A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Genetic analysis of a Nonpungent EMS mutant in pepper (*Capsicum annuum* L.)

무신미 EMS 돌연변이체의 유전적 분석

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SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
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ABSTRACT

Capsaicinoid is the alkaloid compounds produced in peppers (*Capsicum* spp.). They are responsible for pepper pungency or hotness and is one of the important traits in breeding programs. Although many studies have been performed to elucidate its biosynthesis, the biosynthetic pathway is largely based on studies on the similar pathways of other plants. To understand the biosynthesis of capsaicinoid, a non-pungent mutant 221-2-1a, developed from pungent 'Yuwol-cho' were analyzed. 221-2-1a was found to have no mutation in the coding sequence of *Pun1*, but the levels of capsaicinoid in their fruits were drastically decreased compared to that of Yuwol-cho. To identify the

gene(s) responsible for the non-pungent trait in 221-2-1a. Gene expressions of 12 genes involved in capsaicinoid biosynthesis were compared between 221-2-1a and Yuwol-cho together with several selected cultivars. Seven out of 12 genes (*pAMT, BCAT, ACL, KAS, FatA, PAL,* and *Pun1*) showed a significant decrease in their expression levels in 221-2-1a compared to pungent cultivars. Furthermore, the inheritance of pungency was studied in a population derived from a between Yuwol-cho and 221-2-1a. The inheritance study showed that the nonpungency in 221-2-1a is controlled by two recessive genes. To identify the genes responsible for non-pungency trait, samples of Yuwol-cho and bulked F_3 were sequenced and analyzed by MutMap. A total of 11 SNPs were identified in the intergenic sequences and the candidates were annotated. Although candidate genes were not capsaicinoid biosynthesic genes, the candidate genes are believed to be ideal targets in studies to carryout in the future.

Keywords: Capsaicinoid, MutMap, Mutation, EMS, Pungency

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LIST OF ABBREVIATIONS

4CL	4-coumaroyl-CoA ligase
Acl	Acyl carrier protein
ACS	Acyl-CoA synthetase
BCAT	Branched-chain amino acid transferase
BWA	Burrows-Wheeler aligner
C3H	Coumaroyl shikimate/quinate 3-hyroxylase
C4H	Cinnamate 4-hydroxylase
COMT	Caffeic acid O-methyltransferase
dCAPS	Derived cleaved amplified polymorphic sequences
DPA	Day Post Anthesis
ECW	Early California Wonder
EMS	Ethyl methanesulfonate
FatA	Acyl-ACP thioesterase
HCT	hydroxycinnamoyl transferase
HPLC	High Performance Liquid Chromatography
KAS	Ketoacyl-ACP synthase
PAL	Phenylalanine ammonia lyase
pAMT	Putative aminotransferase
PCR	Polymerase Chain Reaction
Q30	Ratio of reads that have Phred quality score of over 30
QTL	Quantitative trait loci
SNP	Single nucleotide polymorphism

LITERATURE REVIEW

1. Capsaicinoid in pepper

Capsaicinoid is a principle pungent component found in pepper. Pure form of capsaicinoid was isolated in early 1876 by Thresh and was named as capsaicin (Thresh, 1876). Capsaicinoid is classified as alkaloid compound composed of vanillylamine and acyl CoA derivatives (Aza- González et al., 2011). The two major capsaicinoid in pepper are capsaicin and dihydrocapsaicin which constitute about 77±98% of total capsaicinoid (Govindarajan et al., 1987; Collins et al., 1995; Zewdie et al., 2001). Other capsaicinoid such as homocapsaicin, homodihydrocapsaicin, nordihydrocapsaicin and nonivamide also constitute in small quantities in capsicum fruits (Constant et al., 1996; Huang et al., 2013). Although the structures of these metabolites slightly vary (Figure 1), all the capsaicinoid moiety feature the pungent taste.

Capsaicinoid have versatile functions with many health promoting and pharmacological properties (Kobata et al., 2008; Luo et al., 2011). The pungency caused by capsaicinoid is one of the most important traits of pepper and has become the reason for growing interest by crop producers (Bosland and Votaba, 2012). However, the pungency of capsaicinoid makes it hard to take advantage of its beneficial properties. Its side effects are controversial and not fully elucidated yet

(Bode and Dong, 2011). Some studies warn it would be a double-edged sword and can cause carcinogenesis rather than healing process (Bernstein et al., 1989; Watson et al., 1993; Backonja et al., 2008; Webster et al., 2010a; Webster et al., 2010b; Irving et al., 2011). Yet, the use of capsaicinoid as medicine will not be superseded as their action bypasses intolerance and several side effects (Yong et al., 2017). Studies for pros and cons on capsaicinoid are still on the march.

Not only to the mankind, but capsaicinoid is beneficial to pepper themselves. It indirectly selects consumers such as birds who can widely and safely disperse the seeds (Tweksbury and Nabhan, 2001). It also gives immunity to pepper fruits against *Fusarium* (Tweksbury et al., 2008). In this way, capsaicinoid have helped prosperity of pepper at the evolutionary point of view.

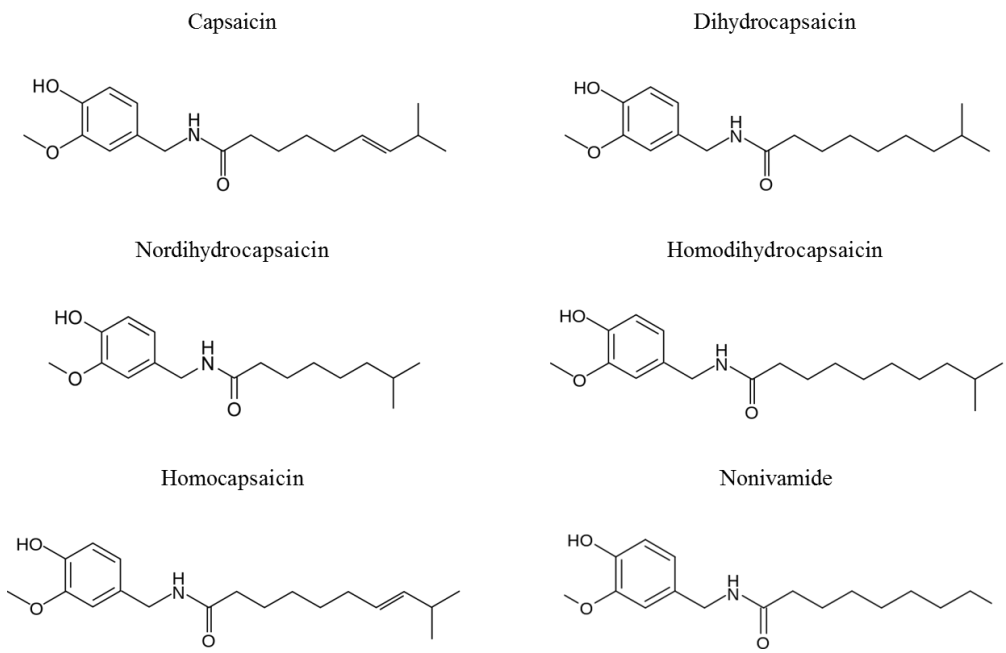

Figure 1. Chemical structures of major capsaicinoid.

2. Studies on capsaicinoid biosynthesis pathway.

The general capsaicinoid biosynthetic pathway was first proposed at the end of the 1960s (Bennet and Kirby, 1968; Leete and Louden 1968). Capsaicinoid synthesis begins approximately 20 days post-anthesis (DPA) in the placental tissues and accumulates in the epidermis of the placenta (Iwai et al., 1979; Stewart et al., 2005). Capsaicinoid biosynthesis involves merging of the phenylpropanoid pathway with precursor phenylalanine and the branched chain fatty acid pathway which involves precursor's valine or leucine. Vanillylamine is synthesized by phenylpropanoid biosynthesis pathway, and CoA derivatives, whose side chains are medium chain fatty acids, are synthesized by fatty-acid biosynthesis pathway. Enzymes and genes related to the phenylpropanoids biosynthesis pathway were studied first, (Fujiwake et al., 1982; Sukrasno et al., 1993; Curry et al., 1999) followed by fatty-acid biosynthesis (Curry et al., 1999; Aluru et al., 2003; Stewart et al., 2005; Mazourek et al., 2009). Even though a capsaicinoid biosynthetic pathway was already proposed with specific enzymes and genes, direct and comprehensive reports were provided only recently (Kim et al., 2014).

3. MutMap analysis

MutMap is a genome-wide analysis of SNP frequencies to find causative SNPs of a trait of interest. Goal of this analysis is to elucidate causative SNP which is responsible for trait of interest. In general, MutMap is a rapid gene identification

method using the cross of the wild type and the mutant line followed by pooling of F_2 segregating population and WGS (Whole Genome Sequencing; Abe et al., 2012; Huang et al., 2013). Thereby it demonstrates that sequencing based SNP identification can be rapid and accelerate identification of the gene controlling a trait.

As described by Abe et al. (2012), more than 10 F_2 plants are suitable for analysis and the coverages of WGS raw data are recommended to be higher than 15 times of the genome size of the target plant species. The MutMap development has two major steps for narrowing down the candidate SNPs. The first step is that the coordinates for all the sliding window are checked whether average of SNP index values are higher than the corresponding average of 95% confidence interval upper side value. If logical operation is true, corresponding sliding window coordinates are selected. Second, all the SNP index coordinates whose SNP index value is close to one are selected as candidate SNPs. Modifications in this procedure can be applied in cases where self-pollination is lethal or when de novo assembly is required because of significant structural variation in reference genome (Fekih et al., 2013; Takagi et al., 2013b). Furthermore, it is also possible to analyze QTLs using MutMap (Takagi et al., 2013a).

There have been many studies of cloning mutated genes using MutMap analysis. MutMap is suitable for traits regulated by single genes, however, several reports adopted MutMap to identify causative SNPs for traits controlled by more than one genes (Lee et al., 2017; Song et al., 2017; Deng et al., 2017; Fan et al.,

2017).

Introduction

Peppers (*Capsicum* spp.) are a native to the North America and have been cultivated since 6000 B.C. (Perry et al., 2007). Currently, peppers are cultivated worldwide and are considered as one of the most economically important vegetable crops with highest market value. Among more than 30 different species in *Capsicum* genus, *C. annuum* is the most extensively cultivated with a wide varieties of shapes and sizes of fruits (Bosland and Votaba, 2012). They are widely used in the food and pharmaceutical industries due to their strong flavor and valuable antioxidants. The secondary metabolites from the pepper fruits have shown to possess anti-inflammatory (Spiller et al., 2008), anticancer (Surh et al., 1995; Chanda et al., 2004; Oyagbemi et al., 2010; Anandakumar et al., 2013) and antimicrobial (Careaga Monica et al., 2003) activities. Among the secondary metabolite capsaicinoid is a alkaloid compound contributing to the pepper pungency.

Capsaicinoid is one of the most important breeding targets in peppers. Thereby, studies on capsaicinoid measurement have been accelerating for variety development (Jeong et al., 2012). Diverse methods have been used for the capsaicinoid quantification in pepper samples among which High Performance Liquid Chromatography (HPLC) is by far the most common technique used with different equipment, columns, gradients and solvents (Kirschbaum-Titze et al., 2002). And Gibb's reagent test is a rapid and simple assay to quantify the presence of

capsaicinoid based on color alteration.

Earliest studies on capsaicinoid pathway were based on tracing radioactive isotopes (Bennet and Kirby, 1968; Leete and Louden, 1968). Eventually, researches have been focused on identifying genes functioning in the enzymatic steps involved for better understanding of pungency control mechanism (Curry et al., 1999; Aluru et al., 2003; Blum et al., 2003). Since then, various studies have been perfomed to elucidate unidentified genes in the pathway and different components regulating the pathway. Several transcription factors which systematically regulate capsaicinoid biosynthetic genes were also identified (Keyhaninejad et al., 2014; Arce-Rodríguez, 2017).

Some of the key studies include cloning and characterization of *AT3* encoding *Pun1*, a key gene responsible for pepper pungency. A 2.5 kb deletion across the promoter and the first exon results in the non-pungency in cultivated varieties (Stewart et al., 2005). Furthermore, a deletion of 4 bp in *Pun1* coding sequence does not normally result in the formation of capsaicinoid-accumulating blister (Stewart et al., 2007). Recently Kim et al. (2014) and Qin et al. (2014) independently published the whole genome sequence and reported the genes involved in the capsaicinoid biosynthesis in *C. annuum*. Also candidate gene association mapping show that *Pun1* acts as a key regulator of capsaicinoid pathway and their expression variation influences capsaicinoid accumulation (Reddy et al., 2014). In addition, *Pun1* regulates the biosynthesis of capsinoid with *pAMT* at the end point of biosynthesis

(Han et al., 2013). Selection efficiency can be improved by evaluating the genes encoding the enzymes involved in pathway regulation and causative mutations within them leading to change in pungency level. For genetic evaluation, a short DNA sequence called DNA marker is used. Using DNA markers, we can identify polymorphisms in mutations or linkage at the locus of the gene.

Despite the extensive works carried out, genes involved in capsaicinoid biosynthesis are still remains unanswered and more direct evidences are required to reveal the biosynthetic pathway. Also for varietal improvement, mutagenesis and cross breeding for improved pungency or non-pungency traits would make development of ideal hot pepper germplasms. Therefore, the objectives of the present study were characterization of a nonpungent muntant and identification of causative SNPs and the candidate genes.

MATERIALS AND METHODS

Plant materials

C. annuum L. 'Yuwol-cho', a Korean landrace, was used in this study to develop a non-pungent mutant line 221-2-1a. Non-pungent mutant line 221-2-1a was selected from an EMS mutant population (Hwang et al., 2014). Placental tissues of 917 M_2 lines consisting of 8,632 plants were screened for nonpungency by Gibb's reagent test followed by high performance liquid chromatography (HPLC) analysis. After several round of selection, six non-pungent mutant lines were obtained and a mutant line named '*221-2*' having the least amount of capsaicinoid among them were selected. This selected line were further self-pollinated until M_5 generation and named 221-2-1a. F_2 and F_3 populations derived from a cross between Yuwol-cho and its mutant line 221-2-1a were used to map nonpungency in 221-2-1a. Plants were cultivated at Seoul National University farm (Suwon, Republic of Korea).

Sample preparation for capsaicinoid analysis

Three fruits were harvested at mature green stage from parental lines and F_2 and F_3 plants together with control plants. The fruits from plants were harvested at 15, 30 and 45 DPA (Fig. 2). Placenta tissue from the dissected fruits were used to prepare the extracts for analysis. Freeze dried placenta tissue were extracted with 7.5

ml of ethyl acetate:acetone (6:4) by vigorous shaking at 37℃ for 24 h. Supernatant of 3 ml were taken and evaporated in a vacuum concentrator AES1010 (Operon, Gimpo, Republic of Korea). The obtained pellet was dissolved in 1 ml HPLC grade methanol (Sigma-Aldrich, Saint Louis, Missouri, USA) and the extracts were filtered with 0.2μm pore syringe filter prior chromatographic analysis.

Gibb's screening and HPLC analysis

For Gibbs screening of pungency, 10 μl of prepared extracts were sprayed onto a filter paper followed by the same volume of 2,6-dichloroquinone-4-chloroimide (Gibb's reagent; Sigma-Aldrich, Saint Louis, Missouri, USA). Ammonia gas was steamed for 30 s on the sprayed spot and the pungency was determined by transition of the spot to blue color.

HPLC analysis of the extracts were performed in National Instrumentation Center for Environmental Management (NICEM, Seoul, Republic of Korea). Capsaicinoid contents were calculated by the sum of capsaicin and dihydrocapsaicin contents in the extracts. Pure capsaicin and dihydrocapsaicin were used as reference standards (Sigma-Aldrich, Saint Louis, Missouri, USA).

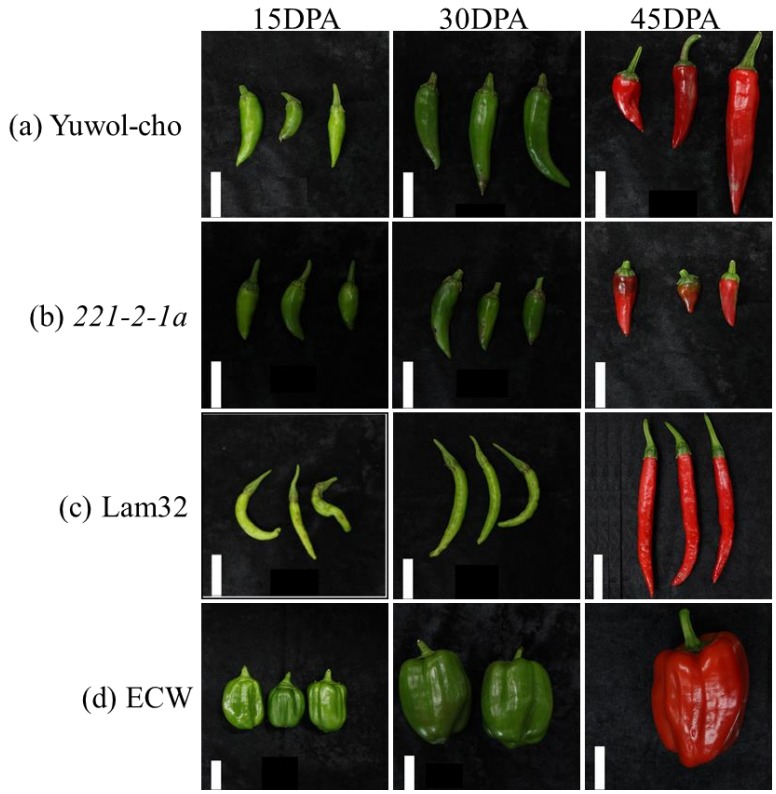

Figure 2. Pepper fruits of 221-2-1a and control plants. Fruits were harvested at developmental stages of 15, 30 and 45 DPA. The scale bar in the picture is 3 cm.

Isolation of RNA and cDNA synthesis

For gene expression study, the placental septum were separated from the collected fruits for RNA extraction. The tissues were immediately frozen in liquid nitrogen and ground to fine powder. The RNA extraction was conducted in triplicates with 100 mg of tissue powder for each RNA extraction. Total RNA was extracted using MG Total RNA Extraction Kit (MGmed Inc., Seoul, Republic of Korea). The integrity of the RNA was analyzed on a 1% agarose gel and the concentration was determined using Take 3 Bio analyzer (BioTek Instruments Inc., Winooski, VT, USA). Approximately 2 µg of RNA were used for cDNA synthesis anchored by oligodT_{18} primers using Moloney Murine Leukemia Virus Reverse Transcriptase (Promega, Wisconsin, US).

Quantitative real-time PCR analysis

The real-time analysis was performed in the Roche Light Cycler480 (Roche Applied Science) using SYTO™ 9 Green Fluorescent Nucleic Acid Strain (Thermofisher Scientific Korea, Seoul, Korea). Coding sequences of 12 target genes involved in the capsaicinoid pathway (**Figure 3**) were obtained from *C. annuum* genome database (http://cab.pepper.snu.ac.kr) and the primers were designed using Primer3 software (http://carbon.bioneer.co.kr/primer3plus/) (**Table 1**). In total, 20 µl of the resulting cDNA was diluted 8-fold and served as the template. The Real-time PCR was performed in a 20 µl reaction volume containing 2 µl of diluted cDNA,

2 µl of 10 mM dNTPs, 2 µl of 10xreaction buffer, 0.5 µl of SYTO 9, 10 pmol of each primer, 0.4 µl of R Taq, and 12.1 µl of nuclease free water. The reaction mixture was heated to 98℃ and denatured for 5 min followed by 45 cycles of 95℃ for 10 s, 60℃ for 20 s, and 72℃ for 20 s. The reliability and reproducibility were ensured with three independent biological replicates per plant. The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to quantify the relative expression levels of the selected target genes. Sigmaplot (Ver 13.0, Systat software Inc., San Jose, USA) was used for making charts of gene expressions.

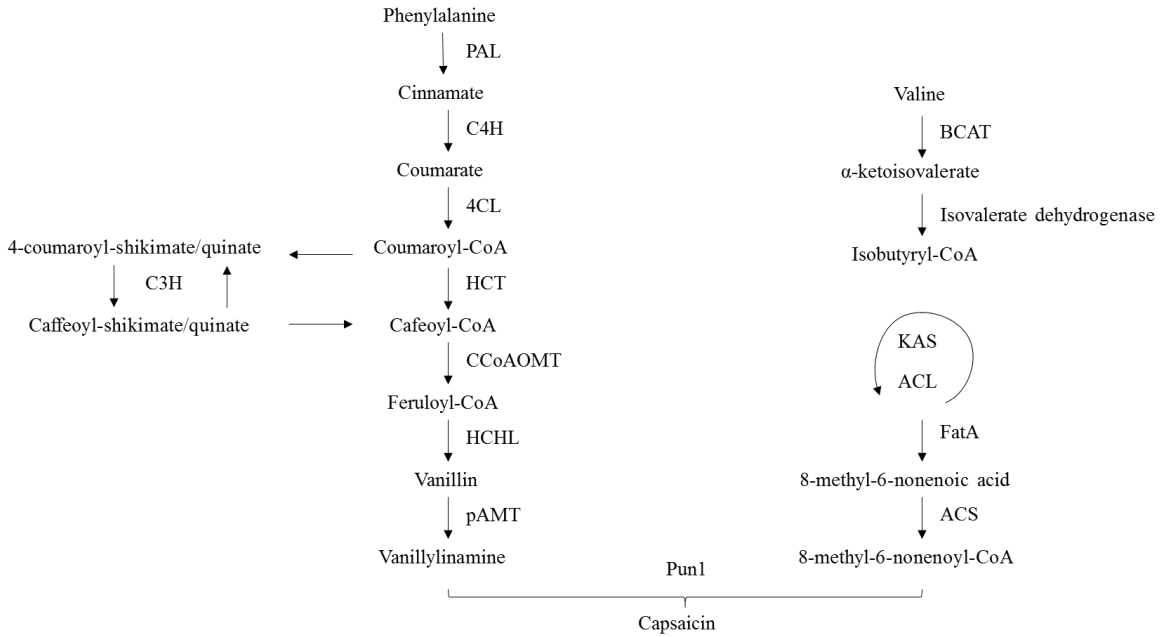

Figure 3. Capsaicinoid biosynthetic pathway and genes analyzed in this study. PAL, Phenylalanind ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl-CoA ligase; HCT, hydroxycinnamoyl transferase; C3H, coumaroyl shikimate/quinate 3-hydroxylase; COMT, caffeic acid O-methyltransferase; pAMT, putative aminotransferase; BCAT, branched-chain amino acid transferase; KAS, ketoacyl-ACP synthase; ACL, acyl carrier protein; FatA, acyl-ACP thioesterase; ACS, acyl-CoA synthetase.

Table 1. Primers used for real-time PCR.

Name	CDS (bp)	Location	Foward primer	Reverse primer
4CL	1,629	CA03g30500	GGACCGATTGAAGGAATTGA	GGACAACAGCAGCATCAGAA
ACL	797	CA06g11050	GCCACTCGTCGCCTCAGTAT	GCGCAGCAAACTTGGACTCTC
ACS	1,900	CA03g18160	TGGCTCAGCTGAATTTGTTG	TAACCCGTGAACGTGAAACA
Actin	1,161	CA00g80270	CTTGTCTGTGATAATGGAACAG	GGGATACTTCAAGGTGAGAATA
BCAT	1,398	CA04g16660	CAAGGAAGGAACAGCACCAT	TCGCCTTTGCTTTCTTCATC
C3H	1,491	CA08g09680	GCCAAAAGAGTGGAGGAGCC	CACCTCAGCCATTGCCCATT
C4H	1,518	CA06g25940	CACCCACAAGCTTCCGTACC	GTTTCTTCCAGTGAGCGGGG
COMT	990	CA03g21160	GCACCAGTTCCACCACCA	TCGAGTACCGTGGCACAGAT
FatA	1,116	CA06g26640	CCAGGACACAAGACGCCTTC	CAGCTCTTCGTGGCACAAGT
KAS	1,911	CA01g00840	GTGTACAAATGCCAGCAAGCTCTG	GATTCCACTTTGTCCCTCGAGAAG
PAL	1,855	CA00g95510	CAACAGCAACATCACCCCATGTTTGC	GCTGCAACTCGAAAAATCCACCAC
pAMT	1,395	CA03g08530	GATGAGGTGGTATGTGGATTTGGAA	ATACTCAACGAGGGGCCTGAAACAG
Pun1	3,752	CA02g19250	ATCTCAACCAGTGCGTACAGAAAAGACT	GTGAACCAACTTTGATGGTAGCATTGAT

Polymerase chain reaction (PCR) amplification and sequence analysis of *Pun1*

PCR was performed in 50 µL reaction volumes containing 10 µL of 5x PCR buffer, 100 ng of template DNA, 100 pmol of each primer, 4 µL of 10mM dNTPs and 5U of Taq polymerase (Prime Star GXL; Takara). PCR cycling conditions for the *Pun1* gene included an initial denaturation step at 98℃ for 5 min, followed bay 30 cycles of 95℃ for 10 s, 55℃ for 15 s and 72℃ for 60 s and a final extension step at 72° for 5 min. All amplified products were resolved in 1% agarose gel (Lonza, Lockland, USA). The products were gel eluted and purified using LaboPass™ Gel and PCR Clean-up Kit (Cosmogenetech, Seoul, Korea). The resulting product were cloned in T-Blunt vector (Solgent, Seoul, Korea) and sequenced using BigDye v.3.1, AB at Macrogen (Macrogen, Seoul, Korea). The DNA sequence was analyzed using SeqMan (Ver 5.00, DNASTAR Inc., Madison, WI, USA).

Genomic DNA extraction

Total genomic DNA was extracted from young leaves of 133 F_2 and 80 F_3 plants along with the parent and control lines using modified cetytrimethylammonium bromide (Sigma-Aldrich, Saint Louis, Missouri, USA) method (Han et al., 2013). The extracted DNAs were quantified using Epoch microplate spectrophotometer (BioTek, Vermont, US) and a fluorescence-based

quantification with Picogreen (Invitrogen, California, US)

Whole genome sequencing of wild type and mutant bulk

Whole genome sequencing was performed for bulked samples of 18 non-pungent F_3 lines. Samples were mixed in equal ratio to give a mixed DNA of 6.038 µg and were used for library construction along with the reference Yuwol-cho DNA sample of 6.022 µg. Two separate paired-end DNA libraries were constructed for 2 µg of Yuwol-cho and F_3 DNA samples using TruSeq DNA PCR-Free kit (FC-121-3001). DNA was sheared, separated and enriched for fragments ranging between 200 to 400 bp in length. Adapter sequence was added to the samples for bar coding as per Illumina guidelines. The libraries were sequenced on the Illumina HiSeq 4000 platform producing reads of 100 bp from forward and reverse ends of the fragment. All samples were demultiplexed using their respective adaptor sequence and the raw data were generated by base calling using HiSeq Control Software (v3.3), Real Time Analysis (v2.5.2). Bcl2fastq conversion software (v2.16.0.10) was applied to convert the base call binary into FASTQ format.

Alignment of reference sequence and MutMap

In order to identify the mutations incorporated by EMS, we generated the reference sequence of Yuwol-cho genome on the basis of publicly available CM334

reference genome (CM334 20140122 (v1.55) CHROMOSOME, http://peppergenome.snu.ac.kr/blast.php). Approximately, 600 million paired-end short reads were obtained from sequencing of wild variety Yuwol-cho and 18 non-pungent F_3 mutants which were pooled. MutMap analysis was conducted by MutMap pipeline as described by Abe et al. (2012) after certain modifications in SNP calling. The raw data processing is performed using software in MutMap pipeline. There are three steps in data processing - read qualification, reference sequence construction and genome wide analysis for SNPs. Burrows-Wheeler aligner (BWA) and Coval software were involved in these procedures. BWA program based on Burrows-Wheeler transform (BWT) was used to align short paired end read to the reference genome (Li et al., 2009). Alignment data were further converted to SAM format using SAMTools (v0.1.8) in order to apply Coval filtering (Li et al., 2009b; Kosugi et al., 2013). Coval software was applied to filter low quality short reads. Coval base calling was improved by modifying mismatch filtering value and depth value to 4 and 5 (Kosugi et al., 2013). SNP indices were calculated using same criteria's in the previous study (Abe et al., 2012), and the SNP index plots were constructed by R (v2.15.0). Genes closer than 50 kb to SNPs were analyzed. Functions of these genes were analyzed by blastx analysis of coding sequence. Gene annotations are summarized by frequency and identification rate to the pre-existing databases.

RESULTS

Capsaicinoid measurement in placenta tissues

To evaluate capsaicinoid contents of the EMS mutant 221-2-1a, capsaicin and dihydrocapsaicin content were measured together with the pungent and non-pungent controls (**Figure 2)**. Capsaicinoid content was quantified by sum of capsaicin and dihydrocapsaicin levels in the extracts of fruit placenta collected at 15, 30 and 45 DPA as shown in **Figure 4**. The HPLC analysis showed significantly high capsaicinoid levels in the selected pungent varieties and relatively negligible amount in the non-pungent varieties. Capsaicinoid contents in the wild variety Yuwol-cho were 266.9±16.64 μg/gDW at 15 DPA, 28510.7±704.57 μg/gDW at 30 DPA, and 19236.9±1033.4 μg/gDW at 45 DPA. The 221-2-1a contained significantly levels of capsaicinoid compared to the pungent varieties with 17.2±2.41 μg/gDW at 15 DPA, 94.6±1.38 μg/gDW at 30 DPA, and 18.3±1.64 μg/gDW at 45 DPA. The pungent control varietyLam32 contained 27555.1±377.77 μg/gDW at 15 DPA, 14231.2±389.1 μg/gDW at 30 DPA, and 19236.9±1033.4 μg/gDW at 45 DPA. the nonpungent control variety ECW showed the lowest capsaicinoid content among all with 6.8±0.13 μg/gDW at 15 DPA, 7.3±0.5 μg/gDW at 30 DPA, and 1.4±0.10

μg/gDW at 45 DPA.

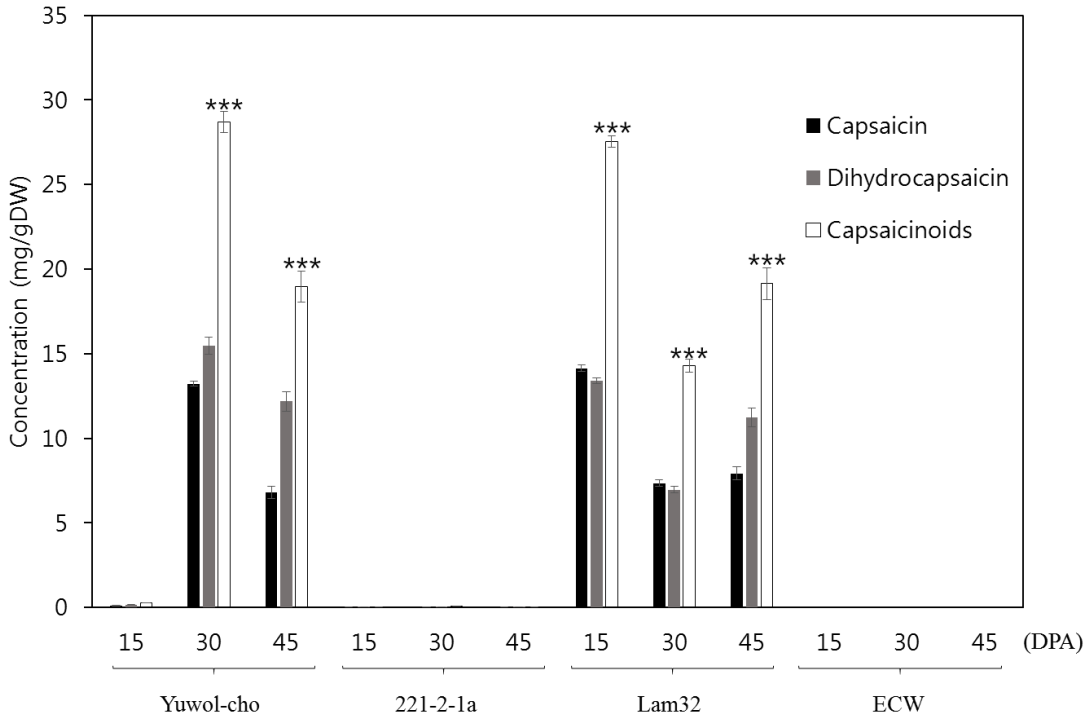

Figure 4. Levels of capsaicinoid in pungent and nonpungent varieties. Placental extracts from pungent and non-pungent varieties were analyzed for their capsaicinoid content at three developmental stages. The capsaicinoid content was quantified by the sum of capsaicin and dihydrocapsaicin levels. *** $p \leq 0.001$ compared with ECW using Dunnett's multiple comparison test.

Expression analysis of capsaicinoid pathway genes

The real-time PCR analysis was performed to analyze the differential expression of the genes involved in the capsaicinoid biosynthetic. Four genes related to fatty acid biosynthesis showed high expression levels in pungent varieties Yuwol-cho and Lam32 whereas the non-pungent varieties ECW and 221-2-1a showed significantly reduced expression levels (**Figure 5**).

In more detail, it was revealed that the expression levels of *BCAT* (branched-chain amino acid transferase) in Yuwol-cho was 10.28 fold higher to control non-pungent ECW and 70.65 fold to 221-2-1a. Also the 221-2-1a showed an 88.59 fold decreased *BCAT* expression levels compared to another pungent control line Lam32. Likewise, the expression levels of *ACL* (acyl-CoA synthetase ECW and 221-2-1a) in was 6.6 fold and 4.88 fold lower than that in Yuwol-cho221-2-1a*, respectively*. The mutant 221-2-1a displayed an 8.6 fold decrease in *ACL* expression level compared to Lam32.

Coming to the *KAS* (β-ketoacyl ACP synt) and *FatA* (acyl-acyl carrier protein thioesterase) gene, although the overall expression levels were relatively lower in the pungent varieties, there was a significant difference between pungent and non-pungent varieties. The expression levels of *KAS* of ECW and 221-2-1a was 38.61 fold and 44.33 fold less that of in Yuwol-cho221-2-1a. Similarly *FatA* expression levels of ECW and 221-2-1a was 18.89 and 31.27 fold less than that of Yuwol-cho221-2-1a. The expression of *KAS* was 12.45 fold and *FatA* was 15.82 fold lower

in the mutant non-pungent 221-2-1a compared to the pungent control Lam32.

On the other hand, three genes from phenylpropanoid pathway of capsaicinoid biosynthesis also showed significant variation in their expression levels between the pungent and non-pungent varieties (**Figure 6**). Gene expressions of *PAL*, *pAMT* and *Pun1* genes showed a positive correlation with pungency levels. The expression levels of the *PAL* gene in ECW and 221-2-1a was 12.94 fold and 5.43 fold lower than that of Yuwol-cho221-2-1a. The expression levels of 221-2-1a was 3.01 fold lower than that Lam32. The *Pun1* gene expression was also significantly reduced in ECW and 221-2-1a. 221-2-1aThe expression levels of *pAMT* gene significantly different between the pungent and nonpungent varieties. The wild type pungent variety Yuwol-cho showed 229.32 fold and 114.21 fold higher expression of *pAMT* than those of ECW and 221-2-1a and . Likewise, the 221-2-1a variety showed a 38.83 fold lower expression of *pAMT* than Lam32.

The remaining genes from both the fatty acid and the phenylpropanoid pathways of the capsaicinoid biosynthesis did not show any significant differences between the pungent and the non-pungent varieties (Figure 7). These genes are *C4H*, *4CL*, *C3H* , *COMT*, and *ACS.* Although participating in capsaicinoid biosynthesis, they may not be regulating the pungency levels in the mutant line.

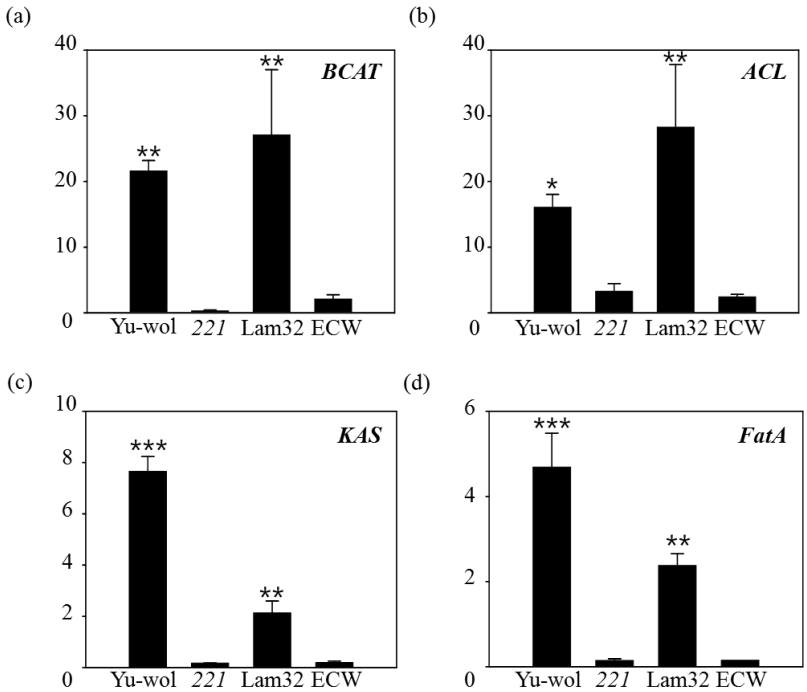

Figure 5. Real-time PCR expression of fatty acid pathway genes in the capsaicinoid biosynthesis from pepper placenta tissues. (a) *BCAT*, (b) *ACL*, (c) *KAS*, and (d) *FatA* belonging to fatty acid biosynthesis pathway were found to regulate the capsaicinoid biosynthesis and thereby the pepper pungency. Mean and Standard deviation values were obtained from triplicates. Ratio of target gene average C_p (x axis) to reference C_p (y axis) are plotted against varieties. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ compared with ECW using Dunnett's multiple comparison test.

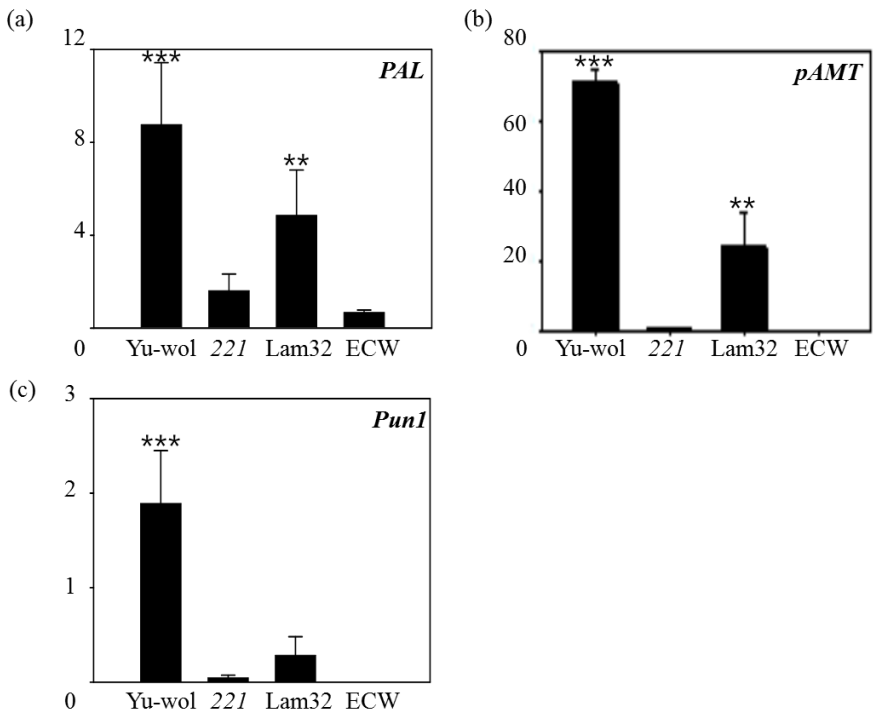

Figure 6. Real-time PCR expression of phenylpropanoid pathway genes in the capsaicinoid biosynthesis from pepper placenta tissues. (a) *PAL*, (b) *pAMT* belong to phenylpropanoid biosynthesis pathway in the capsaicinoid biosynthesis. (c) *Pun1* participates in the downstream mechanism merging the fatty acid and phenylpropanoid pathways. Mean and Standard deviation values were obtained from triplicates. Ratio of target gene average C_p (x axis) to reference C_p (y axis) are plotted against varieties. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ compared to ECW using Dunnett's multiple comparison test.

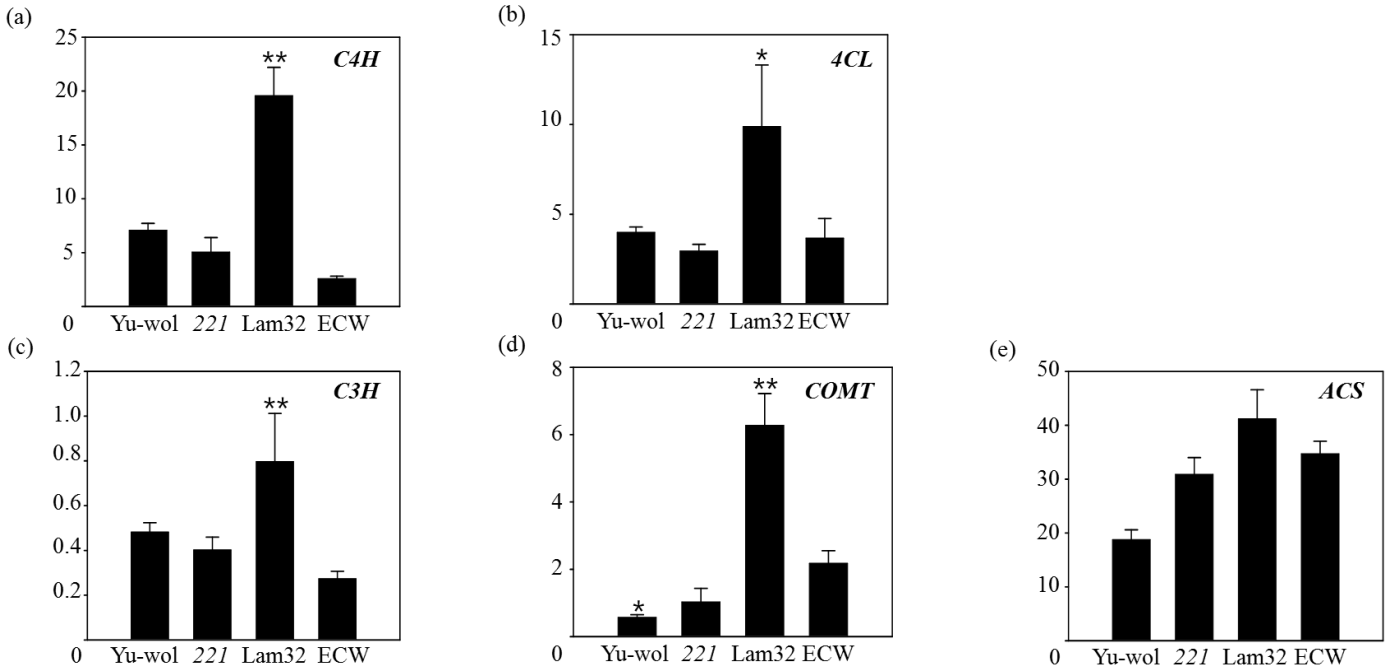

Figure 7. Real-time PCR expression of insignificant genes involved in capsaicinoid biosynthesis. (a) *C4H*, (b) *4CL*, (c) *C3H* and (d) *COMT* belongs to phenylpropanoid pathway, and (e) *ACS* belongs to fatty acid biosynthesis pathway of capsaicinoid biosynthesis. Mean and Standard deviation values were obtained from triplicates. Ratio of target gene average C_p (x axis) to reference C_p (y axis) are plotted against varieties.* $p \leq 0.05$, ** $p \leq 0.01$ compared with ECW using Dunnett's multiple comparison test.

Comparison of *Pun1* exon sequences

The *Pun1* gene has been reported to control presence of the capsaicinoid biosynthesis in pepper fruits (Stewart et al., 2005). In order to detect mutations in the *Pun1* gene, the coding sequences (557 bp of exon 1 and 585 bp of exon 2) were compared between the 221-2-1a and the wild variety Yuwol-cho. It was observed that the 221-2-1a does not carry mutations in the coding sequence of *Pun1* (**Figure 8**).

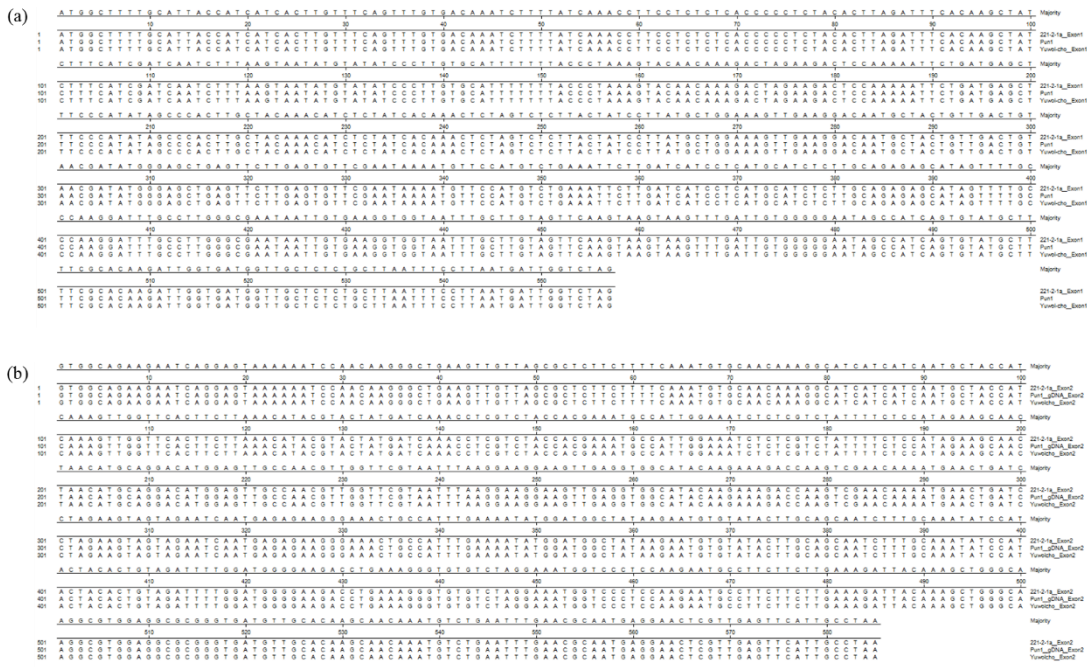

Figure 8. *Pun1* exon sequences of Yuwol-cho and 221-2-1a**.** No mutation was observed in non-pungent mutant 221-2-1a compared to the wild variety Yuwol-cho exon sequences. (a) exon 1, (b) exon 2.

Evaluation of pungency segregation

The F2 progeny of the wild pungent variety Yuwol-cho and the mutant non-pungent variety 221-2-1a was used to reveal the inheritance pattern of nonpungency by Gibb's test (**Table 2**). As expected, all the F_1 progeny produced pungent fruits and nonpungency was segregated in the F_2 generation. The results show that 9 out of 133 pepper lines of F_2 generation was observed to have non-pungent fruits. These results were confirmed by HPLC. The result as shown in **Table 3** confirms the non-pungency of the selected lines with lower capsaicinoid contents similar to the parent mutant line 221-2-1a. The Chi square test showed a good fit to 15:1 ratio (15 - pungent; 1 - non-pungent; p = 0.8055). However, considering the quantitative variation of capsaicinoid within the nine non-pungent F_2 lines, all plants were self-pollinated again to obtain the F_3 lines. Eighteen lines from these were selected for further analysis after HPLC quantification of capsaicinoid (**Table 4**).

Table 2. Inhertitance of pungency in population derived from a cross between wild type and mutant cross.

Plants		Number of plants	Pungency	Expected ratio (p value)
Parents	Yuwol-cho	5	All pungent	1 : 0
	221-2-1a	5	All non-pungent	0 : 1
Controls	Lam32	5	All pungent	1 : 0
	ECW	5	All non-pungent	0 : 1
Cross	221-2-1a X Yuwol-cho	5	All pungent	1 : 0
	F_1	133	124(P) : 9(NP)[z]	15:1 (p = 0.8055)[y]
	F_2			

[z] P – Pungent and NP – Nonpungent.
[y] P – Expected ratio was found to be accurate with p value > 0.05.

Table 3. HPLC quantification and confirmation of non-pungent F_2 lines.

	Yuwol-cho	221-2-1a	F_2 plant line number								
			15	**19**	**25**	**38**	**51**	**68**	**79**	**90**	**91**
CAP[z] (ppm[y])	13230.3	51.2	16.83	408.22	146.56	3.87	115.80	251.06	723.54	23.68	1006.2
DICAP[x] (ppm)	15468.1	43.0	7.3	229.11	71.89	1.32	61.54	122.72	415.56	22.33	548.38
Dry mass (g)	0.1	0.1	0.075	0.076	0.091	0.089	0.102	0.087	0.031	0.074	0.062
Total (ppm)	28698.4	94.2	24	637	218	5	177	374	1139	46	1555

[z] CAP capsaicin content
[y] ppm μg per dry mass of placenta
[x] DICAP dihydrocapsaicin content

Table 4. Capsaicinoid contents of F_3 samples from 221-2-1a X 'Yuwol-cho'.

F3 plant No.	Capsaicinoid (ppm).	**F3 plant No.**	Capsaicinoid (ppm).
10-1	4.6	**38-1**	23.8
10-2	4117.4	**38-2**	1768.5
10-5	30.8	**38-3**	85.2
11-1	163.9	**47-1**	381.6
11-2	49.3	**47-2**	68.0
11-3	2.0	**47-3**	10.2
15-1	27.9	**68-1**	78.3
15-2	3901.3	**68-2**	0.0
15-3	1537.4	**68-3**	20.8
19-1	915.5	**79-1**	0.0
19-2	4.1	**79-2**	32.8
19-3	5.3	**79-3**	143.4
25-1	523.8	**90-1**	34.3
25-2	855.5	**90-2**	385.6
25-3	58.9	**90-3**	1553.3

Seqeuncing of Yuwol-cho genome and bulked F_3 DNA

Genome sequence data of Yuwol-cho and bulked F3 samples 221-2-1awere obtained using Illumina sequencing. Since the bulked samples were derived from F_3 population, the phenotypic change is minimal and therefore segregation of non-pungent trait can be unequivocally observed with small difference. The bulked DNA samples of the recessive mutant F_3 progeny with a substantial genomic coverage >10x should show a 100% mutant and 0% wild type reads. The depth of the sequence of Yuwol-cho was 15.11 and that of F_3 bulk was 13.46. The total number of reads for Yuwol-cho was 693,013,122, and F_3 was 556,859,190. Reads with Phred quality score higher than 30 were used for MutMap analysis. Ratio of reads that have Phred quality score of over 30 (Q30), which means that the chances of having base call error are one in 1000, were 91.99%, 88.29% each. The Yuwol-cho reference sequence was mapped using 404,135,397 reads by applying on CM334 reference genome. Consensus sequence of F_3 was mapped using 203,313,763 reads on the constructed Yuwol-cho reference sequence.

Table 5. Summary of Illumina sequencing of Yuwol-cho and F_3 non-pungent bulked DNA.

Sample name	Number of lane	Reference	Number of F_3 Bulked	Total reads[z]	Total read bases	Aligned data		Coverage
						Mapped	Unmapped	
Yuwol-cho	1	CM334	-	693,013,122	69,994,325,322	404,135,397	79,740,607	15.1134
F_3 Mutant bulk	1	-	18	556,859,190	56,242,778,190	203,313,763	36,008,019	13.4628

[z] The short reads in which less than 10% of nucleotides had Phred quality < 30 were excluded from the analysis. Short reads having their corresponding paired-end reads were used for alignment.

MutMap analysis for candidate region identification

A total number of coordinates, where SNP index value were higher than 0.3, was 153,497. Among them, a total number of positions where SNP index value is 1 were 15,704. These coordinates were distributed through whole chromosomes. To narrow down the sites for causative mutation resulting in non-pungency, the average SNP indices value of sliding window were analyzed (**Figure 9**). The width of sliding window was 2 million bp, and incremented step size was 10,000. In the case the average SNP indices value of sliding window was higher than 95% confidence interval upper side value (green line in **Figure 9**), positions in sliding window interval were regarded as candidate region and analyzed. Four regions were selected as candidates on chromosome 6, 8 and 11. The total number of positions with SNP index value =1 in those candidate regions was 536. In the chromosome six, there are 10 positions in 66.8 Mb to 68.82 Mb region, 160 positions in 98.02 Mb to 101.48 Mb region. In the chromosome eight, there are 147 positions in 82.12 Mb to 84.72 Mb region. In the chromosome 11, there are 219 positions in 33.6 Mb to 35.64 Mb region. Genes whose distance from intergenic candidate positions were closer than 50kb were selected to narrow down candidates. Eleven candidate genes were finally identified using this criteria (**Table 6**). None of them were related to structural genes involved in capsaicinoid biosynthesis pathway. Also, functions of six genes in candidates still remained unknown.

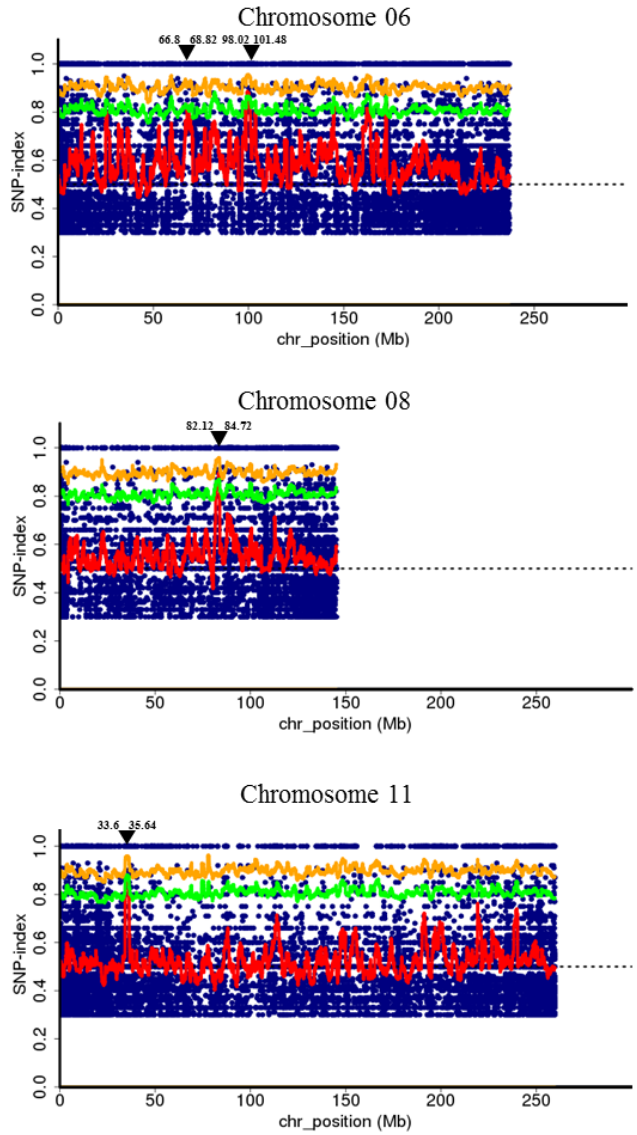

Figure 9. SNP index plots for identifying causal mutation. Blue dot is SNP index, Red line is sliding window average of SNP index. Green line is sliding window average of 95% confidence interval upper side. Orange line is sliding window average of 99% confidence interval upper side. Inverted triangle is the range of candidate regions.

Table 6. Summary of SNPs detected between 'Yuwol-cho' wild type and bulked F_3 DNA by MutMap analysis.

Chromosome : coordinate	Reference base	Altered base	The number of reads covering the site	Mapping quality	SNP index	95% Confidence cutoff	Neighboring gene ID
Chr06 : 68617496	C	T	6	60	1	0.833333	CA06g06490
Chr06 : 99583834	G	A	7	60	1	0.857143	CA06g07320
Chr06 : 101363111	G	A	9	38	1	0.777778	CA06g07330
Chr06 : 101470143	G	A	8	29	1	0.875	CA06g07340
Chr08 : 82394126	C	T	13	58	1	0.769231	CA08g04490
Chr08 : 82459707	C	T	6	29	1	0.833333	CA08g04500
Chr08 : 82487246	G	A	9	34	1	0.777778	CA08g04510
Chr08 : 83152977	C	T	7	60	1	0.857143	CA08g04550
Chr11 : 33844238	G	A	6	52	1	0.833333	CA11g05930
Chr11 : 35548221	C	T	6	60	1	0.833333	CA11g06100
Chr11 : 35568284	G	A	7	27	1	0.857143	CA11g06110

Table 7. Annotations of genes close to candidate SNPs detected by MutMap

Gene ID	Annotation	Gene distance from position
CA06g06490	Glucose-methanol choline (Gmc) oxidoreductase, putative	-25652
CA06g07320	OSJNBa0887O24.13 protein	44089
CA06g07330	PREDICTED: acyl-CoA—sterol O-acyltransferase 1-like [*Solanum tuberosum*]	40382
CA06g07340	F-box protein SKIP19	17889
CA08g04490	PREDICTED: uncharacterized membrane protein At3g27390-like isoform X1[*Citrus sinensis*]	-4483
CA08g04500	At3g17900/MEB5_12-like protein	3724
CA08g04510	PREDICTED: protein PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1-like [*Solanum tuberosum*]	4977
CA08g04550	Detected protein of unknown function	26380
CA11g05930	Actin cross-linking protein, putative [*Theobroma cacao*]	2117
CA11g06100	CW14 protein isoform 1 [*Theobroma cacao*]	6214
CA11g06110	Detected protein of unknown function	10042

DISCUSSION

The domestication of chili pepper (*Capsicum*) for human use is one of the earliest based on archeological evidences (Basu and De Krishna, 2003). Pepper breeders often cross pungent and non-pungent genotypes to introduce exotic traits (Blum et al., 2002). Moreover, research on pungency levels in peppers is beneficial for understanding the biosynthetic mechanism behind this trait. In this study, a new non-pungent EMS mutant line 221-2-1a developed from Korean landrace 'Yuwol-cho' was characterized. The mutant line were self-pollinated for five generations to remove the harmful effects of EMS and for consistency of the mutation (Parry et al., 2009). We compared the capsaicinoid levels of the developed mutant with the wild type and the result showed a remarkable decrease of both the capsaicin and dihydrocapsaicin content equivalent to the control non-pungent line ECW.

It has been reported that capsaicin is the major capsaicinoid and dihydrocapsaicin accounts for approximately 20% of total capsaicinoid in *Capsicum* spp. (Kozukue et al 2005; Choi et al 2006). However, this study shows that the major pungent component in Yuwol-cho was dihydrocapsaicin (**Figure 4**). Thus, the presence of dihydrocapsaicin levels higher than the typical capsaicin in Yuwol-cho and the mutant 221-2-1a having drastic decreased levels of capsaicinoid makes it a suitable material for this study.

The relative expression study to identify the differentially expressed genes

greatly helps in understanding the mechanism involved in capsaicinoid biosynthesis in pepper plants. Twelve genes were selected from the capsaicinoid pathway to analyze the differential transcript profile between the pungent and non-pungent pepper plants. The candidate genes *pAMT*, *BCAT* and *ACL* were found to be highly expressed with many folds higher in pungent cultivars Yuwol-cho and Lam32 compared to the non-pungent cultivars 221-2-1a and ECW. Other genes were also identified to have a significant fold difference in expression between the pungent and non-pungent cultivars such as *KAS, FatA, PAL* and *Pun1*. Same candidate genes were previously reported when analyzing the mechanism of pungency between various *Capsicum* species (Sarpras et al., 2016).

Pun1 has been considered as the most plausible candidate gene in many previous studies such as Stewart et al. (2007) who identified that the deletion on the gene results in decreased capsaicinoid accumulation and Reddy et al. (2014) revealed that *Pun1* acts as a key regulator in capsaicinoid accumulation. With this apprehension, the *Pun1* gene of the mutant cultivar 221-2-1a was sequenced to identify the mutation. However, it was confirmed that the non-pungency of the EMS developed mutant was not caused by any mutation on *Pun1*.

With this, the downstream regulator of the phenylpropanoid pathway converting vanillin to vannilylamine, *pAMT* gene is believed to the causative candidate gene as it shows momentous expression levels in pungent cultivars. Previous knockdown experiments of the *pAMT* gene by VIGS (Virus Induced Gene

Silencing) demonstrated that silencing this gene resulted in reduced capsaicinoid in pepper fruits (del Rosario Abraham-Juárez et al., 2008). It was also observed that the functional loss of *pAMT* gene resulted in the formation of capsinoids, a compound first isolated in CH-19 Sweet' variety of pepper and in mainly present across the non-pungent *C.annuum* genotypes (Lang et al., 2009). Ogawa et al. (2015) confirmed that the accumulation of capsaicin content correlates to the expression profile of *pAMT*. Very recently, Sarpras et al. (2016) did a comparative analysis among various species of *Capsicum* and identified that the *pAMT* gene is highly expressed in highly pungent *C. chinense* compared to other moderate or low pungent *Capsicum* species.

With the intent of identifying the causative mutations in the developed non-pungent variety 221-2-1a, a cross population was developed by crossing it with the wild cultivar Yuwol-cho. All the F_1 hybrid plants showed pungency while segregation was detected in F_2 population satisfying with the classic Mendelian model (15:1). Further F_3 population was developed for more consistency and 18 out of 30 F_3 individuals were used for MutMap analysis. MutMap helps in rapid identification of causal mutations by whole genome sequencing and comparison of SNP index plot of the bulked DNA against the wild cultivar (Abe et al., 2012). Q30 values of Yuwol-cho and bulked F_3 DNA were fixed 90% in MutMap analysis. In the **Figure 9**, there are several regions where sliding window average of SNP index values fluctuates, even though their value were lower than sliding window average of 95% confidence value. This might be caused by the alignment algorithm or since

applying hold value for Phred quality score in MutMap analysis.

With this, 11 SNPs and their position were identified but to a much letdown, they were identified in the intergenic regions of genes which are not involved in the capsaicinoid biosynthesis. Since it is likely that the substitution in intergenic region affects activity of enhancer or promoter, these identified genes were considered as possible candidates. Nevertheless, it is possible that unknown component or precisely a transcription factor from these identified candidate genes may indirectly regulate some of the genes involved in capsaicinoid biosynthetic pathway. As a follow up, this year, a cross population is being developed between 221-2-1a and Lam32 to identify the SNPs and compare with this data.

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ABSTRACT IN KOREAN

캡사이시노이드는 고추에서 생성되는 알칼로이드이다. 이 물질은 고추에서 매운맛이 나게하는 원인 물질이기 때문에, 캡사이시노이드 함량은 육종 계획 수립시 가장 중요하게 고려되는 형질들 중 하나이다. 이 물질의 생합성을 규명하기 위해 많은 연구가 수행되고 있으나, 아직까지는 생합성 경로의 많은 부분을 다른 식물의 유사한 생합성 경로로부터 차용하여 설명하고 있다. 본 연구에서는 캡사이시노이드의 생합성을 규명하기 위하여 신미 '유월초'를 바탕으로 만든 무신미 돌연변이체 221-2-1a를 연구하였다. 221-2-1a은 *Pun1*의 엑손 서열에 돌연변이가 없지만 그 과실에서 유월초와 비교하여 매운맛이 현저히 감소하는 특징을 나타내는 돌연변이체이다. 221-2-1a의 무신미 형질을 조절하는

유전자를 규명하기 위하여, 유월초, 221-2-1a를 비롯한 대조군 태좌조직에서 캡사이시노이드 생합성에 관여하는 것으로 알려진 유전자 12개의 발현량을 비교하였다. 돌연변이체에서는 분석한 12개 유전자 중 7개의 유전자 (*pAMT, BCAT, ACL, KAS, FatA, PAL*, and *Pun1*) 의 발현량이 감소하는것이 관찰되었다. 또한 유월초와 221-2-1a의 교배조합 F_2 집단을 대상으로 신미의 분리양상을 관찰하였다. 그 결과 두 개의 열성 유전자에 의해 조절되는 형질에서 나타나는 분리비를 확인하였다. 이 유전자들을 규명하기 위해 무신미 F_3개체들과 유월초의 DNA를 이용하여 뮤트맵 분석을 수행하였다. 그 결과 11개의 후보 SNP를 유전자간 서열에서 확인되었다. 비록 SNP에 인접한 후보유전자들이 직접적으로 캡사이시노이드 생합성에 관여하는 것으로 판단하기는 어렵지만, 후속 연구를 위한 단초로서 활용될 것으로 기대된다.

Appendix 1. Coordinates where SNP value is 1 in MutMap

Chromosome	Coordinate	Reference base	Altered base	Mapping quality	The number of reads covering the site	SNP index
Chromosome06	66827214	C	T	60	7	1
Chromosome06	66936938	C	T	60	7	1
Chromosome06	66943305	C	T	60	5	1
Chromosome06	67160499	G	A	42	7	1
Chromosome06	67762489	C	T	60	7	1
Chromosome06	68376435	C	T	60	8	1
Chromosome06	68471174	C	T	60	8	1
Chromosome06	68524327	C	T	41	6	1
Chromosome06	68617496	C	T	60	6	1
Chromosome06	68796459	C	T	60	6	1
Chromosome06	98118397	G	A	60	6	1
Chromosome06	98930568	G	A	60	6	1
Chromosome06	99282825	G	A	60	8	1
Chromosome06	99583834	G	A	60	7	1
Chromosome06	99667375	G	A	23	5	1
Chromosome06	99669084	G	A	30	7	1
Chromosome06	99673131	C	T	12	5	1
Chromosome06	99673142	C	T	16	6	1
Chromosome06	99673533	C	T	43	10	1
Chromosome06	99677265	G	A	37	5	1
Chromosome06	99689708	G	A	55	9	1
Chromosome06	99690594	G	A	60	5	1
Chromosome06	99706697	G	A	58	8	1
Chromosome06	99708827	G	A	50	5	1
Chromosome06	99709091	G	A	26	6	1
Chromosome06	99709118	G	A	27	6	1
Chromosome06	99734420	G	A	43	5	1
Chromosome06	99734798	C	T	42	5	1
Chromosome06	99735073	C	T	41	6	1
Chromosome06	99752620	C	T	60	6	1
Chromosome06	99753905	G	A	48	11	1
Chromosome06	99754102	G	A	47	6	1
Chromosome06	99754298	C	T	44	5	1

Chromosome06	99758051	G	A	54	8	1
Chromosome06	99758064	C	T	53	7	1
Chromosome06	99759268	G	A	42	12	1
Chromosome06	99759280	C	T	43	11	1
Chromosome06	99762356	C	T	10	5	1
Chromosome06	99762441	C	T	9	5	1
Chromosome06	99765903	G	A	45	7	1
Chromosome06	99765905	C	T	45	7	1
Chromosome06	99766134	C	T	57	8	1
Chromosome06	99766511	C	T	47	6	1
Chromosome06	99767054	C	T	60	9	1
Chromosome06	99768405	G	A	37	5	1
Chromosome06	99771211	G	A	50	5	1
Chromosome06	99771213	C	T	50	5	1
Chromosome06	99780438	C	T	31	5	1
Chromosome06	99780440	G	A	31	5	1
Chromosome06	99781579	G	A	52	6	1
Chromosome06	99781588	G	A	51	6	1
Chromosome06	99785851	G	A	31	5	1
Chromosome06	99786270	C	T	43	12	1
Chromosome06	99790643	G	A	57	7	1
Chromosome06	99793424	C	T	60	6	1
Chromosome06	99799310	C	T	34	5	1
Chromosome06	99815796	C	T	50	5	1
Chromosome06	99818501	G	A	44	5	1
Chromosome06	99828255	C	T	60	5	1
Chromosome06	99828288	G	A	60	6	1
Chromosome06	99831581	G	A	41	10	1
Chromosome06	99831583	G	A	42	9	1
Chromosome06	99831589	C	T	42	9	1
Chromosome06	99833172	G	A	44	5	1
Chromosome06	99833753	C	T	47	8	1
Chromosome06	99833759	C	T	45	7	1
Chromosome06	99833835	G	A	48	9	1
Chromosome06	99833843	C	T	48	9	1
Chromosome06	99834865	C	T	37	7	1

Chromosome06	99835814	C	T	58	6	1
Chromosome06	99836184	C	T	52	15	1
Chromosome06	99837808	G	A	35	5	1
Chromosome06	99848965	G	A	36	6	1
Chromosome06	99858602	G	A	29	5	1
Chromosome06	99861235	G	A	29	7	1
Chromosome06	99862349	G	A	37	5	1
Chromosome06	99862366	C	T	29	6	1
Chromosome06	99867438	G	A	55	5	1
Chromosome06	99867442	C	T	55	5	1
Chromosome06	99874694	G	A	37	5	1
Chromosome06	99876648	G	A	41	10	1
Chromosome06	99878428	C	T	37	5	1
Chromosome06	99880091	G	A	55	5	1
Chromosome06	99880769	G	A	37	5	1
Chromosome06	99885835	C	T	60	7	1
Chromosome06	99892331	C	T	54	8	1
Chromosome06	99892369	C	T	55	5	1
Chromosome06	99893766	C	T	45	10	1
Chromosome06	99894866	G	A	33	10	1
Chromosome06	99896167	G	A	37	5	1
Chromosome06	99896194	C	T	40	7	1
Chromosome06	99896402	G	A	52	9	1
Chromosome06	99896697	C	T	60	5	1
Chromosome06	99941335	C	T	56	6	1
Chromosome06	99941336	G	A	56	6	1
Chromosome06	99941467	G	A	33	6	1
Chromosome06	99941820	G	A	54	8	1
Chromosome06	99944560	G	A	37	5	1
Chromosome06	99944565	C	T	37	5	1
Chromosome06	99947304	G	A	29	5	1
Chromosome06	99948160	G	A	29	8	1
Chromosome06	99954292	G	A	50	11	1
Chromosome06	99954574	G	A	53	9	1
Chromosome06	99955427	G	A	52	6	1
Chromosome06	99960954	G	A	60	5	1

Chromosome06	99967980	G	A	29	5	1
Chromosome06	99969186	C	T	50	7	1
Chromosome06	99971761	C	T	44	5	1
Chromosome06	99974347	G	A	41	10	1
Chromosome06	99975280	G	A	36	6	1
Chromosome06	99975657	G	A	51	6	1
Chromosome06	99975661	C	T	51	6	1
Chromosome06	99975662	G	A	51	6	1
Chromosome06	99979221	G	A	46	6	1
Chromosome06	99979249	G	A	39	7	1
Chromosome06	99993815	C	T	47	6	1
Chromosome06	99994931	G	A	27	5	1
Chromosome06	100016035	G	A	36	9	1
Chromosome06	100016248	C	T	37	7	1
Chromosome06	100016251	C	T	37	7	1
Chromosome06	100016354	G	A	42	6	1
Chromosome06	100021341	G	A	56	6	1
Chromosome06	100032493	G	A	29	10	1
Chromosome06	100048990	C	T	53	8	1
Chromosome06	100069537	G	A	37	5	1
Chromosome06	100071004	C	T	40	7	1
Chromosome06	100101543	C	T	47	6	1
Chromosome06	100111577	G	A	44	5	1
Chromosome06	100111581	C	T	44	5	1
Chromosome06	100112225	G	A	47	6	1
Chromosome06	100112226	G	A	47	6	1
Chromosome06	100112227	C	T	47	6	1
Chromosome06	100112266	C	T	29	6	1
Chromosome06	100114606	G	A	36	5	1
Chromosome06	100126243	C	T	42	6	1
Chromosome06	100127753	C	T	40	7	1
Chromosome06	100185388	G	A	60	7	1
Chromosome06	100188328	C	T	23	8	1
Chromosome06	100188895	G	A	20	6	1
Chromosome06	100189337	G	A	40	7	1
Chromosome06	100190800	C	T	47	6	1

Chromosome06	100190801	G	A	47	6	1
Chromosome06	100193454	C	T	40	7	1
Chromosome06	100193457	G	A	40	7	1
Chromosome06	100201531	C	T	55	11	1
Chromosome06	100206160	C	T	56	8	1
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Chromosome06	100229289	G	A	55	10	1
Chromosome06	100306167	G	A	36	6	1
Chromosome06	100306171	C	T	29	5	1
Chromosome06	100306175	G	A	29	5	1
Chromosome06	100309244	C	T	35	7	1
Chromosome06	100309251	G	A	36	6	1
Chromosome06	100312509	G	A	44	5	1
Chromosome06	100321655	C	T	42	6	1
Chromosome06	100332632	C	T	60	6	1
Chromosome06	100339430	C	T	51	8	1
Chromosome06	100345941	G	A	44	5	1
Chromosome06	100345976	G	A	52	6	1
Chromosome06	100356901	G	A	60	6	1
Chromosome06	100487414	G	A	48	6	1
Chromosome06	100487426	G	A	45	5	1
Chromosome06	100500948	G	A	36	5	1
Chromosome06	100500963	G	A	35	6	1
Chromosome06	100500978	C	T	34	7	1
Chromosome06	100500981	C	T	27	6	1
Chromosome06	100505807	G	A	49	7	1
Chromosome06	100505813	G	A	49	7	1
Chromosome06	100514365	G	A	60	9	1
Chromosome06	101172827	G	A	29	6	1
Chromosome06	101172830	G	A	29	6	1
Chromosome08	82122825	C	T	38	7	1
Chromosome08	82122845	C	T	33	5	1
Chromosome08	82308185	G	A	40	10	1
Chromosome08	82344251	C	T	24	10	1
Chromosome08	82362014	C	T	48	5	1
Chromosome08	82364936	G	A	44	5	1

Chromosome08	82393763	C	T	35	6	1
Chromosome08	82393765	C	T	35	6	1
Chromosome08	82394016	G	A	45	7	1
Chromosome08	82394126	C	T	58	13	1
Chromosome08	82405560	G	A	30	5	1
Chromosome08	82407016	C	T	35	11	1
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Chromosome12	27551992	G	A	54	5	1
Chromosome12	27552038	G	A	43	5	1
Chromosome12	27557788	C	T	55	5	1
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Chromosome12	27680093	C	T	52	5	1
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Chromosome12	27795703	G	A	53	5	1
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Chromosome12	28061373	G	A	47	6	1
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Chromosome12	28214100	G	A	42	6	1
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Chromosome12	28256634	C	T	34	5	1
Chromosome12	28257466	C	T	39	5	1