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Advances in the biosynthesis, gene mining, and molecular mechanisms of cucurbitacin in Cucurbitaceae crops

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Abstract

Cucurbitacin, a highly oxidized tetracyclic triterpenoid compound, is a common secondary metabolite in Cucurbitaceae crops. Its presence imparts a bitterness to the fruit, significantly reducing its quality. However, the accumulation of cucurbitacin enhances the plants resistance to pests and diseases, earning it the reputation of a 'green pesticide'. In recent years, cucurbitacin has attracted extensive attention from researchers. Therefore, this review summarizes the identification, gene mapping, and marker development, biosynthesis and regulation, transport mechanisms, and the domestication of non-bitter Cucurbitaceae varieties. Additionally, it provides insights into the breeding of Cucurbitaceae crops and the *in vitro* biosynthesis of cucurbitacin.

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Introduction

Cucurbit crops are mostly annual herbaceous climbing plants, comprising 118 genera and 825 species. Common examples include the genera *Cucumis*, *Citrullus*, *Luffa*, *Momordica,* and *Cucurbita*[1,2]. Cucurbitaceae crops not only have significant edible value but also possess important medicinal properties[3,4]. Currently, due to genetic factors and adverse environmental conditions, Cucurbitaceae crops develop a bitterness, which severely affects their flavor and quality, significantly reducing their commercial value.

Bitterness compounds are widely present in plants, such as alkaloids[5], flavonoids[6], and terpenoids[7]. The substance causing bitterness in Cucurbitaceae crops is a highly oxidized tetracyclic triterpenoid compound called cucurbitacin[8–11], with its basic structure shown in Fig.1. The cucurbitaceae crops contain various cucurbitacins, which not only possess high medicinal value in terms of anti-cancer, anti-bacterial, and anti-tumor properties, but also exhibit strong resistance to diseases and pests in cucurbitaceae crops[12,13]. Due to its significant role in human health and plant stress resistance, cucurbitacin has attracted extensive attention. Based on this, this review summarizes the identification of bitterness compounds in Cucurbitaceae crops, gene localization of bitterness traits, biosynthesis of common cucurbitacins, and domestication of non-bitter Cucurbitaceae crops. This provides a reference for future breeding of cucurbit crops with bitter vegetative parts but non-bitter fruits, as well as for the efficient utilization of cucurbitacin.

Identification of bitterness compounds in Cucurbitaceae crops

Research on cucurbitacin began in the 1950s[14]. Since then, it has been widely studied due to its high medicinal value. Currently, based on the molecular structure of cucurbitacin, it is classified into 20 types, from cucurbitacin A to T[15], and the types and compositions of cucurbitacin also exhibit spatio-temporal specificity.

The bitter compounds most abundant in the opened cotyledons and fruit are cucurbitacins C, while cucurbitacin B is present in the roots of cucumber seedlings and unopened cotyledons[16]. Therefore, the substance primarily responsible for the bitterness in cucumber fruits is cucurbitacin[17].

There is a noticeable bitterness in both young fruits and stem vines of melons, with various types of cucurbitacins present in their tissues, among which the content of cucurbitacin B accounts for over 80%[18]. Furthermore, Zhou et al.[19] found that 15 d after pollination in melon fruits, there was a large amount of cucurbitacin B in bitter melons, while no cucurbitacin B was detected in non-bitter melons.

The bitter taste of watermelon root, stem, and vine are closely related to cucurbitin, and the cucurbitin types are different among different species. Lavie et al.[20] identified cucurbitin B and cucurbitin E from the ancestor of cultivated watermelon (*Citrullus colocynthis*). In addition, this study also identified cucurbitin in watermelon leaves, but did not specify the type of cucurbitin in leaves[20]. Later, cucurbitins I, J, and T were identified from the ancestor of cultivated watermelon (*Colocynthis vulgaris*), of which cucurbitins T was firstly reported as a novel cucurbitin compound[21]. Davidovich-Rikanati et al.[22] also identified cucurbitins B and E from *Citrullus lanatus* and cucurbitins from watermelon leaves, suggesting that the types of cucurbitins in leaves may be similar to those in fruits. Studies have shown that these cucurbitins mainly exist in the form of glycosides[23,24]. Cucurbitin B and E were isolated from different germplasm resources of *Citrullus spp*, and the contents of cucurbitins in watermelon fruits and roots were higher than that in leaves and stems[25].

Bitter melon is named for its bitterness and the compounds in bitter melon are primarily present in the form of glycosides or aglycones[26]. Currently, 161 types of cucurbitacins have been detected from various organs of bitter melon. The main identified components responsible for the bitterness of bitter melon are triterpenoid saponins (momordicoside I, momordicoside II, momordicoside K, and momordicoside L)[27]. There are two types of cucurbitane triterpenoids: cucurbitane triterpenoids with a C5 and C19 ring structure and cucurbitane triterpenoids without a C5 and C19 ring structure[28]. In addition to cucurbitacins, alkaloids can also contribute to the bitterness of bitter melon.

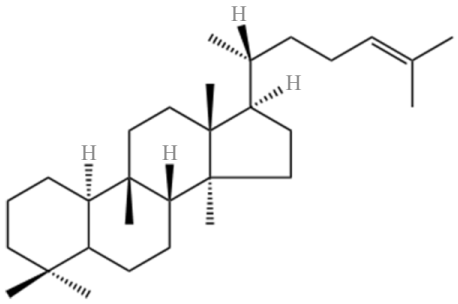

Fig. 1 The basic skeleton structure of cucurbitacin (adapted from Ma[7]).

Additionally, researchers have found that other cucurbit crops, such as zucchini[29], bottle gourd[30], wax melon[31], and sponge gourd[32], are also rich in cucurbitane-type bitter substances, specifically cucurbitacins.

Advances in gene mapping of bitter traits in Cucurbitaceae crops

The genetic characteristics of bitterness in Cucurbitaceae crops

Genetic analysis of bitterness in cucumber

The bitterness in cucumber includes both vegetative and fruit bitterness. There have been numerous studies on the genetic analysis of vegetative bitterness. It has been found that vegetative bitterness vs non-bitterness is controlled by a single dominant gene (*Bi*)[9,17,33,34]. However, Wenher et al.[35] discovered that, in addition to *bi*, *bi-2* also participates in the regulation of bitter foliage in cucumber, the two loci follow a 9:7 genetic segregation ratio, showing the complementary gene action.

The fruit bitterness in cucumber is primarily governed by a single dominant gene *Bt*[8,36], however, *Bt* and *Bt-2* are also responsible for this trait[37]. When *Bt-2* and *Bt* are both present, they exhibit a dominant-recessive epistatic interaction[38]. Additionally, studies have shown that *Bt* is not linked to femaleness gene *F*[39,40], while the *Bt-2* is linked to fruit skin color gene *u* and *D*, and the small spines gene *ss*[37,41]. Proposed by Walters et al.[37], *Bt-2* was present in the wild cucumber Hardiwickii. Since there are no subsequent gene mapping studies on Hardiwickii, this is the only locus with an unknown causal gene.

In addition, vegetative bitterness in cucumber is not influenced by *Bt*, but *bi* exhibits a recessive epistatic effect on *Bt*. When the vegetative bitterness gene is in a heterozygous state, the fruit exhibits bitterness regardless of the presence of *Bt*[42]. Subsequently, Shang et al.[34] demonstrated that *Bt* can regulate the synthesis of cucurbitacin in fruit by directly activating the expression of *Bi*. Therefore, the formation of bitterness in cucumber vegetative and fruits is controlled by a complex genetic network involving multiple genes and various genetic interactions.

Genetic analysis of bitterness in melon

Research has found that the Mendelian segregation ratio for the bitterness trait in melon fruits is 9:7, indicating the bitterness is controlled by two pairs of dominant genes, with an epistatic effect[43–45]. However, other studies have identified that the genes controlling the bitterness in melon fruits are *Bif-1*, *Bif-2,* and *Bif-3*, which exhibit independent inheritance[46]. Additionally, some research has shown that the segregation ratio for the presence or absence of bitterness in melon fruits is 3:1, with the bitterness trait being controlled by a single dominant gene[17,47]. This discrepancy may be attributed to the differences in the varieties of the parental materials used in the studies.

In addition, there are also some studies on the bitterness of melon stems and vines. By crossing bitter and non-bitter parental lines, genetic analysis of F_1, F_2, BC_1 and BC_2 indicates that the segregation of stem and vine bitterness traits conforms to a 3:1 and 1:1 segregation ratio[48,49], which suggests that the stem and vine bitterness is controlled by one pair of genes, with bitterness being dominant over non-bitterness.

Genetic analysis of bitterness in watermelon

The bitterness trait of watermelon fruit is controlled by a single gene. In the 1990s, it was identified that the bitterness of watermelon fruit is governed by a single dominant gene (*Bi*) using wild watermelon materials[50]. Subsequently, there have also been studies reporting that the bitterness trait of watermelon fruit follows a single-gene independent inheritance[51]. Similarly, many studies have utilized hybridization between bitter and non-bitter parents to construct F_2 and backcross populations for genetic analysis, finding that the bitterness trait in watermelon is also controlled by a single gene, with bitterness being dominant over non-bitterness[52–56].

Genetic analysis of bitterness in sponge gourd

The earliest study on the genetic rules of bitterness in sponge gourd was conducted by Thakur et al., who proposed that bitterness in sponge gourd is controlled by the *Bi* gene in *Luffa acutangula* (L.) Roxb. and the *S* gene in *Luffa cylindrica* (L.) Roem.[57]. Through crosses and backcrosses between two reported cultivated varieties, *Luffa acutangula* (L.) Roxb.[58], and *Luffa cylindrica* (L.) Roem.[59], it was found that each of the two cultivated varieties of sponge gourd possesses a single dominant gene, with the dominant genes complementing each other to control the bitterness in the fruit[60–62].

Genetic analysis of bitterness in other Cucurbitaceae crops

In addition, the inheritance patterns of the bitter taste traits of bottle gourds, zucchini, and gourds were also reported[2,63–65]. The bitterness of bottle gourd fruits is jointly regulated by two complementary gene pairs, *Bt* and *I*[63]. Borchers & Taylor[64] crossed 'Green Striped Cushaw' zucchini with 'Goldbar' zucchini and genetic analysis revealed that the bitterness trait is controlled by three dominant complementary genes, with two contributed by 'Goldbar' and the third by 'Green Striped Cushaw'. Zhang[65] discovered that the F_2 generation from the hybridization of multiple high-quality bottle gourd varieties conformed to a 9:7 segregation ratio, indicating that gene complementation is the cause of the severe bitterness in bottle gourd. Wu et al.[2] demonstrated through the hybridization of two local varieties, 'Hangzhou Gourd' and 'Puxian Gourd', that the genetic segregation ratio for the bitterness trait in bottle gourd is also 9:7, with *QBt.1* and *QBt.2* complementing each other to produce the bitter fruit.

Genetic mapping of bitterness traits in Cucurbitaceae crops

Genetic mapping of bitterness traits in cucumber

Cucumber is an important model plant for studying agronomic traits in cucurbit crops, and current research on the identification of its bitterness gene is the most in-depth (Table 1). Wenher et al.[35] used the material 'NCG-093' (short petiole mutant) to find that the gene causing the absence of cucurbitin in leaves was named *bi-2*; however, subsequent gene mapping, cloning, or breeding work did not utilize this locus. It may correspond to the *Bl* gene specifically expressed in the leaves identified by Shang et al.[34] using E3-231 (wild type 406). Huang et al.[66] localized the *Bi* gene of cucumber leaves to a region of approximately 35 kb on chromosome 6. Subsequent comparative genomic analysis revealed that a gene in this

region shares a high homology of 90% with the cucurbitadienol synthase gene in zucchini, suggesting that this gene maybe involved in the synthesis of cucurbitacin C in cucumber leaves. Similarly, the study conducted a genetic linkage analysis and localization of SSR markers by constructing a population of RILs, and eventually also located the *Bi* gene on cucumber chromosome 6, with the nearest flanking markers, SSR02309 and SSR00004, being 1.7 and 2.2 cm away from the bitterness gene, respectively[9].

Combined with BSA-seq and AFLP markers, the *Bt* gene was located between E23M66-101 and E25M65-213, with genetic distances of 5 and 4 cm, respectively[42]. Zhang et al.[10] constructed an SSR linkage map and used 148 F_9 RILs to locate the cucumber fruit bitterness gene *bi-1*, which identified two flanking SSR markers (SSR0004 and SSR02309) with genetic distance of 1.9 cm and 3.3 cm, respectively. Li et al.[36] mapped the cucumber fruit bitterness gene *Bt* to chromosome 5, and the closest markers to *Bt* were SSR12291 and SSR02118, with genetic distances of 1.8 and 1.9 cm, respectively. Zhang et al.[67] located the *Bt* using Indel markers, and obtained a marker (Bt-InDel-1) linked to *Bt* gene, with a genetic distance of 0.8 cm from *Bt*, which laid the theoretical foundation for the fine mapping of the cucumber fruit bitterness gene. In the same year, the cucumber materials of '931' (*btbt*) and '46GBt' (*BtBt*) were used as parents to construct an F_2 population and the *Bt* gene was mapped into a 3.3 cm region on the short arm of chromosome 5 through SSR marker genetic linkage analysis, additionally, the *Bt* was finely mapped into a 1.5 cm region on chromosome 5, achieving the first localization of the cucumber fruit bitterness gene[68]. In 2014, the research group ultimately cloned the *Bt* gene, laying the foundation for the study of the molecular mechanism of the bitterness gene in Cucurbitaceae crops[34].

Previous research on the mapping of cucumber bitterness genes had not developed molecular markers based on the *Bi* gene sequence. Building on previous studies, Venkatesh et al.[33] developed reliable, co-detectable molecular markers using high-resolution melting (HRM) and Kompetitive Allele Specific PCR (KASP) techniques (BiHRM1 and Bi-KASP). These gene-based markers can significantly improve the accuracy and efficiency of breeding non-bitter cucumber lines.

Liu et al.[69] utilized the 9930 v2.0 genome to study the mapping of bitterness genes in cucumber, including the fruit bitterness gene

Table 1. The bitterness genes of cucurbitaceae crops located in related markers.

Species	Chr	Molecular marker	Primer sequence (5'-3')	Populations	Ref.
Cucumber	6	SSR02309 (1.7 cm), SSR00004 (2.2 cm)	SSR02309-F: TGAAATGCCTCTGCAATGAC SSR02309-R: TCATGACTAGACACGCCAGC SSR00004-F: TTCATTGCAAAGCACACACA SSR00004-R: TGAAAAGAGGGAACAAAAGCA	9110Gt (*bibi*) × 9930 (*BiBi*)→RILs	[9]
	5	E23M66-101 (5 cm), E25M65-213 (4 cm)	E23: GACTGCGTACCAATTCTA M66: GATGAGTCCTGAGTA E25: GACTGCGTACCAATTCTG M65: GATGAGTCCTGAGTAAGAG	931 (*Bt*) × 932 (*bt*)→F_2	[42]
	5	SSR10795 (0.8 cm), SSR07081 (2.5 cm)	SSR10795-F: CATCAAAATACCTCCATCTCCA SSR10795-R: GCATGAATAGCATGGGGTTT SSR07081-F: GGCGACTTTGGAGTGTAACAA SSR07081-R: GGAAAGATATTCTCAGGGAATCTAA	46GBt (*BiBiBtBt*) × 931 (*BiBibtbt*)→F_2	[67]
	6	SSR0004 (1.9 cm), SSR02309 (3.3 cm)	–	9110Gt (*bi-1bi-1*) × 9930 (*Bi-1Bi-1*)→RILs	[10]
	5	SSR12291 (1.9 cm), SSR02118 (1.8 cm)	SSR12291-F: CGCACGAGAACCTTTATTGA SSR12291-R: TCACATCAAATTAACACTTTCATCTC SSR02118-F: TGGATTGTCATCTCATTGGC SSR02118-R: GGTGAGTGGTAATTTTATGAATTTTG	D9320 (*Bt*) × D0432-2-2 (*bt*)→BC_1	[36]
Melon	2, 5	2mBiPr21619699, 2mBiPr21653588, 5mBiPr20403004, 5mBiPr20822407, and 5mBiPr21331862	2mBiPr21619699-F: AATGGCATAACCTTTCACCT 2mBiPr21619699-R: CTTTCTATCACCAACCGACT 2mBiPr21653588-F: TTATCTAAGTTTCCTCGGTC 2mBiPr21653588-R: CTTCAACTTGGATGTTTTCT 5mBiPr20403004-F: GGAATAGGAATAGGAAGAATGT 5mBiPr20403004-R: AAAAGGGTTAATGATAAGAGAC 5mBiPr20822407-F: TAGGTTTAACCTGTTTTCACC 5mBiPr20822407-R: GCATACAAAGCATTTTTTCTT 5mBiPr21331862-F: ATGGTGAGCATTGTTTTCGA 5mBiPr21331862-R: TCTTTTGGGTCTTGGGCTTC	C68 (*Bt*) × C69 (*bt*)→BC_1	[45]
Watermelon	1	W01-2 (0.93 cm), W01-3 (0.99 cm)	–	W1-1 (*bt*) × PI 186490 (*Bt*)→BC_1	[52]
Watermelon	1	SNP3162335, SNP3278961	SNP3162335-F: TGTCAAATGGGTTCATGAAGTT SNP3162335-R: TTCCTGTCTTTTGTGGTTTGG SNP3278961-F: TTCGCACTAACCTGGAAAAG SNP3278961-R: ATTTGAAACCCGCCCTTAAA	9904(*Bt*) × Handel (*bt*)→RILs	[54]
Sponge gourd	7	LuBt1-2 (1.9 cm), LuBt1A (1.2 cm)	–	48-1-0-0 (*bt*) × 4-0-0-0 (*bt*)→BC_1	[61]
Sponge gourd	7	SGE292 (6.08 cm), SGC196 (3.11 cm)	SGE292-F: TGGGGACAACCCGGCTT SGE292-R: GACTGCGTACGAATTCTG SGC196-F: AGCGAGCAAGCCGGTGG SGC196-R: GACTGCGTACGAATTATG	48-1-0-0 (*bt*) × 4-0-0-0 (*bt*)→BC_1	[62]
Bottle gourd	6, 7	BGReSe_09031-BGReSe_09068, BGReSe_11107-BGReSe_11032	–	Hangzhou Gourd (*bt*) × Puxian Gourd (*bt*)→F_2	[2]

(*Bt*) and the leaf bitterness gene (*Bl*) in the bHLH gene cluster. However, Shang et al.,[34] found that the Bt locus contains gene clusters of bHLH93(*Bt*) and bHLH95 (*Bl*) through the modified 9930 v3.0 genome[70], *Bi* is located on chromosome 6 and encodes enzymes of the oxysqualene cyclase (OSC) family, which catalyzes the generation of cucurbitadienol. *Bt* and *Bl* are homologous genes located on chromosome 5, which regulate the formation of bitterness in fruits and leaves, respectively. The discovery of these genes provides an important foundation for the study of cucumber bitterness traits and helps further understand the evolutionary process and genetic mechanisms of cucumbers.

Genetic mapping of bitterness traits in melon

Research on the mapping of bitterness genes in melon started relatively late, and there are few studies currently (Table 1). Zhou et al.[19] clarified the biosynthesis pathway of cucurbitacin B in melon, discovering three genes on chromosome 9 that regulate the formation of bitter substances. Li et al.[47] constructed a genetic map comprising 10 linkage groups using 477 SNP markers, with a total length of 337.79 cm and an average marker interval of 0.71 cm, the whole-genome QTL mapping on linkage group 8 (corresponding to chromosome 9) detected a bitterness QTL explaining 20% of the phenotypic variation. Moreover, a genome-wide association study identified seven SNPs related to bitterness traits, all of which were also located in the genomic region of the bitterness QTL on chromosome 9[47]. Shang et al.[45] mapped the candidate genes for melon bitterness into a 7.3 Mb region on chromosome 2 and a 2.2 Mb region on chromosome 5.

Genetic mapping of bitterness traits in watermelon

Research on the genetic mapping of the bitterness gene in watermelons has been quite extensive (Table 1). As early as the 1990s, researchers identified the bitterness gene (*Bi*) from wild watermelons, which is closely linked to the isoenzyme marker Pgm-1 at a distance of 11.3 cm[50]. Zhang et al.[52] used CAPS markers to construct a genetic map and located the bitterness gene within a 1.01 Mb interval between two markers w01-2 and w01-3 on chromosome 1. Sun et al.[55] mapped a QTL controlling fruit bitterness in watermelon into the interval between markers w01-2 and w01-3 on chromosome 1, with a high LOD value of 95.0931, explaining 99.5904% of the phenotypic variation, and the distances to the flanking markers were 0.93 cm and 0.99 cm, respectively. Li et al.[53] used '9904' (bitter) and 'Handel' (non-bitter) as parents to map the *Bt* gene into a 6.16 Mb candidate interval on chromosome 1. The following year, Li[54] further narrowed the interval into a 116.7 kb segment between the two markers SNP3162335 and SNP3278967. This interval contains four candidate genes (*Cla011507*, *Cla011508*, *Cla011509*, and *Cla011510*). Since then, the study further utilized 16 watermelon materials, combined with qRT-PCR results, to hypothesize that the bHLH gene *Cl011508* may regulate watermelon fruit bitterness[56].

Genetic mapping of bitterness traits in sponge gourd

The sponge gourd variety '48-1-0-0' was utilized as a parent crossing with '4-0-0-0' to obtain F_2 and a backcross population, the bitterness gene *Bt* was integrated into linkage group 3 and was initially located between SGE292 and SGC196 with genetic distances of 6.08 and 3.11 cm, respectively[63] (Table 1). Qin[61] located a bitterness gene *Labt* in *Luffa acutangula (*L.*)* Roxb. on chromosome 7, between markers Lubt1-2 and Lubt1A, with the genetic distances of 1.9 and 1.2 cm, respectively; moreover, a bitterness gene *Lcbt* in *Luffa cylindrica* (L.) Roem was also identified with a genetic distance of 6.3 cm[61] (Table 1). The results also indicated that the bitterness gene of *Luffa acutangula* (L.) Roxb. was probably homologous to cucumber *Csa1G044* (GenBank Accession: KM655), which encodes oxysqualene cyclase (OSC)[61]. Given this, it was hypothesized that this gene might be the *Bi* gene reported by Thakur et al.[57]. Since then, few studies related to the localization of bitter taste genes in sponge gourd have been reported.

Genetic mapping of bitterness traits in other Cucurbitaceae crops

There are reports on the mapping of bitterness genes in bottle gourd (Table 1). Wu et al.[2] crossed 'Hangzhou bottle Gourd' with 'Puxian bottle Gourd' to construct an F_2 population, conducted bitterness gene mapping, and detected two QTLs, with QBt.1 locating in a 17.62 cm interval on LG2, corresponding to a 1.6 Mb region on chromosome 6, and QBt.2 locating in an 8.44 cm interval on LG9, corresponding to a 1.9 Mb region on chromosome 7.

Biosynthesis of cucurbitacins in cucurbit crops

Formation and modification of cucurbitacin skeletons in Cucurbitaceae crops

Cucurbitacins are synthesized through the mevalonate (MVA) pathway to forms the basic skeleton of cucurbitane-type triterpenoids[34]. Starting with acetyl-CoA as the substrate, MVA is formed through a series of enzyme-catalyzed reactions, which then produces dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP). Two molecules of IPP and one molecule of DMAPP, under the action of farnesyl pyrophosphate synthase (FPS), form farnesyl pyrophosphate (FPP). Subsequently, under the action of squalene synthase (SQS) and squalene epoxidase (SQE), 2,3-oxidosqualene is produced. Finally, the basic skeleton structure of cucurbitacins is formed through the catalysis of the oxidosqualene cyclase (OSC) family.

After the formation of the basic skeleton of cucurbitane-type triterpenoids, various cucurbitane-type triterpenoids are produced through the action of multiple modifying enzymes. These multi-site modifying enzymes include oxidoreductases and acyltransferases[71,72]. The most common oxidoreductases in this process are the cytochrome P450 (CYP-450) family, which can modify multiple sites to produce hydroxyl, carboxyl, aldehyde, keto, and epoxy groups (Fig. 2)[73]. This lays the foundation for further modifications by acyltransferases and other enzymes.

Analysis of the cucurbitacin biosynthesis pathway in Cucurbitaceae crops

The biosynthesis of cucurchinin in melon, cucumber and watermelon all involves enzymes such as squalene oxidizing cyclase (OSC) and cytochrome P450 (CYP450), which are regulated by specific transcription factors[19]. *CmBt* and *CmBr* in melon regulate the synthesis of cucurbitin B in fruits, and roots, respectively[19]. *CsBl*, *CsBt,* and *CsBr* genes in cucumber regulate cucurbiturin C synthesis in leaves, fruits, and roots, respectively[34]. The *ClBt* and *ClBr* regulate the synthesis of cucurbitin E in watermelon fruits, and roots, respectively[19].

In melon, the biosynthesis of cucurbitacin B involves one *OSC* gene (*CmBi*), six *CYP450* genes, and one *ACT* gene. These eight genes are co-expressed in various tissues of melon, regulating the biosynthesis of cucurbitacin B[19]. The key steps in cucurbitacin B biosynthesis are as follows: First, the cucurbitadiol is produced under the action of *CmBi*, which encodes cucurbitadienol synthase. Second, the *Cm890*-encoded oxidoreductase catalyzes the C-11 carbonylation and C-20 hydroxylation, forming 11-carbonyl, 20-hydroxyl cucurbitadienol. Third, *Cm180*-encoded oxidase generates 11-carbonyl, 2,20-dihydroxy cucurbitadienol. Finally, cucurbitacin B is produced through the action of *CmACT* (Table 2, Fig. 3)[74,75].

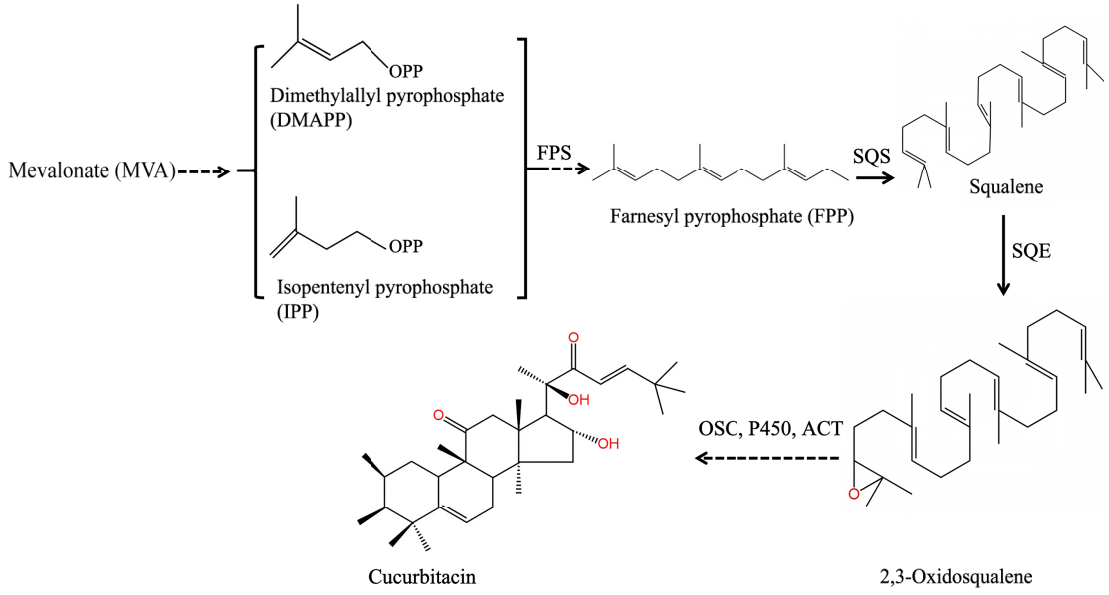

Fig. 2 Cucurbitacin biosynthesis process (adapted from Ma[7]). FPS: Fanesyl pyrophosphate synthase; SQS: Squalene synthase; SQE: Oxidoqualene synthase; OSC: Oxidoqualene cyclase; P450: Cytochrome P450 monooxygenase; ACT: Acetyltransferase. The dotted line indicates that there are multiple steps involved.

The biosynthesis of cucurbitacin C involves one *OSC* gene (*CsBi*), eight *CYP450* genes, and one acyltransferase (*ACT*) gene[76,77]. The first step in the biosynthesis of cucurbitacin C is similar to that of cucurbitacin B. It is catalyzed by an enzyme from the oxidosqualene cyclase (OSC) family encoded by *CsBi*, producing cucurbitadienol. Then, the enzyme encoded by *Cs540* modifies cucurbitadienol at the C-19 position to produce 19-hydroxy cucurbitadienol. Subsequently, the enzyme encoded by *Cs160* catalyzes the C-25 position, resulting in 19,25-dihydroxy cucurbitadienol. Finally, cucurbitacin C is produced through the action of the acyltransferase encoded by *CsACT* (Table 2, Fig. 3)[76].

The genes involved in the biosynthesis of cucurbitacin E include one *OSC* gene (*ClBi*), seven *CYP450* genes, and one *ACT* gene[19]. In the first step of CuE biosynthesis, the enzyme catalyzing the production of cucurbitadienol is encoded by *ClBi*. In the second step, both *Cl890A* and *Cl890B* encode cytochrome P450 oxidases that form 11-hydroxy cucurbitadienol and 11-carbonyl-20β-hydroxy cucurbitadienol. This is followed by the oxidation catalyzed by the enzyme encoded by *Cl180*, forming 11-carbonyl-2β,20β-dihydroxy cucurbitadienol. Finally, cucurbitacin E is produced through the action of the acetyltransferase encoded by *ClACT* (Table 2, Fig. 3)[15].

'Switch' genes regulating cucurbitacin biosynthesis

The bHLH transcription factors include a basic region and a helix-loop-helix domain, comprising a class of transcription factors with a basic helix-loop-helix structure[34,69]. bHLH TFs activate the transcription of genes related to gourd toxin synthesis by binding to the promoter regions of these genes[78]. Due to its key role in the regulatory process, it can turn the cucurbitine biosynthesis pathway on or off, so it is called the 'switch' gene[79]. Xu et al.[78] conducted a homology and phylogenetic tree analysis of the *Bt* gene cluster regulating the biosynthesis of cucurbitacin C. They found that the genes in this cluster originated from three ancestral genes following a shared whole-genome tetraploidization event in the Cucurbitaceae family. Additionally, a new conserved gene cluster, which is paralogous to the *Bt* cluster, was identified. This new cluster includes two tandemly repeated *bHLH* genes. The evolutionary relationship and gene expression characteristics of these two genes in the paralogous cluster indicate that one of the genes (*Brp*) is involved in regulating the biosynthesis of cucurbitacin C in roots[78]. These findings provide new insights into the function and evolution of *bHLH* genes in cucurbit crops and offer new perspectives on the regulation of cucurbitacin biosynthesis.

'Switch' gene regulating cucurbitacin B biosynthesis

CmBr and *CmBt* can regulate the expression of cucurbitacin B biosynthesis genes, with *CmBt* and *CmBr* specifically regulating the biosynthesis of bitter substances in melon fruits and roots, respectively. Zhou et al.[19] discovered *CmBr* and *CmBt* in the bHLH gene cluster on chromosome 9, the expression and content of *CmBr* and *CmBi* were detected by different melon materials, and the expression of *CmBr* and *CmBi* were positively correlated. In addition, Wang et al.[80] knocked out the *CmBr* using CRISPR/Cas9 and obtained a *CmBr* near-isogenic line combined with backcross breeding, the cucurbitin B content in wild-type fruits was significantly increased after CPPU (a crop growth regulator that can induce bitter taste in cucurbitae) treatment, and the content of cucurbitin B in mutant fruit did not change significantly, but the fruit was still not bitter. Based on this, it can be concluded that *CmBt* is the 'switch' gene regulating the formation of bitterness in melon fruits, while *CmBr* is the 'switch' gene regulating the formation of bitterness in melon roots.

'Switch' gene regulating cucurbitacin C biosynthesis

Nine genes involved in cucurbitacin C biosynthesis are directly regulated by two bHLH-type transcription factors (*Bl* and *Bt*). The *Bl* controls the formation of bitterness in cucumber leaves, while *Bt* controls the formation of bitterness in cucumber fruits, through re-sequencing of cucumber mutants, it was found that the *Bl* gene interacts with the *Bi* promoter to regulate *Bi* expression[34]. Based on a genome-wide association analysis of 115 cucumber core germplasms, an SNP site closely linked to leaf bitterness was identified, this SNP causes the amino acid substitution from cysteine to tyrosine at position 393 amino acid in *Csa6G088690*, resulting in leaves from bitter to non-bitter[34]. Therefore, it can be concluded that *Bl* is the 'switch' gene specifically regulating the synthesis of bitterness in cucumber leaves.

Based on 115 cucumbers, *Csa5G157230* and *Bi* were highly expressed in wild fruits, but not expressed in cultivated fruits, and

Table 2. Gene related to biosynthesis and regulation of cucurbitacin B, C, and E in melon, cucumber, and watermelon.

	Gene name	Gene ID	Gene types	Notes	Structure of Cucurbitacins
Cucurbitacin B	*CmBi*	*Melo3C022374*	OSC	Cucurbitadienol synthase	
	CmACT	*Melo3C022373*	ACT	Acyltransferase	
	Cm160	*Melo3C022377*	CYP81Q58	–	
	Cm170	*Melo3C022376*	CYP89A140	–	
	Cm180	*Melo3C022375*	CYP81Q59	C2 hydroxylase	
	Cm710	*Melo3C022372*	CYP87D19	–	
	Cm890	*Melo3C002192*	CYP87D20	C11 carbonylase + C20 hydroxylase	
	Cm490	*Melo3C023960*	CYP712D8	–	
	CmBt	*Melo3C005611*	bHLH TF	Specifically expressed in fruits	
	CmBr	*Melo3C005610*	bHLH TF	Specifically expressed in roots	
Cucurbitacin C	*CsBi*	*Csa6G088690*	OSC	Cucurbitadienol synthase	
	CsACT	*Csa6G088700*	ACT	Acyltransferase	
	Cs160	*Csa6G088160*	CYP81Q58	19,25-Dihydroxy-cucurbitadienol	
	Cs170	*Csa6G088170*	CYP89A140	–	
	Cs710	*Csa6G088710*	CYP89D19	–	
	Cs490	*Csa3G698490*	CYP712D8	–	
	Cs540	*Csa3G903540*	CYP88L2	19-Hydroxy-cucurbitadienol	
	Cs550	*Csa3G903550*	CYP88L3	–	
	Cs890	*Csa1G044890*	CYP87D20	–	
	CsBt	*Csa5G157230*	bHLH TF	Specifically expressed in fruits	
	CsBl	*Csa5G156220*	bHLH TF	Specifically expressed in leaves	
Cucurbitacin E	*ClBi*	*Cla007080*	OSC	Cucurbitadienol synthase	
	ClACT	*Cla007081*	ACT	Acyltransferase	
	Cl160	*Cla007077*	CYP81Q58	–	
	Cl170	*Cla007078*	CYP89A140	–	
	Cl180	*Cla007079*	CYP81Q59	C2 hydroxylase	
	Cl710	*Cla007082*	CYP89D19	–	
	Cl890A	*Cla008355*	CYP87D20	C11 carbonylase + C20 hydroxylase	
	Cl890B	*Cla008354*	CYP87D20	C11 carbonylase + C20 hydroxylase	
	Cl490	*Cla017252*	CYP712D8	–	
	Cl510	*Cla016164*	CYP88A60	–	
	ClBt	*Cla011508*	bHLH TF	Specifically expressed in fruits	
	ClBr	*Cla011510*	bHLH TF	Specifically expressed in roots	

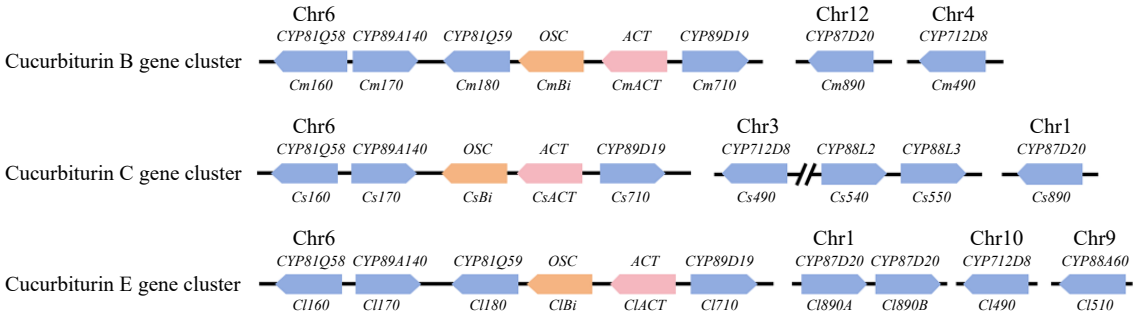

Fig. 3 Cucurbitacin B, C, and E gene clusters (adapted from Ma[7]). Orange squares represent OSC family genes, pink squares represent ACT genes, and blue squares represent CYP450 family genes.

Csa5G157230 was positively correlated with *Bi* expression. It was inferred that *Csa5G157230* may be a *Bt* gene regulating the formation of bitter taste in cucumber fruits. It is the 'switch' gene that regulates the synthesis of bitter fruit[78].

'Switch' gene regulating cucurbitacin E biosynthesis

In cucumber, *CsBl* and *CsBt* are specific transcription factors that regulate the biosynthesis of cucurbitacin C in leaves and fruits, respectively. The mutation of *CsBt* leads to the domestication of the wild type with bitterness. The biosynthesis of cucurbitacins is conserved among Cucurbitaceae crops[34]. Based on this, it can be speculated that the biosynthesis of cucurbitacin E in watermelon is also regulated by conserved transcription factors. In 2016, Zhou et al.[19] established a transient *Agrobacterium* infiltration expression system in cotyledons and confirmed that transient expression of *ClBt* or *ClBr* in watermelon cotyledons induces the biosynthesis of cucurbitacin E. Therefore, Zhou et al.[19] speculated that *ClBt* and *ClBr* are the 'switch' genes regulating the synthesis of bitterness in watermelon fruits and roots, respectively.

Transport mechanism of cucurbitacins

Although cucurbitacins are hailed as 'green pesticides', they can also be toxic to the plant's cells. To prevent self-toxicity, plants have gradually evolved a detoxification mechanism[15], wherein cucurbitacins produced by the cells are transported to adjacent cells or even other tissues or organs through specific transport proteins, thereby reducing self-toxicity. With the advancement of molecular biology, researchers have begun to pay more attention to the study of transport proteins within plants.

Through transcriptome analysis of wild and cultivated cucumbers, Shang et al.[34] discovered a transport protein co-expressed with cucurbitacin C biosynthesis genes, named *CsABC1*, which is located on the vacuolar membrane of cucumber leaf cells. This transport protein can transport cucurbitacin C from the cytoplasm to the vacuole, thereby protecting the cell from self-toxicity. Zhong et al.[81] further confirmed the results of Shang et al.'s experiment.

Additionally, in 2022, Zhong et al.[82] discovered the transport proteins for cucurbitacin B and E in melon and watermelon through gene mining and comparative genomics. These *MATE* genes, *Melo3C002190* and *Cla008357*, were named as *CmMATE1* and *ClMATE1*, respectively. Cucurbitacin B is transported to the rhizosphere *via CmMATE1*, selectively enriching two bacterial genera in the soil (*Enterobacter* and *Bacillus*). The enrichment of these two bacterial genera, in turn, increases resistance to the soil-borne fungal pathogen *Fusarium oxysporum*, thereby enhancing plant adaptability[82].

Domestication of non-bitter Cucurbitaceae crops

In the genetic analysis of bitterness traits in Cucurbitaceae crops, a high similarity was observed among crops such as cucumber, melon, watermelon, and bottle gourd. The research identified a genetic segregation ratio of 9:7 for bitterness across multiple species[2,35,43–45,65]. This phenomenon suggests the existence of a conserved regulatory network for cucurbitacin biosynthesis in Cucurbitaceae crops. For instance, the *CsBt* in cucumber and the *ClBt* in watermelon can induce the synthesis of cucurbitacin E[19,45], demonstrating their functional conservation across different species. This synergistic effect of gene regulation not only supports the hypothesis that bitterness domestication occurred before the diversification of Cucurbitaceae but also provides new perspectives for understanding the complex regulatory mechanisms of cucurbitacin biosynthesis through cross-species validation. In the future, these findings may offer crucial evidence for improving bitterness traits of Cucurbitaceae crops through molecular breeding and gene editing technologies, thereby supporting a variety improvement and production optimization on a global scale.

Prospects

Breeding of bitter-free Cucurbitaceous crops

Currently, the genetic populations used for the positioning research of bitter genes in Cucurbitaceae crops are mostly F_2 and backcross populations, which has resulted in a series of issues such as inaccurate positioning of bitterness genes, poor experimental repeatability and stability. It is necessary to continuously overcome difficulties and utilize permanent populations to construct genetic maps, moreover, there should be a vigorous collection of wild and foreign germplasm resources for germplasm innovation. In addition, when constructing genetic maps for Cucurbitaceae crops, outdated markers such as RFLP, AFLP, RAPD, and SSR are still being used, which places the genetic markers relatively far from the target genes. There should be ongoing development of functional SNP markers and continuous technological innovation to accelerate the fine mapping and cloning process of bitter genes in cucurbit crops. Although a large number of genes related to the bitter traits in cucurbit crops have been identified, the lack of a mature genetic transformation system has led to insufficient research on the functional analysis of these related genes. Therefore, optimizing the genetic transformation system of cucurbit crops and improving gene editing efficiency is also one of the key focuses for the future breeding of new varieties of cucurbit crops without bitterness.

Cucurbitacins produced by cucurbit crops have certain disease and pest-resistance capabilities. Over the years, researchers have domesticated these crops to gradually eliminate fruit bitterness while retaining vegetative bitterness to resist pests and diseases[81]. However, due to incomplete domestication, cucurbit crops still exhibit bitterness when subjecting to environmental stress. Therefore, researchers have proposed a new breeding approach for non-bitter cucurbit crops by regulating the 'switch' genes of cucurbitacin biosynthesis, it is possible to cultivate superior varieties with bitter vegetative parts but non-bitter fruits. This approach ensures that cucurbit crops are protected from pests and diseases while significantly improving fruit quality.

Development of the medicinal value of cucurbitacins

The medicinal values of cucurbitacins, such as anti-tumor, anti-inflammatory, antioxidant, and blood sugar-lowering effects, are well known. However, the extremely low content of cucurbitacins in cucurbit crops pose a significant challenge for extraction. Therefore, while optimizing extraction processes, large-scale *in vitro* synthesis is also necessary. Currently, the high cost of synthesis hinders large-scale production. Some researchers have proposed introducing the entire cucurbitacin biosynthetic pathway into the yeast genome, enabling rapid and efficient synthesis and modification of cucurbitacins through fermentation. This approach offers new insights and references for developing new anticancer drugs in the future[83]. Additionally, due to the large molecular weight and complex structure of cucurbitacins, there are few reports on the physiological and biochemical mechanisms of cucurbitacin production, which significantly hinders the *in vitro* synthesis process. By thoroughly studying the physiological and biochemical mechanisms of cucurbitacin production in various cucurbit crops, we can provide a theoretical foundation for the large-scale *in vitro* synthesis of cucurbitacins and greatly enhance their medicinal value.

Author contributions

The authors confirm contribution to the paper as follows: draft manuscript preparation: Chai Y; concept design and manuscript revision: Sun Y. All authors reviewed the results and approved the final version of the manuscript.

Data availability

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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Conflict of interest

The authors declare that they have no conflict of interest.

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