22.17±4.18	26.61±11.38	48.78±15.53	2160.44±216.49	382.49±35.82	2542.93±242.4

204	CS^{H}/CS^{H}	63.74±10.24	54.37±10.99	118.11±21.12	2408.69±201.49	348.91±24.3	2757.59±213.94
205	CS^{S}/CS^{H}	27.37±7.71	56.53±15.54	83.91±23.25	1890.82±359.9	419.54±161.25	2310.35±521.15
213	CS^{S}/CS^{S}	31.36±2.46	45.63±3.66	77±5.99	1998.59±277.64	311.94±33.04	2310.53±307.36

Capsaicinoid and capsinoid concentration at 30 days after fruit set was measured.

Four plants (22, 51, 134 and 184) were not determined. * indicates that this experiment was not repeated.

S SNU11-001 *H* Habanero

DISCUSSION

cDNA sequence structure of SNU11-001 was similar to Aji Dulce strain 2 (Tanaka et al 2010b), hence SNU11-001 had *Tcc* element in third intron and eight bp insertion. However SNU11-001 was distinguished from Aji Dulce strain 2 because SNU11-001 contained additional 45 bp deletion and capsinoid contents was much higher than that of Aji Dulce strain 2 (6855.98±1795.53 μg/gDW), even though capsinoid was extracted from whole fruits in SNU11-001 while it was extracted from placenta and seeds in Aji Dulce strain 2 (Tanaka et al ., 2010b). We expect that SNU11-001 can be used as a practical breeding material and research material due to high contents of capsinoid.

Segregation ratio of *pAMT* genotype (*pAMT/pAMT* vs *pamt/pamt*) in SNU11-001 x Habanero F_2 population was approximately 3 : 1 as expectedly. This result corresponded to the segregation ratio of capsaicinoid vesus capsinoid plants in HPLC analysis. However segregation ratio of this population did not correspond to expected 1:2:1 ratio (*pAMT/pAMT* : *pAMT/pamt* : *pamt/pamt*). It was speculated that open pollinated seeds was contaminated when F_2 generation was developed.

CS transcriptional levels might be related to pungency levels because *CS* expression patterns were different in five tested cultivars (Fig. 2.). *CS* genotypes of SNU11-001 and Habanero did not show differences in capsinoid contents. Plants with CS^S/CS^S, CS^H/CS^H and CS^S/CS^H contained almost similar concentration of capsinoid (3033.95±383.82 μg/gDW, 2622.69±207.26 μg/gDW and 2933.66±309.53 μg/gDW, respectively. Habanero was selected to generate a population because of high level of capsaicinoid. We assumed that the factors resulting in high contents of capsaicinoid could contribute to capsinoid contents. If the factors causing high capsaicinoid concentration in Habanero had have an effect on increasing capsinoid contents in *pAMT* mutant plant of F_2 population, *pAMT* mutant plant would have contained higher capsinoid concentration than SNU11-001. However the highest level of capsinoid in *pAMT* mutant individual (No. 76) is 6050.75±698.74 μg/gDW, which is lower than that of SNU11-001.

A new cultivar ‘Maru Salad’ derived from the cross of nonpungent pepper ‘Murasaki’ x ‘CH-19 Sweet’ contained capsinoid approximately 700 μg/gDW, which is much lower than both ‘CH-19 Sweet’ (5825±286 μg/gDW) and SNU11-001. This result demonstrated that other factors with

respect to capsinoid contents might exist, which is related in quantitative control of capsaicinoid. Other genes in capsaicin or capsiate pathway could be transcript accumulation of *Pal*, *Ca4h*, *Comt*, *pAMT* and *Kas* related to pungency level. (Curry et al., 1999, Aluru et al., 2003). QTL of capsinoid also could be occur like QTL *cap* for capsaicionids. (Blum et al., 2003; Ben-chaim et al., 2006). Futhermore, F_2 populations using SNU11-001 and other three cultivars (ECW, Yuwol-cho and Takanotsume) have to be developed to validate the relationship between *CS* expression levels and capsinoid contents.

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ABSTRACT IN KOREAN

캡시노이드는 맵지 않은 고추에서 발견된 물질로써 캡사이시노이드처럼 항암효과와 항비만 효과 등의 의학적 효능이 있다고 알려져 있다. 캡시노이드의 전구체인 바닐릴알코올은 *pAMT* 돌연변이 유전자에 의해 만들어진다. 선행연구결과에서 캡시노이드의 생합성에는 *CS* 유전자도 관여한다는 것이 밝혀졌다. 그러나 *CS*의 전사체 축적과 캡시노이드 함량의 상관관계에 대해서는 명확하게 알려지지 않았다. 본 연구는 캡시노이드의 양적 조절에 관여하는 유전적 인자를 찾기 위하여 수행되었다. 유전자원 검정을 통하여 *C. chinense* 'SNU11-001'가 이전에 보고된 *C. annuum* 'CH-19 Sweet' 보다 캡시노이드를 많이 함유하고 있다는 것을 밝혀내었다. SNU11-001의 *pAMT* 유전자의 mRNA에 403bp의 뉴클레오타이드 서열 삽입과 45bp의 삭제로 종결 코돈이 생겨 기능을 하지 못하는 것을 알아냈다. SNU11-001과 각각 다른 매운 맛을 지닌 *Capsicum* 품종들 간의 *CS*와 *pAMT* 유전자 발현을 확인할 결과 *CS*의 transcript 축적은 매운맛이 높은 품종일수록 높았다. 캡시노이드의 함량을 조절하는 것으로 알려진 capsaicin

synthase (*CS*)와 putatiave amino transferase (*pAMT*) 유전형을 구분할 수 있는 분자마커를 개발하고, *C. chinense* 'SNU11-001'과 'Habanero'를 양친으로 F_2 집단을 구축하여 개체별 유전형을 분석하였다. *CS*의 유전형과 캡시노이드 함량의 상관관계를 분석한 결과 *CS*의 종류는 캡시노이드의 양적인 형질에 영향을 미치지 않았다. 본 연구는 캡시노이드 고함유 품종의 육종에 이용할 수 있는 유전자원을 발굴하고 육종에 필요한 분자마커를 개발하고자 하였다.