Original Contribution

INFLUENCE OF THE PUN1 GENE ON CAPSAICIN SYNTHESIS IN HYBRID LINES OF THE GENUS CAPSICUM

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ABSTRACT

The fruits of the Capsicum genus plants are valued for their spicy taste, unique only to them, due to the capsaicin alkaloid and its analogs, named capsaicinoids. Capsaicinoids are absent in sweet peppers, which are sometimes preferred due to the lack of pungency. Their presence or absence is a genetically controlled process, and many of the genes that affect capsaicin synthesis are known. The *Pun1* gene plays a lead, and mutations in this locus are the most common cause of loss of pungency in the three related species *Capsicum annuum*, *Capsicum chinense*, and *Capsicum frutescens*. In the present study, we analyzed the effect of the *Pun1* gene on the synthesis of capsaicin in hybrid lines of the genus *Capsicum*. This analysis was performed by comparing the fruit spiciness profile (determined organoleptically) and the allelic state of the *Pun1* gene (determined by PCR reaction, using allele-specific primers). The comparative analysis confirms our hypothesis that the pungent and lack of pungent in the selected hybrid lines is entirely controlled by the action of this *Pun1* gene only.

Key words: *Capsicum*, *Pun1*, *pun1-1*, Hybrid lines, Pungent Pepper, Non-pungent Pepper

INTRODUCTION

The pungency of pepper fruits is due to a group of alkaloid compounds - capsaicinoids, synthesized only in the species *Capsicum* (1-3). After biosynthesis, capsaicinoids are secreted into the outer epidermal cells of the fetus and accumulate in structures called vesicles "blisters" located on the surface of the placenta that traps the seeds. Although seeds are not the source of hotness, they sometimes absorb capsaicinoids due to their proximity to the placenta (4-7). No other plant part produces capsaicinoids. They give a feeling of spiciness only in mammals (humans). The mechanism of action of capsaicinoids in mammals studied in recent decades. Capsaicinoids bind to *TrpV1* receptors responsible for heat sensation (8, 9).

The capsaicin synthesis main path was elucidated in the late 1960s (10-11). Biosynthesis and accumulation of capsaicinoids is a genetically determined trait in *Capsicum* fruits. Most of the genes of the enzymes involved in biosynthesis have been identified and are presented in (**Figure 1**) (12).

Two pathways are involved in the biosynthesis of capsaicin. The first is the phenylpropanoid pathway derived from phenylalanine, leading to vanillalamine. The second is a branched-chain fatty acid pathway derived from valine leading to 8-methyl-6-nonenoyl-CoA (13-15). The condensation reaction of vanillalamine with 8-methyl-6-nonenoyl-CoA is catalyzed by coenzyme A-dependent acyltransferase (7).

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Figure 1. Capsaicin biosynthesis model: The genes whose alleles have a qualitative effect on capsaicin synthesis are underlined, and other enzymes are associated with quantitative changes.

The gene encoding acyltransferase (AT3) is called *Pun1*, formerly known as capsaicin synthase (CS), located on chromosome 2 (16, 17). The locus has a qualitative effect on the biosynthesis of capsaicin. Mutations in the *Pun1* gene have been identified that lead to loss of pungency. Several mutant alleles have been identified as responsible for the loss of pungency. So far, four alleles are known to cause a loss of *Pun1* function in the homozygous state: *pun1-1* in *C. annuum* L., *pun1-2* in *C. chinense* Jacq., *pun1-3* in *C. frutescens* L., and *pun1-4* in the Japanese non-pungency variety "Nara Murasaki "- *C. annuum*. The recessive *pun1-1* has a 2.5-kb deletion in the putative promoter region to most of the first exon (18). The second type of mutant *Pun1* allele *pun1-2* in *C. chinense* has a 4-bp deletion in the first exon that creates an early stop codon. There is transcription in this allele, but no protein product is produced (19). In the *pun1-3* allele in *C. frutescens*, a large deletion in the second exon results in the loss of 70 amino acids in the *Pun1* protein. This allele is neither transcribed nor translated (20). The origin of the *pun1-4* allele in the Nara Murasaki cultivar of *C. annuum* is unknown. Although there is a full promoter and exon 1, one insertion into a second exon causes a shift in the reading frame (21). A mutation was found through research and mapping that causes a loss of pungency in the spicy *Capsicum chacoense*. It is located on the site PI260433-np, which is not allelic to other known alleles *Pun1*. This gene is designated *Pun 2* (20). In 2019, a new locus called *Pun 3* was identified that controls the spiciness of *C. annuum*. It is

homologous to the transcription factor *CaYMB31*. *Pun3* is a significant regulator of capsaicinoid biosynthetic genes in the *Capsicum annuum*. It controls the expression of structural genes in the capsaicinoid pathway. In the sweet pepper *C. annuum* "YCM334", a recessive non-functional allele with a mutation with loss of meaning in the first exon and a premature stop codon leading to low gene expression was found (22). Differences in the content and amount of capsaicinoids in plants of the genus *Capsicum* depend on differences in gene expression of the capsaicinoid pathway genes and environmental influences. The accumulation of transcripts of several capsaicinoid biosynthetic genes (*p-AMT*, *Pal*, *Kas*, *BCAT*, *FAT*) is associated with the level of pungency (18). In some of them, quality control of capsaicin synthesis has been established. In 2009, a mutation in *p-AMT* was reported in the sweet pepper CH-19 Sweet, which causes the capsaicinoid pathway to switch to the capsinoid pathway and reverse the ratio between the two analogs (23). A mutation causing loss of function of the *p-AMT* gene was also found a little later in Capsicum chinense, in which fruit hotness decreased (24). In C. frutescens pepper S3212, a deletion was reported in the coding region of *p-AMT*, which is responsible for the reduced spiciness and accumulation of capsinoids instead of capsaicinoids (25). In 2020, a mutation in *CaKR1* was observed, causing loss of pungency in *C. chinense* (26). The effects of the BCAT gene, which is at the base of the branched-chain fatty acids, have been observed (27). Another critical gene in the biosynthetic pathway has been identified as *Kas*, a key for regulating the significant precursors for acyl parts of capsaicinoids (28). Two transcription factors (*Erf* and *Jerf*) have been identified, the expression of which shows a correlation with the capsaicinoid content and the intensity of the spiciness, respectively (29). Environmental factors that affect the level of spiciness are light, temperature, carbon dioxide, altitude, soil, precipitation, relative humidity, and fertilization (30-38).

Despite the many genetic and physical factors responsible for capsaicin biosynthesis, few of them have a qualitative effect, i.e., control the complete absence of capsaicin and spiciness in the fruit. The *Pun1* gene is the general gene that qualitatively controls the synthesis of capsaicin by encoding the primary precursor (acyltransferase) of the condensation reaction of the final substances of the phenylpropanoid pathway and branched-chain fatty acid pathway. Mutations in the *Pun1* locus are the most common, preferred, and used in selecting sweet peppers due to their qualitative inheritance. Allele-specific markers were used to determine the allelic state of the *Pun1* gene in the selected hybrid lines. By comparing the data from them with the data from the organoleptic analysis, it will be analyzing the Influence of the *Pune1* gene on capsaicin synthesis, and it will be determined whether this is the only gene that controls their pungency.

MATERIALS AND METHODS
PLANT MATERIAL
Two sweet Bulgarian varieties (Familiya and IZK Delicates) of the species *C. annuum* L., hot pepper (type Habanero) of the species *C. chinense* Jacq, and hot pepper of the species *C. frutescens* L. was chosen for parental forms. The selected cultivars, F1 and F2, in selected hybrids were tested in the experimental plot at the Agricultural university-Plovdiv during the period 2018-2020. The parent forms were cross-pollinated and were obtained F1 plants from 4 crosses *C. annuum* - Familiya x *C. chinense*, *C. annuum* - Familiya x *C. frutescens*, *C. annuum* - IZK Delicates x *C. chinense*, *C. annuum* - IZK Delicates x *C. frutescens*. Controlled self-pollination was carried out during the next growing season by isolating the flowers in the button phase of crosses F1 to obtain seeds F2. The parental forms, plants F1 of the four crosses and F2 of the crosses *C. annuum* - Familiya x *C. chinense* and *C. annuum* - IZK Delicates x *C. frutescens* were grown and analyzed. Organoleptically, the presence and absence of capsaicinoids in the fruits of the parental forms, F1, and F2 of the crosses, were explored.

STATISTICAL ANALYSIS
The data from the performed organoleptic analysis were used to compare the results obtained from the experiment and the predictions of the selected hypothesis (a single dominant gene, Pun1, controls fruit spiciness). A statistical indicator Chi-squared ($\chi2$) was used

to estimate the deviations. Determining the critical value for a significance level of 0.05 (5% confidence level) is based on the frequency distribution of probabilities. The critical values for a different number of classes are determined depending on the degrees of freedom of the equation. The degree of freedom of the equation is equal to (n-1), where n is the number of considered phenotypic classes. In this case, we have two phenotypic types (plants with non-pungent fruits and plants with pungent fruits), and we compare the value of χ2 at a degree of freedom: n-1 = 2-1 = 1 (**Table 1**).

Table 1. *The chi-square distribution table: critical values for different probability levels (P) and degree of freedom (DF) = 1. Underlined - critical values of χ2 at 5% confidence threshold.*

degrees of freedom (DF)	Probability of a larger value of χ2 (P)								
	0,99	0,95	0.90	0.75	0.50	0.25	0.10	0.05	0.01
1	0.00	0,004	0.016	0.102	0.455	1.32	2.71	<u>3.841</u>	6.63

GENOMIC DNA EXTRACTION AND MOLECULAR ANALYSIS

To perform genetic analyses, high-quality genomic DNA was extracted from tissue from the young leaves of pepper plants using the standard OmegaBio-Tek chemicals. The DNA obtained from the extraction is of similar quality (without degraded fragments) and in approximately equal quantities.

Allele-specific PCR primers designed by Wyatt et al. (39) were used to establish the allelic state of the *Pun1* gene in the parental pepper forms, F1 in crosses between sweet and spicy parents, and F2 in the crossbreed *C. annuum* - Familiya x *C. chinense* (**Table 2**).

Table 2. *Primers to determine the allelic state of the Pun1 gene (Pun1 or pun 1-1)*

Primer	Primer sequence (5' → 3')
pun1-1 fwd 1	TCCTCATGCATCTCTTGCAG
pun1-1 fwd2	GCTCCACGGAAAAGACTCAT
pun1-1 rev	CAAATGGCAGTTTCCCTTCTCTCATT

PCR was carried out in a QB-96 Thermal Cycler (Quanta Biotech, London, UK). The PCR reaction to determine the allelic state of the *Pun1* gene was performed in the following reaction mixture: 12.5μl PCR ready Mix (Bioline Meridian Life Science Inc.), 0.25 μl of 10 μM *pun1-1*fwd1 primer, 0.25 μl of 10 μM *pun1-1*fwd2 primer, 0.25 μl of 10 μM *pun1-1*rev primer, 1 μl solution of genomic DNA and H2O to a final volume of 25 μl. The PCR cycles are as follows: 94 ° C for 4 minutes, 35 cycles at 94 ° C for 30 seconds, 60 ° C for 1 minute, 72 ° C for 2 minutes, and final extension at 72 ° C for 10 minutes. The products were visualized on a 1% agarose gel stained with ethidium bromide and photographed under UV light. DNA fragment sizes were determined by comparing with the 100bp DNA marker.

RESULTS AND DISCUTION

The presence and absence of capsaicinoids in the fruits of the parental forms were analyzed organoleptically. Their presence was found in the fruits of all plants of *C. chinense* - Habanero and *C. frutescens* (is felt pungency) and their absence in the fruits of all plants of the species *C. annuum* - Familiya and IZK Delicates (is no felt pungency). To confirm the organoleptic analyzes and accurately determine the allelic state of the *Pun1* gene, a PCR reaction with a 3-primers PCR marker was performed. It is

designed, so that primer *pun1-1*fwd1 is positioned in the first exon in the absence of a *Pun1* mutation. The second example, *pun1-1*fwd2, cover the deleted region and connects only when the *pun1-1* mutation is present. When combining, the *pun1-1*fwd1 with *pun1-1*rev is amplified by 1063 bp, which indicates the availability of wild type *Pun1* allele. When *pun1-1* fwd2 is combined with the same reverse example, *pun1-1*rev amplifies a 746 bp product, an indication of *pun1-1* allele (40). After PCR reaction with the primers used to determine the presence of the recessive allele *pun1-1* were amplified products with a size of 746 bp in the Bulgarian varieties non-pungent pepper of the species *C. annuum*. According to the literature, these fragments correspond to the homozygous state of the *pun1-1* allele (*pun1-1 / pun1-1*), i.e., the lack of pungency, respectively, of capsaicin synthesis due to the transition of this mutation to a homozygous state. In the case of pungent peppers of the species *C. chinense* and *C. frutescens*, bands with a size of 1064 bp were observed, corresponding to the homozygous state of the allele *Pun1* (*Pun1/Pun1*) - the wild type (**Figure 2 and Figure 3**).
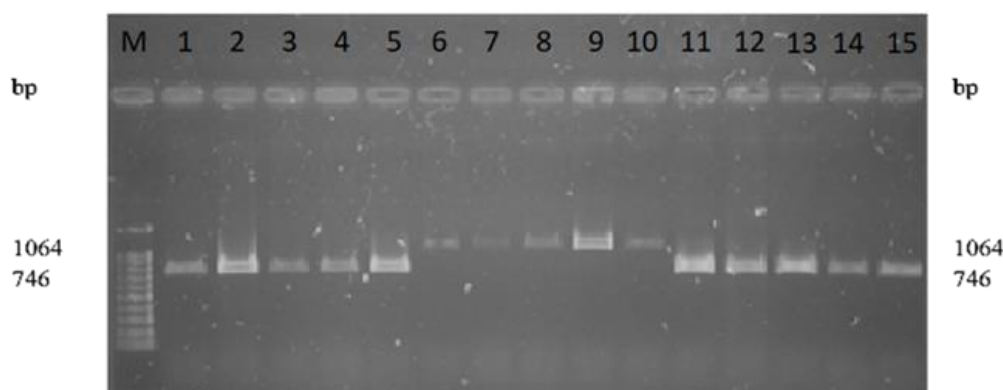

Figure 2. PCR reaction with primers: *pun1-1*fwd1, *pun1-1*fwd2 and *pun1-1*rev to determine the allelic state of *Pun1* in the parental forms - M – 100bp Molecular marker, start 1 – 5 - *C. annuum*, non-pungent variety Familiya - bands of 746 bp, start 6 – 10 - *C. chinense* - pungent, bands of 1064 bp, start 11– 15 - *C. annuum*, non-pungent variety IZK Delicates - bands of 746 bp.
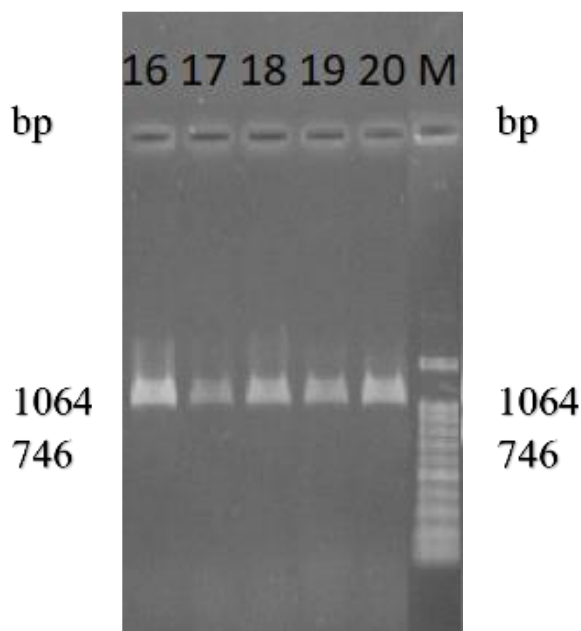

Figure 3. PCR reaction with primers: *pun1-1*fwd1, *pun1-1*fwd2 and *pun1-1*rev to determine the allelic state of *Pun1* in the parental forms: M - 100bp Molecular marker, start 16 – 20 - *C. frutescens*, pungent, bands of 1064 bp.

The obtained results guarantee the purity of the pungent and not-pungent paternal lines of peppers. The parental lines were crossed (*C. annuum* - Familiya x *C. chinense* - Habanero; *C. annuum* - Familiya x *C. frutescens*; *C annuum* – IZK Delicates x *C. chinense* - Habanero and *C. annuum* – IZK Delicates x *C. frutescens*) and during the next growing season, the F1 generation was grown. The fruits of the F1 plants were also analyzed organoleptically. Organoleptic analysis showed that the fruits of all F1 plants are pungency. The allelic composition of the *Pun1* gene was determined by PCR reaction with an allele-specific marker. Both 746 bp and 1064 bp products were amplified (**Figure 4**).
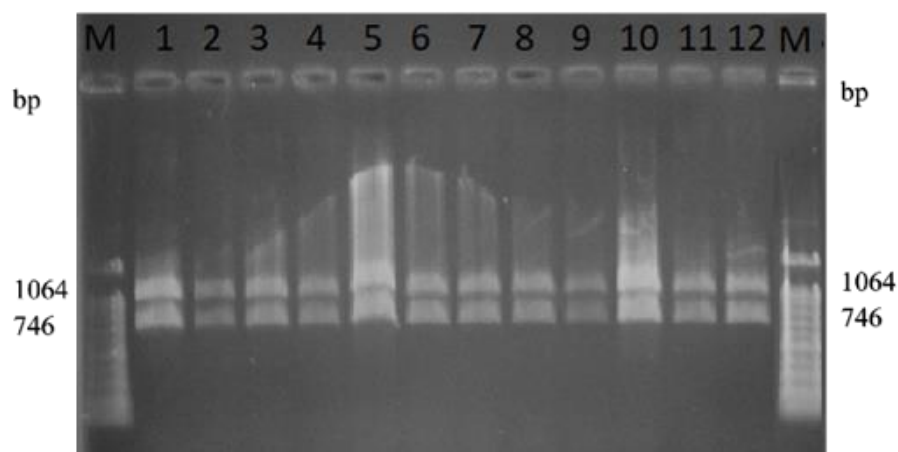

Figure 4. PCR reaction with primers: *pun1-1*fwd1, *pun1-1*fwd2 and *pun1-1*rev to determine the allelic state of *Pun1* in F1 of the different crosses: M – 100bp molecular marker; start 1 – 3 - *C. annuum* - Familiya x *C. chinense* - Habanero -bands of 746 bp and 1064 bp; start 4 – 6 - *C. annuum* - Familiya x *C. frutescens* - bands of 746 bp and 1064 bp; start 7 – 9 - *C. annuum* - IZK Delicates x *C. chinense* - Habanero - bands of 746 bp and 1064 bp; start 10 – 12 *C. annuum* - IZK Delicates x *C. frutescens* - bands of 746 bp and 1064 bp.

The results prove that the crosses were conducted successfully, and the hybrid plants are heterozygous with the *Pun1*/*pun1-1* genotype. Controlled self-pollination of selected plants from crosses F1 (*C. annuum* - Familiya x *C. chinense* - Habanero and *C. annuum* - IZK Delicates x *C. frutescens*) was performed and seeds were collected. During the next growing season, F2 plants were grown from them. Organoleptically, it has been found that plants of both hybrid lines have plants with pungent and non-pungent fruits. The data from the analysis were used to calculate their ratio. Under the influence of a single dominant gene (in this case *Pun1*), are expected ¾ from the plants to have pungent fruits and ¼ non-pungent fruits. The statistical indicator Chi-square (χ^2) was used to compare the data obtained in the experiment with the expected results and to estimate the deviations (**Table 4 and Table 5**).

Table 4. *Calculation of the indicator χ2 in the experiment with organoleptically determined plants (with pungent and non-pungent fruits) F2 from the cross C. annuum – Familiya x C. chinense – Habanero*

Parameters	pungent	non-pungent	$\sum$
p (observed)	185	59	244
q (expected)	183	61	244
$\dfrac{(p-q)^2}{q}$	0,022	0,066	$\chi^2 = 0,088$

Table 5. *Calculation of the indicator χ2 in the experiment with organoleptically determined plants (with pungent and non-pungent fruits) F2 from the cross C. annuum - IZK Delicates x C. frutescens*

Parameters	pungent	non-pungent	$\sum$
p (observed)	64	22	86
q (expected)	65	21	86
$\dfrac{(p - q)^2}{q}$	0,015	0,047	$\chi^2 = 0,062$

In hybrid line *C. annuum* - Family x *C. chinense* - Habanero χ2 = 0.088, and in hybrid line *C. annuum* - IZK Delicates x *C. frutescens* χ2 = 0.062. In both hybrid lines, the value of the indicator χ2 is below the critical value at the 5% confidence threshold. We can assume that the decay of the manifestation of the sign - pungency in fruit is in the expected ratio of 3:1, and the data from the experiment are following the predictions of the selected hypothesis. Although the indicator χ2 is an objective measure of the accuracy of the selected hypothesis, an analysis was performed at the molecular level - determining the genotype of 177 plants from F2 of the cross *C. annuum* - Familiya x *C. chinense* - Habanero. By PCR reactions, the allelic composition of the *Pun1* gene of each plant was determined, and the results coincide entirely with the results of the organoleptic analysis **(Figure 5)**.
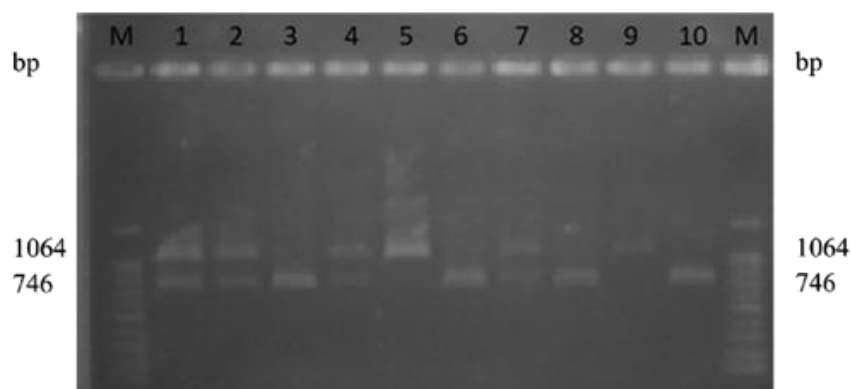

Figure 5. PCR reaction with primers: *pun1-1*fwd 1, *pun1-1*fwd2 and *pun1-1*rev to determine the allelic state of *Pun1* in F2 population of the cross *C. annuum* - Familiya x *C. chinense* **-** M – 100bp molecular marker; start - 1,2,4,5,7,9 – pungent; start – 3,6,8,10 – non-pungent – coincide with a specific allelic state of *Pun 1***:** Band of 746 bp – *pun1-1/pun1-1*; Band of 1064 bp. – *Pun1/Pun1*; Bands of 746 bp and 1064 bp. – *Pun1/pun1-1.*

CONCLUSIONS
Organoleptic and laboratory analyzes proved the hybrid nature of F1. We found a 3:1 (pungent/ non-pungent) ratio in F2 of crosses of *C. annuum* - Familiya x *C. chinense* - Habanero and *C. annuum* - IZK Delicates x *C. frutescens*, typical of complete gene dominance. We established co-segregation of the *Pun1* genotype and a pungency trait in the selected hybrid lines. The *pun1-1/pun1-1* genotype phenotypically determines non-pungent peppers. The *Pun1/Pun1* and *Pun1/pun1-1* genotypes phenotypically determine pungent peppers. Establishing the influence of the *Pun1* gene on the pungency and lack of pungency, tracking its mode of inheritance and its phenotypic manifestation, allows the use of marker-assisted selection of peppers with pungent and non-pungent fruits.

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