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GENETIC AND BIOCHEMICAL REGULATION OF CAPSAICIN BIOSYNTHESIS IN *CAPSICUM* SPP.

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Abstract

Peppers (Capsicum spp.) are widely cultivated and consumed worldwide, with uses ranging from fresh vegetables in human diets to condiments, natural colorants, and medicinal products. Their sensory appeal is largely defined by pungency, a trait perceived differently among individuals but highly valued for both culinary and health-related properties. Pungency in peppers is primarily determined by the accumulation of capsaicin and related capsaicinoids in the fruit. Understanding the biochemical pathways responsible for capsaicinoid biosynthesis and the genetic mechanisms regulating this process has therefore been a major focus of Capsicum research. Advances in this area have provided critical insights into key biosynthetic enzymes, regulatory genes, and transcription factors controlling capsaicin production. Such knowledge is essential for pepper breeding programs, as it enables the selection of parental lines with targeted pungency levels. This review synthesizes recent progress in capsaicin biosynthesis, including pathway regulation, associated genes and transcriptional networks, and the development of molecular markers linked to pungency in pepper fruits.

Key words*: bioactive compounds, hot pepper, pungency, spiciness.*

INTRODUCTION

Pepper (*Capsicum* spp.) is a well-known vegetable, popular for its flavour, cultivated worldwide for fresh fruit and dried as a spice. Originally from tropical and humid regions of South and Central America, peppers are now grown and consumed worldwide. Studies indicate that pepper domestication began in central-eastern Mexico (Kraft et al., 2014). This region has numerous genetic varieties, including local populations with significant value for breeding and high potential as germplasm (Ramirez-Rodas et al., 2026).

After the Columbus expeditions, pepper cultivation spread to Europe and subsequently rapidly expanded worldwide (Koeda et al., 2019).

Hot or sweet, peppers belong to the Solanaceae family. Around the world, there are five domesticated species: *Capsicum annuum* L., *Capsicum bacatum* L., *Capsicum chinense* Jacq., *Capsicum frutescens* L., and *Capsicum pubescens* Ruiz & Pav.; all species are cultivated in numerous countries with variations in shape, colour, and levels of spiciness (Kraft et al., 2014).

Pungent peppers have different degrees of pungency, and if they are very pungent, they cannot be used as fresh vegetables. Still, they can be used for pain relief, anti-inflammatory detumescence, appetite regulation, and rheumatism prevention, and they have also been proven to be effective as a repellent against pests and are very popular as a green protection pesticide (Zhang W. et al., 2022).

Important for humans' diet, bioactive compounds such as vitamins, anthocyanins, flavonoids, carotenoids, and capsaicinoids from peppers have been described as having protective effects on many aspects of human health. Peppers have been used for ages for their analgesic, anti-obesity, cardioprotective, memory-enhancing, and anti-metastatic activity; diabetic and gastroprotective, antimicrobial, anticancer, pharmacological, and neurological properties (Agarwal et al., 2017; Batiha et al., 2020; Clark & Lee, 2016; de Sá Mendes & Branco de Andrade Gonçalves, 2020;

Hayman & Kam, 2008; Sanati et al., 2018; Singh et al., 2022; Stoica et al., 2016).

Hot peppers are important for human health not only because of their capsaicinoid content and spiciness, but they are also important for other metabolism products like vitamin C, carotenoids, flavonoids, phenols, essential nutrients, and vitamins (Agapie et al., 2020). The high levels of vitamin C and carotene are enough to include peppers in the medicinal plant category (Pandia et al., 2012), and the production of foods that include hot pepper powder is a creative challenge for researchers. Sensory analyses of food products (jelly candy) that contain chili powder were conducted by Pop and colleagues, with good acceptance reviews from consumers (Pop et al., 2014).

Found exclusively in the *Capsicum* genus, capsaicin is an alkaloid responsible for pungency (Palma et al., 2020). Usually, pungency is graded on the Scoville scale, a tool invented by a pharmacologist. The Scoville scale measures the heat units based on the number of times the extracted compounds are diluted in sugar water until the pungency is undetectable. Pure capsaicin can go up to $16x10^6$ on the Scoville scale (Hamed et al., 2019).

The most recent studies on capsaicin biosynthesis are compiled in this review, along with the transcriptional networks, pathway regulation, genes linked to pepper pungency, and molecular markers applicable for selecting Capsicum pungency levels.

CAPSAICINOID BIOSYNTHESIS

At every fruit development stage in the biochemistry and physiology of peppers, numerous changes occur. Volatile compounds that come with increased respiration and protein synthesis bring changes that include taste and flavour transformations in the later stages of maturation (Hamed et al., 2019).

Capsaicinoids such as capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homodihydrocapsaicin, and homocapsaicin are responsible for pungency in chili peppers. The phenolic compounds responsible for 90% of the pungency are capsaicin and dihydrocapsaicin. Capsaicin (8-methyl-N-vanillyl-6-nonenamide) is a crystalline, lipophilic, colorless, and odorless alkaloid produced in various quantities among pepper cultivars (Reyes-Escogido et al., 2011).

Capsaicin is found especially surrounding the seeds in placental tissue to protect them from animals. Birds don't feel the pungent taste, and it's easy to disseminate the seeds. Capsaicin is responsible for pungency, for the burning effect, and it produces pain, but capsaicin can also be used to relieve pain by boosting algesic pathways (Fattori et al., 2016). Initially, it was considered that the capsaicinoids were only synthesized in epidermal cells on the septum and stored in placental tissue. New findings reveal that the extremely pungent cultivars produce vesicles containing capsaicinoids on the inner surface of the pericarp (Jang et al., 2021; Park et al., 2019; Zhu et al., 2019).

Two different pathways produce capsaicinoids: the synthesis of vanillylamine by the phenylpropanoid pathway, giving the phenolic structure, and also through fatty acid metabolism derived from L-valine, leading to 8-methyl-6-noneoyl-CoA (Kaiser et al., 2017; Srebcheva & Kostova, 2022). Their concentrations are related to fruit development (Reyes-Escogido et al., 2011).

Although the Scoville scale is widely known for measuring pungency, the main procedure to determine capsaicin and analogues is high-Performance Liquid Chromatography (HPLC), which allows quantification of capsaicin at under 100 Scoville units. HPLC can be combined with gas chromatography (GC) to determine pungency in raw spices, extracts, and pharmaceutical products (Reyes-Escogido et al., 2011).

The localization of capsaicin is mainly in vesicles of the epidermal cells of the placenta, but some pepper varieties also accumulate capsaicinoids in white ribs or other tissues (Milde et al., 2022). Because some species of pepper are pungent while others are not, environmental conditions are among the main factors influencing the levels of capsaicinoids inside fruits (Arce-Rodríguez & Ochoa-Alejo, 2019; Lagunovschi-Luchian et al., 2016). It is known that hydric stress reduces the fruit yield and increases capsaicinoid levels; the fruits are smaller with a higher content of pungent alkaloids (Naves et al., 2019). Water deficit affects the phenylpropanoid pathway and increases capsaicin levels by raising the activity

of enzymes involved in the capsaicinoid biosynthesis pathway (Reyes-Escogido et al., 2011). A high quantity of moisture has the same effect, increasing the pungency levels to protect the seeds from fungi, which are known for thriving under high moisture conditions (Naves et al., 2019).

GENES RESPONSIBLE FOR CONTROLLING THE CAPSAICINOID BIOSYNTHESIS PROCESS

The identification of genes responsible for controlling the capsaicinoid biosynthesis process was performed using genetic fine mapping and biochemistry methods (Sun et al., 2020). Both pathways of synthesizing capsaicinoids were the subject of many studies trying to find the genes responsible for pungency in *Capsicum* spp., mostly by comparing pungent with non-pungent cultivars (Koeda et al., 2019).

Pun1, PAMT, KAS, and *BCAT* genes were associated with capsaicinoid production and are strongly linked with the level of spiciness in pepper fruits (Tanaka et al., 2017).

The main candidate for pungency seemed to be *Pun1* (Stewart et al., 2007; Stewart Jr et al., 2005), but the latest experiments demonstrated that there are many other genes responsible for the hot pepper's pungency. Understanding the pathways of capsaicin biosynthesis was the key to finding the genes involved in pungency. The reference genome analysis, RNA sequencing, and genome-wide association studies showed that there are many genes involved in capsaicin biosynthesis pathways, but only *Pun1* and *pAMT* have been proven to majorly affect the quantity of capsaicin (Kabita et al., 2019).

Pun1 encodes a BAHD-type acyltransferase, AT3 (capsaicin synthase), and is considered to be capable of catalyzing the amide bond between trans-8-methyl-6-nonenoyl-CoA (derived from branched-chain amino acid biosynthesis) and vanillylamine (derived from the phenylpropanoid pathway), resulting in the formation of capsaicin (Milde et al., 2022). Although it is not the only gene involved, *Pun1* is directly linked to capsaicinoid biosynthesis in its final step. Most non-pungent peppers have non-functional *pun1* alleles, and *Pun1* is also related to the biosynthesis of capsinoids, non-pungent analogs of capsaicinoids (Han et al., 2019).

In a previous study, it was determined that crossing a non-pungent cultivar with several pungent cultivars will reveal that the loss of pungency is based on a single recessive gene, *CaKR1* (ketoacyl-ACP reductase) involved in the biosynthesis of an intermediate in the branched-chain fatty acid pathway that limits the entire capsaicinoid biosynthesis (Koeda et al., 2019). The regulatory genes responsible for capsaicin metabolism are still being studied, but so far it has been proven that silencing *Pun1* leads to a decreased level of capsaicin in *C. annuum, C. Chinense*, and *C. frutescens*, and silencing *pAMT* results in a decreased level of capsaicin in *C. frutescens* and *C. Chinense* (Zhang W. et al., 2022).

The opposite is also available, and crossing the pungency trait from *C. chinense* to a donor of *C. annuum* is possible. The presence of the *Pun1* and *Pun3* alleles from *C. chinense* is shown to significantly increase capsaicin and dihydrocapsaicin in the progeny. Interspecific hybridization between these species can increase pungency in *C. annuum* through dominance and interaction effects (Ahn et al., 2025).

An interesting study was conducted by Rathnayaka et al. (2021), in which two Japanese chilli pepper cultivars were subjected to drought stress. It is known that the levels of capsaicinoids in pepper fruits are related to fruit development and environmental conditions. The study analyzed the levels of capsaicin using the PCR technique for 18 genes involved in the capsaicinoid biosynthesis pathway, grouping the genes into two categories (Table 1). The first group of genes was related to the branched-chain fatty acid pathway, and the second group was related to the phenylpropanoid pathway in capsaicinoid biosynthesis. The results showed that drought stress increased the levels of capsaicinoids only in genes related to the first group. The genes from the second group are related to the biosynthesis of other secondary metabolites, such lignin, flavonoids, or phenolic acids, and this could be the reason for no significant difference in gene expression (Rathnayaka et al., 2021).

Sixteen of these genes were also studied with two pungent cultivars, relating eight of them to

capsaicinoid variation within the cultivars. All of the genes related to capsaicinoid variation are part of the branched-chain fatty acid pathway (*Pun 1, PAMT, KAS, CaMYB31, BCAT, CaKR1, ACL, FAT*) (Kondo et al., 2021)

Numerous genes correlated with capsaicinoid levels were identified by Zhang (2022), who analysed 59 capsaicin synthesis-related genes. Distributed on 11 chromosomes, the genes showed different expression levels depending on the stage of fruit maturity (Zhang W. et al., 2022).

Table 1. Genes encoding proteins involved in capsaicinoid biosynthesis - Rathnayaka et al. (2021)

Pathway	Gene name
Branched-chain fatty acid pathway	*ACL* - acyl carrier protein
	pAMT- putative aminotransferase
	Pun 1 - acyltransferase
	WRKY 9
	CaKR1 - ketoacyl-ACP reductase
	CaMYB31
	FAT – ketoacyl-ACP reductase
	KAS I – ketoacyl-ACP synthase
	KAS III – ketoacyl-ACP synthase III
	BCKDH – branched-chain α-ketoacid dehydrogenase
	ACS – acyl-CoA synthase
	BCAT – branched-chain amino acid transferase
	4CL – a-coumaroyl-CoA ligase
Phenylpropanoid synthesis pathway	*PAL* – phenylalanine ammonia-lyase
	C3H – coumaroyl shikimate/quinate 3-hydroxylase
	HCT – hydroxycinnamoyl transferase
	C4H – cinnamate 4-hydroxylase
	COMT – caffeic acid O-methyltransferase

It was thought that capsaicinoids were exclusively biosynthesized only in the septum and stored in the placental tissue, but lately it was revealed that capsaicinoid accumulation is a quantitative trait regulated by the expression of BCAT, KAS, CS, KR, C4H, and FatA genes, and the same structural genes might control capsaicinoid biosynthesis both in the pericarp and placenta (Park et al., 2019).

New findings suggest that the capsaicinoid accumulation must be evaluated not only by studying the genes within the metabolic pathway, but also by studying the ones involved in cellular processes associated with capsaicinoid accumulation, such as transport and capsaicin storage. Their mechanisms could potentially influence indirect pathways (Burgos-Valencia et al., 2024; Fei et al., 2024).

TRANSCRIPTION FACTORS INVOLVED IN THE BIOSYNTHESIS PROCESS

The transcription factors (TFs) associated with regulatory genes were studied to determine which of them control pungency. Several *MYB* transcription factors and *AP2/ERF* are involved in the capsaicinoid biosynthesis pathway (Díaz et al., 2023).

Analysing the genes involved in regulating capsaicinoid biosynthesis, a pattern emerged, linking genes with higher expression to transcription factors. *KAS, PAMT,* and *AT3* genes showed a similar expression profile to *CaMYB31*, suggesting a strong link between them, even though *CaMYB31* was also found in vegetative tissue, indicating additional roles in other biological processes derived from the phenylpropanoid pathway (Arce-Rodríguez & Ochoa-Alejo, 2017; Burgos-Valencia et al., 2020).

Studying genes involved in the ethylene biosynthesis pathway and discovering that they exhibited a similar expression pattern to the genes associated with capsaicinoid biosynthesis, *COMT, pAMT*, and *KAS I* (the genes that enhance capsaicinoid biosynthesis) are activated by the *CaMYB108* transcription factor. Also, *CaMYB48* is possible to participate in capsaicinoid biosynthesis, but it has not been fully studied. The gene that induces endogenous ethylene production, *AP2/ERF* with the *CcERF2* transcription factor, is confirmed to be involved and associated with capsaicinoid biosynthesis (Wen et al., 2022).

Belonging to Apetala2/Ethylene (*AP2/ERF*) family, *ERF* transcription factors have an important role in plant growth and development processes. Related to responses to environmental states, the TFs are highly involved in all fruit developmental processes, stress responses, and defence against diseases, and are closely associated with capsaicinoid biosynthesis. Because capsaicinoid biosynthesis is affected by environmental stimuli, 142 members of the ERF family identified in pepper were studied, and four of them (*CaERF102, CaERF53, CaERF111, and CaERF92*) were strongly linked to the regulation of capsaicinoid biosynthesis (Song et al., 2020).

MYB is a large and important transcription factor family, including four groups: *4R-MYB, 3R-MYB, R2R3-MYB*, and *MYB-related* subfamilies. *R2R3-MYB* is a dominant group, highly conserved, with 16 duplicated genes involved in the development processes of pepper fruit and ripening, and that makes a valuable contribution to capsaicinoid biosynthesis (Wang et al., 2020). *R2-R3-MYB* regulates multiple processes such as tissue development, abiotic stress response, and it metabolism, and also controls the phenylpropanoid pathway responsible for secondary metabolism products like anthocyanins (Han et al., 2019).

Altering the environmental conditions also alters the expression of key genes involved involved in capsaicinoid biosynthesis, which affect the levels of spiciness, and this represents important information for breeders. *MYB* transcription factors, especially *MYB31*, are related to Solanaceae-specific *MYB*, which is involved in capsaicinoid biosynthesis. *CaMYB48* plays a crucial role in regulating capsaicinoids by encoding a protein with a transcriptional activator function and can directly activate the transcription of capsaicinoid biosynthesis genes (Sun et al., 2020).

Previous studies have confirmed that *MYB31* and *MYB48* play key roles in capsaicin regulation, one of the precursors for capsaicinoids (Arce-Rodríguez & Ochoa-Alejo, 2017). Silencing *MYB48* reduces the expression of capsaicin biosynthesis pathway pathway genes such as *AT3* and *KAS, which* affect the entire capsaicin biosynthesis. *AT3* gene, called *Pun1* (Srebcheva & Kostova, 2022), is an important point in the synthesis of capsaicin from vanillylamine and 8-methyl-6-nonenoyl-CoA, and *CaMYB37* is confirmed to bind to the *AT3* gene, activating its transcription, and both are linked in capsaicinoid biosynthesis. Liu proved that silencing *CaMYB37* changes the expression of capsaicin biosynthesis genes, and the capsaicin content is reduced (Liu et al., 2022). Studying 14 TFs that control *AT3* gene expression, Zhang et al. determined an undeniable link between the *AT3* gene and pungency in pepper fruit (Zhang B. et al., 2022).

Silencing *CaMYB31* showed a significant reduction of capsaicinoid levels for the genes involved in capsaicinoid biosynthesis, such as Kas, *BCAT, C4H, Comt*, and *pAMT*. Silencing *Pun1* has the same effect as silencing *CaMYB31*, reducing capsaicinoid production (Han et al., 2019).

Having an important role in response to environmental factors, a study based on drought stress applied to pepper plantss determined a significantly higher expression for TFs *CaMYB31* and *WRKY9*, both involved in the branched-chain fatty acid biosynthesis pathway (Rathnayaka et al., 2021).

Recent studies confirm the important role of R2R3-MYB transcription factors in the regulatory role in the biosynthesis of secondary metabolites, and analyzing the promoter of the CcMYB330 gene suggests that hormones and light may have an impact on capsaicin levels in peppers. Silencing CcMYB330 impacted expression levels of *CcFatA, CcHCT, CcKAS, CcPAL*, and *CcBCAT,* suggesting that CcMYB330 positively regulates the capsaicin biosynthesis (Cheng et al., 2025).

MYB TFs are related to the regulation of capsaicinoid accumulation, but are also important for the regulation of glucosinolates, carotenoids, and the phenylpropanoid metabolic pathway. Related to capsaicinoid biosynthesis, CcMYB330 binds to the *CcPAL* structural gene with a positive impact, but CcMYB4 TFs negatively regulate the expression of structural genes in the capsaicinoid biosynthesis pathway by binding with the *CcPAL2* promoter in *Capsicum chinense* fruits (Yu et al., 2024; Zhang M. et al., 2025).

The bHLH (basic helix-loop-helix) TFs constitute the second largest family of transcription factors and play crucial roles in several growth and development stages. Extensive research shows that bHLHs are highly involved in the regulation of plants' secondary metabolite biosynthesis, linked with salt tolerance in peppers and cold resistance. The CcbHLH68 is a TF that has been demonstrated to influence the production of capsaicinoids (Cheng et al., 2026).

MOLECULAR MARKERS

Pungency is not fully elucidated at the genetic and molecular levels, but it is important for breeding programs that require the targeted use of compounds found only in *Capsicum*. The

Pun1 gene is highly related to pungency, although it is not the only gene that controls it, and marker-assisted selection helps to develop new lines of peppers and better understand the mechanisms behind pungency. Molecular markers allow the detection of pungency at the early stages of development, but identifying and describing a single,universal marker linked with capsaicinoid compounds is still a complex challenge. Using the PCR technique, specific markers based on detected polymorphism were developed, and *MAP1* was identified as a marker linked with pungency in accessions from *C. annuum, C. bacatum, C. chinense, C. frutescens and C. pubescens,* and also in wild species of *Capsicum* (Rodríguez-Maza et al., 2012).

A mutation in a ketoacyl-ACP reductase (*CaKR1* gene) was related to the loss of pungency in Capsicum, and using molecular markers to target the gene region with additional markers, also used to narrow down the specific DNA region, helped determine where the gene is located (Koeda et al., 2019).

Trying to find the genes responsible for pungency, a study from Bulgaria used 3 allele-specific primers (*Pun 1-1 fwd1, Pun 1-1 fwd2,* and *Pun 1-1 rev*) to identify the allelic state of the *Pun1* gene in parental peppers. Allele-specific markers were used to analyse the influence of the *Pun1* gene on capsaicin synthesis to determine if this could be the only gene responsible for pungency (Srebcheva & Kostova, 2022).

Breeding peppers with different levels of capsaicinoids remains a challenge for researchers, and using genomic selection and molecular markers could improve selection. A study using genome-wide markers and markers from GWAS (genome-wide association studies) was conducted. Using markers associated with QTLs for genes known to influence capsaicinoid biosynthesis, it was determined that they are located within the same QTL block as the capsaicinoid biosynthesis genes (Kim et al., 2022).

QTL mapping and GWAS studies were able to identify loci related to capsaicinoid content in peppers, mostly located on chromosomes 3, 6 and 10. The same locations were used for identifying the genes responsible for capsaicinoid biosynthesis. QTL analysis also searched chromosomes 4, 6, and 11, and genes related to pungency were identified on chromosome 6. *Erf (Ethylene responsive factor*), *Jerf (Jasmonic acid and ethylene responsive factor*), *CaMYB31, Ankyrin,* and other genes encoding transcription factors are known to be responsible for capsaicinoid levels in the placenta and pericarp. SNPs, insertions, or deletions in exons of the *pAMT* gene generated mutations, and these mutations have been used for marker-assisted selection to control capsinoid contents in peppers, developing new varieties with high capsinoid contents (Jang et al., 2021).

Marker-assisted selection (MAS) is an important technique to select the desired traits from germplasm collections and to reduce breeding times. SSR (Simple Sequence Repeat) markers are highly polymorphic and are used mostly in genome mapping, variety identification, and genetic diversity estimation. SCAR (Sequence-Characterized Amplified Region) markers are used to detect traits of interest in genotypes for breeding programs. For instance, the SCAR marker *BF6-BR8* proved to be linked to capsaicinoid synthesis and pungency (Başak et al., 2022).

GWAS analysis suggests that structural genes in the capsaicinoid biosynthetic pathway determine capsaicinoid content. Next-generation sequencing tools that include whole genome resequencing, genotype-by-sequencing, and RNA-Seq are easier to use if a reference genome is available. Studying two accessions, pungent and non-pungent, it was demonstrated that other loci named *Pun3* are also related to capsaicinoid contents and encode the CaMYB31 transcription factor, known to regulate *Pun1* expression and other fatty-acid-related genes. *CaMYB31* may be a candidate gene for *Pun3*, with a very important role in regulating the capsaicinoid biosynthetic genes (Han et al., 2019).

The quantitative trait locus *Capsaicinoid1* (*Cap1*), which is identical to *Pun3,* makes a major contribution to pungency accumulation. *Cap1/Pun3* encodes a specific transcription factor, MYB31, with an important role in regulating capsaicinoid levels in peppers, and it is related to *WRKY9,* both contributing to the evolution of highly pungent peppers (Zhu et al., 2019).

The aminotransferase (*pAMT*) encodes a protein in the phenylpropanoid pathway, and a mutation of *pAMT* was associated with the loss of pungency by producing capsinoids instead of capsaicinoids. In a QTL mapping study study, the *pAMT* gene is associated with *Pun2*, a locus on chromosome 3 that contains the *pAMT* gene (Yi et al., 2022).

CONCLUSIONS

Capsaicinoid levels are highly dynamic during fruit development, being affected by many factors such as environmental conditions, biotic stress, and nutrient availability.

An important crop, cultivated worldwide for many beneficial traits, especially for human health, peppers are intensely studied for breeding purposes. Understanding pungency, the genes involved in pungency, the genes that encode the transcription factors, and the molecular markers that can be used for selecting the pepper pungency level are hot subjects on researchers' tables. The ultimate step in trying to completely understand and control spiciness has not yet been made, and only time and hard work will reveal the secrets of pungency mechanisms.

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