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ORIGINAL ARTICLE

Genetic analysis of pungency deficiency in Japanese chili pepper 'Shishito' (*Capsicum annuum*) revealed its unique heredity and brought the discovery of two genetic loci involved with the reduction of pungency

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Abstract

The sensation of pungency generated by capsaicinoids is a characteristic trait of chili peppers (*Capsicum* spp.), and the presence or absence of pungency is central in determining its usage as a spice or a vegetable. In the present study, we aimed to clarify the heredity and genetic factors involved in the deficiency of pungency (quite low pungency) that is uniquely observed in the Japanese chili pepper 'Shishito' (*Capsicum annuum*). First, the F_2 population ('Shishito' × pungent variety 'Takanotsume') was used for segregation analysis, and pungency level was investigated using capsaicinoid quantification with high-performance liquid chromatography. Also, restriction site associated DNA sequencing of the F_2 population was performed, and genetic map construction and quantitative trait locus (QTL) mapping were implemented. The results indicated that the F_2 population showed varying capsaicinoid content and two major QTLs were detected, *Shql3* and *Shql7*, which explained 39.8 and 19.7% of the genetic variance, respectively. According to these results, the quite low pungency of 'Shishito' was a quantitative trait that involved at least the two loci. Further, this trait was completely separate from general non-pungent traits controlled by individual recessive genes, as described in previous studies. The present study is the first report to investigate the genetic mechanism of pungency deficiency in Japanese chili peppers, and our results provide new insights into the genetic regulation of pungency in chili pepper.

Keywords Chili pepper · Capsaicinoids · Non-pungency · QTL mapping · RAD-seq

Introduction

Chili peppers (*Capsicum* spp.) are members of the Solanaceae family and are characterized by their pungent taste. The sensation of pungency is derived from chemical constituents known as capsaicinoids (Suzuki and Iwai, 1984), and their amounts determine the pungency levels of chili pepper fruits and whether they are utilized as a spice or vegetable. Capsaicinoids are indispensable ingredients in food cultures around the world, e.g., as a spice, and recently their functional health effects, e.g., anti-inflammatory, antioxidant, antitumor, and weight-loss properties, have been a subject of focus (Naves et al. 2019). In chili pepper fruits, capsaicinoids are mostly biosynthesized in the epidermal cells of the placental septum (Fujiwake et al. 1982) and their biochemical and genetic mechanisms have been investigated. First, capsaicinoid biosynthesis has been shown to consist of two intermediate pathways using radiotracer studies (Bennett and Kirby 1968). One is the phenylpropanoid pathway that synthesizes vanillylamine from phenylalanine, and the other is the branched-chain fatty acid pathway that synthesizes a series of branched-chain fatty acid moieties from valine or leucine. Capsaicinoids are eventually generated by the condensation of vanillylamine and branched-chain fatty acids. In investigating the genetics of capsaicinoids, several genome and transcriptome analyses have profiled a host of genes involved in capsaicinoid biosynthesis (Kim et al. 2014; Martínez-López et al. 2014; Qin et al. 2014;

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Zhang et al. 2016). In particular, several genes have been identified as necessary in generating capsaicinoids. First, *Pun1*, which is located on chromosome 2, is the most critical gene for synthesis. This gene was once known as the *C* locus controlling the presence of pungency (Deshpande 1935), and the responsible gene was identified by Stewart et al. (2005). *Pun1* encodes an acyltransferase that is responsible for the final reaction of capsaicinoid biosynthesis, and mutation of the *Pun1* gene results in complete deficiency of pungency (Kirii et al. 2017; Stellari et al. 2010; Stewart et al. 2007). Moreover, *putative aminotransferase* (*pAMT*) is also critical as it is responsible for synthesizing vanillylamine from vanillin in the phenylpropanoid pathway (Lang et al. 2009). *pAMT* is located on chromosome 3 and its various gene mutations are known to cause a drastic reduction of pungency (Tanaka et al. 2019). In addition, Koeda et al. (2019) recently discovered a novel gene called *ketoacyl-ACP reductase* (*CaKR1*) located on chromosome 6. *CaKR1* is involved in branched-chain fatty acid elongation, and also is necessary for generating capsaicinoids in chili peppers. Besides these biosynthesis genes, Arce-Rodríguez and Ochoa-Alejo (2017) initially proposed that the R2R3-MYB transcription factor encoded by *CaMYB31* significantly affected the expression of capsaicinoid biosynthesis genes. Around the same time, Han et al. (2019) identified the locus (chromosome 7) and dysfunctional allele of *CaMYB31* (*Pun3*) that resulted in deficiency of pungency.

As mentioned above, *Pun1*, *pAMT*, *CaKR1,* and *CaMYB31* are qualitative genes responsible for the presence of pungency in chili peppers. Therefore, it is suggested that the mechanism of pungency deficiency in most chili peppers could involve the dysfunction of these four genes; however, several Japanese chili peppers could be exceptions. In Japan, there are several quite low pungency varieties of *C. annuum,* such as 'Shishito', 'Manganji' and 'Fushimi amanaga', which are utilized as vegetables. Although these varieties appear to be the same as typical non-pungent sweet chili peppers, they are distinguished by the occurrence of pungency. Specifically, capsaicinoids are never or hardly synthesized in general non-pungent varieties due to the dysfunctional genes, and the occurrence of pungency is rarely observed. Conversely, Japanese non-pungent varieties are known to become pungent depending on environmental factors, and the presence of these pungent fruits are known to cause problems in cultivation and distribution in Japan (Ishikawa et al. 2004; Minamiyama et al. 2012; Murakami et al., 2006). Our research group investigated this phenomenon using the variety 'Shishito' and that revealed parthenocarpy, lack of seeds fruits, was related to the occurrence of pungency (Kondo et al. 2021a, b). We also observed that several capsaicinoid biosynthesis genes, including *Pun1*, *pAMT*, *CaKR1,* and *CaMYB31*, are hardly expressed in non-pungent 'Shishito' fruits but highly expressed in pungent fruits. This implied that the loss of pungency in 'Shishito' was not dependent on dysfunctional gene mutation in the capsaicinoid biosynthesis pathways described above. Therefore, these Japanese varieties presumably repress their pungency according to unique mechanisms. In an effort to clarify these mechanisms, we focused on the quite low pungency trait observed in 'Shishito' and investigated the heredity and genetic factors associated with pungency. In the present study, we prepared a F_2 population ('Shishito' × pungent variety 'Takanotsume'), and the pungency level was investigated by quantifying the capsaicinoid content. Next, we implemented quantitative trait locus (QTL) mapping involving pungency level using the F_2 population, and investigated the candidate genetic loci responsible for pungency deficiency in 'Shishito'.

Materials and methods

Plant materials

For the segregation analysis of a low pungency in 'Shishito' (*C. annuum*), we constructed F_1 progeny by crossing 'Shishito' and the pungent *C. annuum* variety 'Takanotsume', with the F_2 progenies being obtained by self-pollination. Plants were field-cultivated in 2020 and 2021 at a farm at Shinshu University (Nagano, Japan). In each year, ten F_1 individuals and 160 F_2 individuals were cultivated. Further, two kinds of F_1 progeny were generated by crossing 'Shishito' and two non-pungent cultivars: 'Shishito' × 'California Wonder' (*C. annuum*), which is a completely non-pungent cultivar owing to the dysfunctional *Pun1* allele (*pun1*[1]), and 'Shishito' × 'Himo' (*C. annuum*), which exhibits quite low pungency attributable to a dysfunctional *pAMT* allele (*pamt*[2]). Ten F_1 plants were cultivated for each cross in 2020 only.

Capsaicinoid extraction and quantification

For phenotyping of pungency traits in several progenies, we performed high-performance liquid chromatography (HPLC) to quantify capsaicinoid content in the placental septum. To normalize the stage of fruit development, we referenced the color of the fruit surface and only collected fruits in which the color began to change from deep green to brown. Capsaicinoids were extracted from grouped placental septa of ten fruits per plant. In the capsaicinoid extraction, the placental septa were initially lyophilized using a freeze-drier (FDU-200, EYELA, Tokyo, Japan). The capsaicinoids were then extracted from 200 mg of the powdered dried tissue using acetone, and the contents were quantified by HPLC as described by Kondo et al. (2021a). In the present study, capsaicinoids were defined as the total contents of

capsaicin and dihydrocapsaicin, then the capsaicinoid content per unit dry weight of placental septa (μg • gDW^{-1}) was calculated.

RAD-seq and genotyping of the F_2 population

Genetic analysis was conducted by restriction site associated DNA sequencing (RAD-seq) using 104 F_2 individuals that were randomly selected from a total of 160 F_2 progenies in 2020. As for their two parents, three plants were used for RAD-seq respectively. gDNA was extracted from young leaves of individual plants using a DNeasy Plant Pro Kit (Qiagen, Hilden, Germany). Library construction for Next Generation Sequencing (NGS) was performed by digesting gDNA with *EcoR*III and *EcoR*I and subsequently ligating adaptors with different barcodes. On average, 400 bp DNA fragments were obtained and pair-end sequencing was performed using a HiseqX (Illumina, San Diego, CA, USA). After sequencing, adaptor trimming and quality filtering were conducted using Fastp v0.20.0 (Chen et al. 2018), and sequence reads with quality values less than Q15 were trimmed. Burrows-Wheeler Alignment (BWA v0.7.8; Li and Durbin 2009) was used to map the trimmed sequence reads to the *C. annuum* genome of 'Zunla 1' (Ref_v1.0), and variant call was subsequently performed using the Genome Analysis Toolkit (GATK v4.1.2.0; McKenna et al. 2010). The raw genotyped data were then filtered according to the following criteria: QD < 2.0, QUAL < 30.0, SOR > 4.0, FS > 60.0, MG < 40.0, MQ < 40.0, MQRankSum < − 12.5, ReadPosRankSum < − 8.0. Finally, we obtained Single Nucleotide Polymorphism (SNP) data in a Variant Calling Format (VCF) file. To determine the genotype of the F_2 progenies, we screened parents-endemic SNPs from the VCF files of the two parents using TASSEL 5 (Bradbury et al. 2007), then the SNPs genotyped in less than 70% of F_2 progenies (73 individuals) were removed. Consequently, a total of 17,085 SNPs were obtained that distinguished the genotype of the parents.

Genetic map construction and QTL mapping involved in pungency level

QTL mapping of pungency level factors was performed by constructing a linkage map using the R package 'qtl' (Broman et al. 2003). Initially, the segregation suitability of each SNP in the F_2 population was investigated using the chi-square test, and SNPs in which segregations corresponded to the expected ratio (1:2:1, $p > 0.01$) were identified. A genetic map was then constructed using the Kosambi function according to the following criteria: maximum recombination fraction of 0.35, and threshold of LOD score of 3.0. Subsequently, QTL mapping of capsaicinoid content (μg • gDW^{-1}) was also conducted with the R package 'qtl'. Mapping was performed using the logarithm of the odds (LOD) score calculated by composite interval mapping (CIM), and the threshold was determined by 1000 permutation tests at a significance level of $p < 0.05$. We detected QTLs based on the threshold and estimated the additive effect, dominant effect, and genotypic variance explained (PVG) for each QTL. Furthermore, the physical positions of detected QTLs were compared with those of known QTLs and capsaicinoid biosynthesis genes. The physical positions of the detected QTLs in the 'Zunla 1' genome were converted to those in the 'CM334' genome (Pepper.v.1.55) using the genome browser of the Solanaceae Genomics Network (https://solgenomics.net) and the physical position data of capsaicinoid biosynthesis genes and known QTLs in the 'CM334' genome which was integrated by Han et al. (2018), was referenced. Finally, we merged the physical position data of the known QTLs and the QTLs detected herein; the merged data are listed in Tables S1, S2.

Verification of genotypic effects in the vicinity of candidate QTLs

In the present study, two high LOD peaks were observed on chromosomes 3 and 7, and we designed a Cleaved Amplified Polymorphic Sequences (CAPS) marker and Amplification Refractory Mutation System (ARMS) markers adjacent to the two loci, respectively. Details of the marker design method are described in Figs. S1, S2, and the primer sequences employed are listed in Table S3. Using these two markers, the genotypes of the F_2 progenies were investigated. In 2020, 56 F_2 individuals that were not used for RAD-seq were investigated, and 160 plants cultivated in 2021 were subsequently investigated.

Genomic variant analysis in several capsaicinoid biosynthesis genes

We targeted seven capsaicinoid biosynthesis genes (*ketoacyl-acyl carrier protein synthase III* (*KASIII*), *4-coumaroyl coenzyme A ligase* (*4CL*), *caffeoyl shikimate esterase* (*CSE*), *hydroxycinnamoyl transferase* (*HCT*), *CaMYB31*, *Pun1*, and *pAMT*) located mainly around the two detected QTLs, and explored the gene mutations in their isoforms reported in previous studies (Table S2). First, the gDNAs of 'Shishito' and 'Takanotsume' were subjected to whole-genome resequencing by a sequencing service (Macrogen Japan, Tokyo, Japan); on average, 350 bp DNA fragments were applied for pair-end sequencing using a NovaSeq 6000 (Illumina, San Diego, CA, USA). Sequence reads were subsequently mapped to the 'Zunla 1' genome as described in the RAD-seq analysis, and genomic variants were detected by two steps. First, we broadly explored large Indels within a 40 kbp genomic region including the gene isoform at the center

using Pindel v0.2.5 (Ye et al. 2009), which can detect structure variants (SVs) based on the mapping files (BAM format files). In the analysis, we detected variants that were supported by at least 4 reads (read coverages). Then, we specified Indels and SNPs in the narrow region ranging from the gene upstream region (2000 bp from the initial point of transcription) to the 3' untranslated region (UTR), using not only Pindel v0.2.5 but also GATK v4.1.2.0 as described in the RAD-seq analysis. Among the detected variants, those harboring polymorphism between 'Shishito' and 'Takanotsume' were selected. Then, we trimmed the variants that were non-allelic between 'Shishito' and 'Zunla 1' (pungent variety), as these variants rarely seemed to induce gene mutations that resulted in dysfunction.

Results

Pungency traits in 'Shishito' and the various F_1 progenies

Capsaicinoid contents in 'Shishito' fruits were 218 and 424 µg • gDW^{-1}, while those in 'Takanotsume' were 23,108 and 35,005 µg • gDW^{-1} in 2020 and 2021, respectively (Table 1). While the contents varied among individuals in both cultivars, pungency levels of 'Shishito' were at least 30 times lower than 'Takanotsume' in both years. The F_1 progenies 'Shishito' × 'Takanotsume' had 10,820 and 13,602 µg • gDW^{-1} of capsaicinoid contents in 2020 and 2021, respectively, which were in between those of the two parents. In comparison, the F_1 progenies 'Shishito' × 'California Wonder' [$pun1^1/pun1^1$] showed 118 µg • gDW^{-1} of capsaicinoid content, which was intermediate pungency level relative to the two parents. Conversely, the F_1 progenies 'Shishito' × 'Himo' [$pamt^2/pamt^2$] contained 12,541 µg • gDW^{-1}, which largely exceeded their parents.

Pungency traits in the F_2 population of 'Shishito' × 'Takanotsume'

In each cultivation year, the F_2 population 'Shishito' × 'Takanotsume' showed varying capsaicinoid contents; contents were in the range of 113–35,211 µg • gDW^{-1} in 2020 and 476–37,074 µg • gDW^{-1} in 2021. This indicated that the low pungency trait of 'Shishito' was a quantitative trait. Then, multiple peaks were observed in the frequency distribution (Fig. 1). In 2020, three clear peaks were observed at 800, 12,000, and 20,000 µg • gDW^{-1}, whereas the frequency distribution in 2021 showed two peaks at 5950 and 17,850 µg • gDW^{-1}.

QTL mapping related to pungency level

QTL mapping was conducted using 104 F_2 individuals ('Shishito' × 'Takanotsume') cultivated in 2020. A genetic map was constructed using 221 SNPs obtained by RAD-seq, which consisted of 12 linkage groups with a total length of 2,750 cM and the mean distance of the marker intervals was 13.2 cM. QTL mapping detected two significant LOD peaks ($p < 0.05$) (Fig. 2). One peak showed an LOD of 6.96 within 50.0–71.0 cM on chromosome 3, and the other peak had an LOD of 6.69 within 202.0–225.0 cM on chromosome 7; these major QTLs were named *Shql3* and *Shql7,* respectively (Table 2). The physical position of *Shql3* was in the marker interval between ST3.4 and ST3.5 (5,167,786–20,615,780 bp on chromosome 3), while *Shql7* was located in the marker interval between ST7.9–ST7.13 (218,675,433–221,842,745 bp on chromosome 7). Assessment of the genetic effects indicated that the additive effect and dominant effect of *Shql3* were − 6294 and − 646, while those of *Shql7* were − 4155 and 2220, respectively. The genotypic variance explained (GVE) for *Shql3* and *Shql7* was 39.8 and 19.7%, respectively. This implied that the 'Shishito'

Table 1 Pungency traits of chili pepper varieties and their F_1 progenies

Parents and cross combination of F_1 progenies	Cultivation year	Population size (n)	Capsaicinoid content (µg • gDW^{-1})	
			Mean ± SD[a]	Range
Shishito	2020	3	218 ± 95.7	143–353
	2021	5	424 ± 194	173–764
Takanotsume	2020	4	23,108 ± 9160	13,270–35,939
	2021	5	35,005 ± 7948	25,101–43,348
Himo	2020	3	81.8 ± 18.2	56.1–95.4
California Wonder	2020	3	ND	
Shishito × Takanotsume F_1	2020	10	10,820 ± 4798	4728–21,895
	2021	10	13,602 ± 3520	7106–20,221
Shishito × Himo F_1	2020	10	12,541 ± 3449	8150–19,647
Shishito × California Wonder F_1	2020	10	118 + 168	8.38–545

[a]Standard deviation

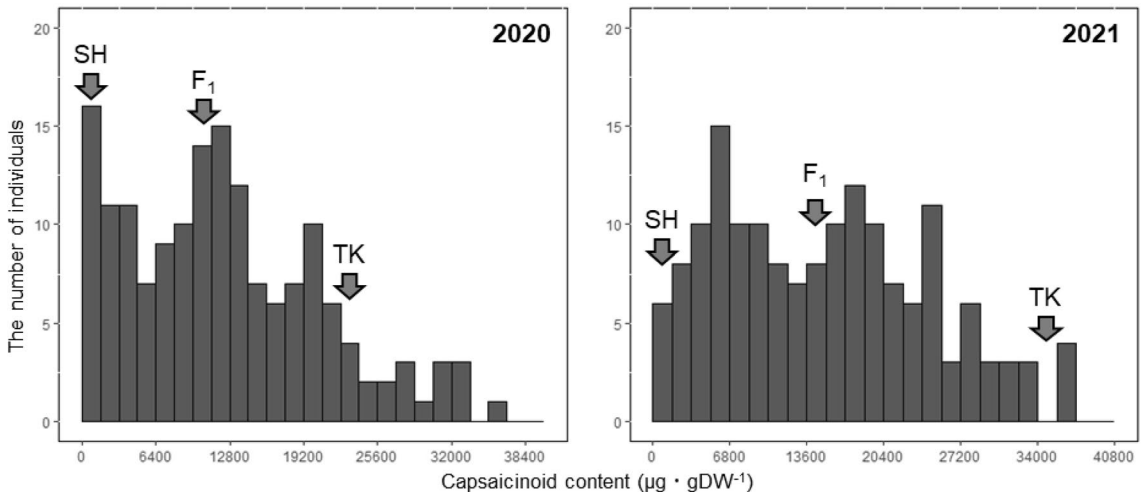

Fig. 1 Frequency distribution of capsaicinoid content in an F_2 population ('Shishito' × 'Takanotsume') cultivated in 2020 and 2021. Arrows labeled SH, F_1, and TK indicate the mean values in Shishito, F_1 progenies, and Takanotsume, respectively

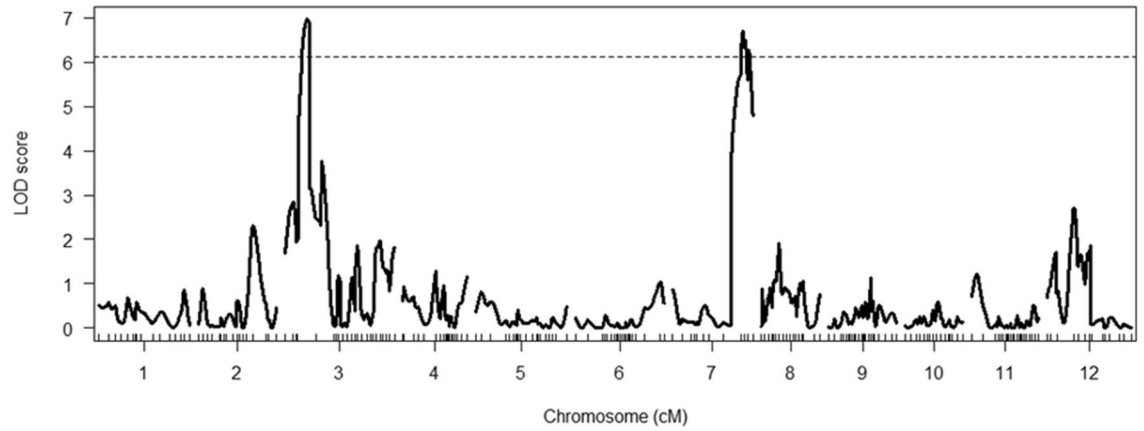

Fig. 2 Logarithm of the odds (LOD) scores calculated by the quantitative trait locus mapping related to capsaicinoid content in the F_2 population in 2020 ($n = 104$). The black line indicates LOD scores in the composite interval mapping, and the black dotted line is the threshold determined by 1000 permutation tests at a significance level of $p < 0.05$

Table 2 QTLs involved with capsaicinoid concentration, which were detected in F_2 population

QTL name	Linkage group (chromosome)	Locus[a] (cM)	LOD score	Marker interval[b] (bp)	Additive effect	Dominant effect	GVE[c] (%)
Shql3	3	50.0–71.0	6.96	5,167,786–20,615,780	− 6294	− 646	39.8%
Shql7	7	202.0–225.0	6.69	218,675,433–221,842,747	− 4155	2220	19.7%

[a]Confidence interval in the genetic map, which fulfills the LOD thresholds ($p < 0.05$)

[b]Ranges among physical positions of the two genetic markers, which included the confidence intervals in the genetic map

[c]Genotypic variance explained by QTL

alleles of *Shql3* and *Shql7* were two of all genetic factors related to the low pungency trait of 'Shishito'.

In proximity to the *Shql3* and *Shql7* loci, we investigated known QTLs and capsaicinoid biosynthesis genes involved in the amount of capsaicin, dihydrocapsaicin, nordihydrocapsaicin or total capsaicinoid content. Figure 3a, b shows the physical positions of known QTLs on chromosomes 3 and 7 in the 'CM334' genome. Nine QTLs exist along with four capsaicinoid biosynthesis genes in proximity to *Shql3* (237–252 Mbp, chromosome 3) (Fig. 3a). Notably,

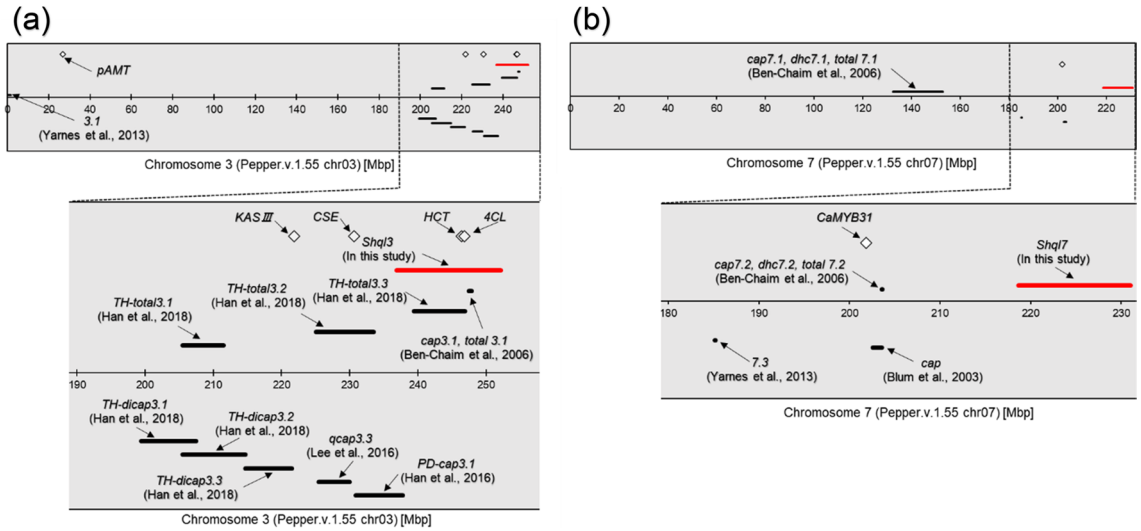

Fig. 3 Physical position of known quantitative trait loci (QTLs) and capsaicinoid biosynthesis genes around *Shql3* and *Shql7*. The physical positions in chromosomes 3 **a** and 7 **b** of the 'CM334' genome (Pepper.v.1.55). Horizontal lines indicate the positional range of QTLs, and diamonds indicate those of capsaicinoid biosynthesis genes. The complete data are also shown in Tables S1, S2

TH-total3.3 (Han et al. 2018), *cap3.1* • *total3.1* (Ben-Chaim et al. 2006), *4CL*, and *HCT* were within the *Shql3* region. Additionally, in proximity to *Shql7* (219–231 Mbp, chromosome 7), three QTLs were located along with *CaMYB31*, which were more than 15 Mbp from that of *Shql7* (Fig. 3b).

Effect of genotypes linking *Shql3* and *Shql7* to pungency level

To verify the genotypic effects of *Shql3* and *Shql7*, we determined the genotypes in the vicinity of the two loci and investigated the relationship between these genotypes and capsaicinoid contents in the F_2 population. Figure 4a illustrates the capsaicinoid contents in the three genotypes for each locus. Over two cultivation years, the capsaicinoid content in the F_2 population changed depending on the genotypes of both loci. Regarding *Shql3*, the capsaicinoid contents were respectively 17,817 and 18,245 μg • DW^{-1} in the homozygous allele of 'Takanotsume' (TK/TK), while those in the homozygous allele of 'Shishito'(SH/SH) were 7958 and 10,658 μg • DW^{-1} in 2020 and 2021. As for *Shql7*, TK/TK individuals showed 16,003 and 18,556 μg • DW^{-1}, while SH/SH individuals showed 6444 and 11,741 μg • DW^{-1} in 2020 and 2021, respectively. Integrating the above, the genotypic change from TK/TK to SH/SH in *Shql3* resulted in 55.3 and 41.6% reduction of capsaicinoid in the F_2 population. Meanwhile, 59.7 and 36.7% reductions were observed in the case of *Shql7*. Additionally, there was a significant positive correlation ($p < 0.001$) in the average contents of capsaicinoid between the two years (Fig. 4b); the contents became gradually lower along with the increase in the number of 'Shishito' alleles in each locus. This demonstrates that the genotype of *Shql3* and *Shql7* certainly affected pungency levels regardless of differences in the cultivation environment.

We also investigated the relationship between capsaicinoid content and genotypic combinations of the two loci in the F_2 population (Fig. 5a). In 2020, we clearly observed genotypic effects of *Shql3,* i.e., the SH allele resulted in reductions of capsaicinoid contents for each *Shql7* genotype. Similarly, possessing the SH/SH genotype of *Shql7* also resulted in reductions in all of the *Shql3* genotypes. Accordingly, both genotypes seemed to independently change the pungency levels, and extraordinary epistasis was not observed in 2020. A similar tendency was observed in 2021, but the phenotypic changes were sometimes unclear (Fig. 5a). For example, in the TK/TK individuals of *Shql7*, capsaicinoid contents hardly differed among the three *Shql3* genotypes. Moreover, compared to 2020, large differences in the capsaicinoid contents between SH/SH and other *Shql7* genotypes were not observed in the TK/TK genotype of *Shql3*. However, a significant positive correlation ($p < 0.001$) in the average contents between the two years was observed (Fig. 5b), indicating similarity of their genetic effects in both years. Notably, the F_2 individuals possessing both TK/TK genotypes in the two loci showed 21,153 and 20,996 μg • gDW^{-1} of capsaicinoid in each year, which decreased to 5290 and 8998 μg • gDW^{-1}, respectively, when both loci were replaced with SH/SH genotypes. This corresponded to 75.0 and 57.1% reductions in capsaicinoid contents, respectively, which were large reductions compared to the case of the independent locus alone, as described above.

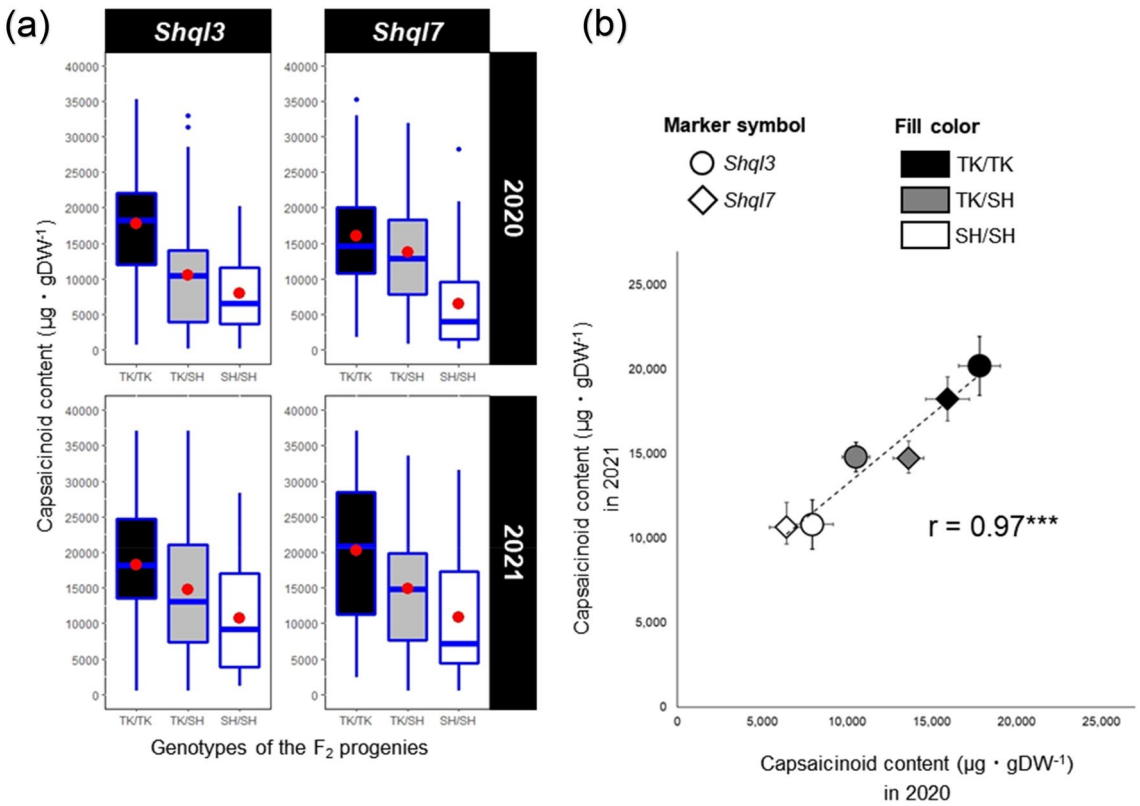

Fig. 4 Genotypic effects of *Shql3* and *Shql7* on the capsaicinoid contents in the F_2 population in 2020 and 2021. **a** Boxplots show capsaicinoid contents in three genotypes of each locus and red circles indicate the mean values. **b** The scatter plot shows a comparison of the average contents between the two years. TK/TK, TK/SH, and SH/SH denote the homozygous allele of 'Takanotsume', heterozygous, and homozygous allele of 'Shishito', respectively. In the scatter plot, values indicate the mean ± standard error in each year, and their genotypes are represented by combinations of marker color and symbol. The Pearson's correlation coefficient is shown as *r*. ***$p < 0.001$

Genomic variants in several capsaicinoid biosynthesis genes

As another attempt to explore the genetic factors involved in the quite low pungency of 'Shishito', we implemented whole-genome resequencing of 'Shishito' and 'Takanotsume', and explored genomic mutations in known capsaicinoid biosynthesis genes (*KASIII*, *4CL*, *CSE*, *HCT*, *CaMYB31*, *Pun1*, and *pAMT*), which, except for *Pun1* and *pAMT,* were located mainly around *Shql3* and *Shql7*. In the whole-genome resequencing, a total of 436,167,062 and 603,425,568 reads were obtained for 'Shishito' and 'Takanotsume' after filtering, respectively, which were equivalent to approximately 65.8 and 91.0 Gb. Then, genomic variants in 'Shishito' were detected within the 40 kbp region, including the gene isoform at the center, which were allelic with both 'Takanotsume' and 'Zunla 1' (Table 3). At this scale, several large Indels with variant sizes that exceeded 100 bp were observed in 'Shishito'; however, such Indels were never observed in the narrow genomic region upstream of the gene (− 2000 bp from the transcription start site) to the 3'UTR region (Table 3). When we focused on this region, most of the variants existed upstream of the gene or in introns at modifier levels (Tables S4, S5), which rarely caused mutations in the splice acceptor or donor sites. Then, only nine variants were detected in exons, including seven SNPs and two Indels (Table 4). Among the nine variants, most were in the 3'UTR and 5'UTR or synonymous substitution in exons, which could never affect the coding sequences of amino acids after translation. On the other hand, two significant variants were observed in *CSE* and *pAMT*. First, 'Shishito' had a 5 bp deletion in the 5'UTR region in one of the CSE isoforms (XM_016710287), which could induce a frameshift mutation. However, another *CSE* isoform (XM_016710288) never showed such variants. Meanwhile, one SNP at exon 15 of *pAMT* could cause a non-synonymous substitution in 'Shishito'.

Discussion

Chili pepper varieties can be broadly defined as either a spice or a vegetable depending on the presence of pungency. Thus, the genetic regulation of pungency has been the focus of many researchers. According to previous studies, the

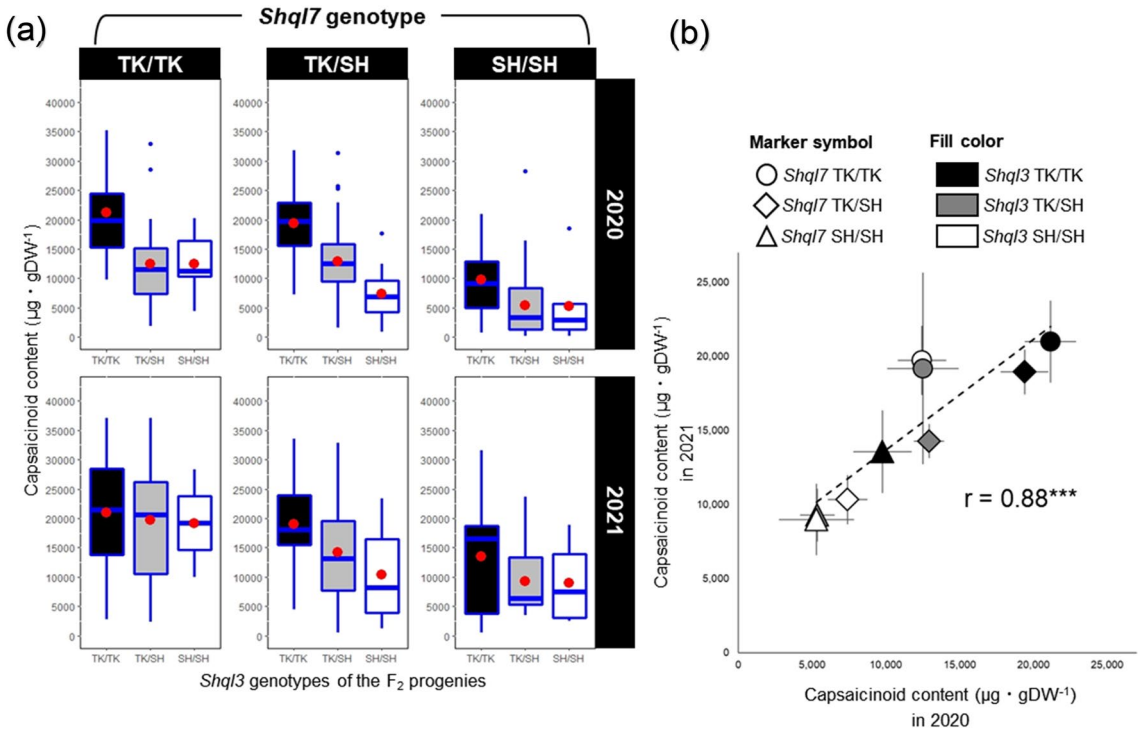

Fig. 5 Effects of the genotypic combination of *Shql3* and *Shql7* on capsaicinoid contents in the F_2 population in 2020 and 2021. **a** Boxplots show capsaicinoid contents in the nine genotypic combinations, and red circles indicate the mean values. **b** The scatter plot shows a comparison of the average contents between two years. TK/TK, TK/SH, and SH/SH denote the homozygous allele of 'Takanotsume', heterozygous, and homozygous allele of 'Shishito' respectively. In the scatter plot, values indicate the mean ± standard error in each year, and their genotypes are represented by combinations of marker color and symbol. *r* shows Pearson's correlation coefficients. ***$p < 0.001$

presence of pungency is known as a qualitative trait that is controlled by individual recessive genes; four genes: *Pun1*, *pAMT*, *CaKR1,* and *CaMYB31* were discovered as the related genes (Stewart et al. 2005; Lang et al. 2009; Koeda et al. 2019; Han et al. 2019). The recessive alleles could have been used for breeding non-pungent cultivars, i.e., the genetic mechanism of pungency deficiency might be explained by the effect of any of the genes in most sweet cultivars. However, our research groups previously proposed the feasibility of the Japanese variety 'Shishito' as an exception, due to the occurrence of highly pungent fruits that were rarely observed in non-pungent cultivars (Kondo et al. 2021a, b). Therefore, we attempted to clarify the genetic regulation of pungency deficiency in 'Shishito' by investigating the inheritance and genetic factors of pungency.

In the present study, segregation analysis of pungency traits using F_1 and F_2 progenies ('Shishito' × 'Takanotsume') revealed that pungency deficiency in 'Shishito' was a quantitative trait. Thus, this trait is completely separate from qualitative traits controlled by recessive single genes such as those described above. This was also assisted by phenotypic analysis in other F_1 progenies. The results showed that the 'Shishito' × 'Himo' F_1 progenies exhibited obviously higher capsaicinoid contents than their parents due to genetic complementation, demonstrating that 'Shishito' possessed a functional *pAMT*, although 'Shishito' had several genomic variants compared to 'Takanotsume' (Tables 3, 4, S4, S5). Further, capsaicinoids were also detected in 'Shishito' × 'California Wonder' F_1 progenies, at a level not exceeding that in 'Shishito'. Although it could not be determined that 'Shishito' possessed a functional *Pun1* allele according to this result alone, it is possible that 'Shishito' has a normal allele. This is because $pun1^1$ of 'California Wonder' is known as an allele that results in a complete deficiency of pungency (Stewart et al. 2005). Then, we were unable to detect a significant QTL in chromosome 2 where *Pun1* is located (Fig. 2), and significant genomic mutations were never observed in *Pun1* and the proximal region in 'Shishito' (Table 3). Accordingly, it was considered that 'Shishito' possessed either a functional and semi-functional *Pun1* allele, except for $pun1^1$ at least, and *Pun1* or *pAMT* might not be a major genetic factor in the low pungency of 'Shishito'.

To elucidate the genetic factors of low pungency in 'Shishito', we implemented QTL mapping of capsaicinoid content in the F_2 population. As the result, we identified two loci involved in the reduction of capsaicinoid content, designated *Shql3* and *Shql7* (Fig. 2, Table 2). The total GVE of

Table 3 The number of genomic variants based on whole-genome resequencing in the seven capsaicinoid biosynthesis genes

Variant region	Variant detection method	Variant type	Gene name						
			CaMYB31	*KAS III*	*4CL*	*CSE*	*HCT*	*pAMT*	*Pun1*
						XM_016710287[a]	XM_016710288[a]	XM_016707492[a]X M_016707493[a]	
Broad region (40 bp region surrounding the gene)	SV detection by Pindel	Deletion			1[b] (1)	18 (1–56)		9 (1–4,397)	1 (10)
		Insertion			1 (14)	9 (1–27)		2 (54–119)	4 (1–2)
Narrow region (Upstream of the gene to the 3'UTR region)	SV detection by Pindel	Deletion			1 (1)	7 (1–56)	4 (1–56)	5 (1–8)	
		Insertion			1 (14)	2 (1–27)	1 (1)	2 (1–2)	
	SNP/Indel detection by GATK	SNP	4		6	22	17	21	
		Deletion		1 (1)	1 (1)	6 (1–12)	3 (1–5)	3 (1–4)	
		Insertion		1 (1)	1 (14)	2 (1–27)	1 (1)	11 (1–16)	

[a]Multiple gene isoforms (Gene accession in 'Zunla 1' genome database (Ref_v1.0)) in *CSE* and *HCT*

[b]Number of unique variants in 'Shishito' that are allelic with both 'Takanotsume' and 'Zunla 1'. Numbers in parentheses indicate ranges of the variant size (bp)

the two loci was approximately 60%, suggesting that *Shql3* and *Shql7* were major genetic factors responsible for the pungency level of the F_2 population. Although several QTLs related to pungency level have been elucidated in previous studies, most were identified from QTL analysis using interspecific crossed progenies, such as *C. annuum* × *C. frutescens* and *C. annuum* × *C. chinense* combinations (Blum et al. 2003; Ben-Chaim et al. 2006; Yarnes et al. 2013; Lee et al. 2016; Han et al. 2018). Therefore, it should be mentioned that the present QTLs derived from intraspecific crossed progenies (*C. annuum* × *C. annuum*) seem to be uncommon. Also, when we considered their high GVEs, there is a high possibility that the responsible genes will be identified in a future study. Moreover, the genetic effects of these loci were verified regardless of differences in the cultivation environment via genotyping analysis in the vicinity of *Shql3* and *Shql7* (Fig. 4a, b). Then, it was revealed that the 'Shishito' allele of the two loci additively decreased capsaicinoid contents in the absence of obvious epistasis (Fig. 5a, b). Therefore, it was suggested that changes in the pungency level of the F_2 population resulted mainly from the independent accumulation of *Shql3* and *Shql7* alleles. However, some exceptions were also observed in 2021 with several genotypic combinations (Fig. 5a); therefore, the presence or absence of epistasis among the two loci remains controversial.

Finally, we arranged the existence of QTLs around *Shql3* and *Shql7*. Surprisingly, the edge of chromosome 3 was the QTLs-enriched region which was contained not only *Shql3* but also various QTLs related to the capsaicinoid content, regardless of differences in the mapping population used in individual QTL analyses (Fig. 3a, Table S1). This suggested that the edge of chromosome 3 was one of the critical regions involved in the regulation of pungency levels in chili peppers, which is indirectly supported by the high density of capsaicinoid biosynthesis genes: *KASIII*, *4CL*, *CSE*, *HCT* (Fig. 3a, Table S2). Relatively fewer QTLs seemed to be in proximity to *Shql7*, with only *CaMYB31,* which is responsible for the presence of pungency, being in the vicinity (Fig. 3b, Tables S1, S2). These five proximal capsaicinoid biosynthesis genes (*KASIII, 4CL, CSE, HCT,* and *CaMYB31*) were considered to be the responsible genes for the two loci; thus, we conducted whole-genome resequencing and explored their genomic variants in 'Shishito'. As the result, significant variants that apparently induced gene dysfunction were not observed in most genes (*KASIII, 4CL, HCT*, and *CaMYB31*), although they had various Indels and SNPs in the upstream region and in introns at modifier levels (Tables 3, S4, S5). This implied the normality of their gene functions, which was supported by our previous studies. Our research group previously revealed that *4CL* and *HCT*, located in the physical range of *Shql3*, were normally expressed in non-pungent 'Shishito' fruit; moreover, their

Table 4 Genomic variants[a] based on whole-genome resequencing in the exon region of seven capsaicinoid biosynthesis genes

Gene	Variant detection method	Variant type	Physical position in Zunla 1 genome (Ref_v1.0)		Variant region	Estimated variant effect
			Chromosome	Position (bp)		
KAS III	SNP/Indel detection by GATK	Deletion (1)[c]	NC_029979.1	32,473,270	3'UTR	–
CSE (XM_016710287[b])	SV detection by Pindel & SNP/Indel detection by GATK	Deletion (5)[c]	NC_029979.1	232,133,416-232,133,420	5'UTR	Frameshift mutation
	SNP/Indel detection by GATK	SNP	NC_029979.1	232,133,426	5'UTR	–
		SNP	NC_029979.1	232,133,433	5'UTR	–
		SNP	NC_029979.1	232,133,448	5'UTR	–
pAMT	SNP/Indel detection by GATK	SNP	NW_015961157.1	213,690	Exon12	Synonymous substitution
		SNP	NW_015961157.1	212,124	Exon15	Synonymous substitution
		SNP	NW_015961157.1	212,123	Exon15	Non-synonymous substitution (A→G)
		SNP	NW_015961157.1	210,407	3'UTR	–

[a]Unique variants in 'Shishito' that are allelic with both 'Takanotsume' and 'Zunla 1'

[b]Gene isoform (Gene accession in 'Zunla 1' genome database (Ref_v1.0)) in *CSE*

[c]The deletion size (bp)

expression levels had no significant correlation with capsaicinoid content when 'Shishito' experienced pungency fluctuation (Kondo et al. 2021a, b). In contrast, *KASIII* and *CaMYB31* (linked to *Shql3* and *Shql7,* respectively) showed low expression in non-pungent 'Shishito' fruits and high expression in pungent fruits, thereby implying they were functional (Rathnayaka et al. 2021; Kondo et al. 2021b). Therefore, it was difficult to definitively determine that these genes were responsible for *Shql3* and *Shql7*. However, transcriptional levels do not always correspond with the phenotype in the case of nonsense-mediated mRNA decay (Shaul 2015), or detected Indels and SNPs in the upstream region and in introns of these genes could furtively affect their function; thus, our hypothesis should be thoroughly investigated in future studies. On the other hand, we remarkably found one isoform of *CSE* (XM_016710287) in 'Shishito' with a probable frameshift mutation due to the 5 bp deletion in the 5'UTR region (Table 4). Thus, this variant was considered as a candidate gene of *Shql3*, while another isoform (XM_016710288) did not show such mutation, and it was also considered that this isoform retained its normal function. To demonstrate validity, transcriptional and gene function analyses via reverse genetic approaches will be necessary. Regardless, since it was difficult to determine the responsible genes for *Shql3* and *Shql7* in the present study, further narrowing of their candidate genomic regions is required. Actually, more than 800 and 200 genes were located on *Shql3* and *Shql7,* respectively, based on the 'Zunla 1' genome database (data not shown), indicating that further precise and elaborate QTL mapping will be necessary for screening candidate genes.

In conclusion, the present study was the first to investigate the inheritance and genetic factors of pungency deficiency in Japanese chili peppers. The present results provide novel insights regarding the genetics of pungency traits in *Capsicum* spp. The absence of pungency in chili peppers has been generally acknowledged as a qualitative trait controlled by individual recessive genes; however, the present study has newly identified an exception. The results of our study will provide helpful information for further research in clarifying the genetic regulation of pungency in chili peppers.

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