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Promoting the glycosylation of drug-like natural products in a *Saccharomyces cerevisiae* chassis by deletion of endogenous glycosidases

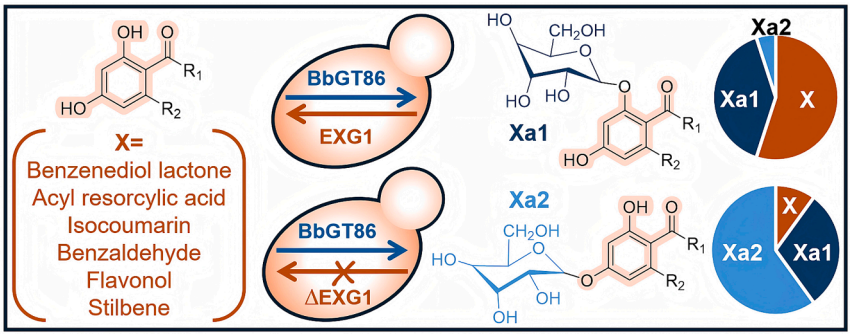
Yingying Huang [a,b,c,d,1], Weimao Zhong [a,1,2], Kinga E. Varga [e], Zsigmond Benkő [e], István Pócsi [e], Chenglong Yang [b,c,d], István Molnár [a,f,*]

[a] Southwest Center for Natural Products Research, The University of Arizona, Tucson, AZ, United States
[b] Institute of Food Science and Technology, Fujian Academy of Agricultural Sciences, Fuzhou, China
[c] Key Laboratory of Subtropical Characteristic Fruits, Vegetables and Edible Fungi Processing (Co-construction by Ministry and Province), Ministry of Agriculture and Rural Affairs, Fuzhou, China
[d] Fujian Key Laboratory of Agricultural Products (Food) Processing, Fuzhou, China
[e] Department of Molecular Biotechnology and Microbiology, Institute of Biotechnology, Faculty of Science and Technology, University of Debrecen, H-4032 Debrecen, Hungary
[f] VTT Technical Research Centre of Finland, Espoo, Finland

HIGHLIGHTS

- Natural and unnatural product glucosides were produced in *Saccharomyces cerevisiae*.
- Knockout of endogenous glycosyl hydrolases improved glycoconjugate production.
- The total yield and the distribution of glucoside regioisomers were both modulated.
- Exoglucanase deletion and glucoside methylation are complementary strategies.
- Structural motifs affecting glycosylation and glycoside hydrolysis were identified.

GRAPHICAL ABSTRACT



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ABSTRACT

Glycosylation is an effective strategy to improve the absorption, distribution, metabolism, excretion, and toxicity of natural product (NP) pharmacophores. While heterologous production of broad-spectrum fungal glucosyltransferases such as BbGT86 of *Beauveria bassiana* yields varied phenolic glucoconjugates in *S. cerevisiae*, endogenous yeast glycosidases diminish the conversion yields and limit the structural diversity of the products. We set out to improve the efficiency and broaden the regiospecificity of the glucosylation of NPs or their unnatural product analogues (uNPs). Using yeast strains deficient in exoglycanases EXG1 or SPR1, we evaluated total biosynthetic and biocatalytic synthetic biology platforms to produce glycoconjugates from polyketides of the benzenediol lactone family, and polyphenols of the phenylpropanoid class. We show that for 13 out of the 18

* Corresponding author at: Industrial Biotechnology and Food Solutions Area, VTT Technical Research Centre of Finland, P.O. Box 1000, FI-02044 VTT, Espoo, Finland.
E-mail address: istvan.molnar@vtt.fi (I. Molnár).
[1] Equal contribution.
[2] Present address: School of Chemistry and Biochemistry, Georgia Institute of Technology, 30332 Atlanta, GA, United States.

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aglycons tested, exoglycanase deletions improve glucoside yields and/or alter glucoconjugate regioisomer distributions, while macrolactone glycoconjugates with an aryl methylene ketone moiety are impervious to hydrolysis by EXG1. We demonstrate that elimination of EXG1 or biosynthetic methylation of glucosides are efficient alternative strategies to differentially modulate glycoside regioisomer profiles for future pharmaceutical, nutraceutical or crop protection applications.

1. Introduction

Drug-like natural products (NPs) such as polyketides or polyphenolics display wide-ranging bioactivities as therapeutic agents and food supplements (Tao et al., 2024). Glycosylation modulates the physicochemical characteristics and the biological activities of such NPs, and may improve their pharmacokinetic and pharmacodynamic (PK/PD) profiles (Elshahawi et al., 2015; Johnson et al., 2021; Vasudevan & Lee, 2020). Optimization of NPs for pharmaceutical applications may require glycodiversification: the production of novel glycoconjugate "unnatural products" (uNPs) with altered regio- and stereochemistry and/or alternative sugar components (glycorandomization) (Goel et al., 2021; Thibodeaux et al., 2008). Chemical glycosylation may be complex and less economically competitive in many instances, while in vitro glycosylation with purified glycosyl transferases (GTs) is also challenging (Masada et al., 2007; Wang et al., 2023). Synthetic biology promises scalable, cost-effective, and environmentally friendly glycoside production (Crowe et al., 2024) using an engineered microbial chassis that produces GTs (Jiang et al., 2024; Wang et al., 2024b). *Saccharomyces cerevisiae* is well suited to produce GTs, and offers GRAS (generally recognized as safe) status and advanced metabolic engineering and bioprocess development tools (Wang et al., 2024a).

Secreted *exo-β*-glucan hydrolases are important for *S. cerevisiae* growth and sporulation (Aspeborg et al., 2012). Among these, EXG1 is involved in cell wall biosynthesis (de Aldana et al., 1991), while SPR1 is sporulation-specific (Wang et al., 2016). These glycoside hydrolases (GHs) also hydrolyze flavonoids glycosylated at OH-7 or OH-4′, but not at OH-3 (Lyu et al., 2020; Schmidt et al., 2011; Wang et al., 2016; Xu et al., 2022). Additional GHs such as the broad-spectrum intracellular sterol glycosidase EGH1 (YIR007W) has not been described to hydrolyze glycosides of macrocyclic polyketides (Huang et al., 2021; Li et al., 2024b ; Watanabe et al., 2015; Ye et al., 2023; Zhao et al., 2024), while the significance of EGH1 for in vivo flavonoid *β*-glycoside production is questionable (Schmidt et al., 2011, Wang et al., 2016).

We have been developing combinatorial synthetic biological methods to produce benzenediol lactone (BDL) polyketides, including glycosylated uNPs, in *S. cerevisiae* (Bai et al., 2016; Li et al., 2024a; Wang et al., 2019 and 2020; Xie et al., 2018; Yue et al., 2023). Fungal BDLs and their non-macrocyclic congeners show diverse biological activities (Bang & Shim, 2020). BDLs feature a dihydroxybenzene functional group fused to a macrolactone. For resorcylic acid lactones (RALs), the fusion is at C2-C7, while for dihydroxyphenylacetic acid lactones (DALs), a C3-C8 bond forms (Thomas, 2001; Xu et al., 2013c). BDL congeners are biosynthesized by pairs of sequentially acting iterative Type I polyketide synthases (PKSs) (Wang et al., 2008; Xu et al., 2013a; Xu et al., 2014; Zhou et al., 2008). The highly reducing PKS (hrPKS) of the synthase pair assembles a linear polyketide chain. This is further elongated by the nonreducing PKS (nrPKS) partner that also catalyzes aromatic ring closure and releases macrocyclic, linear, or pyrone BDL analogues as products (Wang et al., 2020; Xu et al., 2013b).

We demonstrated that BbGT86, a GT from *Beauveria bassiana* ARSEF 2860 is a flexible biocatalyst. When produced in *S. cerevisiae*, BbGT86 glucosylates, with significant regioselectivity, a broad range of phenolic NPs, including phenylpropanoids and BDL analogues (Xie et al., 2018). BbGT86 forms a functional module with BbMT85 in *B. bassiana*. BbMT85 is an *S*-adenosylmethionine (SAM)-dependent methyltransferase (MT) that methylates OH-4 of the glucose moiety of glucosides (Xie et al., 2019; Xie et al., 2018). The resulting methylglucoside BDLs may display improved bioactivities and resistance to glycoside hydrolysis (Xie et al., 2018). However, the glucoconjugates biosynthesized by BbGT86 are also hydrolyzed by *S. cerevisiae* GHs, creating a futile cycle and severely compromising the production of some glucoside regioisomers in this chassis (Hofer, 2016; Xie et al., 2018).

In contrast to GTs from prokaryotes and plants (He et al., 2022; Schmid et al., 2016), the application of fungal GTs is much less studied for NP glycodiversification in synthetic biology systems (Feng et al., 2017; Ren et al., 2022; Xie et al., 2017, 2018 and 2019). The role of polyketide glycoside hydrolysis in *S. cerevisiae*, and the interplay of endogenous GHs and heterologous GTs has received even less attention. Here, we investigated the influence of *exg1* and *spr1* deletions on the glucosylation and methylglucosylation of phenylpropanoids and polyketides in *S. cerevisiae* producing BbGT86 alone, or the BbGT86 – BbMT85 module. We show that the ΔEXG1 strain (and to a much lesser extent, the ΔSPR1 strain) is an excellent chassis for polyketide, flavonol, and stilbene glucosylation, and yields a modulated glucoconjugate regioisomer profile. We also show that GH deletion and glucoside 4-*O*-methylation are alternative strategies to protect glycoconjugates from chassis-mediated hydrolysis upon the production of NPs or uNPs for pharmaceutical and nutraceutical development.

2. Materials and methods

2.1. Strains and culture conditions

All strains used in this study were derived from *S. cerevisiae* BJ5464-NpgA (Ma et al., 2009). The ΔEXG1 and the ΔSPR1 strains were constructed by deleting the *exg1* and *spr1* genes, respectively, using CRISPR/Cas9 (Lee et al., 2015). Plasmids used in this study are listed in the Electronic Supplementary Information (ESI) and have been described previously (Bai et al., 2016). The expression vectors for BbGT86, the BbGT86 – BbMT85 pair, and the hrPKS – nrPKS pairs were co-transformed into the *S. cerevisiae* host cells using the LiCl-PEG procedure (Gietz & Woods, 2002).

2.2. Quantitative determination of glycoside products in engineered yeast strains

Three to five independent transformants for each recombinant *S. cerevisiae* strain were tested for the bioconversion of polyphenol aglycons fed to the cultures, or for the total biosynthesis of glycosylated polyketides. Fermentations with representative isolates were then repeated at least three times with four replicates each for quantitative analysis (ESI). Extraction of products, isolation from scaled-up cultivations (3–10 L), and structure elucidation of the glucoconjugates by NMR followed previously described protocols (ESI) (Xie et al., 2018). LC-MS analysis and quantification of the products were performed on a Waters AutoPurification semi-preparative HPLC/MS system (ESI). ^{1}H, ^{13}C, and 2D NMR (^{1}H–^{1}H COSY, HSQC, HMBC) spectra were obtained in methanol-d_4 on a Bruker Avance III 400 spectrometer (ESI).

The peak areas (mAU) in HPLC chromatograms, recorded at the wavelengths corresponding to the maximum absorption of the target analytes, were used to calculate the yields and the distributions of the glucosides and 4-*O*-methylglucosides. Total conversion yields (in percents) at the end of the cultivation were calculated as: $C_T = A / (A + As) * 100$ %; where C_T is the total conversion yield of all glycoconjugate products from the aglycon substrate; A is the sum of peak areas of all

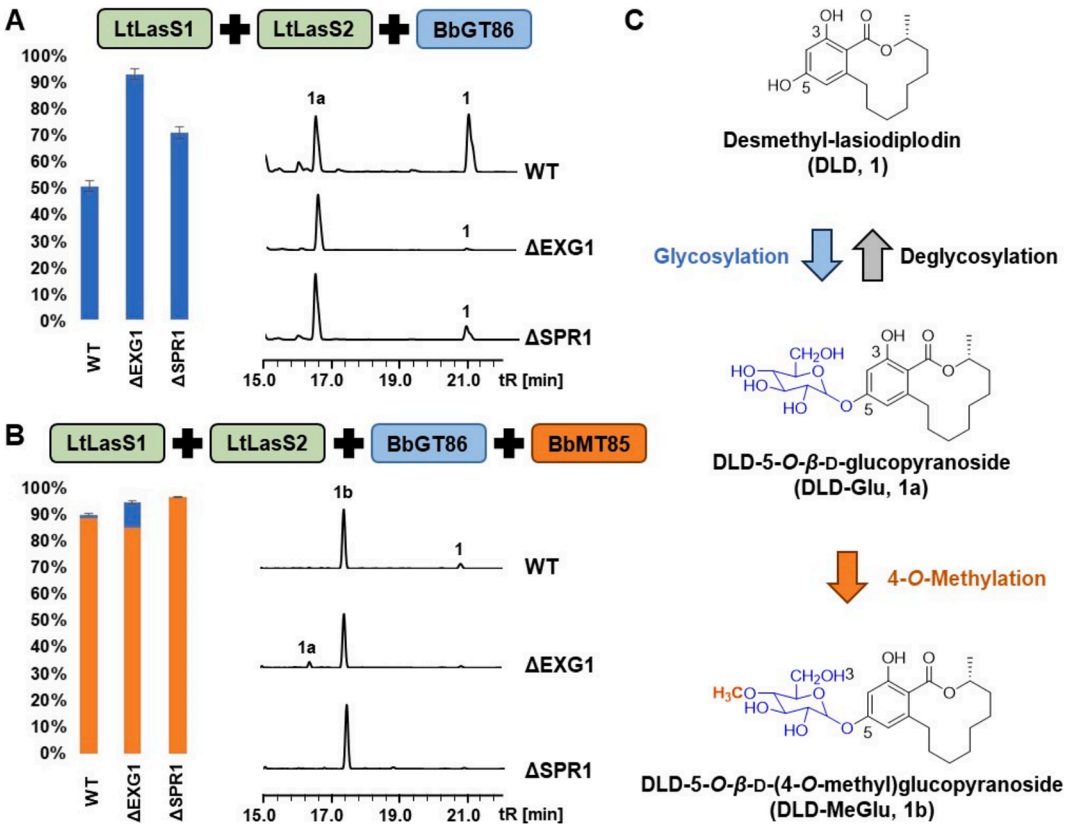

Fig. 1. Conversion of DLD 1 to its glycosides in engineered *S. cerevisiae* strains. Bar charts show the conversion yields of DLD-Glu **1a** (*blue*) and DLD-MeGlu **1b** (*orange*) from DLD **1** in three chassis strains carrying plasmid combinations that encode: (A) LtLasS1, LtLasS2, and BbGT86; and (B) LtLasS1, LtLasS2, BbGT86, and BbMT85. The values are presented as the means, and the error bars indicate standard error with four biological replicates in experiments repeated three times (n = 12). See the ESI for more details. Product profiles shown are HPLC traces of extracts recorded at 318 nm. (C) Chemical structures and bioconversions of DLD **1**, DLD-Glu **1a** and DLD-MeGlu **1b**. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

glycoconjugates (glucosides and methylglucosides); and As is the peak area of the remaining substrate. Product distributions are expressed as the percentages of the specific glycoconjugates within all glycosylated products obtained: Ax / A * 100 %; where Ax is the chromatographic peak area of the specific glucoside or methylglucoside.

2.3. Statistical analyses

The results are presented as the mean ± standard deviation (SD, n = 12). The chi-squared test was used for the statistical analysis of differences in the percentages of total conversion yields and the product distributions of the different strains (**, $p < 0.0001$, and *, $p < 0.05$). Total conversion yields and product distributions obtained with the wild type chassis were the baseline for the comparisons.

3. Results and discussion

3.1. Deletion of S. cerevisiae BJ5464-NpgA exoglycanases affects the production of desmethyl-lasiodiplodin glycosides

Desmethyl-lasiodiplodin (DLD) is a non-steroidal mineralocorticoid receptor antagonist and pancreatic lipase inhibitor that shows prostaglandin biosynthesis inhibitory activities in animals (Skellam et al., 2024). We had demonstrated that recombinant *S. cerevisiae* BJ5464-NpgA producing the *Lasiodiplodia theobromae* hrPKS – nrPKS pair LtLasS1 – LtLasS2 affords DLD (compound **1**, Fig. 1) (Xu et al., 2014). When the glycosyltransferase BbGT86 from *B. bassiana* is co-produced with the PKSs in the host, desmethyl-lasiodiplodin 5-*O*-*β*-D-glucopyranoside (DLD-Glu, compound **1a**) accumulates in the culture supernatant. DLD-Glu **1a** can be further methylated by additionally co-producing BbMT85 to yield desmethyl-lasiodiplodin 5-*O*-*β*-D-(4-*O*-methyl)glucopyranoside (DLD-MeGlu, compound **1b**; Fig. 1). However, since DLD-Glu **1a** is also hydrolyzed by the producer host (Xie et al., 2018), we hypothesized that the elimination of specific GHs will increase the product yield. To evaluate this hypothesis, the exoglycanase-encoding genes *exg1* and *spr1* of *S. cerevisiae* BJ5464-NpgA were individually deleted using CRISPR/Cas9 (ESI). The resulting strains and the parental BJ5464-NpgA were used as chassis to co-produce LtLasS1 – LtLasS2 with either BbGT86 to biosynthesize DLD-Glu **1a**, or with both BbGT86 and BbMT85 to yield DLD-MeGlu **1b**. The conversion of *in situ*-produced DLD **1** to its glycoconjugates **1a** and **1b** was quantified by HPLC-MS (Fig. 1 and ESI).

All recombinant strains co-expressing LtLasS1 – LtLasS2 with BbGT86 produced a single DLD glucoside (compound **1a**) as the product (Fig. 1 and ESI). While the total productivity of DLD congeners did not differ among the strains (approximately 12 mg/L), the conversion of DLD **1** to DLD-Glu **1a** showed significant differences ($p < 0.0001$), with the ΔEXG1 chassis affording the highest conversion yields (93 % conversion, approx. 11 mg/L, Fig. 1). This indicated that the ΔEXG1 strain lost most of its ability to hydrolyze DLD-Glu **1a** and consequently increased the production of this glucoside. Strain ΔSPR1 also showed an improved DLD-Glu conversion efficiency (71 % vs. 51 % in the wild type; ca. 8.5 mg/L). This indicated that SPR1 may also hydrolyze some of the **1a** produced, although not to the extent that was seen with EXG1.

When LtLasS1, LtLasS2, BbGT86, and BbMT85 were co-produced in the three chassis, most (86–98 %) of the polyketide products accumulated as DLD-MeGlu **1b** (Fig. 1 and ESI), confirming our previous observation that *O*-methylation protects the glucoside from host GH enzymes (Xie et al., 2018). The polyketide productivities of all strains were reduced as compared to the DLD-Glu **1a**-producing strains (7–8

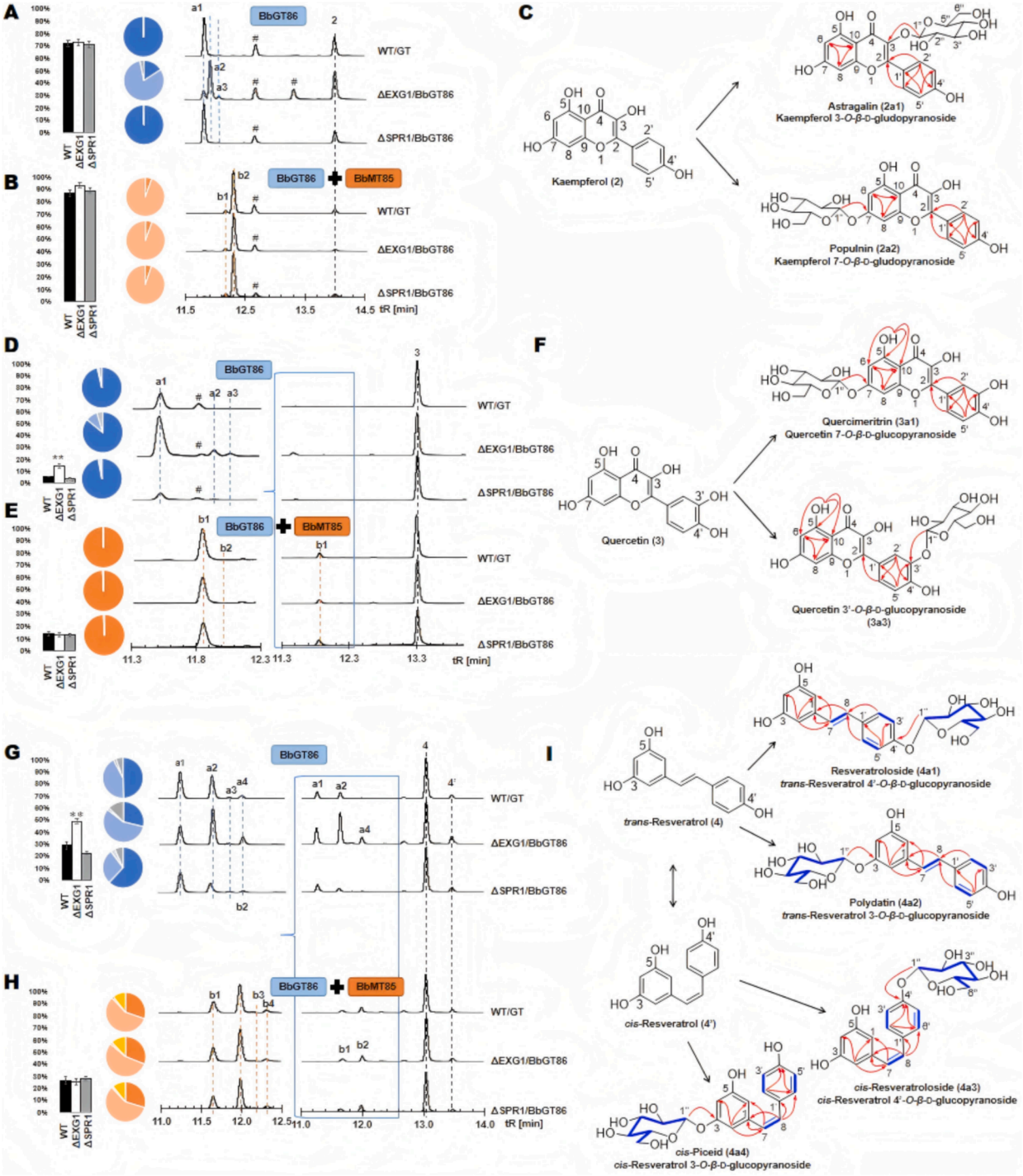

Fig. 2. Glucosylation and methylglucosylation of polyphenols. (A, B, D, E, G, H) Bioconversion of substrates **2**–**4** into their glucosides by *S. cerevisiae* strains producing BbGT86 alone (A, D and G), or to their methylglucosides by strains producing BbGT86 and BbMT85 together (B, E and H), respectively. Reversed-phase LC-MS traces were recorded at 364 nm (compounds **2**, **3** and their derivatives) or 305 nm (**4**, **4′** and their derivatives). #, Host metabolites not related to the fed substrates. Bar charts show the total conversion yields of the glucosides (A, D and G) or methylglucosides (B, E and H) from the fed substrates in the wild type (*black*), the ΔEXG1 (*white*), and the ΔSPR1 (*gray*) strains. **, Significantly different ($p < 0.0001$) from that of the wild type. The pie charts indicate the glucoside (A, D and G) or the methylglucoside (B, E and H) product distributions (products from **2** to **4**: glucoside a1, *blue*; a2, *pale blue*; a3, *pale grey*; a4, *grey*; methylglucoside b1, *orange*; b2, *pale orange*; b3, *grey*, b4, *yellow*). The values presented are the means from four independent experiments with three technical replicates each (n = 12; ESI), and the error bars indicate standard error. (C, F and I) Chemical structures of kaempferol **2**, quercetin **3**, resveratrol (*trans*-resveratrol **4** and *cis*-resveratrol **4′**), and their glucosides detected in this study. Key HMBC and COSY correlations are indicated by *red arrows* and *blue lines*, respectively (ESI). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mg/L vs. 12 mg/L), likely because of the increased metabolic burden of the extended biosynthetic pathway. Among the strains producing all four enzymes, the total polyketide productivities and the yields of DLD-MeGlu **1b** were similar for ΔEXG1 and the wild type strain. Around 9 % of the total polyketides produced were recovered as the aglycon **1** in the wild type strain, with only minor amounts of glucoside **1a** detectable. Just the opposite, most of the non-methylglucosylated polyketides in the ΔEXG1 strain were in the form of glucoside **1a** ($p < 0.0001$). We conclude that some of the nascent DLD-Glu **1a** escaped methylation, likely due to the inadequate methyltransferase activity of the cells. This **1a** was then mostly hydrolyzed in the wild type chassis, leading to the accumulation of **1**. In contrast, compound **1a** persisted in strain ΔEXG1, showing that the hydrolysis of this glucoside is effectively eliminated by the deletion of *exg1*.

The total polyketide productivity was the lowest of all strains when all four genes were expressed in strain ΔSPR1 (approx. 7 mg/L; Fig. 1 and ESI). However, almost all these compounds were the methylglucoside **1b** (98 % of all polyketides produced). We hypothesize that the available methyltransferase activity of the ΔSPR1-derived cells was sufficient to efficiently convert all nascent **1a** to **1b**, precluding accumulation of either glucoside **1a** or aglycon **1**.

3.2. Modulation of phenylpropanoid glycosylation by exoglycanase deletions

The utilization of plant-derived phenylpropanoid polyphenols is often limited by their poor aqueous solubility and instability (Hofer, 2016; Ren et al., 2023). Kaempferol **2** and its structural analogue quercetin **3** are prominent representatives of the flavonol class, naturally found in green tea, fruits, and leafy vegetables. *trans*-Resveratrol **4** (3,5,4′-trihydroxystilbene) is a phytoalexin of the stilbenes class that accumulates in grape skin and seeds (Fig. 2). The glycosylation of kaempferol, quercetin, and resveratrol can improve their stability, bioavailability, antioxidant and other bioactivities (Ren et al., 2023). While our previous experiments demonstrated the glucosylation and methylglucosylation of compounds **2**–**4** by recombinant *S. cerevisiae* producing BbGT86 and BbMT85, the yields of these products were limited by hydrolysis due to the GHs produced by the host (Xie et al., 2018).

Here, we compared the product yields of the wild type BJ5464-NpgA strain and the two deletion strains ΔEXG1 and ΔSPR1, each producing BbGT86, or BbGT86 and BbMT85, respectively. We utilized an in vivo biocatalytic platform where recombinant yeast cultures were fed with kaempferol **2**, quercetin **3**, or resveratrol **4**. Bioconversion products were quantified by LC-MS (Fig. 2 and ESI) and when product yields were sufficient, structures were elucidated by analyzing the 1D and 2D nuclear magnetic resonance (NMR) spectra of the purified compounds (ESI).

The biotransformation of kaempferol **2** was equally efficient (71–73 %) in all three strains producing BbGT86. Three glucosides were detected. Structure elucidation showed that **2a1** was astragalin (kaempferol 3-*O*-*β*-D-glucopyranoside), while **2a2** was populnin (kaempferol 7-*O*-*β*-D-glucopyranoside) (Fig. 2 and ESI). The yield of product **2a3** was too low for isolation and structure elucidation. The distribution of these glucosides was significantly affected by the deletion of *exg1* ($p < 0.0001$). Astragalin **2a1** (isolated yield 3.5 mg/L), a trypanocidal plant metabolite, was the only glucoside produced by feeding **2** to the wild type and the ΔSPR1 strains producing BbGT86. In contrast, the ΔEXG1 strain predominantly converted **2** to populnin **2a2** (81 %; isolated yield: 3.94 mg/L), with astragalin **2a1** (16 %; isolated yield: 0.77 mg/L) and kaempferol glucoside **2a3** (3 %; isolated yield: 0.15 mg/L) as minor products. This indicated that the preferred site for glycosylation of BbGT86 is, in fact, the phenolic alcohol OH-7 and not OH-3 on the pyrone (ESI), in agreement with the regioselectivity of this enzyme on BDL polyketide compounds (Xie et al., 2019). The shift in the kaempferol glucoside spectrum upon deletion of *exg1* is also in concert with previous observations on the regiospecificity of EXG1 that was reported to efficiently hydrolyze flavonoid C7 glucosides, but were seen to be inefficient against C3 glucosides (Schmidt et al., 2011; Wang et al., 2016). Thus, populnin **2a2**, preferentially produced by BbGT86, was likely hydrolyzed by EXG1 in the wild type and the ΔSPR1 strains, allowing the minor glucosylation product, astragalin **2a1**, to accrue instead in these strains.

In contrast, kaempferol **2** was predominantly converted to the 7-*O*-*β*-D-(4-*O*-methyl)glucopyranoside **2b2** (Xie et al., 2018) (approximately 95 %) and to minor amounts of methylglucoside **2b1** (5 %) in all three strains producing BbGT86 and BbMT85, just as in our previous study (Xie et al., 2019). Neither the total conversion yields, nor the product distributions of these strains were significantly different ($p > 0.95$), indicating that kaempferol methylglucosides are recalcitrant to hydrolysis by EXG1 and SPR1. Interestingly, the total conversion yields obtained with the strains co-producing BbGT86 and BbMT85 were higher than those observed for the strains producing BbGT86 alone (87–93 % vs. 71–73 %). While the reason for this improved bioconversion efficiency is unclear, we hypothesize that methylation of the reaction product creates a kinetic pull for the glucosylation reaction.

Next, we used quercetin **3** as the substrate for bioconversion by the three recombinant yeast strains producing BbGT86. Kaempferol **2** and quercetin **3** differ only in the presence of a hydroxyl group at C3′ in quercetin. Since ring B in kaempferol **2** is at most a marginal target for the BbGT86-catalyzed reaction, we expected that quercetin **3** glycosylation will show a similar outcome to that of **2**. However, both the yields and the product distributions were quite different for this flavonol (Fig. 2). First, the glycosylation efficiency of quercetin **3** was low, with the wild type and the ΔSPR1 strains converting less than 6 % of substrate **3** to its glucosides. The ΔEXG1 strain displayed improved yields for the glycosylated products (15 %; $p < 0.0001$ compared to the wild type), in agreement with another study showing an increase of quercetin glucoside production upon the deletion of the *exg1* gene (Schmidt et al., 2011). Three glucosides were detected in all three chassis, with the major product **3a1** confirmed as quercimeritrin (quercetin-7-*O*-*β*-D-glucoside; isolated yield: 0.55 mg/L; ESI), a plant natural product with anti-inflammatory and antioxidant activities (Anuradha & Sukumar, 2013; Legault et al., 2011). Quercimeritrin was accompanied by small amounts of quercetin-3′-*O*-*β*-D-glucoside **3a3** (isolated yield: 0.83 mg/L; ESI) and quercetin glucoside **3a2** in insufficient yields for structure elucidation, likely identical to isoquercitrin (quercetin-3-*O*-*β*-D-glucoside) (Ren et al., 2022). The product distribution of the ΔEXG1 strain significantly differed from those of the wild type and the ΔSGR1 strains ($p \leq 0.05$), with improved production of the minor glucosides **3a2** and **3a3**. Thus, exoglycanase EXG1 does not only hydrolyze the major OH-7-glucoside product of BbGT86 (Ren et al., 2022), but also degrades minor glucoconjugates with the sugar moiety at the B ring of the flavonoid skeleton (Schmidt et al., 2011) such as **3a3** (ESI). Neither the total conversion ($p \geq 0.05$) nor the product distribution ($p \geq 0.05$) was significantly affected in strain ΔSPR1 relative to the wild type.

Strains producing both BbGT86 and BbMT85 converted up to 14 % of **3** to its methylglucosides, with no differences in total conversion yields or product distributions among the three strains ($p \geq 0.05$) (Fig. 2). Quercetin methylglucoside **3b1** represented more than 99 % of the products in all three strains, reflecting that (4-*O*-methyl)glucosides are impervious to hydrolysis by yeast GHs. However, the low overall conversion indicates that product yields are not primarily depressed by hydrolysis in this case. Instead, quercetin is a marginal substrate for BbGT86, likely due to the interference of the 3′ alcohol with substrate binding and/or catalysis.

Resveratrol was biotransformed to two pairs of glucosides (Fig. 2; **4a1** and **4a2** [t_R 11.22 and 11.62 min] vs. **4a3** and **4a4** [t_R 11.83 and 11.96 min]) showing the same mass-to-charge ratio (*m/z*) but different UV spectra. These glucoside pairs were derived from *trans*-resveratrol **4** (t_R 12.98 min) and *cis*-resveratrol **4′** (t_R 13.43 min), respectively. *cis*-Resveratrol **4′** originates from the spontaneous isomerization of the

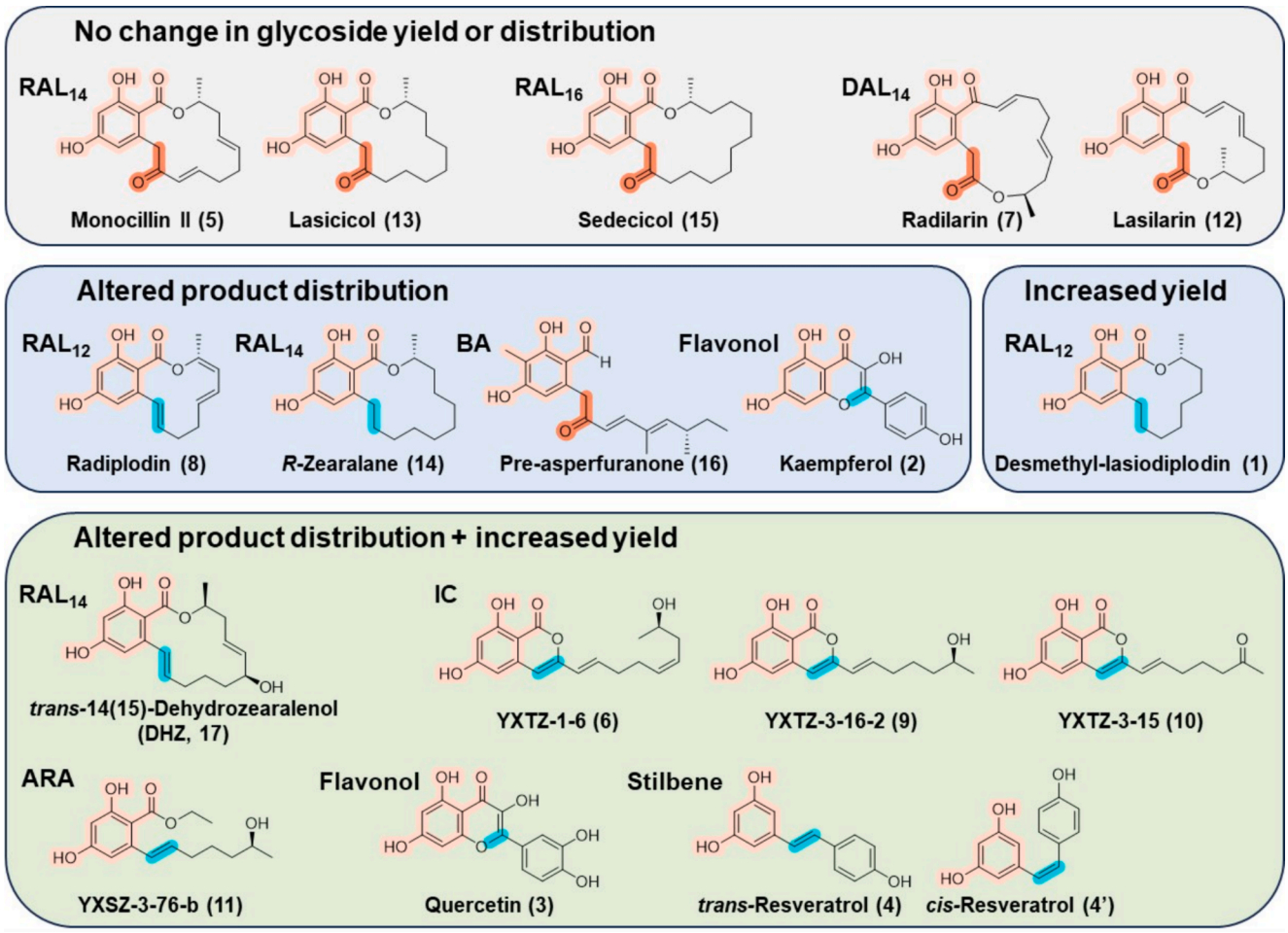

Fig. 3. Total biosynthesis of glucosides in exoglycanase-deficient strains of *S. cerevisiae*. BDL aglycone substrates shown were obtained by in vivo total biosynthesis by producing the appropriate hrPKS – nrPKS pairs in the same cells that also produce the BbGT86 glucosyl transferase. Flavonol and stilbene aglycons were fed to the BbGT86-producing strains. *Pink highlight*, the 2,4-dihydroxybenzaldehyde (or for **4** and **4′**, the 1,3-dihydroxybenzene) moiety; *orange highlight*; the methylene ketone moiety; and *blue highlight*, replacement of the methylene ketone moiety with other functional groups. ARA, acyl resorcylic acid; BA, benzaldehyde; DAL, dihydroxyphenylacetic acid lactone; IC, isocoumarin; and RAL, resorcylic acid lactone. See the ESI for glucoside yields and product distributions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

double bond of **4** under the cultivation conditions, regardless of the presence of the yeast host (Zhao et al., 2015). The structures of **4a1**, **4a2**, **4a3**, and **4a4** were confirmed as resveratroloside (*trans*-resveratrol-4′-*O*-*β*-D-glucoside; isolated yield: 1.10 mg/L), polydatin (a.k.a. piceid; *trans*-resveratrol-3-*O*-*β*-D-glucoside; isolated yield: 0.60 mg/L), *cis*-resveratrol-4′-*O*-*β*-D-glucoside (*cis*-R4′G; isolated yield: 0.66 mg/L), and *cis*-piceid (*cis*-resveratrol-3-*O*-*β*-D-glucoside; isolated yield: 3.3 mg/L), respectively (Fig. 2 and ESI). Bioconversion to the glucosides was most effective with the ΔEXG1 strain (49 % vs. 29 % with the wild type, $p < 0.0001$). An increase in the production of polydatin **4a2** (57 % of all glucosides vs. 43 % with the wild type, $p < 0.05$) and *cis*-piceid **4a4** (13 % vs 6 %, $p < 0.05$) indicated that BbGT86 has a preference to modify OH-3 vs. OH-4′, counteracted by glycoside hydrolysis by EXG1 in the wild type strain (ESI). Whereas the total conversion yields of the ΔSPR1 strain was slightly decreased relative to the wild type strain, this change was not significant (22 % vs. 29 %, $p > 0.1$). However, the glucoside distribution was significantly affected, with both OH-4′ products, **4a1** (62 % vs. 49 % in the wild type, $p < 0.05$) and **4a3** (4 % vs. 2 %, $p < 0.05$) over-represented. This may indicate that the SPR1 exoglycanase can hydrolyze some of the OH-4′ glucoconjugates produced by BbGT86 in the wild type strain. Meanwhile, the bioconversion yields, and the product distributions of the four resveratrol methylglucosides (**4b1**-**4b4**) were not different ($p > 0.5$) in the three strains co-producing BbGT86 and BbMT85 (Fig. 2).

3.3. Combinatorial biosynthesis of glycosidic benzenediol lactone congeners in S. cerevisiae exoglycanase-deficient strains

Next, we leveraged our library of strains that produce BDL congeners for the total biosynthesis of glucosides by co-producing BbGT86 with the various hrPKS – nrPKS pairs. As opposed to our previous study (Xie et al., 2018), we did not use the BbMT85 methyltransferase to protect the glucosides from hydrolysis but relied on the exoglycanase-deficient chassis strains ΔEXG1 and ΔSPR1. We challenged BbGT86 with 14 BDL congeners as potential substrates, representing different classes of NPs or uNPs, including macrolactones (seven RALs and two DALs) and non-macrocyclic BDL congeners (three isocoumarins, one acyl resorcylic acid [ARA], and one benzaldehyde) (Fig. 3). Gratifyingly, 28 polyketide glucosides (up to three per aglycon) were detected in culture supernatants, many in improved amounts.

For five aglycons, deletion of neither of the two GHs improved glucoside productivity, nor did these chassis mutations modulate the product distribution (Fig. 3 and ESI). This indicates that the produced glucoconjugates were largely recalcitrant to hydrolysis by the two host-derived GHs. This category included two 14-membered resorcylic acid lactones (RAL_{14}), monocillin II **5** and lasicicol **13**; the RAL_{16} sedecicol **15**; and two 14-membered dihydroxyphenylacetic acid lactones (DAL_{14}), radilarin **7** and lasilarin **12** (ESI). All these aglycons were good to excellent substrates for BbGT86 (conversion of 53–96 % of the

produced aglycons; ESI). Four aglycons were converted to a single major product each (**5a1**, **7a1**, **12a1**, and **13a2**, each representing at least 84 % of the glycosylated products of the strain, 4 to 8 mg/L each), while for sedecicol **15**, the two glucosides **15a1** and **15a2** were present in similar amounts (ca. 0.1 mg/L each; ESI).

In the case of three BDL congeners, the product distribution changed significantly in at least one of the mutant chassis, but no differences in the overall glucoconjugate yields were seen as compared to the wild type (Fig. 3 and ESI). This indicated that one of the glucosides produced by BbGT86 was a substrate for the host GHs, but BbGT86-catalyzed glucosylation could be efficiently channeled towards the more exoglycanase-resistant glucoconjugate regioisomer to maintain the total conversion yields. This category included the RAL_{12} radiplodin **8**, the RAL_{14} *R*-zearalane **14**, and the benzaldehyde ABH-6 **16**, all of which were excellent substrates for BbGT86 (conversion of the aglycons exceeding 83 %; ESI). For radiplodin **8**, the wild type and the ΔSPR1 strains afforded overwhelmingly **8a1** (95 % of all glucosides; approx. 8 mg/L), while the ΔEXG1 strain favored **8a2** (62 % of the glycoconjugates, $p < 0.0001$; ca. 5 mg/L; ESI). For *R*-zearalane **14**, ten times more of the minor glucoside **14a2** was produced in the ΔEXG1 (but not in the ΔSPR1) strain compared to the wild type (13 % vs. 1 %, $p < 0.002$). For the benzaldehyde substrate **16**, the production of the major glucoside **16a2** increased not only in the ΔEXG1 strain, but also in ΔSPR1 (78–79 % in the mutants vs. 63 % in the wild type, $p < 0.05$), indicating that both GHs hydrolyze some of this glucoside (ESI). As shown earlier (Fig. 1), both the ΔEXG1 and the ΔSPR1 strains afforded more glucoside compared to the wild type with desmethyl-lasiodiplodin **1** as the substrate, but **1a** remained the only glucoside detected (ESI).

Finally, for five potential substrates, including a RAL_{14} (*trans*-14(15)-dehydrozearalenol **17**), three isocoumarins (YXTZ-1–6 **6**, YXTZ-3–16-2 **9**, and YXTZ-3–15 **10**), and an ARA (YXSZ-3–76-b **11**), both the glucoside yields and the product spectra were significantly affected by the deletion of *exg1*, but not that of *spr1* (ESI). This suggests that EXG1 efficiently hydrolyzed the major glucoconjugate preferred to be synthesized by BbGT86. The resulting futile cycle of glucosylation/hydrolysis depressed the overall product yield, and allowed only the minor glucoside regioisomer (modified at the hydroxyl group less preferred by BbGT86) to accrue.

Based on our previous results where 4-*O*-methylation was used to protect glucoconjugates, we hypothesized that BbGT86 disfavors BDL substrates featuring an exocyclic methyl group in the *S*-configuration, such as dehydrozearalenol **17** (Xie et al., 2018). Indeed, **17** appeared to be a weak substrate for glucosylation (18 % conversion of the aglycon; ca. 2 mg/L; ESI) when using the wild type or the ΔSPR1 chassis. However, the total conversion yield was significantly better with the ΔEXG1 strain (41 %, $p < 0.001$; approx. 4 mg/L), although remaining well below those obtained with RAL_{14} substrates featuring an *R*-configured exocyclic methyl group such as *R*-zearalane **14** or lasicicol **13**. Further, the major glucoside produced by the ΔEXG1 strain changed to **17a3** (65 % vs. 6 % in the wild type, $p < 0.0001$) from **17a1** (28 % vs. 79 % in the wild type, $p < 0.0001$; ESI). It is also remarkable that all BbGT86-producing strains afforded three glycosylation products with **17** as the substrate, indicating that not only the two phenolic alcohols (OH-3 and OH-5), but the secondary alcohol on the macrocyclic ring (OH-13) could also be targeted by BbGT86.

Surprisingly, all three isocoumarins appeared to be at best marginal substrates for BbGT86 when produced in the wild type and the ΔSPR1 strains (0–5 % conversion of the aglycons; ESI), despite observing very good conversions to the corresponding methylglucosides in our previous study (Xie et al., 2018). This discrepancy was resolved by detecting moderate (19 % vs. 5 % in the wild type for **6**, $p < 0.0001$) to good conversion to the glucosides (45–58 % vs. 0 % in the wild type for **9** and **10**, $p < 0.0001$; approx. 1 mg/L) in the ΔEXG1 strain, indicating that the failure of the wild type and the ΔSPR1 strains to afford substantial amounts of glucosylated isocoumarins is likely due to the hydrolysis of the nascent glucosides by EXG1. For isocoumarin **6**, the product distribution between the two observed glucosides completely reversed to favor **6a2** in the ΔEXG1 strain (94 % of all glucosides, $p < 0.0001$), compared to the wild type or the ΔSPR1 strains where the small amounts of glucosides produced were almost all **6a1** (ESI). For **9**, two products were present in almost equal amounts, while **10a1** was the only detected product with strain ΔEXG1 for isocoumarin **10**. This is surprising since these isocoumarins differ only in the replacement of an alcohol group with a carbonyl at the distal end of the alkyl tail in **10**, far from the target phenolic alcohols. For the ARA ethyl ester **11**, the yield of glycosylated products doubled in the ΔEXG1 strain (92 % vs. 43 % in the wild type, $p < 0.0001$; ca. 6 mg/L vs. approx. 2.8 mg/L). The product distribution also shifted, with minor glucoside **11a2** increasing at the expense of **11a1**, while the proportion of the major glucoside **11a3** remained constant (ESI).

3.4. Glycosylation vs. glycoside hydrolysis in *S. cerevisiae*

To improve the production of bioactive glucosides with the broad-spectrum glucosyltransferase BbGT86 in a *S. cerevisiae* chassis (Xu et al., 2013b; Xu et al., 2013c), we wanted to break the futile cycle of biocatalyst-mediated glucosylation and host exoglycanase-catalyzed glucoconjugate hydrolysis. Although the major exoglycanases of *S. cerevisiae* that hydrolyze flavonoid glucosides have been identified, there is little information on the use of GH-deficient yeast hosts for the glycodiversification of polyketide NPs or uNPs (Schmidt et al., 2011; Wang et al., 2016). In the current study, we investigated the effects of the deletion of the genes for two such GHs, EXG1 and SPR1, on the glucosylation or methylglucosylation of selected BDL congeners and representative polyphenols of the flavonoid and stilbenoid classes. Except for resveratrol, a stilbene with a 1,3-dihyroxybenzene moiety, all these aglycons share a 2,4-dihydroxybenzaldehyde motif (Fig. 3). These moieties identify candidate aglycons for glucosylation by BbGT86. Since such motifs are not present in sterols and related triterpenoids, the natural substrates of the EGH1 glycosidase, these aglycons were not considered for BbGT86-mediated glycosylation in this study (Watanabe et al., 2015). Similarly, we elected not to investigate Δ*egh1* (i.e., ΔYIR007W) mutants since the sterol endoglucanase EGH1 has not been described to target BDL congeners and did not show significant in vivo flavonoid glycosidase activity (Huang et al., 2021; Schmidt et al., 2011; Wang et al., 2016; Ye et al., 2023; Zhao et al., 2024). Further, its intracellular localization may render EGH1 less effective against glycosides secreted by our synthetic biology chassis (Watanabe et al., 2015). In contrast, we found that the secreted EXG1 glycosidase was the major determinant for the hydrolysis of glucoconjugates in BbGT86-producing *S. cerevisiae*, with the deletion of *exg1* allowing the production of glucosides in much improved yields and/or in altered regioisomer distributions for 13 out of the 18 aglycons investigated. Conversely, the deletion of *spr1*, encoding the SPR1 exoglucanase, rarely affected glucoside yields or product distribution (only three out of the 18 aglycons tested). Further studies addressing the effects of the deletion of additional host-derived glycosidases, or in vitro studies with isolated GHs may prove fruitful avenues to optimize the in vivo production of NP and uNP glycosides in synthetic biology systems (Crowe et al., 2024; Huang et al., 2021; Li et al., 2024b; Ye et al., 2023; Zhao et al., 2024).

We previously showed that the biosynthesis of (4-*O*-methyl)glucosides by co-producing the *O*-methyltransferase BbMT85 with the BbGT86 glucosyltransferase provides efficient protection against glucoside hydrolysis by the host exoglycanases, leading to improved productivity and a different glycoconjugate product spectrum (Wang et al., 2019). Here, we attempted to combine this strategy with exoglycanase deletions, but saw no differences, relative to the wild type, in the production of 4-*O*-methylglucosides when using the exoglycanase mutant strains to decorate selected BDL or polyphenol aglycons. This indicates that elimination of EXG1 or SPR1 does not provide additional benefits when the sugar moiety is protected from hydrolysis by 4-*O*-methylation. However, this protection group is not always desirable,

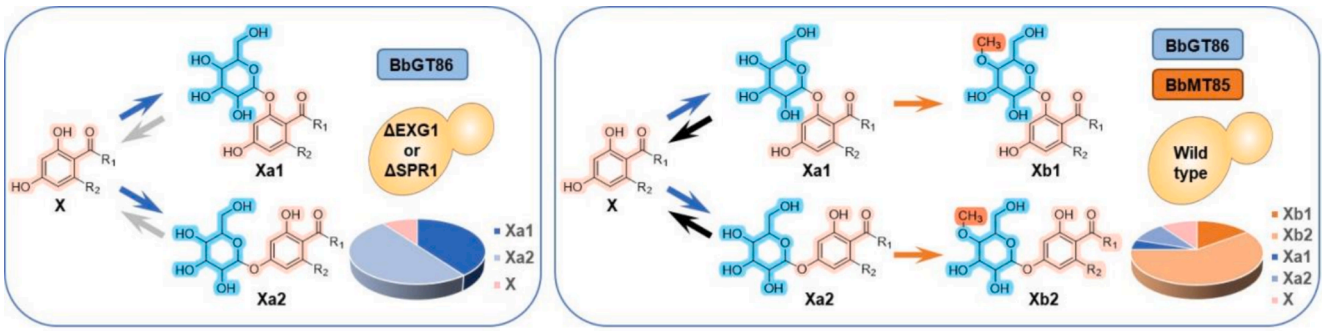

Fig. 4. Comparison of BbGT86-catalyzed glucosylation in exoglycanase-deficient *S. cerevisiae* strains vs. methylglucosylation with BbGT86 and BbMT85 in the wild type strain. Schematic structures represent aglycons (**X**; BDL analogues, polyphenols) with a 2,4-dihydroxybenzaldehyde moiety (*pink highlight*); their regioisomeric glucosides (**Xa1** and **Xa2**) and methylglucosides (**Xb1** and **Xb2**). *Blue arrows*, glucosylation by BbGT86; *black arrows*, glucoside hydrolysis by host exoglycanases EXG1 or SPR1; *grey arrows*, reduced or absent glucoside hydrolysis in the ΔEXG1 or ΔSPR1 strains; and *orange arrows*, 4-*O*-methylation of the glucose moiety by BbMT85. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

especially when the glycoconjugate is intended to be used as a prodrug that is then expected to undergo hydrolysis by the host or its associated microbiota. Further, total biosynthetic methylglucosylation is not always complete, affording a more complex product mixture with unmethylated glucosides beyond the unmodified aglycon (Fig. 4 and ESI). Thus, elimination of host GH vs. methylation of the glucoside are alternative strategies to be employed judiciously, considering the intended application of the glycoside.

Elimination of the EXG1 or the SPR1 exoglycanase may modulate the total yield of the glycoconjugates, the distribution of the regioisomeric glucosides in the product mixture, or both of these parameters upon the biocatalytic or total biosynthetic production of glucosides. The aglycons whose glycosylation was significantly affected by the exoglycanase deletions included a variety of RALs, ARAs, isocoumarins, flavonols and stilbenes (Fig. 3). Importantly, meaningful amounts of the desired isocoumarin glucosides could only be produced in the ΔEXG1 host, revealing that these glucoconjugates were easily hydrolyzed by EXG1 in the wild type yeast. Thus, the α-pyrone ring of the isocoumarin ring system is a structural indicator that correlates with improved yield and/or altered product distribution upon GH deletion. For the remaining compounds, the presence and size of a macrocycle, the existence, length and constitution of an aliphatic side chain, or the occurrence of an additional aromatic moiety were all compatible with both BbGT86-catalyzed glucosylation, and glucoside hydrolysis by at least one of the GHs. Thus, elimination of EXG1 (or in a few cases SPR1) was necessary to obtain improved and/or altered glycosylation outcomes.

Deletion of the exoglycanases had no significant effect on glucoside production in only a minority of cases (five aglycons out of 18 tested; Fig. 3). The corresponding aglycons were all BDLs: these RAL_{14}, RAL_{16} and DAL_{14} macrocycles all share a methylene ketone moiety attached to the carbon in the *ortho*-position of their 2,4-dihydroxybenzene rings (Fig. 3). This structural motif was absent in all but one of the remaining aglycons whose glucosylation was significantly affected by the deletion of one or both exoglycanases. This sole exception was pre-asperfuranone **16**, a (non-macrocyclic) benzaldehyde where the major glycosylated product **16a2** became even more dominant in the ΔEXG1 strain. As part of a macrocycle, this methylene ketone moiety may inhibit EXG1, thus protecting the glucoconjugates from hydrolysis. Conversely, BDL aglycons featuring this methylene ketone motif were excellent substrates for BbGT86, affording high glucoside conversion yields (ESI).

Production of the phenylpropanoid glucoconjugates posed the most significant challenge to our BbGT86-based glucosylation system. Here, the low product yields of the wild type strain for quercetin and resveratrol glucosides were significantly improved in the ΔEXG1 strain. Comparing the product profiles of the wild type and the exoglycanase-deleted strains allowed us to deduce that BbGT86 preferentially glucosylates a phenolic alcohol on the dihydroxybenzene ring, *para* to the aromatic ketone moiety (ESI) (Xie et al., 2018). This also appears to be a favored site for *O*-glycoside hydrolysis by EXG1 (Wang et al., 2016). For stilbenes, glucosylation on either of the two rings is feasible, with the deletion of *exg1* improving productivity and favoring OH-3 glucosylation on the benzenediol ring (ESI). As noted above, GH deletions do not affect methylglucosylation of phenylpropanoid substrates (ESI).

4. Conclusions

Exoglycanase-deficient *S. cerevisiae* chassis such as the ΔEXG1 strain (and to a lesser degree, the ΔSPR1 strain) are useful tools for biocatalyst-mediated glucosylation of bioactive NPs, including polyketides and polyphenols. Using these strains for the glycodiversification of BDLs, isocoumarins, benzaldehydes, flavonols, and stilbenes with BbGT86 and similar broad-spectrum glucosyltransferases (Feng et al., 2017; Ren et al., 2022; Xie et al., 2017; Xie et al., 2019) will help to adjust the bioactivities, and the absorption, distribution, metabolism, excretion and toxicity (ADME-Tox) characteristics of these privileged pharmacophores for their potential uses as nutraceuticals, pharmaceuticals, and crop protection agents.

5. Compliance with ethics requirements

This article does not contain any studies with human or animal subjects.

CRediT authorship contribution statement

Yingying Huang: Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Weimao Zhong:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Kinga E. Varga:** Methodology, Investigation, Data curation. **Zsigmond Benkő:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **István Pócsi:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Chenglong Yang:** Methodology, Investigation. **István Molnár:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: I. M. has disclosed financial interests in TAPI Hungary Industries LLC., which are unrelated to the subject of the research presented here. All other authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2025.132258.

Data availability

Data will be made available on request.

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