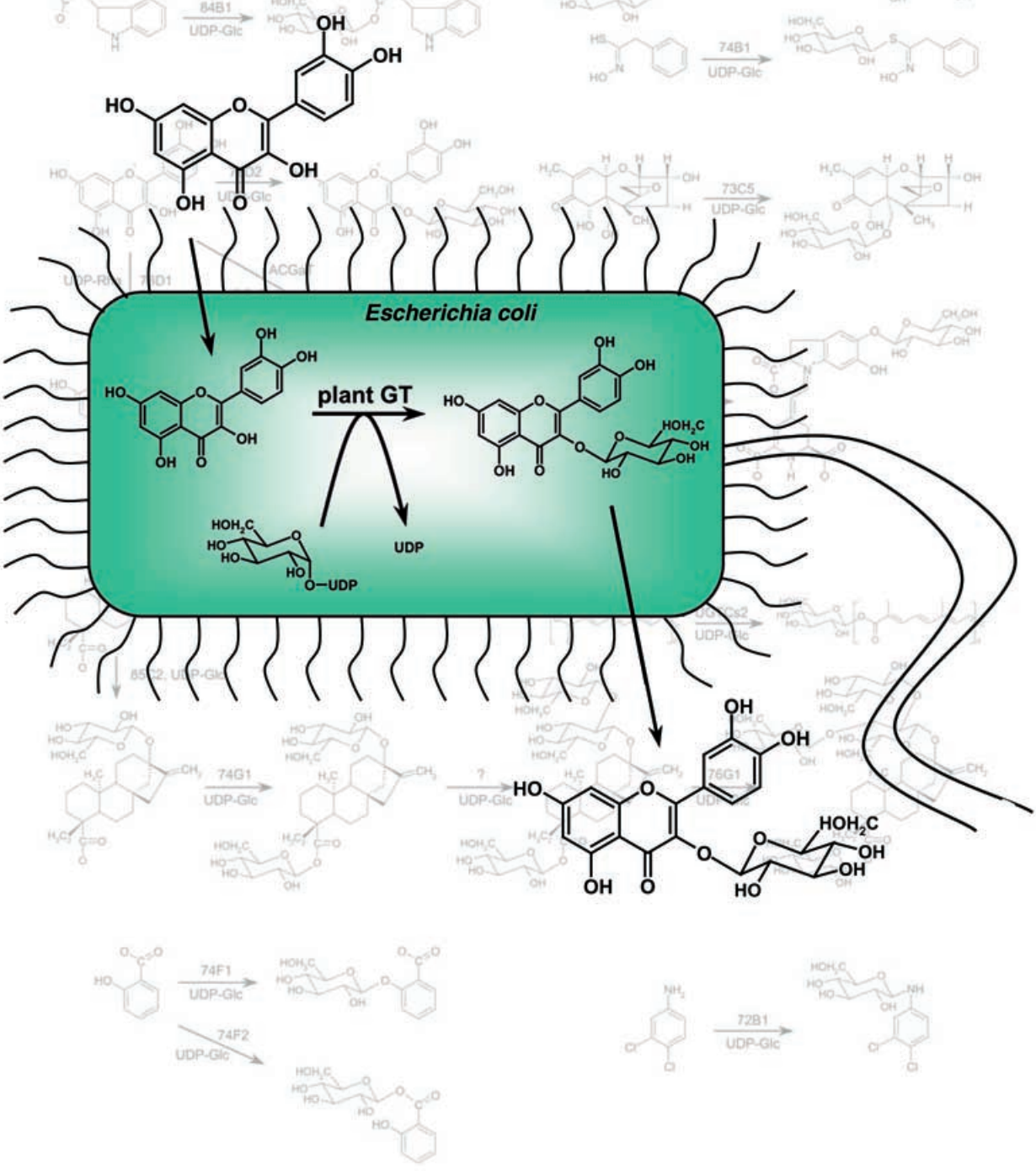
Plant Glycosyltransferases

Their Potential as Novel Biocatalysts



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Plant Glycosyltransferases: Their Potential as Novel Biocatalysts

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Abstract: The potential application of glycosyltransferases in glycoconjugate synthesis has attracted considerable interest from the biotechnology community in recent years. This concept article focuses on the current understanding of the chemistry of a family of plant enzymes capable of glycosylating small lipophilic molecules. These enzymes are discussed in terms of their regio- and enantioselective substrate recognition, sugar-donor selectivity and their utility as biocatalysts in whole-cell systems.

Keywords: biocatalysts · biosynthesis · glycoconjugates · plant glycosyltransferases · regioselectivity

Glycosyltransferases: An Introduction

Glycosylation has long been recognised as a mechanism to improve the hydrophilicity of lipophilic compounds, thereby increasing their pharmacokinetics. This reaction can be carried out through enzymatic or chemical methods. A comparison of the advantages among these two synthetic approaches has been reviewed by Køen and Thiem.[1] Enzymatic glycosylation usually employs glycosyltransferases, glycosidases or "glycosynthases".[2,3] Glycosidases typically catalyse the hydrolysis of glycosidic linkages. However, under appropriate reaction conditions, these enzymes can also be used in reverse hydrolysis and transglycosylation for glycoconjugate synthesis, although the yields are generally low. Glycosidases can be engineered such that the enzymes no longer possess the hydrolysis ability; the resulting mutants, named glycosynthases, thus become elegant biocatalysts with higher catalytic activity to synthesise glycosidic linkages.[3]

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Unlike glycosidases and glycosynthases, glycosyltransferases (GTs) are enzymes that have evolved naturally for glycosylation reactions. GTs transfer sugar moieties from activated sugar donors to acceptor molecules with high efficiency and regiospecificity (Figure 1).[4,5] Many mammalian and microbial GTs have been employed for the synthesis of oligosaccharides and antibiotic glycosides.[2,6] In contrast, due to the small number of plant GT sequences that were available, their use in biocatalysis has been limited. In recent years, many plant GT sequences have been identified and their corresponding recombinant proteins analysed in vitro. The advanced knowledge in the catalytic activities of these

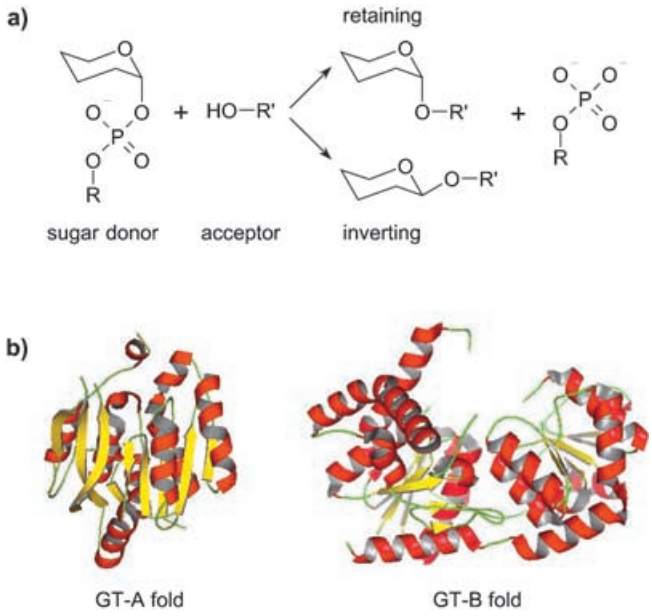


Figure 1. a) The retaining and inverting catalytic mechanisms of GTs, depending on the relative anomeric configuration of the sugar-donor and the glycosidic linkage formed at the acceptor molecule. b) The representative GT protein structures. These structures either consist of a single domain with parallel β-strands flanked on either side by α-helices (GT-A fold), or two Rossmann foldlike domains separated by a deep cleft (GT-B fold).[5]

enzymes has broadened the use of GTs in glycoconjugate biosynthesis.

Plant GTs in CAZy database: Over 12000 sequences encoding GTs in different organisms have now been collected in a carbohydrate-active enzyme database (CAZy, http://afmb.cnrs-mrs.fr/CAZY/).[7] These sequences were identified and classified into 77 families through biochemical studies of their gene products or through sequence homology comparison to the genes encoding enzymes of known catalytic activity. In the genetic model plant *Arabidopsis thaliana*, 429 GT sequences have been identified (Table 1); these sequences occupy 1.6% of the total *Arabidopsis* genome. This is much higher than the 0.7% ratio found in the complete genome of human cells (raw statistical data obtained from the CAZy database, MIPS *Arabidopsis thaliana* database [http://mips.gsf.de/proj/thal db^{-1}/], and The GDB Human Genome database [http://www.gdb.org/]), and probably reflects the need of sedentary organisms to synthesise wider ranges of glycoconjugates to respond to the environmental conditions and to communicate with other organisms, as well as to defend themselves from pathogen infection.

Table 1. The GT families of CAZy classification that contain *Arabidopsis thaliana* sequences.

Family	Mechanism	No. of members	Known activities
1	inverting	121	glycosyltransferase of small molecules (flavonoids etc)
2	inverting	42	cellulose synthase
4	retaining	24	sucrose synthase; digalactosyldiacylglycerol synthase
5	retaining	7	soluble starch synthase
8	retaining	42	galactinol synthase
10	inverting	3	α-1,4-fucosyltransferase; core α-1,3-fucosyltransferase
13	inverting	1	β-1,2-*N*-acetylglucosaminyltransferase
14	inverting	11	*N*-acetylglucosaminyltransferase; β-xylosyltransferase
16	inverting	1	*N*-acetylglucosaminyltransferase
17	inverting	6	*N*-acetylglucosaminyltransferase
19	inverting	1	
20	retaining	11	trehalose-6-phosphate synthase
21	inverting	1	
22	inverting	2	
24	retaining	1	
28	inverting	4	monogalactosyldiacylglycerol synthase
29	inverting	3	
30	inverting	1	
31	inverting	33	
32	retaining	6	
33	inverting	1	
34	retaining	8	
35	retaining	2	
37	inverting	10	xyloglucan α-1,2-fucosyltransferase
41	inverting	2	
43	inverting	4	
47	inverting	39	xyloglucan β-galactosyltransferase
48	inverting	12	callose synthase
50	inverting	1	
57	inverting	2	
58	inverting	1	
59	inverting	1	
61	inverting	7	β-1,2-xylosyltransferase
64	retaining	3	
65	inverting	1	
66	inverting	2	
68	inverting	3	
75	inverting	5	reversibly glycosylatable polypeptide

The sugar acceptors recognised by plant GTs include proteins, lipids, polysaccharides and a vast diversity of small molecules.[8–10] Among these metabolites, the pharmaceutical and nutraceutical properties of small molecules have attracted considerable interest from chemical and food industries. The biofunctionalities of small molecules may be enhanced by increasing their hydrophilicity and stability through glycosylation.[11] In this context, GTs capable of glycosylating small molecules may become useful catalysts, acting as isolated enzymes or applied in whole-cell systems, for synthesising glycosides of high utility.

Plant GTs for small molecules: A large proportion of plant GTs recognise small molecules. For example, based on sequence analysis, 120 *Arabidopsis* GT sequences have been grouped into Family 1 (Table 1), and assigned as GTs for small molecules.[12–14] In the last few years, many of these GTs have indeed been shown to glycosylate small molecules, such as benzoates and flavonoids, in vitro.[10] Within Family 1, the plant GTs can be further classified into two subsets. One subset is defined by the presence of a 44–46 amino acid consensus sequence thought to be involved in nucleotide sugar binding; this consensus is also present in the mammalian GTs conjugating endogenous metabolites, such as steroids and bile acids, and exogenous xenobiotics, such as dietary flavonoids and drugs.[10,15] Through phylogenetic analysis, plant GTs carrying this consensus are grouped into 15 clusters, as shown in Figure 2.[13,16]

The other subset is relatively small and does not contain the consensus. The plant GTs in this subset glycosylate sterols and glycerolipids.[9] It is worth noting that the microbial GTs, such as those involved in the synthesis of antibiotics urdamycin, vancomycin and vicenistatin,[17] are also grouped into Family 1. The reader is referred to the CAZy database (http://afmb.cnrs-mrs.fr/CAZY/) for up-to-date information on the activities described in Family 1.

The Chemistry of Plant Family 1 GTs

Regioselective glycosylation and sugar-donor selectivity: The acceptor recognition of plant Family 1 GTs has been investigated through a functional genomic approach analysing >100 *Arabidopsis* recombinant GTs in vitro against a number of small molecules. This has proven to be an efficient strategy to explore the chemical features of the acceptors recognised by these GTs, as well as the regioselectivity of the enzymes towards their substrates. For example, 48 GTs from six phylogenetic clusters (B, D, E, F, H, L, Figure 2) were found to glycosylate esculetin in the presence of UDP-glucose (UDP-Glc) (Scheme 1a),[18] suggesting that

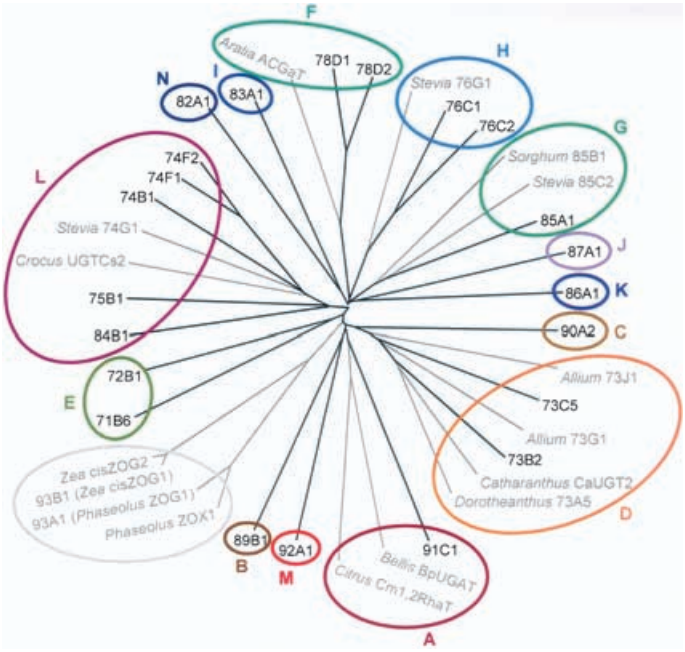
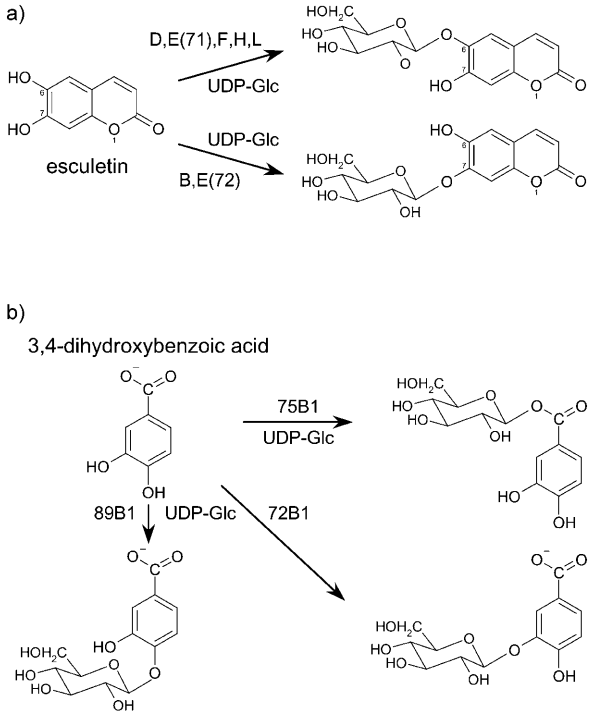


Figure 2. An unrooted phylogenetic tree of the plant Family 1 GTs. In each cluster, the representative *Arabidopsis* sequences are shown in solid line and the GTs reported recently from other plant species are shown in dotted line (accession numbers available in the CAZy database). The detailed phylogenetic analysis and the *Arabidopis* sequences in each branches were described by Li et al.[13] and Lim et al.[19] previously.



Scheme 1.

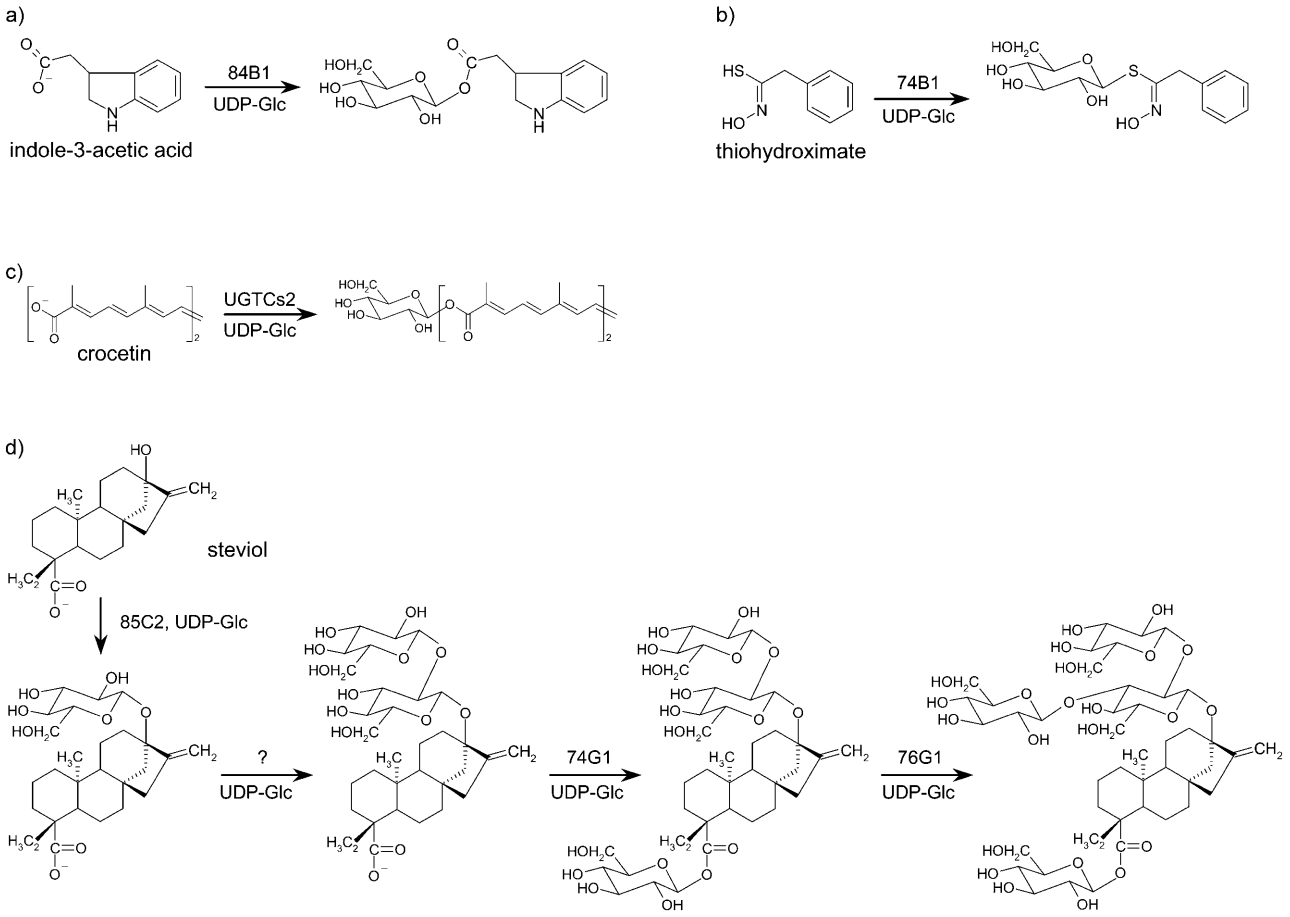
the GTs within these clusters are capable of recognising phenolics with similar chemical structures. When other phenolic compounds, such as benzoates and flavonoids, were analysed, the active GTs were again found within these six clusters.[19,20] Whilst some GTs recognise the same substrate, their regioselectivity can be different. A clear example of regioselective glycosylation is given in Scheme 1b, which shows that three different *Arabidopsis* GTs transfer sugar onto different positions of 3,4-dihydroxybenzoic acid. In this example, GTs in clusters B, D, E, and F glycosylate the OH groups on the benzene ring, whereas GTs in cluster L transfer sugar onto the carboxyl side chain to form a glucose ester.[19]

The capability of cluster L GTs, including those from *Arabidopsis* and other plant species, in forming a glucose ester bond has also been demonstrated in other studies using indole-3-acetic acid, thiohydroximate, crocetin, and steviolbioside as substrates (Scheme 2a–d).[21,22] It is worth noting that conjugation of steviolbioside catalysed by 74G1 leads to the formation of a low calorie natural sweetener stevioside.[22] Two other GTs in cluster L, 74F1 and 74F2 that are 82% identical at the amino acid sequence level, interestingly display different regioselectivity towards salicylic acid; one produces *O*-glucoside and the other produces a glucose ester (Scheme 3a).[19] Further investigation in the functional domains of these two proteins will certainly provide new insights into the enzyme architecture that determines the regioselectivity.

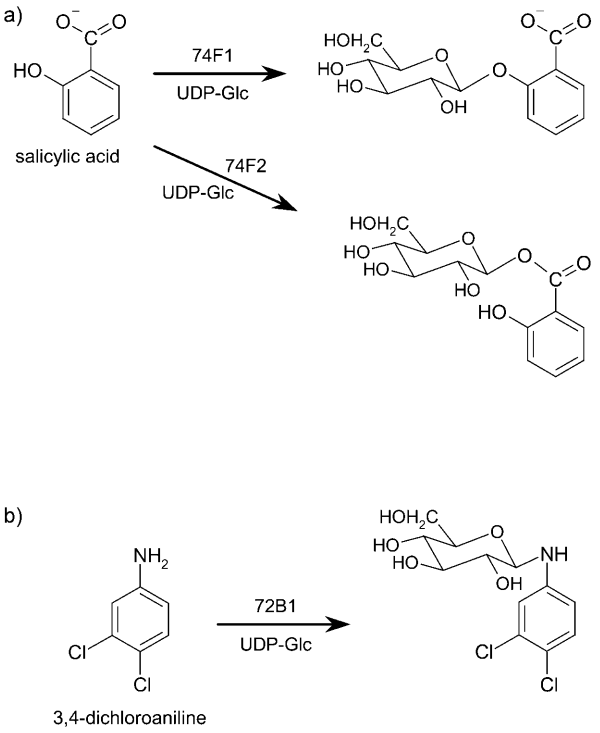
In addition to the O- and S-linkages shown in Schemes 1 and 2, GTs in Family 1 are also capable of catalysing N-linkages (Scheme 3b). Furthermore, a GT may be able to form different linkages with the substrates it recognises. This has been demonstrated using *Arabidopsis* GT 72B1, which forms an *O*-glucosidic bond with 3,4-dihydroxybenzoic acid (Scheme 1b) and an *N*-glucosidic bond with a pollutant 3,4-dichloroaniline (Scheme 3b).[19,23]

Sugar-donor selectivity is another interesting feature of GTs. Currently several sugar donors, such as UDP-Glc, UDP-galactose (UDP-Gal) and UDP-glucuronic acid (UDP-GlcUA), are commercially available for routine biochemical analyses; however, other sugar donors typically used in plants, such as UDP-rhamnose (UDP-Rha), are not available as yet. This makes a comprehensive analysis on the sugar-donor selectivity of the entire multigene family impossible. Nevertheless, several closely related GTs have been found to recognise the same acceptor molecule, display the same regioselectivity, but catalyse the glycosidic linkage by means of different sugar donors. Two *Arabidopsis* cluster F GTs are capable of glycosylating the 3-OH of quercetin in vitro. Interestingly, whilst GT 78D2 uses UDP-Glc as the sugar donor,[20] GT 78D1 prefers UDP-Rha (Scheme 4).[24] Another GT in cluster F, ACGaT from *Aralia*, forms quercetin-3-*O*-galactoside with UDP-Gal as the sugar donor.[25] Although it is likely that these three GTs have a common ancestor, they have evolved to recognise different sugar donors.

Whilst many plant GTs glycosylate the aglycone moiety of glycosides to form products such as quercetin-3,7-di-*O*-glucoside,[20] some other Family 1 GTs are found to glycosylate the sugar moiety of glycosides. Two interesting exam-

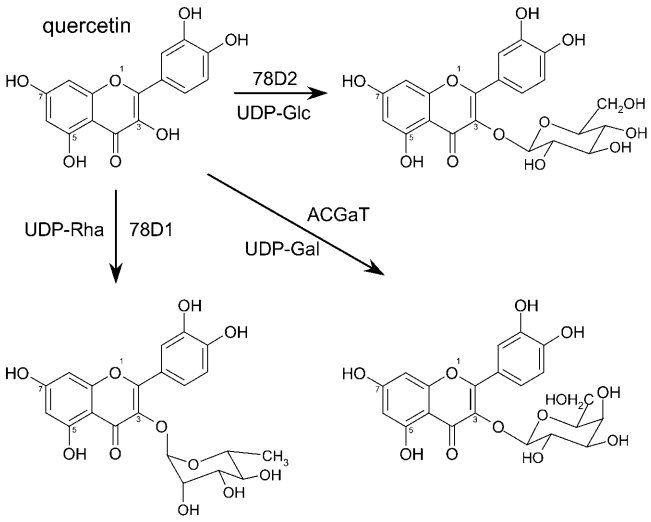


Scheme 2.

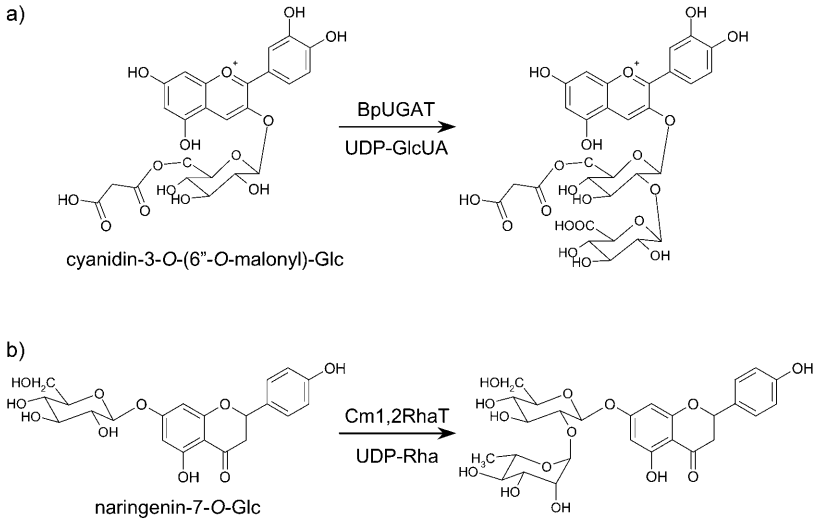


Scheme 3.

ples are shown in Scheme 5. Cyanidin-3-*O*-(6″-*O*-malonyl-2″-*O*-glucuronyl)glucoside is a stable red pigment in the *Bellis* flower. A GT was recently isolated from the *Bellis* flower and confirmed to be able to transfer glucuronic acid from UDP-GlcUA onto the 2-OH group of the malonylglu-



Scheme 4.



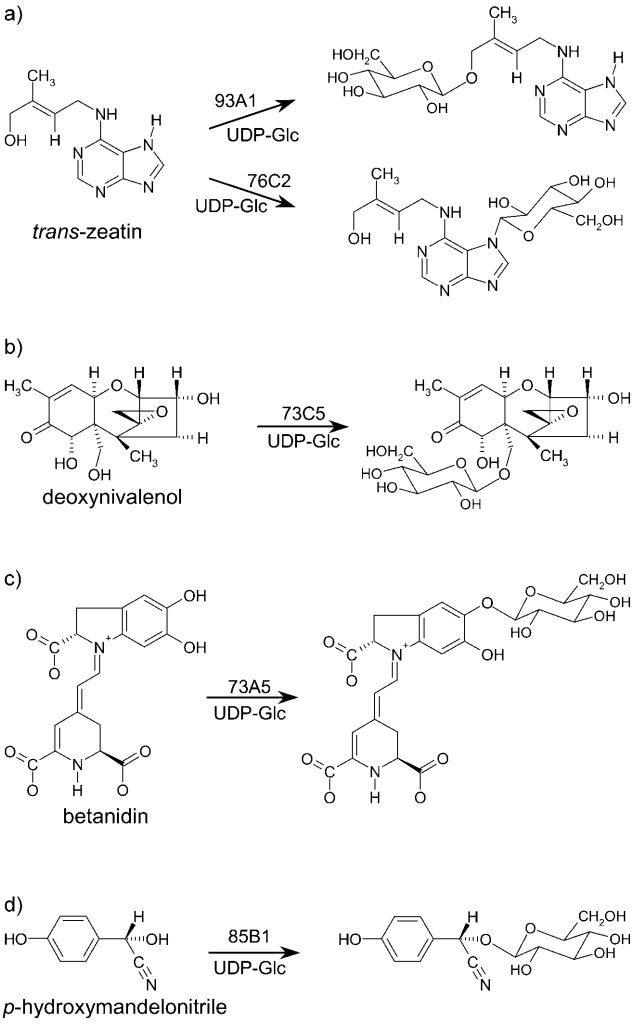
Scheme 5.

cosyl moiety of cyanidin-3-*O*-(6″-*O*-malonyl)glucoside (Scheme 5a).[26] Another example is the *Citrus* Cm1,2RhaT that transfers a rhamnose molecule onto the 2-OH group of the glucose moiety attached to the 7-OH group of naringenin, resulting in the formation of a bitter tasting glycoside in some *Citrus* species (Scheme 5b).[27] There is also a GT in *Citrus* species such as mandarin, that transfers rhamnose onto the 6-OH group of the glucose moiety of naringenin-7-*O*-glucoside and forms a tasteless glycoside.[27] The formation of a low calorie and noncariogenic sweetener, rebaudioside A, in *Stevia* also involves a GT that glycosylates the glucosyl side chain of the precursor (Scheme 2d).[22] These GTs will be useful catalysts to form a diglycosyl chain on acceptor molecules. As yet, no in vitro activities have been reported for any *Arabidopsis* GTs in cluster A related to *Bellis* BpUGAT and *Citrus* Cm1,2RhaT. It is of interest to investigate the activities of these *Arabidopsis* GTs towards glycosides of small molecules.

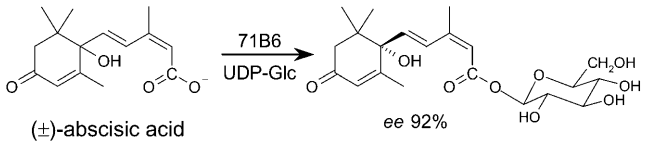
Scheme 6 shows several additional acceptor molecules recognised by Family 1 GTs. *trans*-Zeatin is glycosylated by *Arabidopsis* GTs 76C1 and 76C2 (cluster H) on the adenine moiety to form *N*-glucoside,[16] and can also be glycosylated by *Arabidopsis* GT 85 A1 (cluster G) and *Phaseolus* ZOG1 (93 A1) at the N^6 side-chain to form *O*-glucoside (Scheme 6a).[16,28] Deoxynivalenol is a mycotoxin known to be conjugated by *Arabidopsis* GT 73C5 in cluster D (Scheme 6b).[29] Betanidin and *p*-hydroxymandelonitrile are natural metabolites present in glycosylated form. GTs capable of conjugating these aglycones have also been identified (Scheme 6c and d).[30]

Enantioselectivity: A recent study has demonstrated the capability of an *Arabidopsis* GT, 71B6, in distinguishing between enantiomers, and the use of GT 71B6 in purification of (+)-abscisic acid from (±)-abscisic acid.[31] When incubated with (±)-abscisic acid and UDP-Glc, the enzyme was found to glycosylate (+)-abscisic acid with an enantiomeric excess of 92% (Scheme 7). Thus, a new utility of GTs in discriminating enantiomers as a means of chiral separation has been suggested, in addition to their use in the synthesis of regiospecific glycosides.

The aim of the examples shown above has been to illustrate the acceptor features, the regio- and enantioselectivity and the sugar-donor selectivity of Family 1 GTs. There are yet other plant GT activities that have been reported and reviewed in the last few years, but are not included in this article.[10,32] There is no doubt that many new activities will be revealed in the near future, and our understanding in the



Scheme 6.



Scheme 7.

chemistry of this GT family will be broadened through these studies.

Plant Family 1 GTs as Biocatalysts

Use as purified enzymes: Recombinant technologies have provided a way to use GTs as biocatalysts in synthesising glycosides of small molecules. In this approach, plant GTs can be heterologously expressed in bacterial cells at fermenter scale and purified as recombinant enzymes for large-scale glycosylation reactions. To achieve the reactions more economically, the recombinant enzymes can be recycled through enzyme immobilisation. Various enzyme immobilisation techniques have been applied to microbial and mammalian GTs for glycoconjugate synthesis.[33] In this context, a plant GT partially purified from pummelo fruits has been immobilised and used in bioprocessing for debittering citrus juice by converting limanoids into tasteless glycosides.[34]

A constraint to the use of isolated GTs for biocatalysis applications is the requirement of supplementary activated sugar donors, which are usually expensive and not available in large quantity. Several methods have been reported for in vitro regeneration of sugar donors;[1,33,35] however, the cost of these systems is still considered to be expensive for general large-scale applications.

Use in plant whole-cell systems: Plant whole-cell biocatalysis is a distinct system in comparison to the use of purified enzymes. In a whole-cell system, exogenous aglycones are applied to living cells and are glycosylated by the endogenous GTs that are either present as "house-keeping" enzymes or induced by the exogenous aglycones. The major advantage of this whole-cell system is that living cells can provide activated sugar donors for the glycosylation reactions. In particular in plant cells, specific sugar donors such as UDP-Rha become available. The use of plant whole-cell systems for the glycosylation of small molecules has more than 30 years of history; various types of plant materials such as seedlings and suspension cell cultures have been reported as biocatalysts.[1]

The aglycones applied to plant whole-cell systems are not limited to the natural plant metabolites. Xenobiotics such as pollutant 3,4-dichloroaniline can also be glycosylated through plant whole-cell biocatalysis.[24] This capability has allowed plant cells to act as biocatalysts in phytoremediation.

A major concern in plant whole-cell systems is that the glycosides synthesised in the cells are often stored in vacuoles. Additional extraction steps are therefore required to obtain the products, as described in several examples using *Catharanthus* suspension cell cultures.[36] Thus, plant cells are more suitable to be used in a batch process. Nevertheless, there are also other examples that show the glycosides synthesised in plant cells can be exported into the culture medium.[24] Since many GT sequences are now available, the next step in the development of the plant whole-cell biocatalysis is likely to involve the use of transgenic cells overexpressing GT sequences.

Use in microbial whole-cell systems: Microorganisms are simple but efficient whole-cell biocatalysts widely used in oligosaccharide synthesis.[33] In recent years, progress has been made to use microbial fermentation involving *E. coli* cells heterologously expressing plant Family 1 GTs to produce glycosides of small molecules.[20,37,38] For example, a number of *Arabidopsis* GTs were expressed in *E. coli* and were used as whole-cell biocatalysts to glycosylate quercetin regioselectively. Several different mono- and diglycosides could be produced and were readily recovered from the culture medium.[20] In an extended application, a combinatorial whole-cell biocatalysis system has been demonstrated for the first time using a combination of methyltransferases and GTs to produce polymethylated quercetin glucosides.[38] Whilst the use of plant GTs in bacterial cells is promising, side products may be formed by bacterial enzymes. Some bacteria are known to possess the ability to glycosylate small molecules such as resveratrol and produce α-glycosides of the acceptors.[39]

As described earlier, GTs capable of distinguishing between enantiomers can be used as a new tool in chiral separation. Thus, *E. coli* cells expressing *Arabidopsis* GT 71B6 were used in a fermentation system to purify (+)-abscisic acid from enantiomeric mixtures; an 84% enantiomeric excess of the purified enantiomer was obtained through this whole-cell biocatalysis.[31]

As the time of writing, the products obtained in *E. coli* whole-cell biocatalysis systems involving plant GTs are mainly glucose conjugates. This is because the major activated sugar donors in *E. coli* recognised by plant GTs are UDP-Glc and UDP-Gal. In the last three years, our understanding of the enzymes involved in the biosynthesis and interconversion of nucleotide sugars in plants has been greatly improved, and many genes corresponding to these enzymes have been isolated.[40] By introducing these plant genes into bacterial cells, it is now possible to manipulate the bacterial nucleotide sugar synthesis pathways and generate new activated sugar donors for use by plant GTs in bacterial whole-cell biocatalysis systems.[40]

Conclusion

Enzymatic synthesis of glycoconjugates has many advantages over conventional chemical methods. The regio- and enantioselectivity of GTs provide a simple means to synthe-

sise stereospecific glycosides without the requirement of protection and deprotection of other functional groups. These processes are often difficult or impossible for chemical synthesis. Furthermore, the application of plant GTs in microbial whole-cell systems offers a "green chemical approach" for glycoconjugates synthesis, not only because this approach avoids the use of hazardous chemical derivatives as blocking and deblocking reagents, but also uses safer solvents and reaction conditions, as well as renewable materials. More importantly, the process involves fewer synthetic steps, thereby reducing the cost of glycoconjugate synthesis. The potential of plant GTs as biocatalysts will be further exploited in the near future as more catalytic activities become defined.

Acknowledgements

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