Genetic regulation and molecular governance of pungency and color in chilli (*Capsicum spp.*)

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Introduction

Chilli (*Capsicum spp.*), an important spice cum vegetable crop, belongs to the genus *Capsicum* within the *Solanaceae* family. Beyond its use in food and spices, pepper is also important in the pharmaceutical and cosmetics industries. Commercial importance of chilli pepper is attributable to pungency and color. These two traits of the fruit are responsible to classify chilli as a commercial spice crop in international spice trade. The color and the pungency of red chili pepper powder, contributed by carotenoid and capsaicinoid contents which are important properties for this food ingredient. To breed pepper plants with enhanced nutritional qualities, a thorough understanding of the genes involved in biosynthetic pathways and their regulatory mechanisms is essential. Advances in molecular biology and biotechnology have enabled the identification of genes related to pungency and color of chilli fruit, offering opportunities to develop new pepper varieties with specific metabolite profiles. Improved high-throughput sequencing technologies and computational methods are enhancing the efficiency and accuracy of gene identification and functional analysis related to these traits.

Capsaicinoid biosynthesis pathway

Capsaicinoids, which are secondary metabolites, give peppers their characteristic pungency. Their synthesis primarily involves two pathways: the phenylpropanoid pathway and the branched-chain fatty acid (BCFA) pathway. These pathways convert simple amino acids and fatty acids into capsaicinoids (Fig. 1). The phenylpropanoid pathway begins with the enzymatic conversion of phenylalanine to cinnamic acid by phenylalanine ammonia lyase (PAL). Cinnamic acid is subsequently transformed into vanillin through a series of enzymatic reactions involving cinnamate 4-hydroxylase (C4H), 4-coumaroyl-CoA ligase (4CL), hydroxycinnamoyl transferase (HCT), caffeic acid O-methyltransferase (COMT), and hydroxycinnamoyl-CoA hydratase/lyase (HCHL). Vanillin is then converted into vanillylamine by a putative acyltransferase (pAMT). In the branched-chain fatty acid (BCFA) biosynthetic pathway, the end products are synthesized from valine or leucine through a series of enzymatic reactions involving aceto-lactate synthase, branched-chain amino acid aminotransferase (BCAT), branched-chain α-ketoacid dehydrogenase, ketoacyl-ACP synthase (KAS), acyl carrier protein (ACL), ketoacyl-ACP reductase 1 (KR1), acyl-ACP thioesterase (FatA), and acyl-CoA synthetase (ACS). The final products of these pathways, vanillylamine and various BCFAs, are then condensed by capsaicin synthase (CS), also known as acyltransferase 3 (AT3), which is encoded by the Pun1 gene. This enzyme catalyzes the production of different capsaicinoid derivatives depending on the specific BCFA involved in the reaction (Stewart et al., 2005).

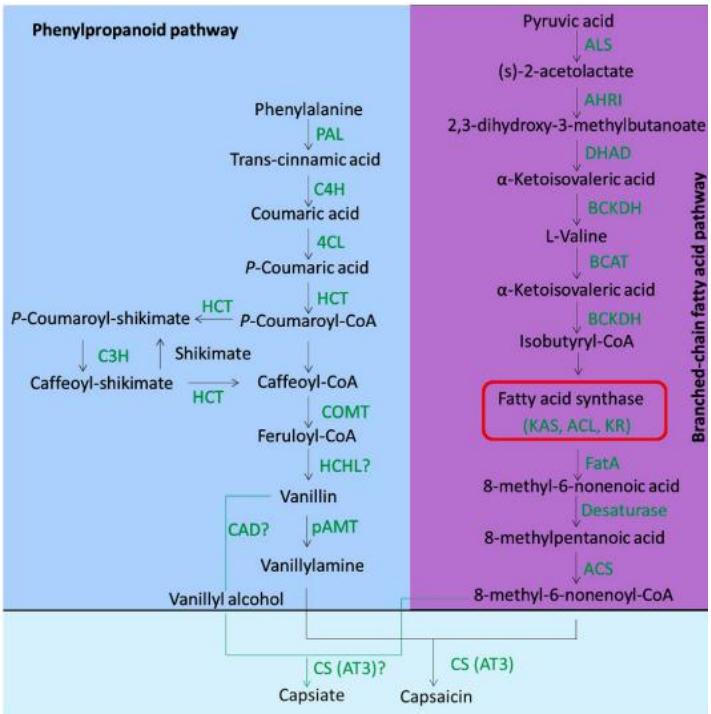

Fig. 1. Schematic flow diagram of the capsaicinoid biosynthetic pathway. Enzymes catalyzing pathway reactions are indicated in green. ACS: acyl-CoA synthetase, ALS: acetolactate synthase, ACL: acyl carrier protein, AHRI: acetohydroxy acid reductoisomerase, AT3: acyltransferase 3 (CS, capsaicin synthase), BCAT: branched-chain amino acid aminotransferase; BCKDH: branched-chain α-ketoacid dehydrogenase, C3H: coumaroyl shikimate/quinate 3-hydroxylase, C4H: cinnamate 4-hydroxylase, COMT: caffeic acid O-methyltransferase, FatA: acyl-ACP thioesterase; DHAD: dihydroxy acid

dehydratase, HCHL: hydroxycinnamoyl-CoA hydratase lyase; HCT: hydroxycinnamoyl transferase, KAS: ketoacyl-ACP synthase, KR1: ketoacyl-ACP reductase, pAMT: putative aminotransferase, PAL: phenylalanine ammonia lyase, 4CL: 4-coumarate CoA ligas (Venkatesh *et al*., 2023)

Molecular regulation of pungency levels in chilli fruit

Capsaicinoids are primarily synthesized in the placenta, where specialized epidermal cells store these compounds in vacuoles. These vacuoles transport the capsaicinoids into subcuticular cavities, which can expand and form blisters along the fruit's epidermis. Eventually, capsaicinoids are excreted and deposited on the seeds and the inner surface of the pericarp (Cisneros-Pineda et al., 2007). However, recent studies have shown that some highly pungent cultivars of *C. chinense*, such as Bhut Jolokia and Trinidad Scorpion, produce vesicles filled with capsaicinoids on the inner surface of the fruit's pericarp (Tanaka et al., 2017) (Fig 2).

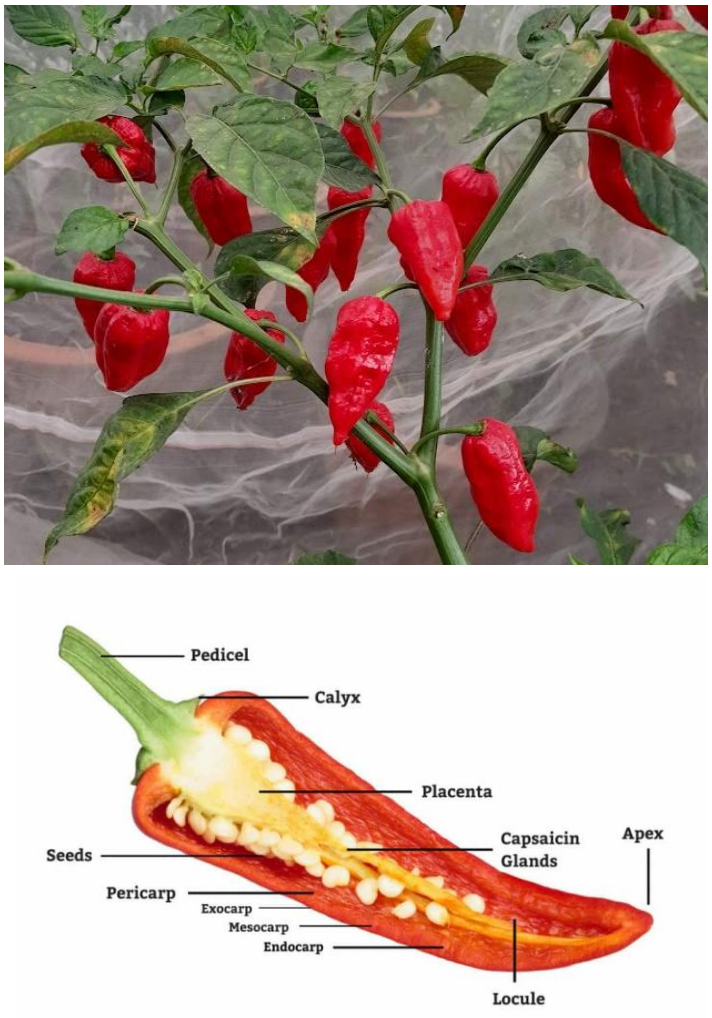

Fig 2: Bhut Jalokia (left), Parts of chilli fruit (right)

The pungency levels in pepper fruit are an inherited trait influenced by the *Capsicum* species, variety, genotype, and environmental growth conditions. The regulation of capsaicinoid accumulation in pepper fruit involves a complex interaction among biosynthetic genes, transcription factors (TF), and various environmental and growth conditions, underscoring the multifaceted nature of this process. Understanding these complex mechanisms is crucial for optimizing capsaicinoid production in peppers. *Pun1, Pun2,* and *Pun3* have been extensively studied as key contributors to capsaicinoid biosynthesis. Specifically, *Pun1* operates in the final stage of capsaicinoid biosynthesis and is expressed only in the fruits. The absence of pungency is caused by a loss of function in *Pun1* due to a 2.5-kb deletion that includes the promoter and exon 1 region (Stewart et al., 2005).

Pepper cultivars exhibit significant variation in capsaicinoid content and pungency levels, suggesting that multiple genetic factors regulate these traits. Previous studies have identified at least 12 quantitative trait loci (QTLs) on six chromosomes associated with capsaicinoid regulation. One major QTL, known as cap (Cap1/cap7.2), has been mapped to chromosome 7 and corresponds to Pun3. Common QTLs have been identified on chromosomes 3, 6, and 10 in various studies (Yarnes et al., 2013). These QTL regions contain genes previously linked to capsaicinoid biosynthesis, such as pAMT, C4H, 4CL, FatA, and Caffeoyl shikimate esterase (CSE), which play important roles in the biosynthesis of capsaicinoids. Two QTLs for capsaicin content were identified on chromosomes 3 and 6, and two separate QTLs for dihydrocapsaicin content were found on chromosome 2 in Bhut Jolokia (Lee et al., 2016).

Carotenoid biosynthesis pathway

Capsicum fruits are abundant in carotenoids, including β-carotene, lutein, β-cryptoxanthin, zeaxanthin, violaxanthin, capsorubin, and capsanthin. The accumulation profiles of these carotenoids can vary significantly depending on factors such as the pepper genotype, the fruit's ripening stage, and its color. The red pepper fruits, the accumulation of two distinctive carotenoid keto xanthophylls, capsanthin and capsorubin, contributes to their red color (Baenas et al., 2019)

The carotenoid biosynthesis pathway is well conserved across most plant species. Precursors for carotenoid biosynthesis are produced through the plastidial methylerythritol phosphate (MEP) pathway, where isopentenyl pyrophosphate (IPP), a precursor for isoprenoid synthesis, along with dimethylallyl diphosphate (DMAPP), are derived from pyruvic acid and glyceraldehyde 3-phosphate. Geranylgeranyl

pyrophosphate (GGPP) is formed by the condensation of three IPP molecules and one DMAPP molecule, a reaction catalyzed by GGPP synthase (GGPPS). GGPP is a crucial molecule serving as a precursor for the biosynthesis of carotenoids, as well as chlorophylls, phylloquinone, tocopherols, and gibberellins (Watkins et al., 2020).

The first step in carotenoid biosynthesis is the condensation of two GGPP molecules to produce phytoene, a process mediated by phytoene synthase (PSY), which acts as a rate-limiting enzyme in carotenoid biosynthesis. Subsequent isomerization and desaturation steps, facilitated by specific desaturase and isomerase enzymes, lead to the production of lycopene. Lycopene can be converted into β-carotene or α-carotene through the action of lycopene β-cyclase (LCYB) and lycopene ε-cyclase (LCYE), respectively. These carotenoids are then hydroxylated to synthesize zeaxanthin and lutein, through the action of β-carotene hydroxylase (BCH) and cytochrome P450 (CYP) enzymes. Zeaxanthin epoxidase (ZEP) converts zeaxanthin into violaxanthin, while violaxanthin de-epoxidase (VDE) can revert violaxanthin back to zeaxanthin (Fig 3).

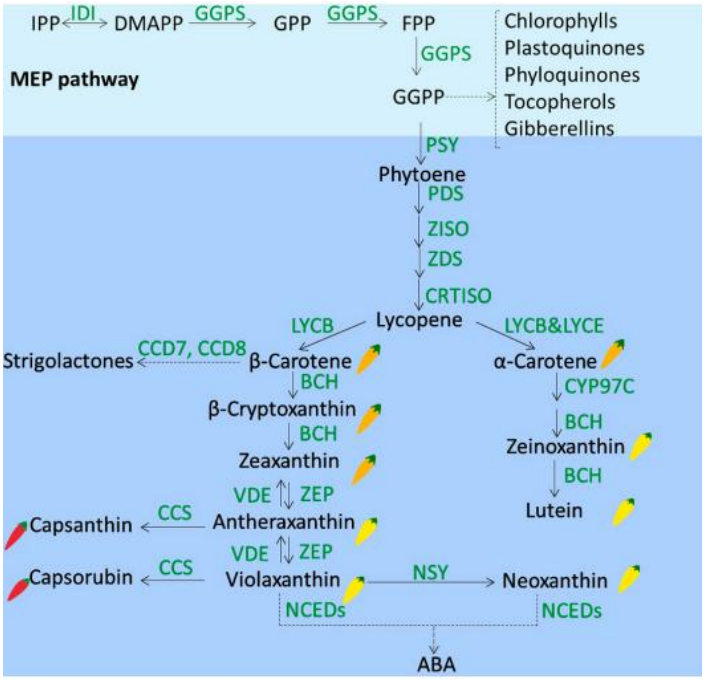

Fig. 3. Schematic flow diagram representing carotenoid biosynthetic pathway in pepper fruit.

In red pepper fruits, the red coloration is due to the accumulation of two distinctive carotenoid keto xanthophylls, capsanthin and capsorubin. This color change occurs when antheraxanthin is converted into capsanthin and violaxanthin is transformed into capsorubin through the catalytic action of the enzyme capsanthin-capsorubin synthase (CCS) (Tian et al., 2015).

Enzymes mediating the biosynthetic pathway reactions are indicated in green. BCH: β-carotene hydroxylase, CCS: capsanthin-capsorubin synthase, CCD7 and CCD8: carotenoid cleavage dioxygenase 7 and 8, CRTISO: carotene isomerase, DMAPP: dimethylallyl pyrophosphate, FPP: farnesyl pyrophosphate, GPP: geranyl pyrophosphate, GGPS: geranylgeranyl pyrophosphate synthase, IDI: isopentenyl diphosphate isomerase, IPP: isopentenyl pyrophosphate, LCYB: β-lycopene cyclase, LCYE: ε-lycopene cyclase, MEP: methylerythritol phosphate pathway, NCED: 9-cis-epoxycarotenoid dioxygenase, NSY: neoxanthin synthase, PSY: phytoene synthase, PDS: phytoene desaturase, VDE: violaxanthin epoxidase, ZDS: ζ-carotene desaturase, ZEP: zeaxanthin epoxidase, ZISO: ζ-carotene isomerase.

Molecular regulation of fruit color in chilli:

Genetically, red is the dominant over yellow and orange in color trait, while light yellow is dominant over white. According to Hurtado-Hernandez *et al*. (1985) the mature color of pepper fruit is determined by three independent loci: C1, C2, and Y. Genetic mapping studies have identified that these loci correspond to the PRR2, PSY1, and CCS genes, respectively. A dominant allele at the Y locus (CCS) is necessary for producing red fruit because it is responsible for the production of capsorubin and capsanthin (Fig 3).

Conversely, a recessive allele at the Y locus results in the accumulation of violaxanthin and antheraxanthin, producing yellow fruit. A mutation at the C2 locus (PSY1) causes the fruit to become orange, while a triple recessive genotype leads to white or ivory-colored fruit. The color of pepper fruit is also influenced by the expression of the LCYB and CrtZ-2 (BCH) genes, with different expression profiles contributing to a range of different colours from yellow to red. Low activity of CrtZ-2 reduces the conversion of β-carotene downstream, causing an accumulation of β-carotene and fewer downstream carotenoids. This change in carotenoid composition helps develop orange and red fruit in peppers (Lee et al., 2020)

Conclusion

The food industry is experiencing a growing demand for natural colours and flavours, and chilli have become an important source of these compounds. Ongoing area of research focuses on developing new pepper cultivars with increased capsaicin and carotenoid levels due to their numerous health benefits and high value in the food industry. Advances in molecular biology and biotechnology have made it possible to identify genes involved in

capsaicinoid biosynthesis, opening up opportunities to create pepper varieties with customized pungency levels. Gaining a deeper understanding of the regulation of capsaicinoids and carotenoids in chilli, combined with technological advancements, will bring substantial agricultural and scientific benefits. Future research should aim to clarify the regulatory mechanisms of these crucial secondary metabolic pathways to enhance and manipulate the agronomic and economic qualities of pepper crops.

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