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REVIEW

Plant-derived isoprenoid sweeteners: recent progress in biosynthetic gene discovery and perspectives on microbial production

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ABSTRACT

Increased public awareness of negative health effects associated with excess sugar consumption has triggered increasing interest in plant-derived natural sweeteners. Steviol glycosides are a group of highly sweet diterpene glycosides contained in the leaves of stevia (*Stevia rebaudiana*). Mogrosides, extracted from monk fruit (*Siraitia grosvenorii*), are a group of cucurbitane-type triterpenoid glycosides. Glycyrrhizin is an oleanane-type triterpenoid glycoside derived from the underground parts of *Glycyrrhiza* plants (licorice). This review focuses on the natural isoprenoid sweetening agents steviol glycosides, mogrosides, and glycyrrhizin, and describes recent progress in gene discovery and elucidation of the catalytic functions of their biosynthetic enzymes. Recently, remarkable progress has been made in engineering the production of various plant-specialized metabolites in microbial hosts such as *Saccharomyces cerevisiae* via the introduction of biosynthetic enzyme genes. Perspectives on the microbial production of plant-derived natural sweeteners are also discussed.

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The increasing incidence of diseases of aging and lifestyle, such as diabetes, has become a social concern in developed countries such as Japan, the United States, and European nations. Obesity, which is a major factor in diabetes, also increases the risk of numerous other lifestyle-related diseases including hypertension. As a result, there is growing interest in non-nutritive natural sweeteners as sugar substitutes that do not raise blood sugar levels. Some of the natural non-nutritive sweeteners are extracts of plants such as stevia (*Stevia rebaudiana*), monk fruit (*Siraitia grosvenorii*), and licorice (*Glycyrrhiza* sp.). These plants have been the subject of extensive phytochemical studies to identify compounds with potent sweetening properties. Moreover, many pharmacological studies have examined the products of these plants to obtain the approval necessary for their use as food additives.

The recent availability of high-throughput sequencing technologies combined with sophisticated bioinformatics methods have enabled plant scientists to conduct genome and transcriptome analyses of non-model plants, including the three above-mentioned genera, and candidate gene mining for gene discovery in plant-specialized metabolic pathways. As a result, many of the genes encoding enzymes related to the biosynthesis of plant-specialized metabolites have been identified. This review focuses on the natural, non-nutritive, isoprenoid sweetening agents produced by stevia, monk fruit, and licorice, and describes the latest findings related to their biosynthesis, focusing on gene discovery and the elucidation of the catalytic functions of biosynthetic enzymes.

The discovery of biosynthetic genes has contributed significantly to advances in synthetic biology, which introduces enzyme genes isolated from plants into microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae* to reconstruct the biosynthetic pathways and produce useful specialized metabolites. Perspectives on the use of biosynthesis genes for the microbial production of plant-derived natural sweeteners are also discussed.

Biosynthesis of steviosides

S. rebaudiana (Bertoni) is a perennial plant from the family Asteraceae, that is native to Paraguay and Brazil, where it is known as "stevia" or "honey leaf" for its powerful sweetness [1]. Although the genus *Stevia* contains over 150 species, *S. rebaudiana* stands out because of its sweetness [2].

Stevia extract is prepared from dried *S. rebaudiana* leaves. It is approved as a food additive in both Japan and the West and is used as a non-caloric sugar substitute in many food products and drinks. The sweet component of stevia extract is a mixture of diterpene glycosides called steviol glycosides, which can accumulate to a level of 10–20% of the dry weight of leaves [3]. More than

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35 different steviol glycosides have been identified in *S. rebaudiana* [4,5]. Steviol glycosides share steviol as the aglycone part and differ in the number and type of sugars attached. Of these, stevioside, containing three glucose molecules linked to steviol, and rebaudioside A, containing four glucose molecules linked to steviol, are present in the highest quantities and are the major substances contributing to the sweetness of stevia extract [6]. Stevioside tastes 150–250 times sweeter than sucrose, whereas rebaudioside A tastes 200–300 times sweeter than sucrose [6]. The overall quality of the taste elicited by rebaudioside A is superior to that elicited by stevioside, because rebaudioside A is not only more potent than stevioside, but is also substantially less bitter [7].

Many molecular studies have examined the biosynthesis of steviol glycosides. A comprehensive analysis of the genes expressed in the leaves led to the isolation of a number of genes encoding the enzymes [8]. Figure 1 shows the proposed pathway for the biosynthesis of rebaudioside A from geranylgeranyl diphosphate (GGPP), a compound with 20 carbon atoms that is the common precursor of diterpenes.

In the biosynthesis of steviol glycosides, GGPP is first cyclized to *ent*-kaurene via an *ent*-copalyl diphosphate (*ent*-CDP) intermediate. Two diterpene cyclases that catalyze these reactions have been isolated: *ent*-copalyl diphosphate synthase (*ent*-CPS) and *ent*-kaurene synthase (*ent*-KS) [9]. Next, *ent*-kaurene is oxidized by the cytochrome P450 monooxygenase (P450) *ent*-kaurene oxidase (*ent*-KO) to produce *ent*-kaurenoic acid. Since *ent*-kaurenoic acid is a gibberellin (plant hormone) precursor, the subsequent production of steviol (corresponding to the non-sugar part of steviol glycosides) results from the hydroxylation of *ent*-kaurenoic acid at C-13 and is the first committed step in the biosynthesis of steviol glycosides. The P450 CYP716D4, denoted as *ent*-kaurenoic acid 13-hydroxylase, is published in GenBank (accession nos. ABD60225, ACD93722, and ACL10147); however, no functional characterization of the enzyme has been reported [4].

After the formation of steviol, a series of glycosylations occur to form the large family of steviol glycosides.

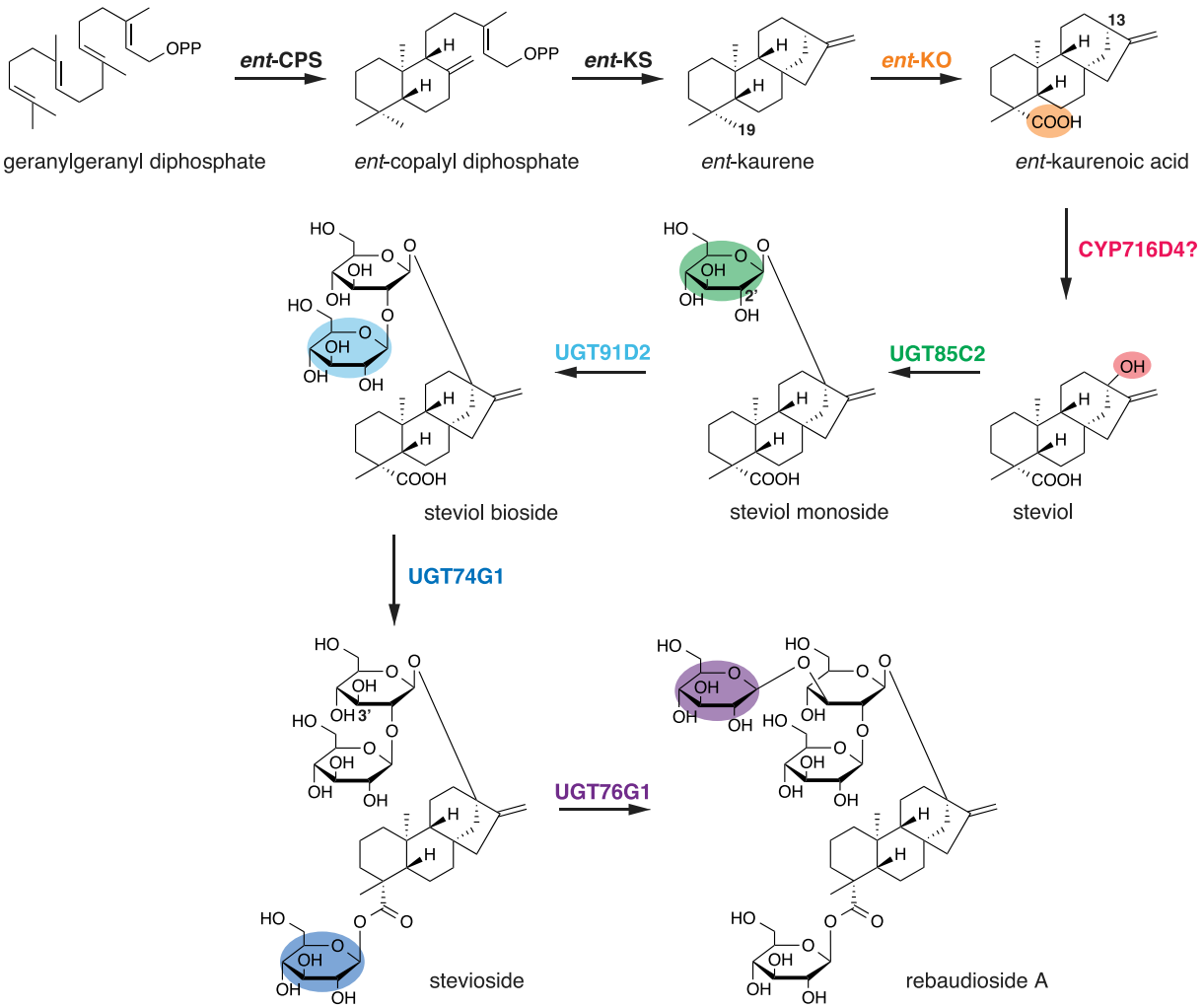

Figure 1. Proposed pathway for the biosynthesis of rebaudioside A in *S. rebaudiana*. *ent*-CPS, *ent*-copalyl diphosphate synthase; *ent*-KS, *ent*-kaurene synthase; *ent*-KO, *ent*-kaurene oxidase. The carboxyl group at C-19 introduced by *ent*-KO and the hydroxyl group at C-13 likely introduced by CYP716D4 are highlighted in orange and pink, respectively. The glucose moieties attached by UGT85C2, UGT91D2, UGT74G1, and UGT76G1 are highlighted in green, pale blue, blue, and purple, respectively.

Four UDP-dependent glycosyltransferases (UGTs) have been identified in the conversion of steviol into rebaudioside A, which contains four glucose molecules linked to steviol. UGTs are enzymes that catalyze the transfer of various sugars from a UDP-sugar to an acceptor molecule. The plant genome contains numerous UGTs catalyzing the glycosylation of a variety of secondary metabolites. For example, 115 UGT genes have been identified in the *Arabidopsis thaliana* genome [10].

Figure 1 depicts sequential glycosylation steps as the main proposed pathway for the biosynthesis of rebaudioside A from steviol. However, in plants, the glycosylation reactions catalyzed by the four glycosyltransferases described below are not always sequential, but form a grid pattern, resulting in a variety of minor products. Steviol has two glycosylation sites: the C-13 hydroxyl group and the C-19 carboxyl group. UGT85C2 transfers glucose to the hydroxyl group at C-13 of steviol to produce steviol monoside [11]. A second glucose molecule is added to C-2′ of the C13-glucose that was added earlier by UGT85C2 to produce steviol bioside. UGT91D2 catalyzes this reaction [12]. The glucosylation of steviol bioside at C-19 catalyzed by UGT74G1 produces stevioside [11]. Of the steviol glycosides, stevioside is present in the highest quantities in stevia leaves. Finally, UGT76G1 transfers another glucose molecule to C-3′ of the C-13 glucose of stevioside, resulting in rebaudioside A, which has four glucose molecules attached to steviol [11]. All of the sugars linked to stevioside and rebaudioside A are glucose. However, there are minor analogous compounds, in which one of the glucose moieties is replaced by rhamnose or xylose. These analogs have different degrees of sweetness compared with stevioside and rebaudioside A. The quality and intensity of sweetness vary depending on the sugar composition of the steviol glycosides. Therefore, UGTs that catalyze the transfer of sugars are important determinants of the sweetness characteristics of stevia.

Biosynthesis of mogrosides

S. grosvenorii (Swingle) is a perennial that belongs to the family Cucurbitaceae. *S. grosvenorii* is native to southern China and its fruit has long been used as a sweetener and as a folk medicine for the treatment of colds, sore throats, and minor stomach and intestinal troubles [13,14]. *S. grosvenorii* is a protected species in China and is cultivated primarily in few areas in Guangxi Province. The export of living plant material is prohibited by law.

The sweetness components in *S. grosvenorii* fruit are mogrosides, which are cucurbitane-type triterpenoid glycosides that are present at levels of approximately 1% in the flesh of the fresh fruit [13]. All mogrosides contain mogrol as the triterpenoid aglycone and differ in the number and type of sugars attached. Among them, mogroside V, which accounts for approximately 20% of mogrosides, is over 300 times sweeter than sucrose [15–17]. Mogrosides are high-potency zero-calorie sweeteners that can be used as sucrose substitutes for diabetic and obese patients. Mogrosides have recently been reported to have a variety of important pharmacological effects such as anti-carcinogenic, anti-diabetic, anti-oxidative, and anti-allergic activities [18,19].

The biosynthetic pathways for mogrosides were mostly unknown until the recent identification of all the enzymes involved in the steps from squalene to mogroside V, made possible by genome sequencing and transcriptome analysis at each developmental stage of the fruit. Figure 2 shows the mogroside V biosynthetic pathway, as proposed by Itkin et al. [20]. The first step in the structural diversification of triterpenoid glycosides is the cyclization of 2,3-oxidosqualene by a group of enzymes called oxidosqualene cyclases (OSCs). More than 80 OSCs have been characterized from plants [21]. Most plant OSCs, including β-amyrin synthase and lupeol synthase, which are found in numerous plant species, catalyze the cyclization reactions of 2,3-oxidosqualene. One of the characteristics of the mogroside V biosynthesis pathway is that cucurbitadienol synthase (CDS), which is an oxidosqualene cyclase, uses 2,3;22,23-diepoxysqualene as its substrate to produce 24,25-epoxycucurbitadienol. Genomic analysis has revealed that *S. grosvenorii* harbors five genes that may encode squalene epoxidases (SQEs). Of these, two are strongly expressed during the initial stages of fruit development, as are CDS, P450 (CYP87D18), and epoxy hydrolases (EPHs), which catalyze the subsequent steps, and are predicted to be involved in the production of 2,3;22,23-diepoxysqualene. In comparison, CDS is encoded by a single gene, and functional analyses using *S. cerevisiae* and transformed tobacco plants indicated that it produces 24,25-epoxycucurbitadienol from 2,3;22,23-diepoxysqualene [20]. The most important discovery of this study was the mechanism behind the hydroxylations at positions C-24 and C-25. Itkin et al. [20] initially hypothesized that these hydroxylations were catalyzed by P450s using cucurbitadienol produced from 2,3-oxidosqualene as the substrate. Therefore, the hydroxylation of cucurbitadienol by over 40 candidate P450s that are strongly expressed in the initial stages of fruit development was investigated. However, none of the P450s examined showed such activity. Itkin et al. [20] then searched for an epoxide hydrolase that could generate 24,25-dihydroxycucurbitadienol from 24,25-epoxycucurbitadienol. The *S. grosvenorii* genome contains eight genes encoding annotated epoxide hydrolases, three of which are strongly expressed in the initial stages of fruit development. These three epoxide hydrolases were investigated and it was determined that 24,25-dihydroxycucurbitadienol was produced from the 24,25-epoxycucurbitadienol substrate [20]. Then, Itkin et al. [20] analyzed the activity of CYP87D18, which had previously been identified by Zhang et al. [22], as being involved in the C-11 oxidation of cucurbitadienol, and

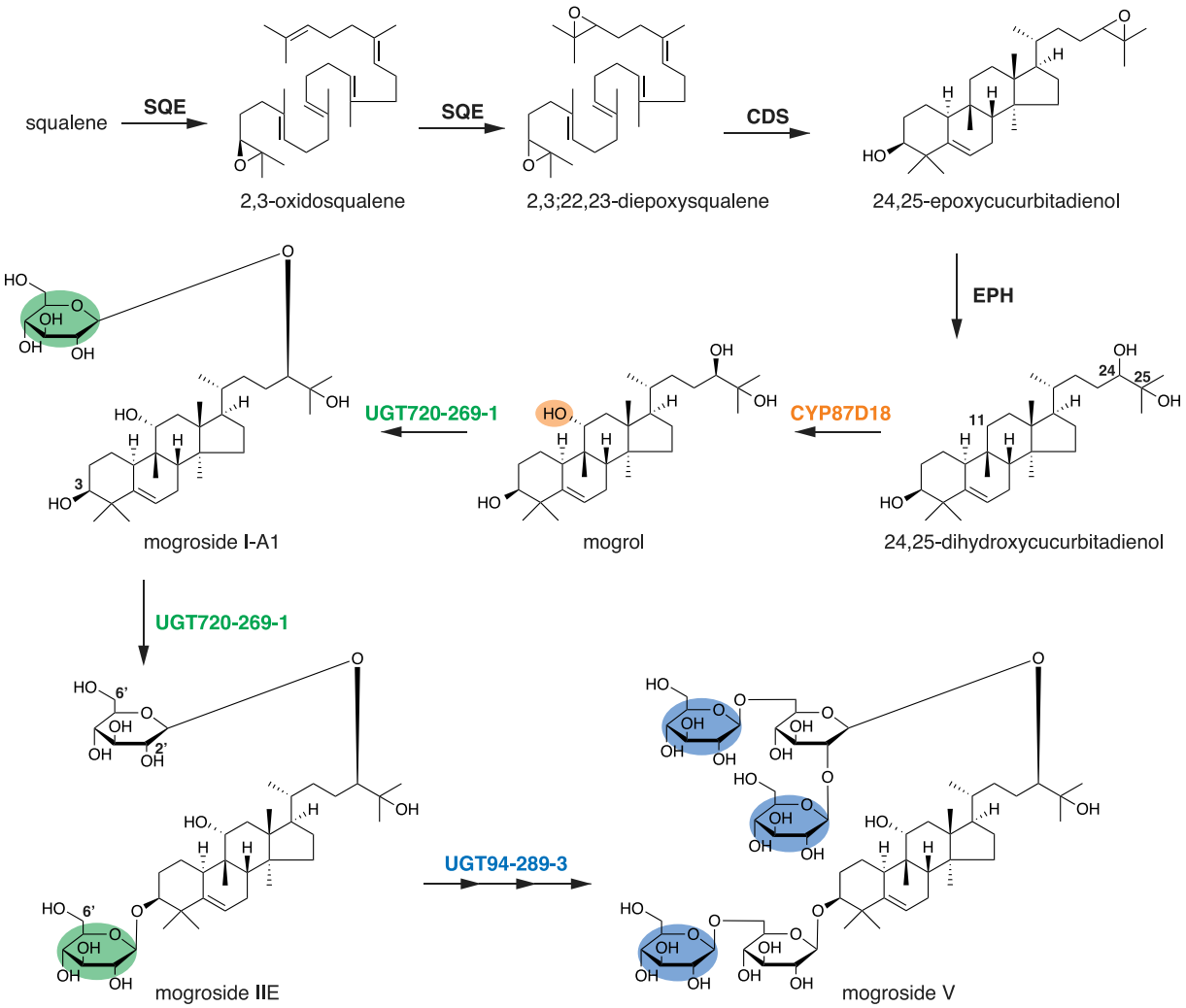

Figure 2. Proposed pathway for the biosynthesis of mogroside V in *S. grosvenorii*. SQE, squalene epoxidase; CDS, cucurbitadienol synthase; EPH, epoxide hydrolase. The hydroxyl group at C-11 introduced by CYP87D18 is highlighted in orange. The glucose moieties attached by UGT720-269-1 and UGT94-289-3 are highlighted in green and blue, respectively.

revealed that CYP87D18 also catalyzes the C-11 hydroxylation of 24,25-dihydroxycucurbitadienol to generate 11,24,25-trihydroxycucurbitadienol, corresponding to the common aglycone part of mogrosides, mogrol.

After the formation of mogrol, a series of glycosylations occurs to add five glucose molecules, two at position C-3 and three at position C-24, to produce the most important sweetness component, mogroside V. Two UGTs were shown to contribute to these steps. One of them is UGT720-269-1, which is strongly expressed in the initial stages of fruit development and transfers one glucose molecule each to the hydroxyl groups at positions C-24 and C-3 of mogrol to produce mogroside IIE (C-3 and C-24 glucosylations) via mogroside I-A1 (C-24 glucosylation) as an intermediate. The second UGT is UGT94-289-3, which is strongly expressed in the latter stages of fruit development and adds sugars to the other sugars already present on the acceptor molecule. UGT94-289-3 was shown to add one glucose molecule each at positions C-2′ and C-6′ of the C-24 glucose of mogroside IIE, which was added earlier by UGT720-269-1. UGT94-289-3 also adds a glucose molecule to position C-6′ of the glucose bound at position C-3 of mogroside IIE, thereby sequentially catalyzing three sugar transfer reactions to produce mogroside V [20].

Biosynthesis of glycyrrhizin

Licorice is the root and stolon of *Glycyrrhiza* plants, which belong to the family Fabaceae. The licorice root is an indispensable ingredient of traditional Kampo medicines in Japan. Approximately, 70% of the 294 Kampo prescriptions approved by the Ministry of Health, Labor, and Welfare of Japan contain licorice. The major bioactive compound contained in licorice is a triterpenoid glycoside, glycyrrhizin. Glycyrrhizin has diverse pharmacological effects and is used as the starting material for pharmaceutical preparations such as liver-protecting remedies. Glycyrrhizin is approximately 150 times sweeter than sugar. Consequently, glycyrrhizin and licorice extracts are used as additives in a variety of foods [23,24]. Of the more than 20 species of *Glycyrrhiza*, three contain large quantities of glycyrrhizin (2–8% of the dry weight): *G. uralensis*, *G. glabra*, and *G. inflata*. In Japan,

G. glabra and *G. inflata* are primarily used as starting materials for food additives, while *G. uralensis* is used as a starting material for pharmaceuticals [24].

Glycyrrhizin, a triterpenoid glycoside, is a conjugate of one molecule of glycyrrhetinic acid (an oleanane-type triterpenoid) and two molecules of glucuronic acid connected to a hydroxyl group at the C-3 of glycyrrhetinic acid (Figure 3). Figure 3 shows the hypothesized biosynthetic pathway for glycyrrhizin. The biosynthesis of glycyrrhizin involves the cyclization of 2,3-oxidesqualene by an OSC, β-amyrin synthase (bAS), to generate the triterpene scaffold, β-amyrin. The subsequent steps are believed to involve a series of oxidative reactions at the C-11 and C-30 positions of β-amyrin to produce the aglycone part, glycyrrhetinic acid. Finally, two molecules of glucuronic acid are transferred to the hydroxyl group at the C-3 position of glycyrrhetinic acid to produce glycyrrhizin.

To identify the oxidizing enzymes and UGTs involved in these reactions that convert β-amyrin to glycyrrhizin, we generated a full-length cDNA library from *G. uralensis* stolon samples and analyzed the nucleotide sequence of approximately 57,000 clones [25]. This analysis identified approximately 10,000 unique genes after eliminating redundant sequences. Five candidate P450s that are highly expressed in the roots and stolons, where glycyrrhizin is specifically accumulated, were selected and their enzymatic abilities to modify β-amyrin were analyzed. CYP88D6, one of the candidate P450s, catalyzed the sequential two-step oxidation at the C-11 position of β-amyrin, thereby converting it to an intermediate product, 11-oxo-β-amyrin [26]. CYP72A154, another candidate P450, catalyzed the three-step oxidation at the C-30 position of 11-oxo-β-amyrin to produce glycyrrhetinic acid [27]. After the formation of glycyrrhetinic acid, a series of glucuronosylations occurs to add two molecules of glucuronic acid to a hydroxyl group at C-3. Recently, Xu et al. [28] reported one unique *Gu*UGAT (inferred to belong to the UGT73 family) that catalyzes the continuous two-step glucuronosylation of glycyrrhetinic acid to yield glycyrrhizin via glycyrrhetinic acid 3-*O*-monoglucuronide as a reaction intermediate, thus uncovering the complete pathway of glycyrrhizin biosynthesis.

Hayashi et al. [29] reported that the productivity of glycyrrhizin in *G. uralensis* varies among strains. As mentioned above, enzymes involved in glycyrrhizin biosynthesis have been identified; however, the genetic basis of glycyrrhizin productivity is still largely unknown. To address this issue, we performed a comparative transcriptome analysis of low and high glycyrrhizin-producing strains of *G. uralensis* and revealed a set of differentially expressed genes, including *bAS*, *CYP88D6*, and *CYP72A154*, of which the transcript levels are 7–15 times higher in the high glycyrrhizin-producing strain than in the low glycyrrhizin-producing strain [30]. Recently, we also succeeded in constructing a draft assembly of the approximately 379-Mb (corresponding to 94.5% of the estimated genome size) whole-genome sequence of the high glycyrrhizin-producing strain of *G. uralensis* [31]. As a result, genes encoding 34,445 proteins have been discovered. These data are considered extremely useful for searching for transcription factors that regulate the expression of the glycyrrhizin

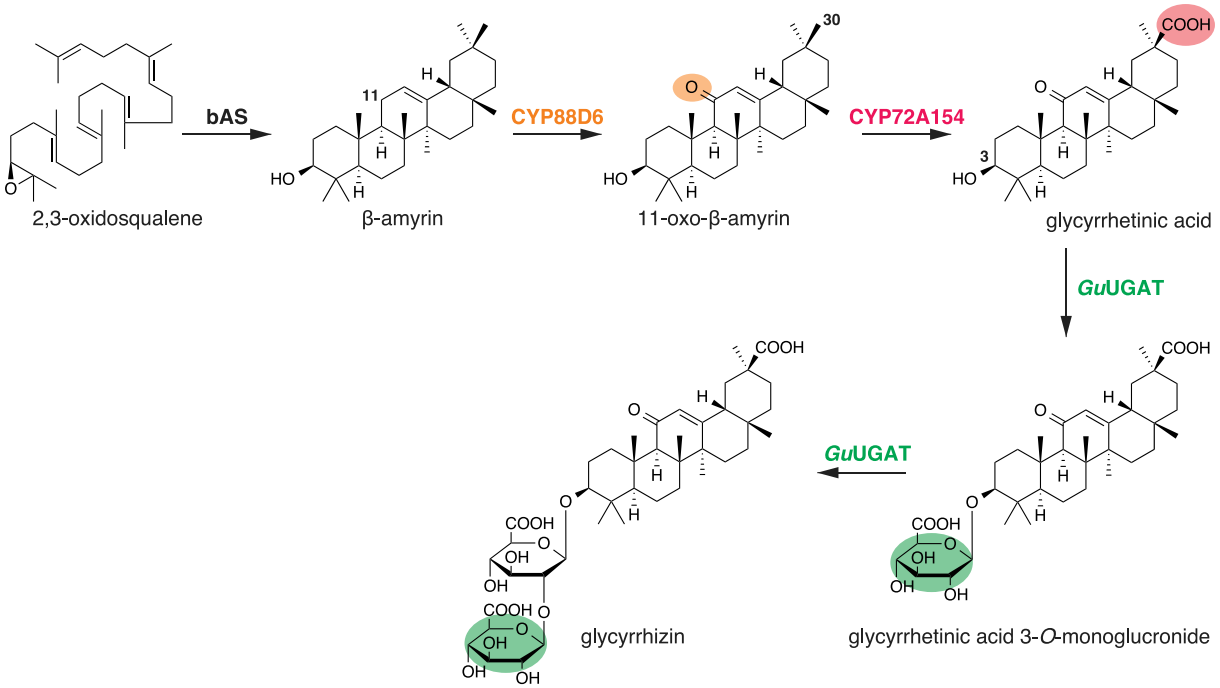

Figure 3. Proposed pathway for the biosynthesis of glycyrrhizin in *Glycyrrhiza uralensis*. bAS, β-amyrin synthase. The carbonyl group at C-11 introduced by CYP88D6 and the carboxyl group at C-30 introduced by CYP72A154 are highlighted in orange and pink, respectively. The glucuronic acid introduced by *Gu*UGAT is highlighted in green.

biosynthetic genes mentioned above, as well as hitherto unidentified factors that might affect glycyrrhizin productivity such as transporters involved in the intracellular accumulation of glycyrrhizin. Moreover, this genome sequencing data will be an important reference for the comparative genome sequencing of different licorice strains and will aid in a better understanding of the genetic basis of different glycyrrhizin productivities and other characteristics.

Perspectives on the microbial production of plant-derived natural sweeteners

Recent progress in the identification of genes encoding enzymes involved in the biosynthesis of natural isoprenoid sweeteners produced by stevia, monk fruit, and licorice is reviewed. As discussed above, most pathway enzymes involved in the biosynthesis of these sweeteners have been identified. It is essential that future research focuses on establishing a biotechnological production system through the introduction of biosynthetic genes of these sweeteners using microbes such as *E. coli* and *S. cerevisiae* as alternative hosts, as these are easy to engineer and inexpensive to grow. The prokaryotic *E. coli* uses an inherent methylerythritol 4-phosphate (MEP) pathway and the eukaryotic *S. cerevisiae* uses the mevalonate (MVA) pathway to produce isopentenyl diphosphate (IPP) and its isomer, dimethylallyl pyrophosphate (DMAPP), which are needed to produce plant-derived isoprenoids. Therefore, both can be employed for the synthesis of isoprenoid sweeteners. *S. cerevisiae* is considered a preferred host because it possesses an endoplasmic reticulum (ER) that allows for the heterologous expression of membrane-localized plant-derived P450s. As mentioned above, the biosynthesis of all three sweeteners involves steps catalyzed by P450s.

Remarkable progress has been made in the engineering of various plant-specialized isoprenoids in *S. cerevisiae*. The production of artemisinic acid, a precursor of the anti-malarial drug artemisinin, enables the low-cost production of artemisinin by semi-synthesis [32,33]. The artemisinin produced by this method is already being supplied to a number of countries in Africa. Another example of a plant-derived isoprenoid in *S. cerevisiae* is the bioactive ginsenoside (compound K), which has received approval from the China Food and Drug Administration to commence clinical trials (CDEL20130379) for arthritis prevention and treatment [34]. This was accomplished by introducing genes that encode ginsenoside biosynthetic enzymes, including dammarenediol-II synthase (an OSC that produces dammarenediol-II from 2,3-oxidosqualene), CYP716A47 (produces protopanaxadiol via the hydroxylation of C-12 in dammarenediol-II), and UGT71A27 (responsible for the glucosylation of the C-20 hydroxyl group).

To produce high levels of desirable isoprenoid products, *S. cerevisiae* has been metabolically engineered to boost flux via the MVA pathway [for more detailed information, see Kampranis and Markis [35] and Moses et al. [36]. This has been achieved by a number of complementary approaches that include the overexpression of a feedback regulation-deficient form of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the overexpression of a mutated version of the transcription factor UPC2 (upc2–1), which leads to the upregulation of most sterol biosynthetic genes, thereby increasing the metabolic flux through the MVA pathway, and by replacing endogenous promoters of competing pathway enzyme genes, such as lanosterol synthase (*ERG7*), for the heterologous triterpenoid pathway with a methionine-repressible promoter. In addition to altering metabolic flux to increase the terpene substrate pool, transforming the subcellular morphology is also a powerful strategy to increase the yield of plant-derived isoprenoids in *S. cerevisiae*. Arendt et al. *has* shown that disruption of the phosphatidic acid phosphatase-encoding gene *PAH1* via genome editing results in dramatic expansion of the ER, which stimulates the production of recombinant ER-associated triterpene biosynthesis enzymes (OSC and P450) and ultimately enhances (up to sixteen-fold) triterpenoid accumulation [37]. A positive effect of *pah1* could also be observed for the production of other terpenoids, depending on ER-associated enzymes for their biosynthesis, such as the sesquiterpenoid artemisinic acid, which was increased two-fold relative to the wild-type strain. Engineering of the yeast strains conducted in the above-mentioned successful examples for altering metabolic flux to increase the terpene substrate pool or expansion of the ER, could also be applied for the production of natural isoprenoid sweeteners highlighted in this review.

The engineering of plant-derived pathway enzymes is also important for the improvement of product yield and/or for specific accumulation of the desired compound. As mentioned in "Biosynthesis of steviosides" the biosynthesis of steviosides includes at least four UGTs to form a metabolic grid of glucosylation reactions, resulting in several minor products *in planta*. The Swiss biotechnology company Evolva is conducting research on the fermentative production of steviol glycosides and recently succeeded in the optimization of UGT76G1 by site-directed mutagenesis based on homology modeling, which enabled the identification of key target amino acids present in the substrate-binding pocket, ultimately diminishing the accumulation of unwanted side products and increasing the specific accumulation of desired compounds in *S. cerevisiae* [12]. Steviol glycosides produced by fermentation under the name EverSweet™ are on course to launch in 2018 in the United States.

The authors of this review have successfully produced glycyrrhetinic acid, which corresponds to the aglycone

part of glycyrrhizin, in *S. cerevisiae* by introducing an OSC, β-amyrin synthase, and two aforementioned P450s [27]. Improvement of the product yield and introduction of glycosylation steps are needed for reconstitution of the full glycyrrhizin biosynthetic pathway.

China is a major producer of licorice as well as monk fruit; however, increased global demand in recent years and/or the imposition of export restrictions for environmental protection purposes have sparked concerns that its stable procurement in the future will become difficult in Japan. Thus, a microbial production method could be a viable alternative to natural sources.

Disclosure statement

No potential conflict of interest was reported by the authors.

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