RESEARCH ARTICLE

Identification of a xyloglucan beta-xylopyranosyltransferase from *Vaccinium corymbosum*

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Abstract

Plant cell walls contain the hemicellulose xyloglucan, whose fine structure may vary depending on cell type, tissue, and/or plant species. Most but not all of the glycosyltransferases involved in the biosynthesis of xyloglucan sidechains have been identified. Here, we report the identification of several functional glycosyltransferases from blueberry (*Vaccinium corymbosum* bluecrop). Among those transferases is a hitherto elusive **X**yloglucan:**B**eta-xylosyl**T**ransferase (XBT). Heterologous expression of *VcXBT* in the *Arabidopsis thaliana* double mutant *mur3 xlt2*, where xyloglucan consists only of an unsubstituted xylosylated glucan core structure, results in the production of the xylopyranose-containing "U" sidechain as characterized by mass spectrometry, glycosidic linkage, and NMR analysis. The introduction of the additional xylopyranosyl residue rescues the dwarfed phenotype of the untransformed Arabidopsis *mur3 xlt2* mutant to wild-type height. Structural protein analysis using Alphafold of this and other related xyloglucan glycosyltransferase family 47 proteins not only identifies potential domains that might influence the regioselectivity of these enzymes but also gives hints to specific amino acids that might determine the donor-substrate specificity of these glycosyltransferases.

KEYWORDS
beta-xylopyranosyltransferase, blueberry GT47, glycosyltransferase, hemicellulose, xyloglucan

1 | INTRODUCTION

One of the major hemicelluloses found in the primary plant cell wall is xyloglucan (XyG) (Pauly & Keegstra, 2016). This polysaccharide has been found not only in land plants including nonvascular plants such as liverworts and mosses (Peña et al., 2008) but also their ancestors, the charophycean green algae (Mikkelsen et al., 2021). The function of XyG in plant growth and development is still under debate (Cosgrove, 2022; Park & Cosgrove, 2015; Talbott & Ray, 1992; Thompson, 2005) as mutant plants lacking XyG do not exhibit obvious growth phenotypes (Cavalier et al., 2008; Kim et al., 2020).

The abundance and fine structure of XyG can vary not only among different plant species but also among different plant tissues (Dardelle et al., 2015; Lampugnani et al., 2013; Liu et al., 2015; Schultink et al., 2014). The core structure of XyG consists of a backbone of β-1,4 glucan that is substituted at *O*-6 with α-xylosyl residues that are often further decorated with other sugar and/or acetyl residues. More than 20 different XyG sidechains have been discovered so far (Pauly et al., 2013; Scheller & Ulvskov, 2010; Schultink et al., 2014), and a one-letter code was established for their description (Fry et al., 1993). Based on this code, G refers to an unsubstituted glucosyl backbone residue, while X represents a xylosylated glucosyl residue, that is, α-D-Xylp-(1->6)-β-D-Glc. Such a XyG core structure

can be further extended on the xylosyl unit at *O*-2 with galactosyl, arabinofuranosyl, arabinopyranosyl, galacturonsyl, or xylosyl residues resulting in L, S, D, Y, and U sidechains, respectively (Hilz et al., 2007; Hsieh & Harris, 2009; Jia et al., 2005; Peña et al., 2008, 2012).

Many glycosyltransferases (GTs) involved in XyG synthesis have been identified that belong to various carbohydrate-active enzymes (CaZy) families (Coutinho et al., 2003; Pauly & Keegstra, 2016). Substitution on the xylosyl residues of the XyG core structure is mediated by members of the GT47 family including MUR3 and XLT2 (Figure 1).
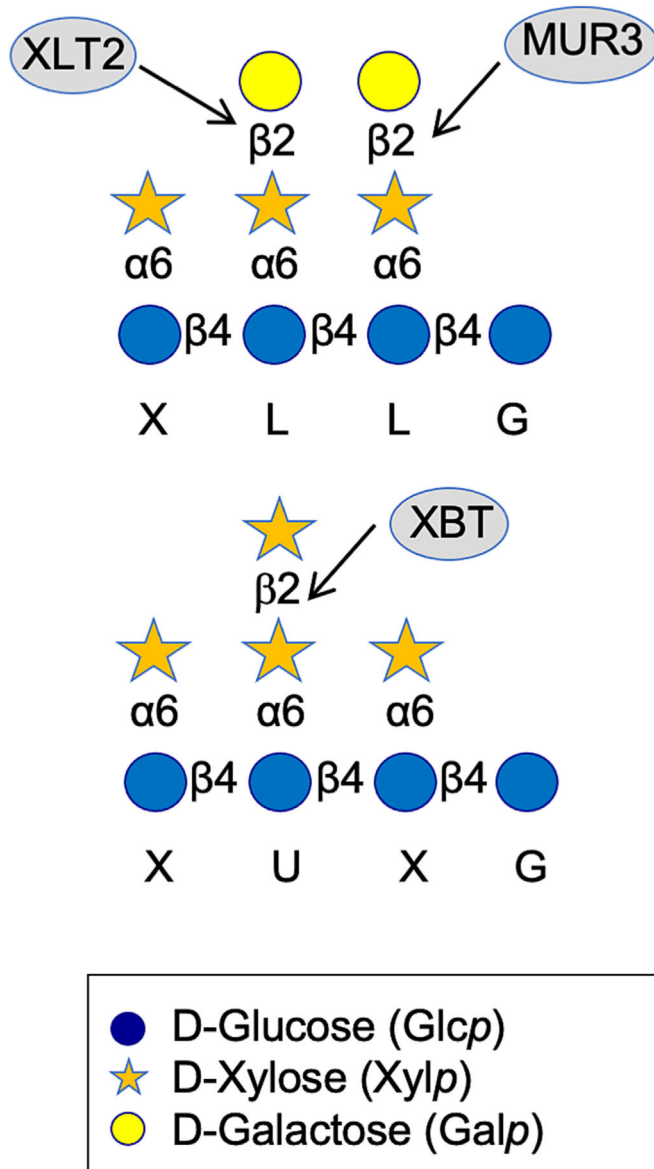

FIGURE 1 Illustration of the xyloglucan oligosaccharides XLLG and XXUG and the corresponding glycosyltransferases. For the one-letter code, see Fry et al. (1993). XyG sidechain galactosyltransferases XLT2 and MUR3 transfer the galactosyl residues to the second and third xylosyl units, respectively, resulting in XLLG. XBT, identified and characterized in this study, represents a xylopyranosyltransferase resulting in XUXG. Blue dots: D-glucose (Glc*p*), orange stars: D-xylose (Xyl*p*), yellow dots: D-galactose (Gal*p*).

MUR3 represents a XyG:galactosyltransferase, which adds a β-galactosyl residue at the *O*-2 position to a xylosyl-core residue resulting in the sidechain L (Madson et al., 2003; Wang et al., 2020). MUR3 transfers the galactosyl moiety to the third xylosyl residue on the XyG chain leading to an XXLG oligosaccharide motive in XyG. In contrast, XLT2 adds the galactosyl residue to the second xylosyl residue leading to a XLXG motive, demonstrating that these GTs exhibit regioselectivity (Jensen et al., 2012). GT47 family members can also transfer galacturonic acid (XUT1), arabinofuranosyl (XST), or arabinopyranosyl moieties (XDT) to the core xylosyl residue (Peña et al., 2012; Schultink et al., 2013; Zhu et al., 2018).

A unique XyG sidechain encompassing a terminal β-xylopyranosyl residue on the XyG core structure, that is, β-D-Xyl*p*-(1->2)-α-D-Xyl*p* (U sidechain), has been identified in leaf tissue of the Argan tree (Ray et al., 2004) and bilberries (Hilz et al., 2007). Here, we describe the identification of a responsible XyG:β-xylopyranosyltransferase among GT47 transferases identified in blueberry (*Vaccinium corymbosum*). Because the letter U had already been used for a GT47 XyG:galacturonsyltransferase (XUT; Peña et al., 2012), we named this enzyme **X**yG:**B**eta-xylosyl**T**ransferase (XBT; Figure 1).

2 | METHODS

2.1 | Plant material and growth

Arabidopsis seeds (*Arabidopsis thaliana* ecotype Columbia [Col-0]; double mutant *mur3.1 xlt2* [Jensen et al., 2012]; complemented Arabidopsis lines) were surface sterilized, stratified, and germinated on soil or half-strength Murashige and Skoog (MS) agar plates. *Nicotiana benthamiana* seeds were surface sterilized and plants grown on soil at 20/19°C under 12-/12-h light/dark cycle with 40%/75% humidity.

2.2 | Phylogenetic analysis and sequences

A Blastp search (Altschul et al., 1990; Camacho et al., 2009) using the Genome Database of Vaccinium (GDV; *V. corymbosum* cv. Draper v1.0 genome peptides; Colle et al., 2019) was performed using the AtXLT2 (gene locus: At5g62220) protein sequence to identify homologous sequences from blueberry (*V. corymbosum*). A cut-off was set at *e* value smaller than $1 * 10^{-40}$. Phylogenetic trees were created using the "One Click" Mode in Phylogeny.fr (Dereeper et al., 2008; Lemoine et al., 2019) using MUSCLE for multiple sequence alignment (Edgar, 2004). Protein sequences were obtained from NCBI, TAIR, and GDV.

The *V. corymbosum* protein sequences used in the study have been deposited in Genebank with the following accession numbers: OQ851743 (VcMUR3), OQ851744 (VcXLT2_1), OQ851745

(VcXLT2_2), OQ851746 (VcXBT_1/VcXBT), OQ851747 (VcXBT_2), OQ851748 (VcXBT_3), and OQ851749 (VcXBT_4).

2.3 | Genetic complementation

The Arabidopsis complementation approach was carried out as described (Jensen et al., 2012; Zhu et al., 2018). Blueberry gDNA was extracted from berries of *V. corymbosum* 'Bluecrop' (Farm Vinnemann in Olfen, Germany) using a CTAB-based extraction. Coding sequences of various GT47 gene candidate genes were amplified (Table S1) and introduced into the pORE-E4 vector (Coutu et al., 2007) using TA cloning (VcXBT, VcMUR3, VcXLT2_1, VcXBT2, and VcXBT 3) or restriction cloning (VcXLT2_2, VcXBT 4). Confirmed plasmids were transformed into *Agrobacterium tumefaciens* GV3101 and subsequently integrated into the Arabidopsis *mur3.1 xlt2* double mutants via floral dip (Clough & Bent, 1998). The transgenic plants were selected on half-strength MS agar plates containing selective antibiotic kanamycin (50 μg/ml). Germinated seedlings were transferred to soil for continuous growth. Homozygous T3 transformants were grown in a Phytotron under 16/8 h light/dark cycles at 21°C with 70% humidity and their phenotypic growth habit determined.

2.4 | Analysis of XyG

Alcohol-insoluble residue (AIR) was prepared from leaf tissue or etiolated hypocotyls of Col-0, *Atmur3.1 xlt2*, and generated Arabidopsis complementation lines by grinding dried plant material followed by an extraction with 70% (v/v) aqueous ethanol and twice with chloroform:methanol (1:1) (Schultink et al., 2013). The AIR material was then subjected to a xyloglucanase (XEG) digestion (Jensen et al., 2012; Pauly et al., 1999). The resulting solubilized XyG oligosaccharides were desalted by solid-phase extraction using Supelclean™ ENVI-Carb™ SPE Tubes (Supelco, 57109-U) (Schultink et al., 2013; Zhu et al., 2018) and analyzed by MALDI-TOF mass spectrometry and High-performance anion-exchange chromatography (HPAEC) equipped with a pulsed amperometric detector (PAD; IC Amperometric Detector, Metrohm) as described (Schultink et al., 2013). XyG oligosaccharides were separated using the following gradient: flow .4 ml/min, 100 mM NaOH to 100 mM NaOH/80 mM sodiumacetate in 22 min followed by a return to 100 mM NaOH within 4 min and a 6 min 100 mM NaOH wash. The XyG oligosaccharide mixture was also subjected to acid hydrolysis followed by monosaccharide compositional analysis by HPAEC as described (Jensen et al., 2012).

2.5 | Mass spectrometry

A MALDI-TOF mass spectrometer (Bruker Daltonik, Rapiflex) was used in positive linear mode with an accelerating voltage of 20,000 V to obtain the mass profiles of released XyG oligosaccharides.

2.6 | Purification of XyG oligosaccharide XUXG/XXUG

The XyG oligosaccharide XUXG/XXUG (*m/z* 1217) was isolated from leaf material of 3-week-old *mur3.1 xlt2* + *VcXBT* transgenic plants. AIR material was digested with XEG and the released oligosaccharides reduced in 1 M ammonium hydroxide containing 10 mg/ml sodium borohydride (Schultink et al., 2013). The reaction was neutralized with acetic acid and desalted as described above. The reduced oligosaccharides were separated by hydrophilic chromatography (Vydac 238TP 5 μl column) on an HPLC system (Knaur, Azura P6.1 C and AS 6.1 L) using an evaporative light scattering detector (SEDEX-LC LT-ELSD, Sedere). Samples were subjected to the following gradient conditions: flow: .5 ml/ml; 6% aqueous MeOH to 6.88% MeOH in 40 min, 6.88% MeOH to 50% MeOH in 10 min, 50% MeOH to 6% MeOH in 5 min, followed by a 6% MeOH wash for 15 min. The oven temperature of the ELSD detector was 41°C utilizing N_2 pressure of 3.1 to 3.3 bar. The content of collected fractions was monitored by MALDI-TOF mass spectrometry, pooled, and freeze-dried.

2.7 | Glycosyl linkage analysis

Glycosidic linkage analysis of XyG oligosaccharides was performed by derivatizing the sample to their partially methylated alditol acetates followed by gas-chromatography electron impact quadrupole mass spectrometry (GC–MS) as described (Liu et al., 2015; Schultink et al., 2013). The GC–MS system consisted of an Agilent 7890B GC System equipped with a 5977A MSD and an SP-2380 Fused Silica Capillary Column (30 m × .25 mm i.d. × 20 μm film thickness; Supelco).

2.8 | NMR analysis

Reduced oligosaccharides were dissolved in D_2O (99.9%), freeze-dried, and dissolved again in .4 ml of D_2O (99.97%) containing .05% of 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt. The ^1H NMR spectra were recorded on a Bruker Avance III 600 MHz NMR spectrometer at 298 K. All chemical shifts were referenced relative to 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid (.00 ppm for ^1H). The NMR data processing and analysis were performed using MestreLab's MestReNova software.

2.9 | Subcellular localization

The subcellular localization was carried out as described (Jensen et al., 2012). The coding sequence of VcXBT was subcloned into vector pMDC83 (Curtis & Grossniklaus, 2003) containing an N-terminal GFP tag. A rat sialyltransferase fused to red fluorescence protein (RnST-RFP) was used as a Golgi marker. These plasmids were transformed into *A. tumefaciens* GV3101. Cultures containing the GFP-

protein fusions and the RFP Golgi marker were used for (co)infiltration of *N. benthamiana* leaves (Sparkes et al., 2006).

2.10 | Protein structural analyses

Available protein models were obtained from the Alphafold2 database (Jumper et al., 2021). The following PDB IDs were used: AtMUR3 Q7XJ98, AtXLT2 F4K6F1, and AtXUT1 Q9SH31. For unavailable models, prediction of protein structures was performed using Colab-Fold (Mirdita et al., 2022). Tomato protein sequences (SlMUR3, SlXLT2, SlXST1, and SlXST2) were obtained from Schultink et al. (2013). Nasturtium protein sequences (TmMUR3 and TmXLT2) were obtained from Jensen et al. (2012). Rice protein sequences (OsMUR3 and OsXLT2) were obtained from Liu et al. (2015). *Physcomitrella patens* sequence (PpXDT) was obtained from Zhu et al. (2018). Exostosin domains in query proteins were identified using the Conserved Domain Database (Lu et al., 2020). Protein similarity was calculated using the Ident and Sim webtool (https://www.bioinformatics.org/sms2/ident_sim.html). Chimera and ChimeraX software were used to perform the superposition of predicted structures and obtain the structure images (Pettersen et al., 2004, 2021). Chimera structural protein alignments were created from structure superposition with a 5 Å residue–residue distance cut-off, allowing for circular permutations and three iterations at most. Protein multiple alignments were analyzed with Jalview 2 (Waterhouse et al., 2009).

3 | RESULTS

3.1 | Identification of GT47 family members in blueberry (*V. corymbosum*)

The β-xylose containing XyG sidechain U has been identified in the fruits of bilberries (*Vaccinium myrtillus* L.), a member of the Ericaceae (Hilz et al., 2007). However, genomic sequence information is only available from another member of the Ericaceae, blueberry (*V. corymbosum*). To confirm the presence of the unusual U sidechain in XyG of blueberry, various tissues of a blueberry plant were collected and subjected to XyG oligosaccharide mass profiling (OLIMP) (Günl et al., 2011). In this procedure XyG oligosaccharides are released from cell wall material by treatment with a XyG-specific endoglucanase and subjected to mass spectrometric analysis by MALDI-TOF (Figure 2). Indeed, numerous XyG oligosaccharides could be identified that contained one or two additional pentoses on the regular XyG oligosaccharides found, for example, in Arabidopsis (Lerouxel et al., 2002). The nature of the additional pentose moieties cannot be deduced by mass spectrometry, and may represent α-arabinofuranosyl or α-arabinopyranosyl residues found in XyG of Solanaceous plants and mosses, respectively, but there is a possibility that they might include β-xylosyl units. Interestingly, no such pentosylation beyond the XyG core structure was found in blueberry root tissue, highlighting again that the fine structure of XyG is tissue specific.
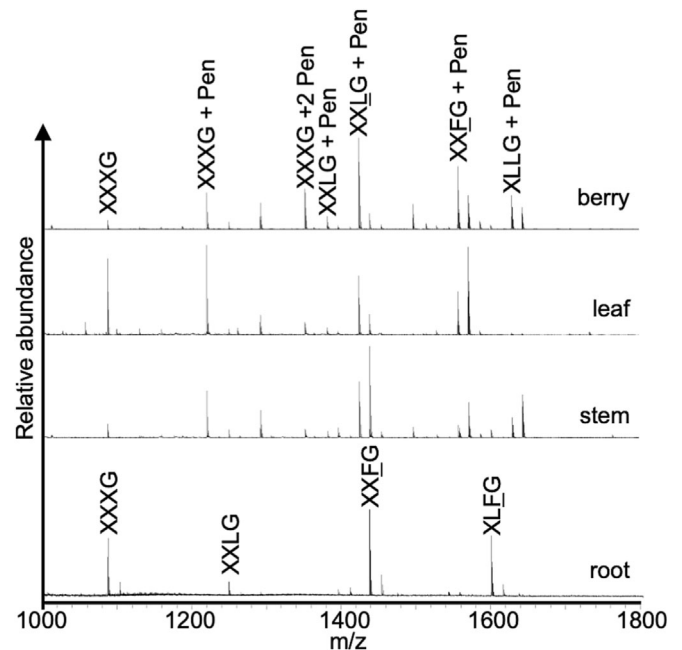

FIGURE 2 XyG oligosaccharide mass profiling of different blueberry tissues. Suggested XyG oligosaccharide structures of selected ion signals are labeled according to the one-letter code. Pen, pentose.

Blueberry may thus contain a XyG:β-xylosyltransferase. It is expected that such an enzyme belongs to GT47 as such an enzyme has the same acceptor substrate and forms the same glycosidic linkage on the same position as the well-characterized GT47 galactosyltransferases MUR3 and/or XLT2. Hence, the amino acid sequence of the Arabidopsis XyG-related GT47 family member AtXLT2 was used as a bait to identify related GT candidates in the GDV (Figure S1). A large number of similar GT47 protein sequences were identified in the blueberry genome. Many of the blueberry proteins in the phylogenetic tree occur in clusters of four, reflecting the tetraploid nature of the sequenced blueberry, that is, they likely correspond to the four allele variants from the same gene. Nevertheless, a number of blueberry proteins are phylogenetically close to functionally characterized XLT2 XyG:galactosyltransferases (AtXLT2, TmXLT2, and OsXLT2) highlighted as an XLT2 clade; others are phylogenetically close to functionally characterized MUR3 XyG:galactosyltransferases (OsMUR3, SlMUR3, EgMUR3, AtMUR3, and TmMUR3) highlighted as a MUR3 clade (Figure S1). In between these two clades are blueberry homologs of many hitherto uncharacterized Arabidopsis GT47s, for example, GT12 and GT15, and the characterized XUT galacturonosyltransferase. In addition, there is a large cluster consisting only of blueberry GT47 sequences whose closest functionally characterized GT is XST1, a XyG:arabinofuranosyltransferase from tomato (Figure S1). Because arabinose and xylose are both pentoses, there is a possibility that this unique *Vaccinium* GT47 clade harbors the elusive XyG:β-xylosyltransferase.

The expression levels of blueberry genes including GT47 genes have been determined in different plant tissues (Colle et al., 2019;

Figure S2). Focusing on GT47s highly expressed in fruit tissue, we selected a few GT47 candidates for functional testing including one putative VcMUR3, two VcXLT2s (1 and 2), and four putative VcXBTs (1 to 4) present in the unique blueberry GT47 clade. Based on the known sequences in GDV, forward and reverse primers were designed, and the corresponding seven coding sequences were cloned from a genomic DNA preparation of blueberry fruits of *V. corymbosum* bluecrop cultivar resulting in a phylogenetic tree including the cloned blueberry bluecrop cultivar GT47 proteins and other functionally characterized GT47s (Figures 3 and S1).

3.2 | Functional complementation in Arabidopsis

The impact of the selected blueberry GT47 enzymes on XyG structure was assessed by individual heterologous expression in the Arabidopsis double mutant *mur3 xlt2*, whose walls contain only the XyG core structure without additional substituents such as galactosyl moieties (Jensen et al., 2012). The XyG structure derived from the various complemented Arabidopsis plants was analyzed by OLIMP (Figures 4 and S3). The OLIMP profile of the untransformed Arabidopsis *mur3.1 xlt2* double mutant walls show the occurrence of a single XyG oligosaccharide with a *m/z* 1085 Da indicative of the XyG oligosaccharide XXXG, thus representing the XyG core structure without any substituents. This OLIMP profile was retained when four of the blueberry genes were constitutively expressed in Arabidopsis *mur3.1 xlt2* (Figure S3), suggesting that when expressed in Arabidopsis, these genes may not be involved in XyG biosynthesis. However, other possibilities of nonfunctionality include the absence, instability, and/or mislocalization of these expressed proteins.

In contrast, expression of *VcMUR3* and *VcXLT2_1* in *mur3 xlt2* resulted in a XyG that contained an oligosaccharide of *m/z* 1247, likely representing a galactosylated XyG oligosaccharide (Figure 4). To ascertain the nature of this substituent, the enzyme-solubilized XyG
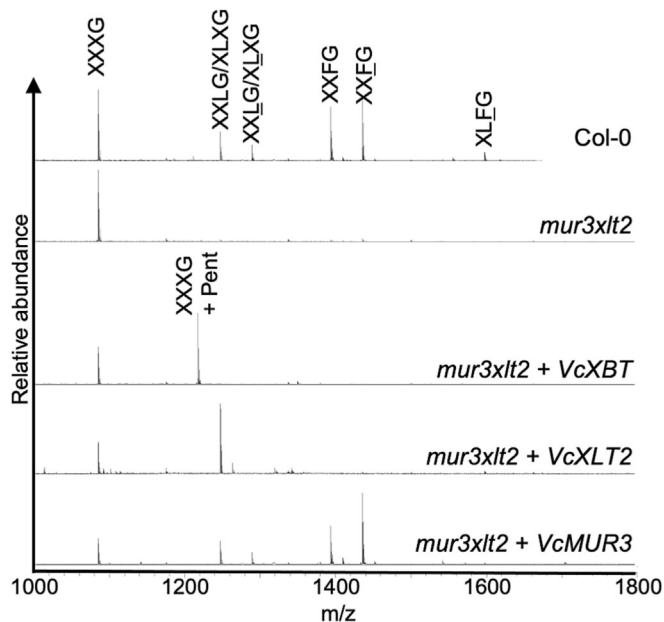

FIGURE 4 XyG oligosaccharide mass profiling (OLIMP) of blueberry GT47 complemented Arabidopsis lines. XyG oligosaccharide profiles derived from leaf tissue of Arabidopsis Col-0, untransformed *mur3 xlt2* mutant, and transgenic lines expressing *VcXBT*, *VcXLT2_1*, and *VcMUR3* in the *mur3 xlt2* genetic background. Suggested oligosaccharide structures of selected ions are indicated by the one-letter code.
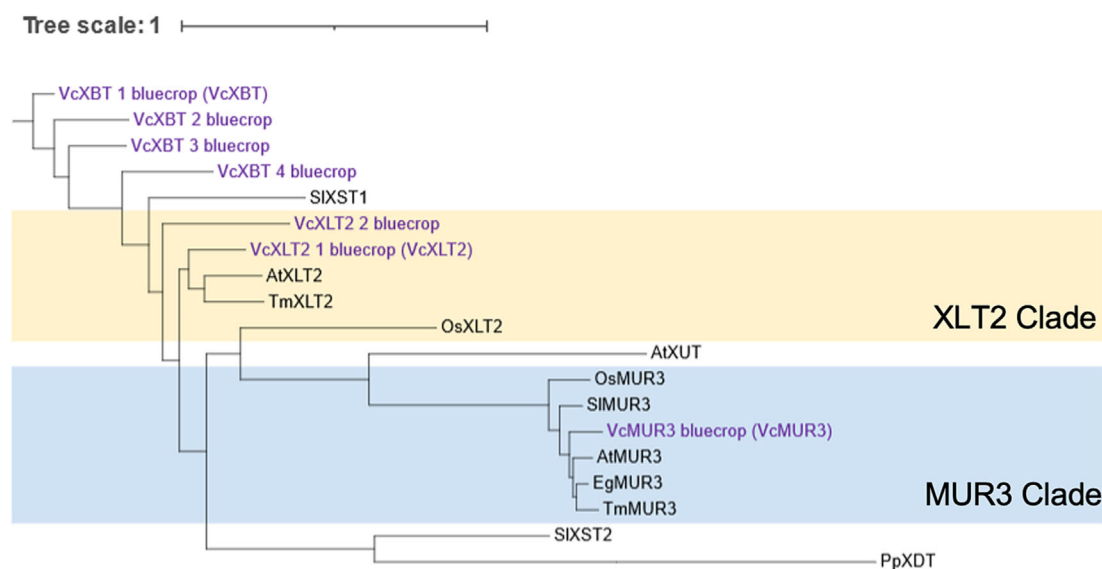

FIGURE 3 Phylogeny of XyG-related GT47 proteins. Black font: protein sequences from known, functionally described GT47 XyG glycosyltransferases from various species. Purple font: blueberry proteins with similarity to the GT47 XyG glycosyltransferases proteins cloned from *Vaccinium corymbosum* bluecrop cultivar. The XLT2 and MUR3 clades are indicated in yellow and blue, respectively. The comprehensive phylogenetic tree is shown in Figure S1. The scale bar indicates the number of substitutions per site, branch lengths are directly related to the amount of genetic change between shown genes. At, *Arabidopsis thaliana*; Os, *Oryza sativa* (rice); Tm, *Tropaeolum majus* (nasturtium); Sl, *Solanum lycopersicum* (tomato); Eg, *Eucalyptus grandis* (Eucalyptus).

oligosaccharide mixtures derived from the Arabidopsis wall materials were also subjected to high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and their elution profile compared with that of a standard XyG oligosaccharide mixture (XXXG, XXLG, XLXG, and XLLG; Figure 5). Based on the retention times, expression of VcMUR3 lead not only to the occurrence of XXLG but also its fucosylated form XXFG, whose ion signal was also observed by OLIMP (Figure 4). Taken together, this provides strong evidence that VcMUR3 indeed represents a functional homolog of XyG:galactosyltransferase MUR3. In contrast, expression of VcXLT2_1 yielded a XyG-containing XLXG and no fucosylated XyG oligosaccharides demonstrating that this enzyme exhibits XyG:galactosyltransferase XLT2 activity when expressed in Arabidopsis. Other observed peaks in mur3 xlt2 transformed with VcXLT2_1 or VCMUR3 (retention time of 23.3 and 25 min; Figure 5) could not be assigned to any XyG oligosaccharide.

When one of the putative VcXBT genes was expressed in the mur3 xlt2 mutant and its wall analyzed by OLIMP, a new XyG oligosaccharide appeared with m/z 1217 representing XXXG with an additional pentose (Figure 5). When this oligosaccharide mixture was subjected to HPAEC several peaks became apparent, whose content was determined by mass spectrometry (Figure S4). The first peak at a retention time of XXXG contained an oligosaccharide with a mass of m/z 1085 confirming the presence of XXXG. The second peak also contained oligosaccharides with a m/z 1085. However, due to a different retention time than XXXG this oligosaccharide must be a structural isomer such as XXGG + 1 pentose. The third peak contained at least two oligosaccharides, one with a m/z 1085 representing another XXGG + 1 pentose, and one with m/z 1217 representing XXXG + 1 pentose. The fourth peak contained only oligosaccharide(s) with m/z 1217, thus representing another XXXG + 1 pentose. Hence, expression of VcXBT lead to the occurrence of several unusual pentosylated XyG oligosaccharides. All HPAEC fractions containing XyG oligosaccharide were pooled and subjected to a monosaccharide compositional analysis (Figure S4). The dominant monosaccharides were glucose and xylose with only trace amounts of arabinose and

galactose (Figure S4). To further confirm the nature of this additional pentose, the enzymatically derived XyG oligosaccharide mixture from mur3 xlt2 plant expressing the VcXBT gene was subjected to hydrophilic chromatography (Figure S5). This type of chromatography avoids solvents such as salts that might interfere with additional analyses. One of the peaks with a retention time of around 27 min was collected and indeed contained carbohydrate(s) with a m/z 1217 indicative of XXXG + 1 pentose. The content of this fraction was subjected to glycosidic linkage analysis (Table 1 and Figure S6). In addition to the expected sugar moieties of 4-Glcp, 6-Glcp, and 4,6-Glcp, representing a partially substituted glucan backbone, only T-Xylp and 2-Xylp representing sidechains were present. Particularly, the 2-Xylp demonstrates that this XyG xylosyl sidechain had an additional substituent on its O-2 position. This substituent is also xylose (T-Xylp), because neither T-Araf nor T-Arap or any other pentosyl unit were observed in the sample.

The collected fraction was also subjected to ^{1}H NMR (Figure 6). The spectrum is consistent with the presence of XyG oligosaccharide alditols, as numerous anomeric signals are observed in the chemical shift region of previously characterized XyG oligosaccharides (Tuomivaara et al., 2015; York et al., 1993). There are several H-1 signals belonging to α-sugars in the region of δ 4.94–5.18 ($J_{1,2}$ 3.2–3.6 Hz) and β-sugars (δ 4.50–4.66 [$J_{1,2}$ 7.8 Hz]). Based on the integral ratios of these signals, it is evident that this fraction contains a mixture of XyG oligosaccharides. Due to the same mass, these are apparent isomers likely representing XyG alditols with a single xylosyl substituent on different position of the three core xylosylated sidechains. Consistent with this hypothesis is the integral ratio of 1:2 of the H-1 signal of a substituted α-Xylp (δ 5.155 [$J_{1,2}$ 3.6 Hz]) to unsubstituted, terminal α-Xylp (δ 4.961–δ 4.949 [$J_{1,2}$ 3.2–3.6 Hz]). Concerning the β-sugars, the H-1 signal δ 4.578 ($J_{1,2}$ 7.8 Hz) was assigned to β-Xylp consistent with published data concerning this sugar (Hilz et al., 2007; Ray et al., 2004). The other signals can be attributed to β-Glcp units of the oligosaccharide backbone (δ 4.646 [$J_{1,2}$ 7.9 Hz]; δ 4.551 [$J_{1,2}$ 7.8 Hz]; δ 4.546 [$J_{1,2}$ 7.2 Hz]). Taken together the mass spectrometrical data, monosaccharide compositional analysis,
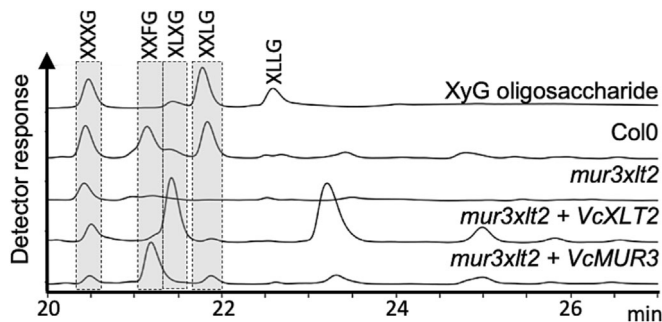

FIGURE 5 XyG oligosaccharide separation by HPAEC-PAD. Standard XyG oligosaccharides, XyG oligosaccharides derived from leaf tissue of Col0, mur3 xlt2, and transgenic lines expressing VcXLT2_1 and VcMUR3 in mur3 xlt2 genetic background. Peaks were assigned according to Schultink et al. (2013) and Hsieh and Harris (2012) as well as based on mass spectrometry.

TABLE 1 Glycosidic linkage analysis of XyG oligosaccharide fraction with m/z 1217 from leaf walls of mur3 xlt2 expressing VcXBT (Figures S5 and S6).

Sugar moiety	Abundance (%)
T-Arap	Not detected
T-Araf	Not detected
T-Xylp	9.5
T-Manp/T-Glcp	1.5
2-Xylp	9.4
6-Glcp	13.8
4-Glcp	4.0
4,6-Glcp	32.4

Abbreviations: 2-Xylp, 2-linked xylopyranose; 4,6-Glcp, 4,6-linked glucopyranose; 4-Glcp, 4-linked glucopyranose; 6-Glcp, 6-linked glucopyranose; T-Araf, terminal arabinofuranose; T-Arap, terminal arabinopyranose; T-Xylp, terminal xylopyranose.

FIGURE 6 Anomeric region of the ^{1}H NMR spectrum of the XyG oligosaccharide fraction (Figure S5A) obtained from leaf walls of *mur3 xlt2* expressing *VcXBT*. The residues corresponding to each group of anomeric proton resonances are indicated at the top of the figure. The spectral region δ 4.67–4.92 containing the strong HDO signal has been deleted for clarity. The location of each of these residues in specific sidechains (X or U) is indicated. Xyl*p*, xylopyranose; Glc*p*, glucopyranose; Gol, glucitol.
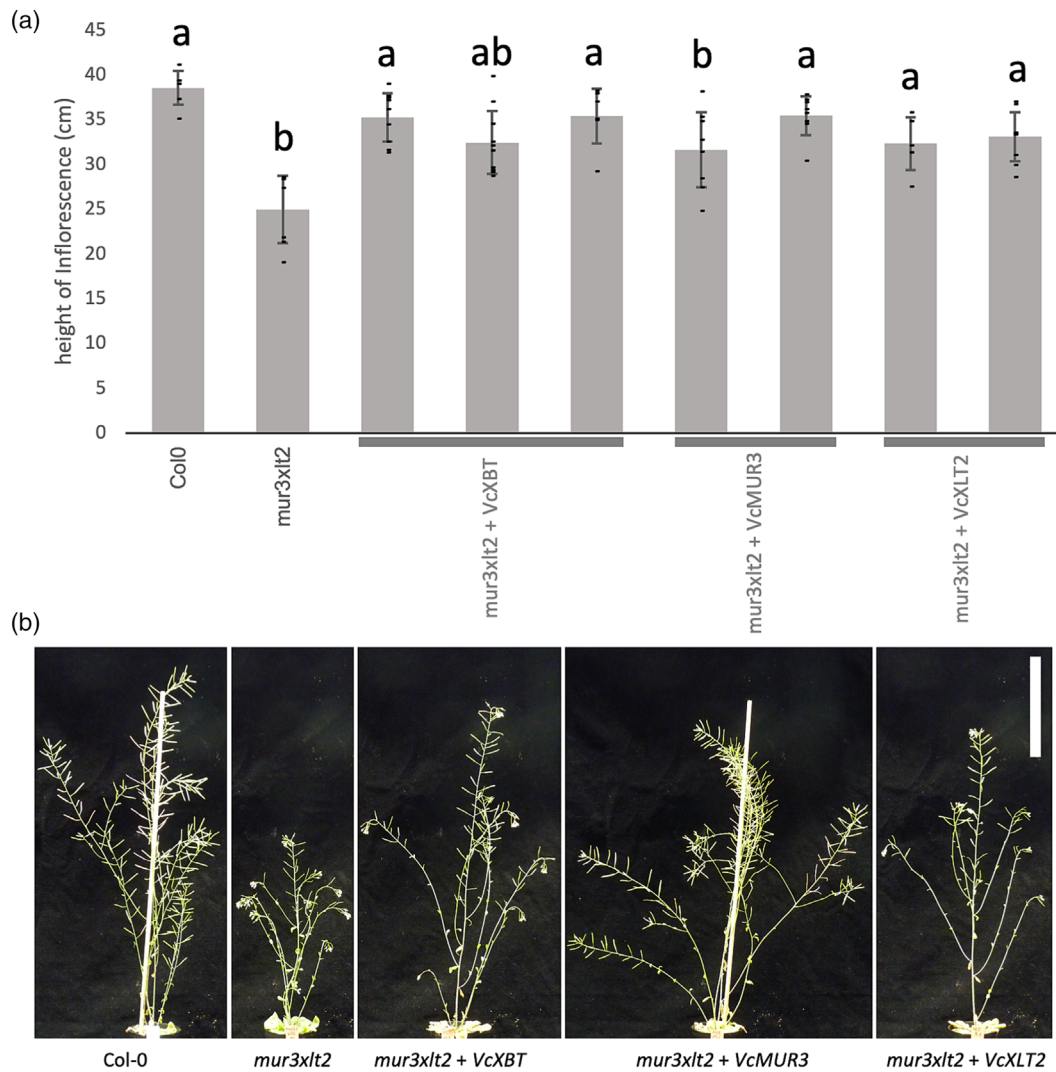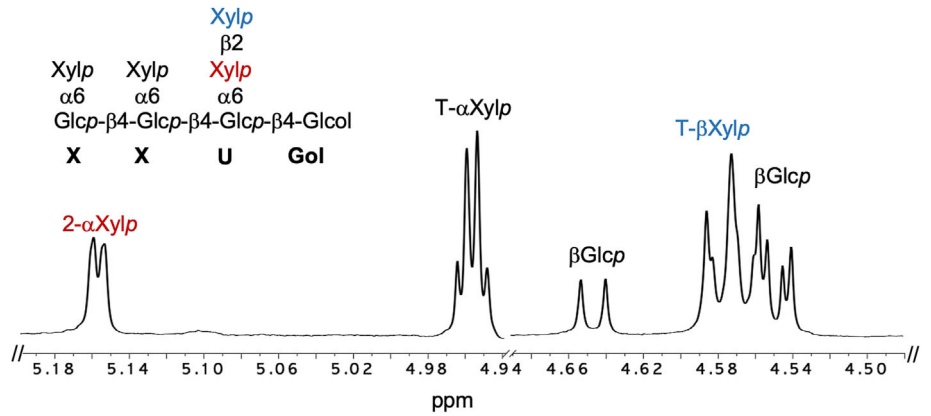

FIGURE 7 Plant height of complemented *mur3 xlt2* plants expressing blueberry GT47s. (a) Height of inflorescence stems of 8-week-old Arabidopsis plants (Col-0, $n = 6$; *mur3 xlt2*, $n = 7$; *mur3 xlt2 + VcXBT*, $n = 25$; *mur3 xlt2 + VcMUR3*, $n = 16$; *mur3 xlt2 + VcXLT2*, $n = 14$). Different letters indicate statistical differences ($p < .05$) based on an ANOVA analysis and subsequent Tukey's test. Error bars indicate standard deviation. (b) Representative pictures from the genotypes shown in (a). Scale indicates 10 cm.

glycosidic linkage, and NMR data of this XyG oligosaccharide fraction are consistent with a XyG oligosaccharide containing a β-Xyl*p*-(1->2)-α-Xyl*p* sidechain—the "U" sidechain (Pauly & Keegstra, 2016). Based on the NMR analysis, this fraction contains a mixture of two isomers of such as XUXG and XXUG.

3.3 | Growth phenotypes of *mur3 xlt2* expressing functional blueberry GT47 genes

The structure of XyG has been shown to affect vegetative growth. The Arabidopsis double mutant *mur3 xlt2* with XyG containing exclusively XXXG units exhibits dwarfism (Schultink et al., 2013; Liu et al., 2015; Zhu et al., 2018; Figure 7). When *VcMUR3*, *VcXLT2_1*, or *VcXBT* were expressed in *mur3 xlt2* using a constitutive promotor, vegetative (stem) growth was restored to nearly normal heights in most of the transgenic lines (Figure 7).

3.4 | VcXBT is localized to the Golgi apparatus

XyG biosynthesis occurs in the Golgi apparatus (Pauly & Keegstra, 2016) as all the known required biosynthetic enzymes have been shown to be localized there (Jensen et al., 2012). The cellular localization of VcXBT was tested by expressing a C-terminal GFP-tagged *VcXBT* transiently in tobacco leaves (Figure S7). Under the confocal microscope, a punctate pattern is visible. Comparison with a coexpressed RFP-tagged Golgi marker (rat sialosyltransferase) indicated cellular colocalization demonstrating that VcXBT is indeed present in the Golgi apparatus.

3.5 | Structural comparison of GT47 proteins

The enzymes involved in substituting the XyG core structure belong to the GT47 family. GT47 enzymes are characterized by the presence of an exostosin domain named after their similarity to the human exostosin-1 (EXT1) GT required for the biosynthesis of heparan sulfate (Lind et al., 1998). The structure of the exostosin transferase domains from EXT1 and related EXT enzymes have been resolved (Leisico et al., 2022). These enzymes adopt a GT-B fold characterized by a β/α/β sandwich structure where two Rossmann-like domains of parallel β-sheets flanked by α-helices are connected by an interdomain cleft containing the active site (reviewed in Li et al., 2023). It is thus likely that the GT47 proteins from plants exhibit a similar fold structure, even though no enzyme-substrate complexes have been experimentally characterized despite the abundant genetic and biochemical data available. The best known XyG GT47 enzymes are MUR3 and XLT2 with several homologs functionally characterized in different plant species (Jensen et al., 2012; Liu et al., 2015; Lopes et al., 2010; Madson et al., 2003; Schultink et al., 2013). In order to compare VcMUR3 and VcXLT2 to previously characterized XyG galactosyltransferases, predicted structures for these two blueberry proteins

were obtained using Alphafold. Models for Arabidopsis and tomato are available in the Alphafold database, but not for the blueberry, nasturtium, or rice homologs, which we generated. As observed when comparing the amino acid sequences (Figure 3), VcMUR3 and VcXLT2 exhibit high structural similarity to other characterized MUR3 and XLT2 homologs, respectively (Figure 8 and Table S2). Although the overall sequence similarity is limited to around 55%, there is a high structural conservation between MUR3 and XLT2 exostosin domains from all of these species, expanding on what has been previously reported in the case of Arabidopsis and tomato proteins (Julian & Zabotina, 2022). Root-mean-square deviation (RMSD) is a measure of the average distance between the positions of the corresponding atoms in the two structures, taking into account both the magnitude and the direction of the differences. It is commonly used to quantify the similarity between two protein structures with the lower RMSD value indicating higher structural similarity in terms of overall shape and orientation. XyG galactosyltransferases show a high overall structural conservation as indicated by a low RMSD between their atomic coordinates (Table S2). Both sequence and structural conservations are particularly high among MUR3 members with values around 90% protein similarity and extremely low RMSD values (.3–.4 Å). In the case of XLT2 proteins, there seems to be less conservation with values ranging 65% to 80% sequence similarity and .7–1.4 Å RMSD. When comparing MUR3 and XLT2 proteins, the predicted structures align for the most part of the exostosin domain, and most of the structural divergence is localized in discrete regions present in the periphery of the GT-B interdomain cleft (regions I to VI; Figure 8). The predicted Alphafold structure of the VcXBT GT47 enzyme shows a high similarity to these XyG galactosyltransferases, particularly regarding the exostosin domain. Similar to what has been observed in the case of the amino acid sequence (Figure 3) the VcXBT structure is closer to XLT2 than to MUR3 as demonstrated by smaller RMSD values (Table S3). While the average distance when comparing the structures of VcXBT1 and AtXLT2 is 1.02 Å, the RMSD values are below 1 when considering only 325 out of 337 structurally similar Cα atoms (RMSD pruned atom pairs). In a similar comparison with AtMUR3, the RMSD values increase to 1.77 and 1.27 Å, respectively (Table S3). This close structural relationship between VcXBT and XLT2 becomes more evident when observing the VcXBT structure in the MUR3-XLT2 divergent regions (Figure 8b). In all six regions, MUR3- or XLT2-specific structural patterns are observed. While MUR3 members show an almost identical overlapping architecture, XLT2s and VcXBT are more variable, presenting extra helices such as in regions I and VI or alternative loop structures (regions II–V). Overall, the VcXBT structure clearly aligns closer to XLT2 than to MUR3 models.

A close inspection of the MUR3-XLT2 structure-based sequence alignment identified multiple residues showing MUR3- and XLT2-specific patterns (Figure S8). In total, 37 residues are differentially conserved between MUR3 and XLT2 exostosin domains. Twelve of them are located in the six previously identified structurally divergent regions (regions I–VI). For example, a serin residue in conserved among MUR3 proteins in region V, where residues $AtMUR3_{S380}$,

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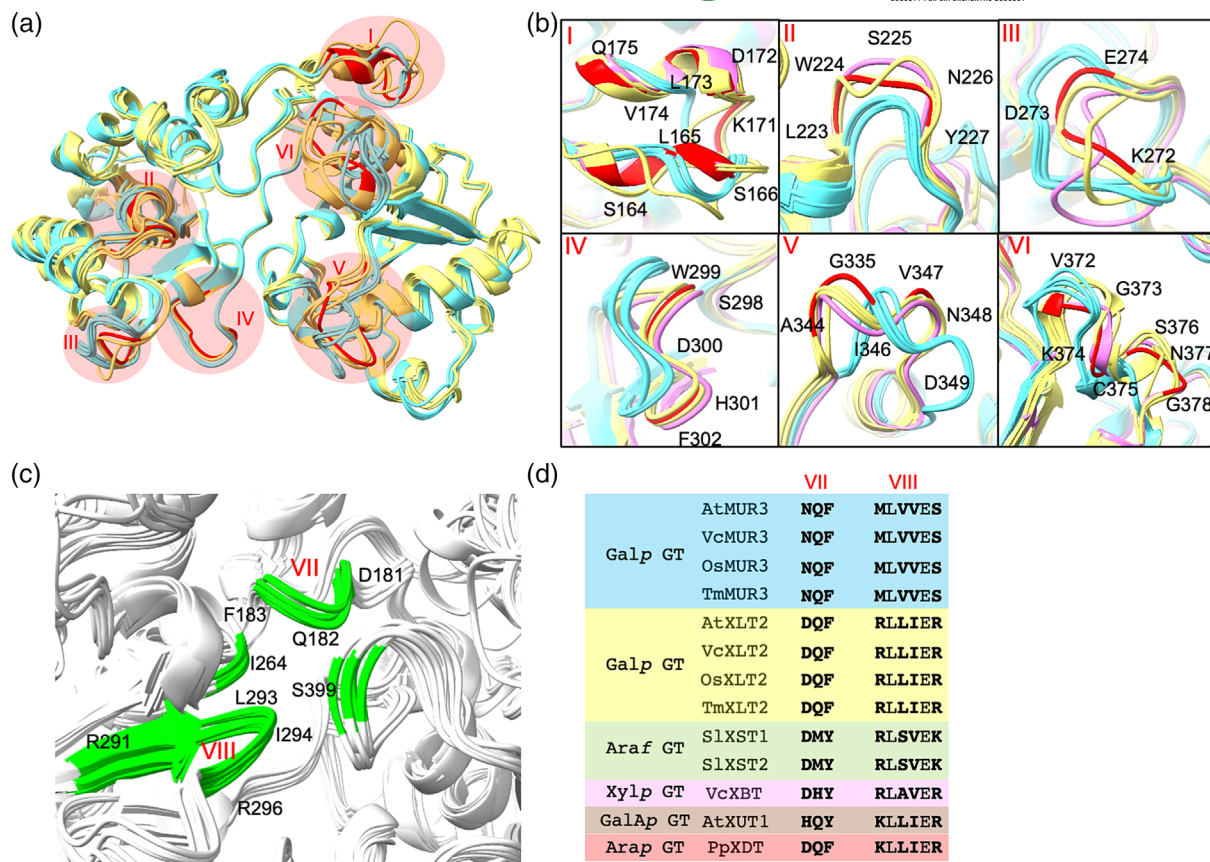

FIGURE 8 Structural comparison of XyG GT47 glycosyltransferases. (a) Superimposed models of characterized MUR3 (blue) and XLT2 (yellow) exostosin domains from diverse plant species (AtMUR3, VcMUR3, OsMUR3, TmMUR3, AtXLT2, VcXLT2, OsXLT2, and TmXLT2). Red circles highlight six regions with high divergence among MUR3 and XLT2 structures. The residues of AtXLT2 included in those six regions were colored in red for reference. (b) Detailed overview of the six regions shown in (a). The predicted structure for VcXBT (pink) was superimposed to MUR3 (blue) and XLT2 (yellow) exostosin domains from diverse plant species. (c) Detailed overview of the structure of superimposed models of AtMUR3, VcMUR3, OsMUR3, TmMUR3, AtXLT2, VcXLT2, OsXLT2, TmXLT2, SlXST1, SlXST2, VcXBT1, AtXUT1, and PpXDT XyG glycosyltransferases. Regions VII and VIII together with two other selected residues forming a possible substrate binding pocket are highlighted in green. Residues corresponding to AtXLT2 are indicated for reference. (d) Sequence details of the two regions shown in (c) for all XyG transferases considered. The bold residues indicate GT-specific patterns.

VcMUR3$_{S361}$, TmMUR3$_{S395}$, SlMUR3$_{S386}$, and OsMUR3$_{S373}$ align almost perfectly (Figures 8b and S8). In the corresponding structures of XLT2 proteins, an alternative architecture is predicted instead, where an aspartic acid residue—AtXLT2$_{D339}$, VcXLT2$_{D314}$, TmXLT2$_{D335}$, and OsXLT2$_{D301}$—is conserved in terms of structure and sequence. There are also 25 residues showing MUR3- and XLT2-specific patterns in regions with a high degree of structural conservation indicated by low RMSD values. For example, a glutamine residue is found in AtMUR3$_{Q427}$, VcMUR3$_{Q408}$, TmMUR3$_{Q442}$, and OsMUR3$_{Q402}$ in the highly structurally conserved C-terminal region of the exostosin domain (Figure S8). The corresponding residue in the overlapping XLT2 structures is an arginine instead—AtXLT2$_{R396}$, VcXLT2$_{R363}$, TmXLT2$_{R382}$, and OsXLT2$_{R348}$.

In order to explore the existence of candidate residues involved in the transfer of specific UDP-sugar substrates to XyG oligosaccharide acceptors, we extended these structural comparisons to other GT47 GTs. Superimposition of XyG:GT47 Alphafold models shows a highly conserved structure of the exostosin domain despite the

different UDP-donor-substrate specificity in these proteins, that is, MUR3 and XLT2—UDP-Gal$p$; XSTs—UDP-Ara$f$; XDT—UDP-Ara$p$; XBT—UDP-Xyl$p$; XUT1—UDP-GalAp (Figure S9A,C and Table S4). Again, the highest structural divergence was concentrated in the same regions described in the XLT2-MUR3 comparison (Figure S9A). Following the same rationale as in the MUR3-XLT2 comparison, we searched for conserved residues showing specific patterns including the two characterized XST. A detailed inspection of the structural alignment revealed 18 residues showing substrate-characteristic patterns (Figure S9C). Twelve of these were located in structurally conserved regions and six more had higher RMSD values indicative of a nonperfect match. We next mapped these residues to the multiple superimposition structure. Eight residues stood out in this analysis, as they are located in close proximity in the core of the exostosin domain, precisely in the GT-B interdomain cleft where the active site is usually found in human EXTs (Figures 8c and S9B). Six of these residues were grouped in two discrete regions, that is, VII and VIII, and the sequence details are shown as an example in Figure 8d. These

residues could show patterns consistent with the diverse substrate requirements of the XyG GT47 enzymes. For example, NQF … MLVVES (MUR3) might be responsible for transferring a galactosyl residue, whereas DMY … RLSVEK (XST) for an arabinofuranosyl residue.

4 | DISCUSSION

4.1 | Identification and functional characterization of a XBT from blueberry

The xylosyl residue of XyG is often substituted at the *O*-2 position with a variety of glycosyl residues including hexose or pentose moieties, both in their pyranosyl or furanosyl form (Pauly & Keegstra, 2016). The availability of the blueberry genome (Colle et al., 2019) allowed us to identify a Golgi localized XBT. Expression of the *VcXBT* gene in the Arabidopsis double mutant *mur3 xlt2* resulted in the synthesis of the XyG U sidechain as evidenced by detailed XyG analysis via OLIMP (Figure 4), HPAEC-PAD (Figure S4), glycosidic linkage analysis (Table 1), and ^{1}H NMR (Figure 6).

The expression of *VcMUR3* or other MUR3 orthologs does not only lead to XyG galactosylation but also additional fucosylation and *O*-acetylation of the galactose residue (Figures 4 and 5; Liu et al., 2015). This does not occur when *VcXBT* is expressed in Arabidopsis suggesting that either the third position of the XyG core structure is not further xylosylated or more likely the Arabidopsis XyG:fucosyltransferase (Vanzin et al., 2002) and XyG:*O*-acetyltransferases AtAXY4/AtAXY4L (Gille & Pauly, 2012) do not recognize the U sidechain as an acceptor substrate.

4.2 | XyG sidechains impact plant growth

The Arabidopsis mutant *mur3* contains a point mutation in the *AtMUR3* gene that renders it inactive (Madson et al., 2003). As a result, XyG in this mutant does not contain the XXLG oligosaccharide but retains the galactosylated XLXG motif. *Mur3* mutant plants show normal plant growth behavior except for minor effects in trichome morphology (Madson et al., 2003). However, the Arabidopsis *mur3 xlt2* double mutant, whose XyG are not galactosylated and only contain the XyG core structure display a dwarfed plant phenotype (Kong et al., 2015; Figure 7). It has been previously shown that structurally aberrant XyG structures such as the unsubstituted XyG core structure impacts the endomembrane morphology likely through XyG self-aggregation (Kong et al., 2015). In contrast, a complete lack of XyG does not affect plant growth (Cavalier et al., 2008; Kim et al., 2020; Kong et al., 2015). Nevertheless, the observed *mur3 xlt2* double mutant growth defect can be rescued by complementation with various XyG GT47 including the expression of various *MUR3* and *XLT2* genes from a variety of species including rice (*OsXLT2*; Liu et al., 2015), the moss Physcomitrella (*PpXLT2*), tomato (*SlXLT2*;

Schultink et al., 2013), and as shown here blueberry (*VcXLT2*; Figure 7). In addition, arabinofuranosylation through expression of SlXST (Schultink et al., 2013), arabinopyranosylation through expression of PpXDT (Zhu et al., 2018), and xylosylation restore the growth phenotype of the double mutant *mur3 xlt2* (Figure 7). This indicates that XyG galactosylation can be functionally replaced by other sugar substituents—which type of substituent does not seem to matter at least under the plant growth conditions used here.

4.3 | Insights into XyG:GT47 regioselectivity

It has been shown that MUR3 adds a galactosyl residue specifically only to the third core xylosyl residue of the XyG oligosaccharide resulting in XXLG, whereas XLT2 adds the residue to the second xylosyl residue generating XLXG (Figure 1). Protein structural analyses show that despite a 55% sequence similarity, MUR3 and XLT2 proteins show a remarkably high degree of structural similarity for the most part of the transferase domain. The structural differences are concentrated in discrete regions near the cleft between the two Rossmann-like domains. Some of these residues might thus be determinants for the different regiospecificity of XLT2 and MUR3 XyG:galactosyltransferases. The VcXBT XyG:xylosyltransferase protein exhibits a higher degree of both structural and sequence similarity to XLT2 proteins. However, when *VcXBT* is expressed in the Arabidopsis *mur3 xlt2* mutant multiple XyG oligosaccharide isomers are observed that contain additional pentosyl units. XBT could potentially transfer other pentoses, such as arabinopyranoses or arabinofuranoses and thus act as an XST (Schultink et al., 2013) or XDT (Zhu et al., 2018). However, based on our results, a more likely scenario is that XBT is able to add the xylopyranose to different positions on XyG core structure resulting not only in the XUXG oligosaccharide but also XXUG, as both have a *m/z* 1217 (Figures S4 and S5 and Table 1). Although the position of these additional xylosyl residues remain to be determined, it seems clear that XBT is more promiscuous than the regioselective MUR3/XLT2. There is a high structural conservation among XyG:GT47 proteins despite the variability of UDP-sugar donor substrates they can utilize and the XyG structure they produce. Our structural comparison identified several residues, which could represent determinants of the wide diversity of substrate specificities found among XyG GT47 enzymes. Some of these residues seem to be located in the interdomain cleft, the region where the active site is present in most GT-B fold proteins in general and exostosin-containing enzymes in particular. Based on this observation, we speculate that these residues might be involved in the binding of specific donor substrates and/or transfer to specific positions in the core XyG structure. Unfortunately, except for MUR3 and XLT2, the dataset is quite limited, and hence, the identification of additional functionally characterized enzymes together with the structural determination of the corresponding enzyme-substrate complexes will be required to establish or dismiss such sequence determinants and decipher the XyG:GT47 substrate specificity code.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

PEER REVIEW

The peer review history for this article is available in the Supporting Information for this article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author, M.P.

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