Applied Microbiology and Biotechnology (2020) 104:6587–6600
https://doi.org/10.1007/s00253-020-10667-z

MINI-REVIEW

Advances on the in vivo and in vitro glycosylations of flavonoids

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Received: 18 March 2020 / Revised: 27 April 2020 / Accepted: 2 May 2020 / Published online: 8 June 2020

Abstract

Flavonoids possess diverse bioactivity and potential medicinal values. Glycosylation of flavonoids, coupling flavonoid aglycones and glycosyl groups in conjugated form, can change the biological activity of flavonoids, increase water solubility, reduce toxic and side effects, and improve specific targeting. Therefore, it is desirable to synthesize various flavonoid glycosides for further investigation on their medicinal values. Compared with chemical glycosylations, biotransformations catalyzed by uridine diphospho-glycosyltransferases provide an environmentally friendly way to construct glycosidic bonds without repetitive chemical synthetic steps of protection, activation, coupling, and deprotection. In this review, we will summarize the existing knowledge on the biotechnological glycosylation reactions either in vitro or in vivo for the synthesis of flavonoid O- and C-glycosides and other rare analogs.

Key points

- *Flavonoid glycosides usually show improved properties compared with their flavonoid aglycones.*
- *Chemical glycosylation requires repetitive synthetic steps and purifications.*
- *Biotechnological glycosylation reactions either in vitro or in vivo were discussed.*
- *Provides representative synthetic examples in detail.*

Keywords Flavonoid glycosides · Glycosylation · Biotechnological glycosylation reactions · Glycosyltransferase

Introduction

Flavonoids are a large number of small molecules with similar structure, 2-phenly ketone moiety, that can be found in the plants' stems, flowers, leaves, or the fruits and exist as their glycosides like galactoside, rhamnoside, arabinoside, or rutinoside (Fang et al. 2013; Pugliese et al. 2013; Taheri et al. 2013; Leonard et al. 2008). Flavonoids possess diverse bioactivities and potential medicinal values, such as antibacterial (Cushnie and Lamb 2011), antimicrobial (Cushnie and Lamb 2005), anticancer (Liu et al. 2010), antiinflammation (González et al. 2011), antioxidant (Burda and Oleszek 2001), antidiabetes (Hii and Howell 1984), and influenza virus neuraminidase inhibition (Liu et al. 2008). Due to the broad medicinal prospect, flavonoids have been attracted considerable attention. To date, there are over 2500 varieties of identified flavonoids (Fig. 1), and the number is still rising sharply.

Flavonoid glycosides, which are conjugated through the linkage between flavonoid aglycones and glycosyl groups, usually show improved properties compared with their flavonoid aglycones, or the properties may be even different (Xiao 2017). Since sugar chain is involved in almost all life processes such as cell differentiation, development, immunity, aging, carcinogenesis, and information transmission, the glycosylation can change the biological activity of flavonoid, increase water solubility, reduce toxic and side effects, and improve specific targeting (Jiang et al. 2008). For instance, rutin (quercetin-3-O-rutinoside) has a higher stability in aqueous solution at 100 °C (Buchner et al. 2006) and a slower degradation rate in phosphate buffer containing Fe^{2+} and Cu^{2+} (Makris and Rossiter 2000) compared with quercetin.

Flavonoids share structures containing a benzene ring A linked with a pyrone ring C and a phenyl ring B in the 2 or 3 position, and glycosylations mostly occur in the positions of

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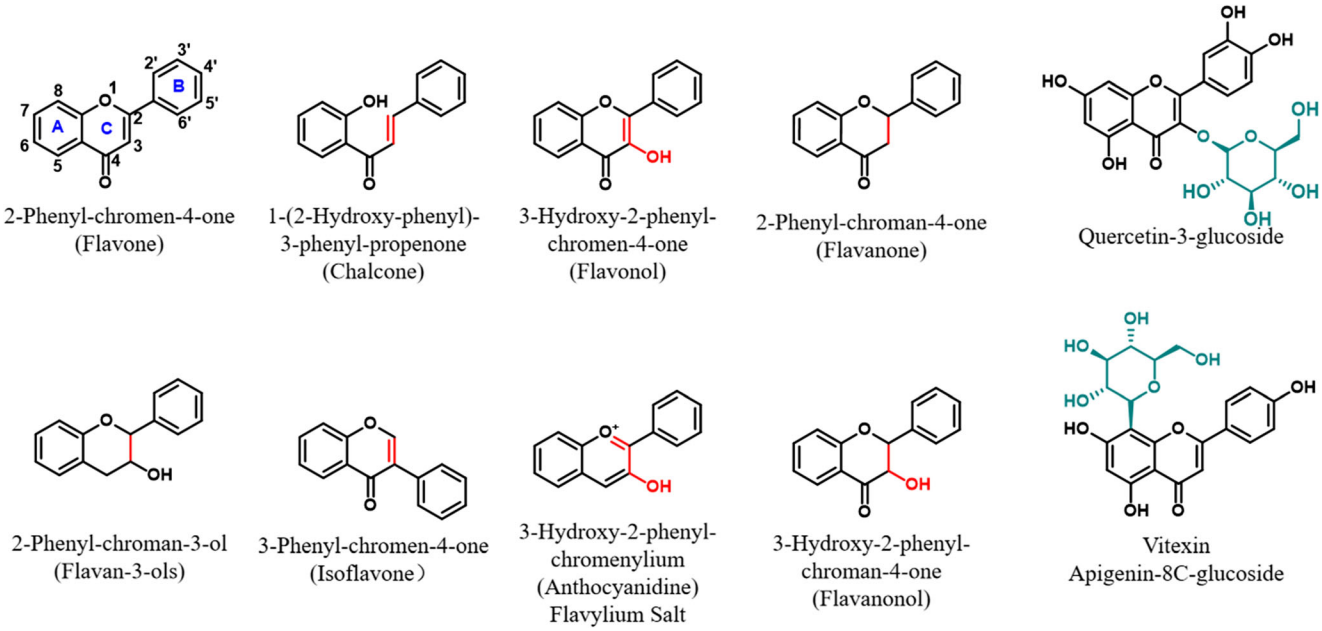

Fig. 1 Chemical structures of representative flavonoids and flavonoid glycosides

C-3, C-5, C-7, C-3′, C-4′, and C-5′ (Xiao 2017) (Fig. 1). Based on the linkage types, flavonoid glycosides are typically divided into two groups: O- and C-glycosides. However, their low content in nature as well as challenging purification process largely limit further investigation on their medicinal values (Qiu et al. 2013; Obmann et al. 2012). Thus, the synthesis, either chemical or biotransformation approach, offers potential alternative to obtain these various flavonoid glycosides, in which glycosylation reaction is the last but most important step and has been of major interest for biochemist to investigate.

However, glycosylations relying on purely synthetic chemical methods, based on repetitive steps of protection, activation, coupling, and deprotection, while highly desirable, are challenging (Nicolaou and Mitchell 2001; Douglas et al. 1998; Grice et al. 1995; Cheung et al. 1997). In addition, the undesirable isomers complicate the desired product isolation and decrease overall yields. Biotransformation, catalyzed by uridine diphosphoglycosyltransferase in the last step in flavonoid biosynthesis (Yonekura-Sakakibara and Hanada 2011), is an environmentally friendly approach that holds a great deal of promise for the bioengineering of glycoconjugates in both prokaryotes and eukaryotes. It offers the opportunity to prepare high-value sugar-based chemicals, biochemicals, and pharmaceuticals from simple feedstocks ranging from carbon dioxide, to glycerol and then glucose; thus, it is applicable in flavonoid glycoside production as well (Koffas and Linhardt 2018). Moreover, this method is capable of production of unnatural compounds which expands flavonoid glycoside library. To date, although hundreds of papers about flavonoid glycoside synthesis were published in the past 10 years, very few reviews paid attention to the summarization of the glycosylation approaches therein (Ati et al. 2017; Yang et al. 2018; Tapas et al. 2008). In this review, we will summarize the existing knowledge on the biotechnological glycosylation reactions either in vitro or in vivo for the synthesis of flavonoid O- and C-glycosides and other rare analogs.

Flavonoid O-glycosides

Flavonoid O-glycoside, in which the anomeric carbon of sugar and flavonoid skeleton are linked through a C–O bond (Roriz et al. 2014), is the most abundant type of flavonoid glycosides (Veitch and Grayer 2011). Glucose, rhamnose, galactose, xylose, arabinose, glucuronic acid, and apiose are the mainly sugar moieties, while glucosylation is the most frequent pattern and mostly presents as β-glucopyranose (Yang et al. 2018). The structures, sources, and activities of representative flavonoid O-glycosides are summarized in Table 1.

Enzymatic O-glycosylations in vitro

Enzymatic glycosylation in vitro is a very attractive strategy due to the enzyme's specificity and the mild reaction condition. During enzymatic O-glycosylation, O-glycosyltransferase is utilized to transfer an activated sugar that containing a nucleoside phosphate or a lipid phosphate

Table 1 Representative flavonoid O-glycosides

Bioactivities	Compound	Source	Tissue
Antifungal	3′-O-β-D-MeGlcAp (vitegnoside) $C_{22}H_{20}O_{12}$	*Vitex negundo* (Sathiamoorthy et al. 2007)	Leaf
	7-O-(6-O-acetyl-β-Glcp)-(1 → 3)[α-Rhap-(1 → 2)]-β-Glcp (saussureanoid) $C_{56}H_{68}O_{34}$	*Saussurea lappam* (Rao et al. 2007)	Root
	5,7-Dihydroxy-3-methoxy-6-C-methylflavone 8,4′-di-O-β-D-glucopyranoside $C_{28}H_{32}O_{17}$	*Picea neoveitchii* (Chen et al. 2012)	Leaf
	Thalassiolin D $C_{22}H_{22}O_{14}S$	*Thalassia hemprichii* (Hawas and Abou El-Kassem 2017)	Whole
Antioxidant	Apigenin-5-O-β-D-(6″-acetylglucopyranosyl-(2 → 1)-α-L-rhamnopyranoside $C_{29}H_{30}O_{14}$	*Cephalotaxus harringtonii* var. *harringtonii Cephalotaxus koreanad* (Mendiratta et al. 2007)	Leaf
	6-O-α-Rhap-(1 → 6)-β-Glcp (nitensoside A) $C_{28}H_{31}O_{15}$	*Pterogyne nitens* (Fernandes et al. 2008)	Leaf
	7-O-(4-O-E-p-coumaroyl-β-Glcp) $C_{30}H_{26}O_{13}$	*Mallotus metcalfianus* (Rivière et al. 2009)	Whole
	6-O-α-Rhap-(1 → 6)-β-Glcp (nitensoside B) $C_{28}H_{31}O_{16}$	*Pterogyne nitens* (Fernandes et al. 2008)	Leaf
	5,7,4′-Trihydroxy-3′,5′-dimethoxyflavone(tricin) $C_{16}H_{12}O_7$	*Erica arborea* (Nazemiyeh et al. 2008)	Aerial part
	Tricin-7-*O*-β-(6″-methoxycinnamic)-glucoside $C_{33}H_{32}O_{14}$	*Saccharum officinarum* (Duarte-Almeida et al. 2007)	Homogenate
Anticancer	3′,5,7-Trihydroxy-4′-methoxyflavone-7-ramnoglucosid (Diosmin) $C_{30}H_{36}O_{13}$	Citrus fruits (Naso et al. 2016)	Whole
Inhibition of lipopolysaccharide-induced NO production	7-O-(2,3-Di-O-acetyl-α-Rhap)-(1 → 6)-β-Glcp (peregrinumin A) $C_{32}H_{36}NaO_{16}$	*Dracocephalum peregrinum* (Fu et al. 2009)	Whole
Inhibition of osteoclast differentiation	5,7,3′,4′-Tetrahydroxyflavone(luteolin) $C_{22}H_{24}O_{11}$	*Cephalotaxus koreanad* (Yoon et al. 2007)	Aerial part
Myeloperoxidase inhibition	6-O-α-Rhap-(1 → 6)-β-Glcp (nitensoside B) $C_{28}H_{31}O_{16}$	*Pterogyne nitens* (Fernandes et al. 2008)	Leaf
Urease inhibition	4′-O-(3-O-E-p-coumaroyl-β-Glcp) $C_{21}H_{20}O_{11}$	*Marrubium anisodon* (Hussain et al. 2009)	Whole
Cytotoxicity	7-O-(6-O-butyryl-β-Glcp) $C_{25}H_{26}O_{11}$	*Phyllanthus emblica* (El-Desouky et al. 2008)	Leaf
	7-O-β-D-GlcAp-(1 → 2)-β-D-Glcp $C_{28}H_{30}O_{17}$	*Stratiotes aloides* (Conrad et al. 2009)	Whole

leaving group as donor to the flavonoid acceptor to form a glycosidic bond. 3-O-glycosyltransferase, 5-O-glycosyltransferase, and 7-O-glycosyltransferase are the most widely investigated enzymes as most glycosylations occur on 3-, 5-, and 7-OH positions of the flavonoids, and especially on the 3-OH (Xiao 2017). We will summarize the most common approaches for O-glycosylations in vitro and discuss the representative synthetic examples in detail.

Representative example: regioselective O-glycosylations

In traditional chemical synthesis of glycosides, repetitive protection and de-protection steps are unavoidable to achieve the regioselective glycosylations (Wang et al. 2013). In contrast, enzymes are capable of catalyzing the reactions with exquisite regioselectivity and stereoselectivity (α- or β-glycosidic

linkages) directly (Chang et al. 2011). Park et al. (Rha et al. 2019) achieved the site-specific glycosylation of hydroxyflavones and hydroxyflavanones by *Deinococcus geothermalis* amylosucrase (DGAS), which shows unique transglycosylation activity on a wide range of acceptor-dependent flavonoids. They found that the enzyme could regioselectively catalyze glucose donors on the 6-OH and 4′-OH positions of hydroxyflavones and hydroxyflavanones while left 3-OH and 7-OH positions intact (Fig. 2a). This regioselectivity may attribute to the interaction between the substrate and enzyme, as the diphenyl propane backbone of flavonoids fits well into the active pocket, and 6-OH and 4′-OH axial positions are more readily accessible for transglycosylation by DGAS compared with 3-OH and 7-OH equatorial positions.

Representative example: stereoselective O-glycosylations

Stereoselective glycosylation can greatly increase the overall yields and facilitate the purifications. Hesperetin is a kind of flavanone extracted from fruits and shows potential antioxidant or anticancer activities. Plou et al. (González-Alfonso et al. 2018) successfully achieved stereoselective α-glucosylation of hesperetin (Fig. 2b), by a transglycosylation reaction catalyzed by free enzyme cyclodextrin glucanotransferase from *Thermoanaerobacter* sp. using soluble starch as glucosyl donor. This is the first report of direct glucosylation of hesperetin employing free enzymes instead of whole cells where the resulting products were glucosides with β-configuration. The synthesized monoglucoside could

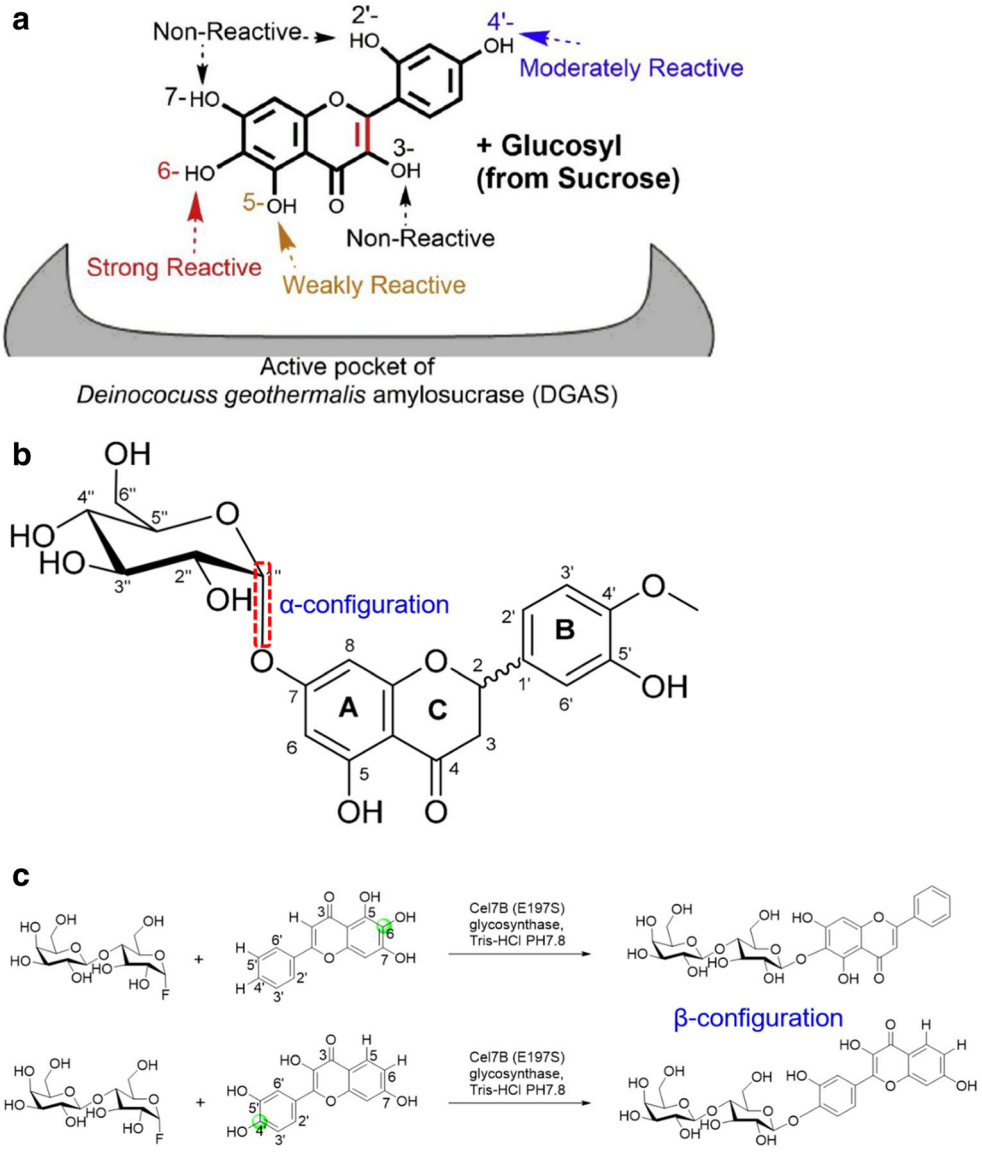

Fig. 2 **a** Site-specific glycosylation of hydroxyflavones and hydroxyflavanones by amylosucrase from *Deinococcus geothermalis*. **b** α-Glucosylation of hesperetin catalyzed by cyclodextrin glucanotransferase. **c** β-Glycosylations between lactosyl fluoride and flavonoids. The glycosidase mutant showed regioselectivities as well, and position O6 was glycosylated instead when O4′ was absent

be of interest in the nutraceutical, cosmetic, and pharmaceutical industries. Davis et al. (Yang et al. 2007) successfully utilized Cel7B-E197S glycosynthase produced from *Humicola insolens* to transfer lactose from lactosyl fluoride to the 4′-OH position of flavonoid with only anomeric β-configuration (Fig. 2c). Interestedly, this enzyme showed no activity with the monosaccharide donor, α-glucosyl fluoride. The glycosylation stereoselectivity depends on the transition state of enzyme-substrate complex, which determines the reaction proceeds in either inversion or retention way at the anomeric position of the donor sugar (Unligil & Rini 2000).

Representative example: various O-glycosyltransferase exploration

Since enzymatic O-glycosylation provides a promising approach to construct O-glycosidic bond, significant efforts regarding O-glycosyltransferase exploration have been made in recent years. Ye et al. (Chen et al. 2019) have characterized 11 new O-glycosyltransferases (GuGTs) from *Glycyrrhiza uralensis*, including isoflavone 7-O-GTs, flavonol 3-O-GTs, and promiscuous O-GTs. Subsequently, they used 8 potential native substrates and 92 compounds of different structural types from a popular medicinal plant licorice to assess the functions of these enzymes (Fig. 3). These enzymes can be utilized to catalyze not only flavones, chalcones, and triterpenoids, but also main licorice compounds such as liquiritin, isoliquiritin, ononin, and 3-O-β-D-glucuronosyl glycyrrhetinic acid efficiently.

Other examples for enzymatic O-glycosylations in vitro

Seo et al. (Jang et al. 2018) successfully synthesized a series of α-flavone glucosides catalyzed by amylosucrase from *Deinococcus geothermalis* (DGAS) using sucrose as sugar donor. Ahn et al. (Ahn et al. 2009) expressed a glycosyltransferase BcGT-3 in *Escherichia coli*, which dominantly glycosylated with the 3-hydroxyl group of flavonols, or coupled with the 7-hydroxyl group if 3-hydroxyl group was not available. Pandey et al. (Pandey et al. 2013) discovered a glycosyltranferase YjiC from *Bacillus licheniformis* DSM-13 that can catalyze the O-glycosylation of five different flavonols, fisetin, quercetin, myricrtin, kaempferol, and 3-hydroxyflavone. Suzuki et al. (Suzuki et al. 2005) isolated and purified the flavonoid 3-O-glycosyltranferase from buckwheat cotyledons, which plays an important role in the quercetin-3-O-glycosylation to synthesize rutin.

Metabolic engineering O-glycosylations in vivo

Metabolic engineering of microorganisms enables productions of flavonoid glycosides through construction and optimization of different metabolic pathways via overexpressing specific gene pathways while suppressing competing pathways, in order to increase the desired product yield (Wang et al. 2011). The overall conversion efficiency of the biosynthetic pathway depends on various factors including precursors, cofactor demand, and optimal expression of the pathway enzymes.

Representative example: microorganisms as biocatalysts in O-glycosylations in vivo

Quercetin is a flavonoid possessing various bioactivities such as inhibition of cancer cells, antioxidative effect, and antiinflammatory activity. However, its glucoside has low content in nature and limit further biological studies. An entomopathogenic filamentous fungus *Isaria fumosorosea* typically used as pesticide was found as biocatalyst to achieve the O-glycosylation efficiently. Dymarska et al. (Dymarska et al. 2018a; Dymarska et al. 2018b) used these species to prepare O-methylglucosides of flavone, 5-hydroxyflavone, 6-hydroxyflavone, 7-hydroxyflavone, and daidzein (Fig. 4a). Furthermore, they utilized *I. fumosorosea* KCH J2 and *I. farinosa* from a spider's carcass as combined catalyst to

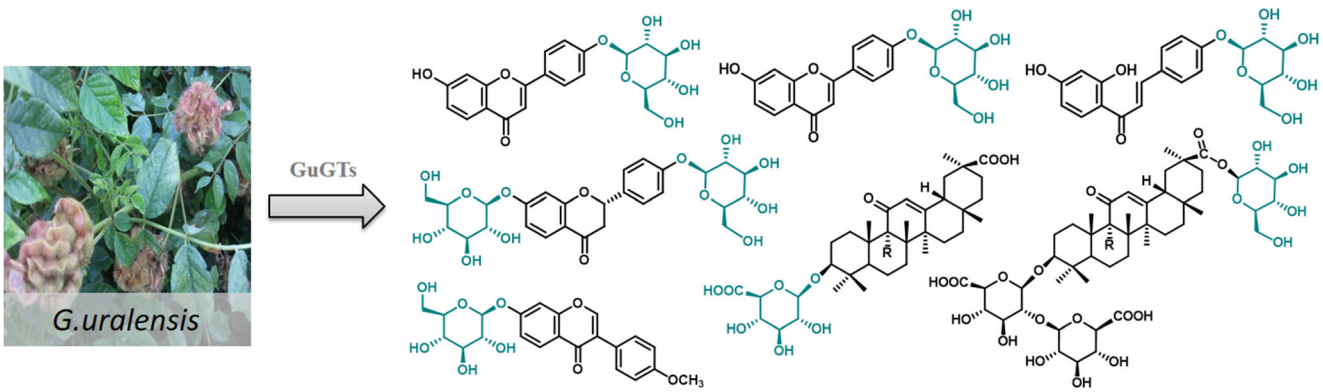

Fig. 3 Possible glycosyltransferase library contributes to the biosynthesis of licorice O-glycosides

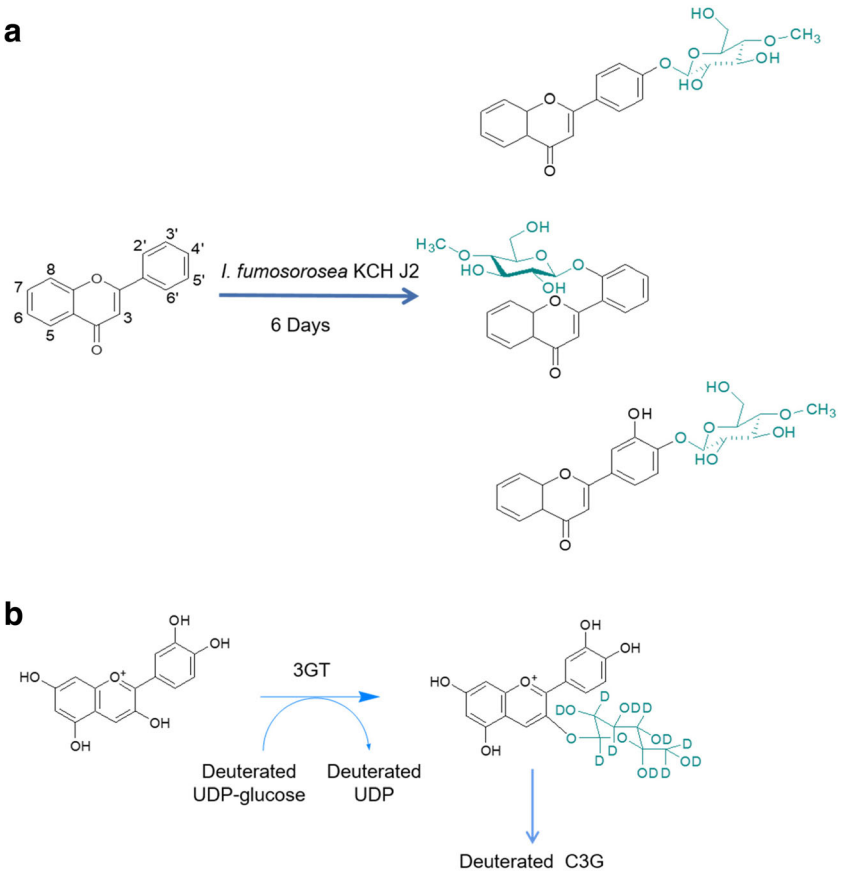

Fig. 4 **a** O-Glycosylation of flavonoid using microorganisms as biocatalysts. **b** Production of deuterated cyanidin 3-O-glucoside from recombinant *E. coli*

successfully prepare 4-O-methylglucopyranosides of 3-hydroxyflavone, 3-methoxyflavone, quercetin, and baicalein in one step. This research paves the way to enlarge the flavonoid glycoside library with compounds that have not been found in nature. Similarly, Xia and Eiteman (2017)) achieved quercetin glucoside formation with engineered *E. coli* in a shake flask culture.

Representative example: deuterated cyanidin 3-O-glucoside production

Cyanidin 3-O-glucoside (C3G) belongs to anthocyanin family that is applied as the colorants of flowers and natural food, such as vegetables and fruits. Although chemically unstable, C3G has received considerable attention due to its potential therapeutic applications, including antioxidant and anticancer properties and neuroprotective effect. Gupta et al. (2018)) reported the biosynthesis of deuterated C3G from recombinant *E. coli* cultured in the presence of deuterated glycerol and deuterated water (Fig. 4b). The in situ–formed deuterated uridine 5′-diphosphate glucose (UDP-glucose) was further incorporated into the deuterated C3G, catalyzed by the glycosyltransferase in the last stage of the anthocyanin biosynthetic pathway. Such deuterated anthocyanin showed improved stability at normal pH, thus will have longer residence in the human body and can be used for the further biological study. In addition, this technique provided an approach for the production of other deuterated anthocyanins using engineered *E. coli* (Jones et al. 2017; Zha et al. 2018).

Other examples for metabolic engineering O-glycosylations in vivo

Miyakoshi et al. (Miyakoshi et al. 2010) efficiently prepared O-glucosylated 3-hydroxyflavone and kaemferol by filamentous fungus *Cunninghamella echinulata*. Dymarska et al. (Dymarska et al. 2017) isolated fungus *Isaria fumosorosea* KCH J2 from a spider's carcass which is an effective biocatalyst for glycosylation of 6-methylflavone to produce 6-methylflavone 8-O-β-D-(4″-O-methyl)-glucopyranoside and 6-methylflavone 4′-O-β-D-(4″-O-methyl)-glucopyranoside. Yoon et al. (Yoon et al. 2012) successfully produced a novel quercetin glycoside 3-O-(6-deoxytalose) in BgalU-rfbD-rffA *E. coli*, by which the production was increased 7-fold with significantly reduced by-products compared with the wild-type strain. Similarly, Ahn et al. (Kim et al. 2012) created two *E. coli* mutant strains deleted in phosphoglucomutase (pgm) or glucose-1-phosphate uridylyltransferase (galU), and the resulting galU mutant produced up to 3-fold more quercetin 3-O-N-acetylglucosamine than wild type. Sordon

et al. (Sordon et al. 2019) found that fungi *Beauveria bassiana*, *Absidia coerulea*, and *Absidia glauca* could regioselectively catalyze O-glycosylation of flavonoids. For instance, the *B. bassiana* AM 278 could convert flavonoids to 4″-O-methyl-7-O-glucosyl derivatives, while *A. coerulea* AM 93 and *A. glauca* AM 177 could catalyze the mono-O-glucosylation reaction of flavonoids.

Flavonoid C-glycosides

Flavonoid C-glycoside is conjugated between aglycon like luteolin or apigenin, and sugar moiety, such as arerhamnoside, arabinoside, and amylaceum through a C-C bond catalyzed by C-glycosyltransferase (Yang et al. 2018). However, there is very limited research about the action pattern comparison between C-glycosyltransferase and O-glycosyltransferase. We speculate that the hydroxyl group from flavonoid is acted as a nucleophile to attack the anomeric position of sugar donor to form C-O bond, while the carbon atom from the aromatic ring of the flavonoid is played as nucleophile in the case of C-C bond formation. The types of glycosidic bonds (C-O or C-C), regioselectivity, and stereoselectivity are related to the inherent property of each glycosyltransferase as well as the transition state of the formed enzyme-substrate complex.

It has been well known that flavonoid C-glycosides typically possess better resistance ability to hydrolysis than O-glycosides due to the more stable C–C bond (Vanegas et al. 2018), thus making these compounds attractive targets in scientific research. Nevertheless, besides their potential therapeutic applications, additional efforts should be put in the future to investigate the function differences of the two kinds of glycosides in the biological process of the plant, which will be beneficial to better understand the whole pathway of flavonoid glycosides. Flavonoid C-glycosides are typically classified into two groups, mono-C-glycosylflavones and bis-C-glycosyflavones, where the glycosylations usually occur at C-6 and C-8 of the A-ring. The bis-C-glycosyl compounds have potential pharmaceutical activity and obvious stability in drug development though naturally occurring bis-C-glycosides are rare. The structures, sources, and activities of representative flavonoid C-glycosides are summarized in Table 2.

Enzymatic C-glycosylations in vitro

C-glycosyltransferases (CGT) can catalyze the glycosylations of flavonoids by formation of C–C bond in which anomeric carbon of the sugar is linked to the carbon of flavonoid aglycone directly (Yang et al. 2018). C-glycosyltransferases are the powerful tools used in C-glycosylation, especially for the bis-C-glycoside production, as chemical synthesis still encounters challenges in handling with two identical or different sugar moieties. However, compared with O-glycosyltransferases, fewer CGT has been characterized so far.

Representative example: 2-hydroxyflavanone as the most common substrate in C-glycosylations

2-Hydroxyflavanone, an intermediate formed by 2-hydroxylase (F2H), is a common substrate that can be glycosylated either directly or indirectly with the use of different types of C-glycosyltransferases. Ferreyra et al. (2013)) characterized a glycosyltranferase UGT708A6 from *Oryza sativa*, which is a bifunctional glycosyltranferase that can produce both O- and C-glycosidated flavonoids from 2-hydroxyflavanone. Vanegas et al. (2018)) have demonstrated a two-step indirect glycosylation to convert 2-hydroxyflavanone intermediates into the 6C-glucoside flavones (isovitexin and isoorientin) and the 8C-glucoside flavones (vitexin and orientin) with the combinations of F2H and C-glycosyltransferases. Furthermore, they established direct glycosylation of flavones with the use of recently identified GtUF6CGT1 from *Gentiana triflora.*

Representative example: regioselective C-glycosylations

He et al. (2019)) have found a brand new C-glycosyltransferase TcCGT1 from *Trollius chinensis* which is the first reported enzyme capable of regioselectively catalyzing the 8-C-glycosylation of flavones, flavonols, and other types of flavonoids (Fig. 5). The crystal structure of this enzyme was elucidated, and the catalytic multifunctional structural mechanism was investigated. Besides, TcCGT1 also can catalyze C-, O-, N-, and S-glycosylation reactions, which presents the possibility of catalyzing the N- and S-glycosylation of flavonoids with the use of TcCGT1.

Representative example: combined enzyme catalysis

Isoorientin and isovitexin are flavone C-glycosides that exhibit a number of bioactivities. Pei et al. (Pei et al. 2020) constructed a recombinant *E. coli* to produce isoorientin while the whole fermentation process took 116 h and the productivity was unsatisfactory. Thus, they chose in vitro enzymatic glycosylation instead. C-glucosyltransferase (Gt6CGT) was expressed from *E. coli* BL21 and combined with *Glycine max* sucrose synthase (GmSUS) to successfully produce isoorientin and isovitexin in an efficient and environmentally safe approach (Fig. 6a). The titer of isoorientin reached 3820 mg/L with a corresponding molar conversion of 94.7%, and isovitexin reached 3772 mg/L with a

Table 2 Representative flavone C-glycosides

Bioactivities	Compound	Source	Tissue
Anxiolytic	5-Hydroxy-7-methoxy-6-C-glycosylflavone $C_{22}H_{22}O_9$	*Sphaeranthus indicus* (Mishra et al. 2007)	Leaf
Antiinflammatory	6-C-(2″-O-sulfato-β-Glcp) (prechafurosideA) $C_{22}H_{22}O_9$	*Camellia sinensis* (Ishida et al. 2009)	Oolong tea
Chloroquine-resistant Plasmodium falciparum	6-C-(2″,3″-di-O-galloyl-β-Glcp) $C_{35}H_{28}O_{18}$	*Clidemia sericea* (Montenegro et al. 2007)	Leaf
Antioxidant	Isoscutellarein 6-C-β-glucopyranoside $C_{21}H_{20}O_{11}$	*Iris pseudopumila* (Rigano et al. 2007)	Rhizome
	2″-O-β-D-xylosylvitexin $C_{28}H_{32}O_{12}$	A. *Heterophyllus* (Wen et al. 2017)	Whole
	Nelumbosides A-D A and B: $C_{35}H_{36}O_{17}$ C and D: $C_{35}H_{36}O_{16}$	*Nucifera* (Jiang et al. 2018)	Embryo
Anticancer	Aciculatin $C_{22}H_{22}O_9$	*Chrysopogon aciculatis* (Carte et al. 1991)	Whole
	Alternanthin B $C_{21}H_{21}O_9$	*Alternanthera philoxeroides* (Fang et al. 2007)	Aerial part

corresponding molar conversion of 97.1% through the optimizing coupled reaction conditions. This combined catalysis is a promising method that can form C-glycosides efficiently.

Representative example: one-pot C-glycosylations

In some instances, it might be more efficient to conduct multiple reaction sequences into one-pot fashion, in which the product is prepared without the required isolation or purification of intermediates (El Amrani et al. 2004). Nothofagin is a flavonoid C-glycoside separated from red-bush herbal tea and showed good antioxidant activity. Bungaruang et al. (2013)) reported a one-pot coupled glycosyltransferase catalysis to produce nothofagin efficiently. The transformation involves selective 3′-C-β-D-glucosylation of naturally abundant phloretin and applies sucrose as expedient glucosyl donor (Fig. 6b). C-glucosyltransferase from *Oryza sativa* (rice) was used for phloretin C-glucosylation from uridine 5′-diphosphate (UDP)-glucose, which was supplied continuously in situ through conversion of sucrose and UDP catalyzed by sucrose synthase from *Glycine max* (soybean).

Other examples for enzymatic C-glycosylations in vitro

Various efforts regarding enzymatic C-glycosylations in vitro were made. Hao et al. (2016)) chemically synthesized over 20 2-hydroxyflavanones as enzyme substrates and found that rice C-glycosyltransferase could produce novel C-glycosylflavones. Sasaki et al. (2015)) found a recombinant protein GtUF6CGT1 from Japanese gentian that could transfer a sugar group to the C6 position of flavone skeleton, which

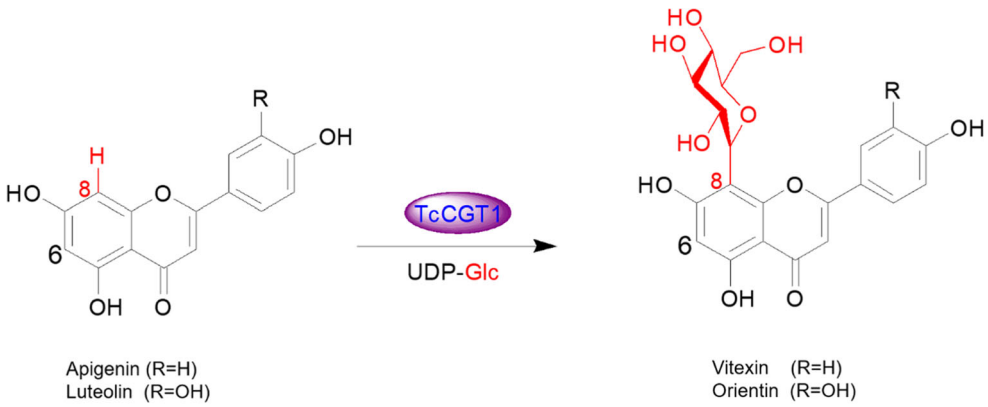

Fig. 5 Regioselective C-glycoslation catalyzed by TcCGT1

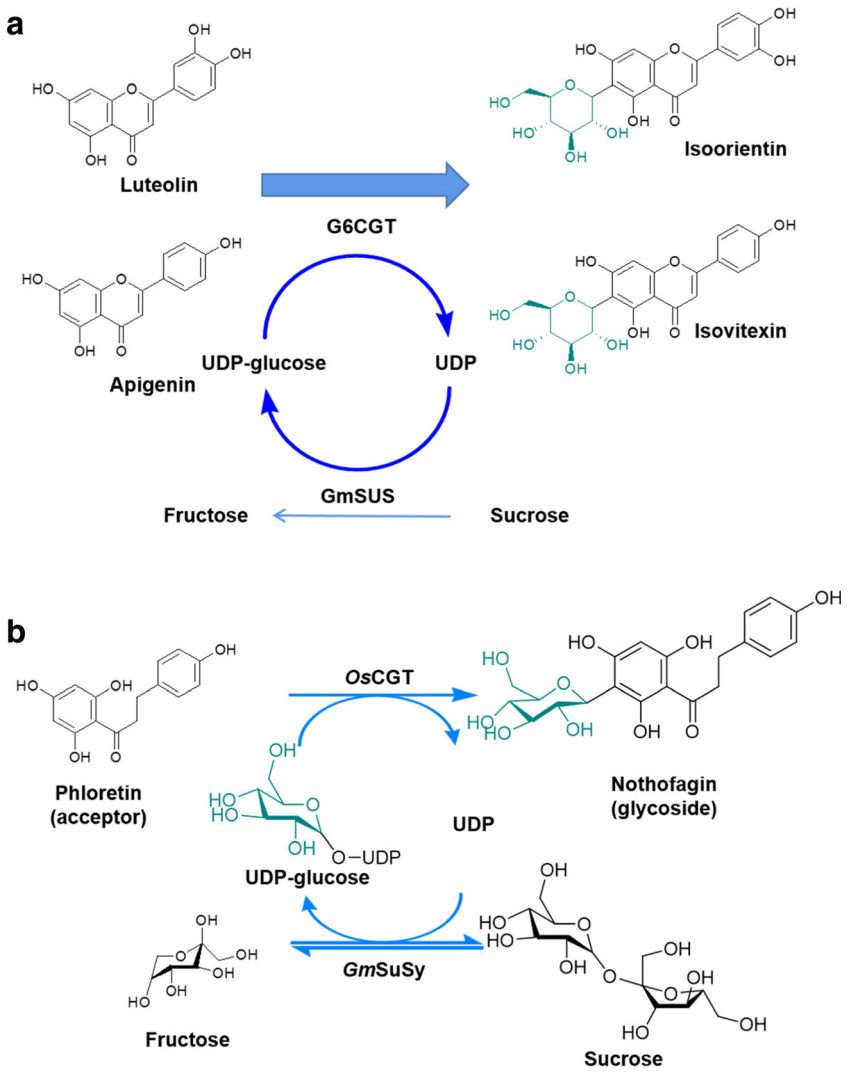

Fig. 6 **a** Combined enzyme catalysis of isoorientin and isovitexin from luteolin. **b** One-pot C-glycosylation to produce nothofagin

is the first reported C-glucosyltransferase that mediates direct C-glucosylation of the flavone skeleton.

Metabolic engineering C-glycosylations in vivo

Representative example: biosynthesis of flavone C-glucosides in engineered *E. coli*

The preparation of flavonoid C-glycosides in microbial cells is seldom reported in comparison with flavonoid O-glycosides. Shrestha et al. (2018)) have successfully produced chrysin 6-C-glucoside and luteolin 6-C-glucoside in *E. coli* with the use of a biotransformation approach (Fig. 7). They developed engineered strains to enhance the conversion rate by nearly 30% (1.5-fold). Subsequently, they significantly improved the conversion rate to 50% in a lab-scale fermentor at 3 L volume, demonstrating the potential of the system to biosynthesize different C-glucosides with the use of engineered *E. coli*.

Representative example: polyprotein expression technology

In recent years, the 2A-based polyprotein system has been used in a variety of eukaryotic systems for transgene co-expression, and in a huge range of different proteins, many with cotranslational and posttranslational subcellular localization signals, have been co-expressed together (El Amrani et al. 2004). Brazier-Hicks and Edwards (2013)) achieved the conversion from dihydrochalcone and flavanone precursors to their C-glycosides by the partially reconstruction of the biosynthetic pathway in tobacco and yeast using polyprotein expression technology (Fig. 8). The pathway includes a flavanone 2-hydroxylase (F2H) co-expressed with CGT, and the development of polyprotein system promoted this efficient co-

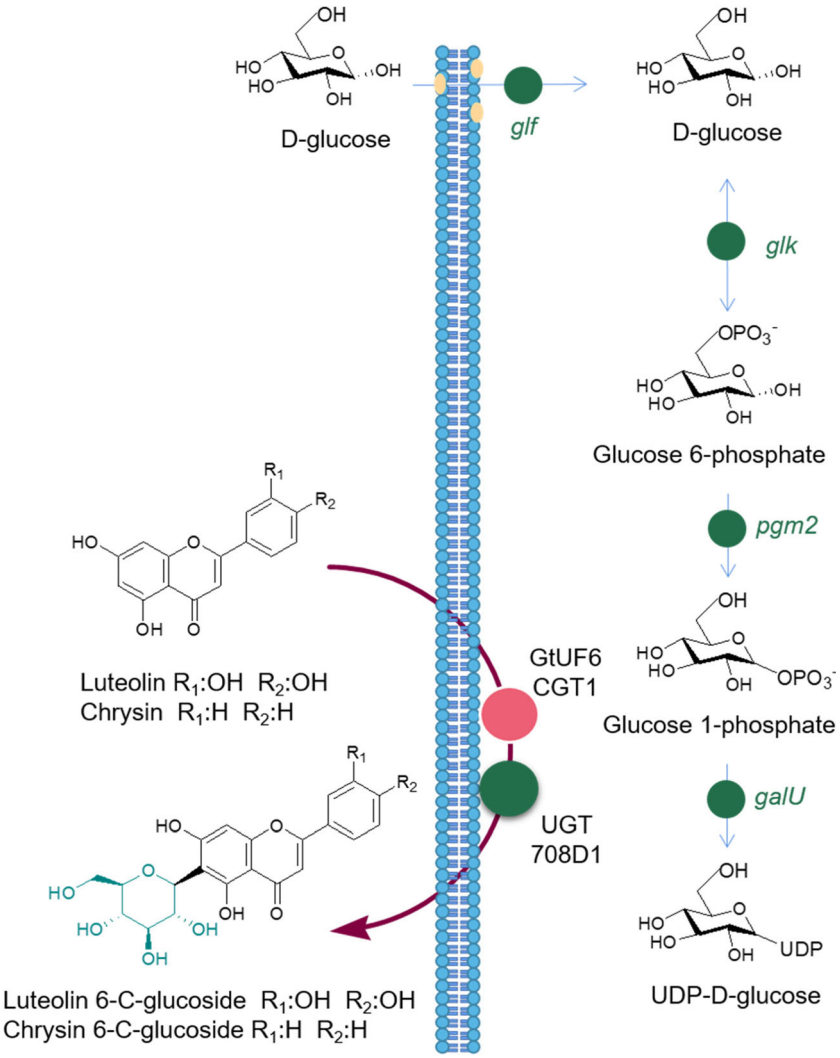

Fig. 7 Biosynthesis of flavone C-glucosides in engineered *E. coli*

expression. Though only very small amount of C-glycoside was produced via metabolic engineering in tobacco, by the treatment of flavanone naringenin, the yield of 2-hydroxynaringenin-C-glucoside was significantly improved in yeast with the use of the F2H-CGT polyprotein construct. This work can achieve the preparation of a series of flavonoid C-glycosides in sufficient amount, thus will facilitate future biological and dietary studies.

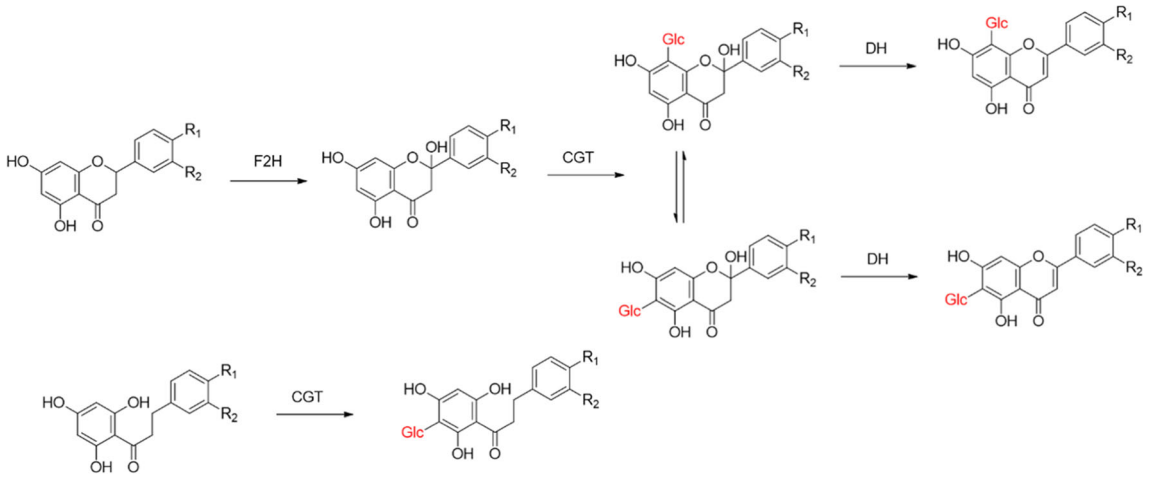

Fig. 8 Polyprotein expression technology involved for C-glycoside formation, where groups R_1 and R_2 can be either hydrogen or hydroxyl

Other flavonoid glycosides

Flavonoid N-glycosides

Flavonoid N-glycosides are formed when a sugar donor is attached to an aglycon through a nitrogen atom, thus establish a C-N-C linkage. Unfortunately, the flavonoid N-glycosides are very rare in nature. It was reported that N-glycosides occur on the nucleosides and peptides occasionally (Brito-Arias 2007) and a few N-glycosyltransferases have been characterized and utilized to perform N-linked glycosylation of proteins. For instance, N-glycosyltransferase ApNGT from *Actinobacillus pleuropneumoniae* is an effective post-translational modification enzyme capable of catalyzing glycosylation with complex proteins (Naegeli et al. 2014), while UGT71E5 is another enzyme extracted from *Carthamus tinctorius* to generate the rare N-glycoside (Xie et al. 2017).

Flavonoid S-glycosides

Similar to flavonoid N-glycosides, there are very few naturally occurring flavone S-glycosides thus very rare S-glycosylations reported as well. However, S-glycosyltransferases have been utilized in the glycosylations of glycopeptide, particularly in the post-translational modifications of proteins. For example, S-glycosyltransferase SunS expressed in *Escherichia coli* was reported to catalyze the conjugation of carbohydrates to the cysteine thiol of proteins selectively (Oman et al. 2011). ThuS, another S-glycosyltransferase can catalyze both S-glycosylation of the thiol of cysteine and O-glycosylation of the hydroxyl group of serine in peptide substrates (Wang et al. 2014). To date, though the glycosylation in the synthesis of flavonoid S/N-glycoside has not been reported, we anticipate that metabolic engineering and cell-based technique will one day be developed to solve these problems.

Conclusions

Flavonoid glycosides are a family of important natural products that perform numerous physiological and pharmacological functions. Besides chemical synthesis that has been well developed over decades, biotechnological techniques, especially enzymatic catalysis and metabolic engineering approaches, mimicing the biosynthetic pathway of flavonoid glycosides, represent an alternative strategy to prepare these biological compounds. Direct enzymatic catalysis in vitro might be more concise and faster than metabolic engineering approach in vivo but requires equimolar quantities of expensive uridine diphosphate (UDP)-sugar. In addition, it is still challenging to elucidate the mechanism of regioselectivity and stereoselectivity of enzymatic glycosylations, which blocks its further applications. We expect the regeneration strategy to prepare various sugar donors in situ (Feng et al. 2020), immobilized enzyme technique, as well as more profound structure analysis to reveal the mechanism of enzymatic reactions, will be developed and significantly reduce the costs and facilitate further rational design for more novel flavonoid glycosides in the future.

Metabolic engineering in vivo may be an alternate way to produce glycosides, as cell metabolism can provide a continual supply of UDP-glucose (De Bruyn et al. 2015). However, this approach sometimes suffers from poor product yields due to redox imbalance and excess metabolic burden, and thus requires compartmentalization of the pathway for optimal function. Fortunately, over the past 5 years, there has been a steady improvement in this area, including better enzyme expression, better enzymes resulting from protein engineering, and better engineered microorganisms used as the carriers. For instance, efforts on co-cultivation of more than one engineered microbial strains to distribute metabolic burden between the co-cultivation partners significantly improved the product yields. Many challenges remain in this young field, for example, large-scale preparation is still challenging and very few work could achieve even milligram scale. However, the promises of the biotechnology are great, offering an alternative approach to meet industrial needs.

Author contributions All authors wrote the manuscript, read, and approved the manuscript.

Funding information This study was supported by grants from National Key Research and Development Project (no. 2019YFC1605801) and Natural Science Research Project of Jiangsu Higher Education Institutions (no. 19KJB150013 to X.Z. and 19KJB150012 to L.L.).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals by any of the authors.

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