Co-ordinated synthesis of gentiobioitol and sorbitol, evidence of sorbitol glycosylation in transgenic sugarcane

Article Type: Full Length Article

Section/Category: Chemistry

Keywords: Saccharum spp.; Gramineae; 6-O-β-D-glucopyranosyl-D-glucitol; gentiobioitol; sorbitol; metabolite profiling; glycosylation; sugar alcohol

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Abstract: Sugarcane (a Saccharum spp. interspecific hybrid) was previously engineered to synthesize sorbitol (designated as sorbitolcane). Motivated by the atypical development of the leaves in some sorbitolcane, the polar metabolite profiles in the leaves of those plants were compared against a group of control sugarcane plants. Eighty-six polar metabolites were detected in leaf extracts by GC-MS. Principal component analysis of the metabolites indicated that three compounds were strongly associated with sorbitolcane. Two were identified as sorbitol and gentiobiose and the third was unknown. Gentiobiose and the unknown compound were positively correlated with sorbitol accumulation. The unknown compound was only abundant in sorbitolcane. This compound was structurally characterized and found to be a sorbitol-glucose conjugate. 13C NMR analysis indicated that the glucopyranose and glucitol moieties were 1,6-linked. Ligand exchange chromatography confirmed that the compound was a β-anomer, thus identifying the compound as 6-O-β-D-glucopyranosyl-D-glucitol, or gentiobioitol.
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Metabolite profiling of sugarcane (a *Saccharum* spp. interspecific hybrid) that was engineered to accumulate sorbitol revealed that the sugar alcohol 6-**O**-**β**-**D**-glucopyranosyl-**D**-glucitol (gentiobiitol) was a major by-product.

\[6-\text{O-β-D-glucopyranosyl-D-glucitol}\]
Co-ordinated synthesis of gentiobiitol and sorbitol, evidence of sorbitol glycosylation in transgenic sugarcane

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Abstract

Sugarcane (a *Saccharum* spp. interspecific hybrid) was previously engineered to synthesize sorbitol (designated as sorbitolcane). Motivated by the atypical development of the leaves in some sorbitolcane, the polar metabolite profiles in the leaves of those plants were compared against a group of control sugarcane plants. Eighty-six polar metabolites were detected in leaf extracts by GC-MS. Principal component analysis of the metabolites indicated that three compounds were strongly associated with sorbitolcane. Two were identified as sorbitol and gentiobiose and the third was unknown. Gentiobiose and the unknown compound were positively correlated with sorbitol accumulation. The unknown compound was only abundant in sorbitolcane. This compound was structurally characterized and found to be a sorbitol-glucose conjugate. $^{13}$C NMR analysis indicated that the glucopyranose and glucitol moieties were 1,6-linked. Ligand exchange chromatography confirmed that the compound was a $\beta$-anomer, thus identifying the compound as 6-$O$-$\beta$-$D$-glucopyranosyl-$D$-glucitol, or gentiobiitol.
1. Introduction

We previously engineered sugarcane (a *Saccharum* spp. interspecific hybrid) to synthesize sorbitol (Fong Chong et al., 2007) by introducing the gene from *Malus domestica* that encodes the enzyme sorbitol-6-phosphate dehydrogenase (MdS6PDH, EC 1.1.1.200) and the gene from *Zymomonas mobilis* which encodes the enzyme glucokinase (ZmGLK, EC 2.7.1.2). S6PDH catalyzes the reduction of glucose-6-phosphate to sorbitol-6-phosphate. Glucokinase was used to manipulate the supply of substrate to the previous reaction. These engineered plants (designated as sorbitolcane) produced up to 120 mg of sorbitol per gram of leaf dry weight, representing approximately 61% of the total soluble sugars in the tissue (Fong Chong et al., 2007).

Whilst the large amount of osmolyte accumulated in the sugarcane plants did not drastically affect growth, it did cause the formation of necrotic lesions at the leaf apex and margins. It has been proposed that this aberrant leaf physiology could be due to disruption of carbohydrate allocation and transport (Sheveleva et al., 1998). Therefore, as an adjunct to physiological phenotyping, the changes in leaf carbohydrates were examined by metabolic profiling. GC-MS metabolite profiling provides a systematic approach to quantify the deviation from metabolic homeostasis that can occur in plants that are subjected to events such as abiotic stress, infection, and genetic modification (Roessner et al., 2001). It can also be employed to reveal the metabolic changes that accompany tissue development. For example, it has been used to relate sucrose accumulation to the metabolic processes along the sugarcane culm.
(Glassop et al., 2007). Metabolite profiling has not yet been used to interrogate the metabolic changes caused by the deliberate overproduction of sorbitol in plants.

In this investigation, we examined the polar metabolites in leaf extracts derived from sorbitolcane. The analysis of those results revealed the presence of a novel sorbitol glucoside, which we subsequently structurally characterized.
2. Results and discussion

2.1. Metabolite profiling of sorbitolcane leaf extracts

Six transgenic sugarcane lines (C-4, C-17, GS-90, S-34, GS-4, and S-76) generated in an earlier study (Fong Chong et al., 2007) were analyzed. The lines C-4 and C-17 were controls that were transformed with the selectable marker neomycin phosphotransferase (nptII). The lines S-34 and S-76 were transformed with mds6pdh + nptII and the lines GS-4 and GS-90 were transformed with mds6pdh + zmglk + nptII.

The polar metabolites extracted from the last fully expanded leaf from six replicates of each line were analysed by GC-MS. A total of 86 metabolites were detected and 27 of these were matched to known compounds in mass spectral libraries. The compounds that could not be identified were labelled as mass spectral tags (MSTs). Analysis of variance of the metabolite abundances revealed that 17 metabolites exhibited statistically significant differences between lines (Supplementary table 1). Interestingly, a minute amount of sorbitol was detected in the control plants which was not detected previously by HPLC (Fong Chong et al., 2007). This can be attributed to the greater sensitivity of GC-MS compared to HPLC. Given the detection limit of the HPLC-based sorbitol assay employed, the sorbitol concentration in the controls was less than 0.1 mg/g dry weight.

The samples were classified according to their metabolite profiles using PCA (Principal Component Analysis). The first two PCs (principal components) explained
the 66% of the variation in the dataset, with 53% assigned to PC1 and the remainder to PC2. Inspection of the score plot revealed that the control (C-4, C-17) and sorbitolcan (S-34, GS-4, GS-90, S-76) plants segregated into two groups on the basis of PC1 (Fig 1A). A loadings plot was constructed to visualize the contribution of each metabolite to the grouping of the sorbitolcan and control plants (Fig 1B). The PC1 values of eighty-one metabolites ranged between ± 0.2 on the loadings plot. The majority of these metabolites were not significantly different between lines. However, significant changes were observed for the five metabolites with PC1 values that were outside this range, namely sorbitol, gentiobiose, MST52, isocitric acid, and galactinol (Fig 1B). Of those, sorbitol, gentiobiose, and MST52 were highly significant (P ≤ 0.001). The result indicated that sorbitolcan was characterized by lower isocitric acid and galactinol but higher sorbitol, gentiobiose, and MST52.

There is typically wide variability in metabolite levels within biological replicates grown under identical conditions. This variation is caused by the intrinsic flexibility of metabolic networks; where small environmental changes can cause a cascade of metabolic fluctuations that ultimately emerge as a pattern of correlations between metabolites (Morgenthal et al., 2006). On this basis, the biological replicates of the lines in this study were treated as independent entities for the purpose of delineating metabolite correlations with sorbitol. MST52 and gentiobiose were positively correlated with sorbitol (Fig 2A and 2B) but isocitric acid and galactinol were poorly correlated with sorbitol (results not shown). Interestingly, visual inspection of the MST52 correlation plot indicated that it was only correlated with sorbitol above a threshold sorbitol level (Fig 2B). The sorbitol concentrations in the controls were
below this threshold. The reason behind the MST52 correlation is unclear. Perhaps MST52 synthesis was only induced by high sorbitol concentrations.

2.2. Preliminary assessment of MST52

Sucrose, glucose and fructose were the main soluble sugars detected by HPLC in control sugarcane leaf extracts (Fig 3A). In addition to these common sugars, sorbitol and MST52 were also detected by HPLC in sorbitolcane leaf extracts (Fig 3B). Pure MST52 was obtained from leaf extracts by liquid chromatography fractionation. The purified MST52 was freeze-dried and subsequently hydrolyzed with trifluoroacetic acid. The hydrolysis products consisted of equimolar quantities of glucose and sorbitol. This stoichiometry indicated that MST52 was a sorbitol glucoside and could be tentatively described as D-glucopyranosyl-D-glucitol.

2.3. $^{13}$C NMR studies indicated MST52 was a 1,6-linked D-glucopyranosyl-D-glucitol

$^{13}$C NMR methods were used to elucidate the structure of MST52. The fully proton-decoupled $^{13}$C NMR spectrum of MST52 contained 12 singlets with chemical shifts ranging from 103 to 60 ppm (Fig 4A). Of the 12 carbons, 9 were found to have resonances between 76 and 69 ppm, while two other resonances were located upfield at 62.39 and 60.69 ppm. The remaining carbon produced a singlet downfield at 102.81 ppm. In addition to the fully proton-decoupled spectrum, an off-resonance decoupled $^{13}$C spectrum, where only one-bond C-H couplings are preserved, was obtained to determine the multiplicity of each $^{13}$C resonance (Supplementary table 2). Three distinct $^{13}$C singlets were found to partially overlap between 69-70 ppm in the fully
decoupled spectrum. In the same region, the multiplets in the off-resonance decoupled spectrum were distorted and experienced strong coupling effects. Therefore, a DEPT (Distorsionless Enhancement by Polarization Transfer) experiment was conducted at 135° to ascertain CH, CH₂, and CH₃ carbon signals (Fig 4B). Polarization transfer methods give clear and unambiguous separation of decoupled $^{13}$C signals according to multiplicity with sensitivity comparable to straightforward decoupled $^{13}$C spectroscopy (Macomber, 1998). With a 135° pulse, the CH and CH₃ signals are positive while the CH₂ signal is negative. Both DEPT and off-resonance decoupled $^{13}$C spectra confirmed the presence of nine methine (CH) carbons and three methylene (CH₂) carbons. Hence, each carbon was attached to at least one hydrogen atom and there was no quaternary carbon. The triplet obtained downfield for one of the methylene carbons (71.31 ppm) was distorted by interfering doublets in that region but the negative signal obtained from the DEPT experiment confirmed its multiplicity. The triplets for the other two methylene carbons resonated upfield (62.39 and 60.69 ppm) with no interference.

In general, the spectral assignments were accomplished by comparison with chemical shift data for simple sugars, oligosaccharides, and polyol derivatives of disaccharides and also by comparing the $^{13}$C shielding data for related compounds such as hexose aldopyranoses and their methyl glycopyranosides (Pochert and Behnke, 1993). Specifically, the published $^{13}$C NMR chemical shift data for reduced isomaltooligosaccharides and nigerooligosaccharides facilitated the assignment of $^{13}$C signals obtained from MST52 (Shimamura et al., 1991; Shimamura et al., 1992). In the following explanation of the MST52 assignments, unprimed carbons (C)
correspond to the pyranose ring while primed carbons (C’) represent those of the polyol chain.

It was expected that D-glucopyranosyl-D-glucitol spectra would display a pronounced downfield shift for C-1, the anomeric carbon of the pyranose ring. The two oxygen atoms (one in the pyranose ring and the other in the glycosidic link) are responsible for this deshielding. Of the eight possible D-glucopyranosyl-D-glucitols (2-, 3-, 4-, 6-O-α- and 2-, 3-, 4-, 6-O-β-) that can account for the nine CH and three CH₂ carbons in the spectrum, six (2-, 3-, 4-O-α- and 2-, 3-, 4-O-β-) were excluded. These six have two terminal primary (CH₂) carbon atoms (C-1’ and C-6’) in the glucitol moiety that resonate at higher field but downfield from the exocyclic primary carbon atom (C-6) of the pyranose ring. Thus, all six conformations would display three upfield resonances corresponding to the three methylene (primary) carbons; as exemplified by the spectrum for 3-O-α-D-glucopyranosyl-D-glucitol (Shimamura et al., 1992). However, 6-O-α-D-glucopyranosyl-D-glucitol and similarly 6-O-β-D-glucopyranosyl-D-glucitol would exhibit only two upfield resonances representing C-1’ and C-6 (Shimamura et al., 1991). The two upfield resonances at 62.39 and 60.69 ppm for MST52 resembled the 6-O-α- or 6-O-β-isomer of D-glucopyranosyl-D-glucitol.

The attachment of a glucopyranose ring to C-2’, C-3’ or C-4’ (secondary carbons) of the glucitol entity would cause a downfield shift of the resonances of those substituted carbons; as the electronegativity of the glycosidic oxygen tends to deshield the carbon. These resonances usually appear in the 80-85 ppm range, which is well separated from the signals of the unsubstituted secondary carbons (Shimamura et al., 1992). Such downfield displacements are also observed with substitution at C-6’.
(primary carbon) but they generally appear amongst the resonances (69-76 ppm) arising from the secondary carbons (Shimamura et al., 1991). Therefore, 6-O-α- and 6-O-β- residues resulting from C-6’ substitution are unlikely to produce downfield signals in the vicinity of 80-85 ppm. This spectral difference can be used to discriminate the two 1-6-linked isomers (6-O-α- and 6-O-β-) from the other six isomers. The C-6’ carbon resonance of MST52 was located in the vicinity of the resonances of the secondary carbons. Therefore, this suggested that MST52 was a 6-O-α- or 6-O-β-isomer of D-glucopyranosyl-D-glucitol.

Although the spectrum for MST52 was similar to the published spectrum for 6-O-α-D-glucopyranosyl-D-glucitol, the sequence of the resonances for the glucitol residue was different (Supplementary table 2). The order of chemical shift assignments (upfield to downfield) for the glucitol residue in 6-O-α-D-glucopyranosyl-D-glucitol are C-1’, C-6’, C-5’, C-3’, C-4’, and C-2’. For MST52, the order was C-1’, C-5’, C-3’, C-4’, C-6’, and C-2’. This difference maybe related to the nature of the glycosidic linkage between glucopyranose and glucitol and could indicate that MST52 is 6-O-β-D-glucopyranosyl-D-glucitol.

2.4. Ligand exchange chromatography confirmed MST52 was a β-anomer

D-glucopyranosyl-D-glucitols can be synthesized in the laboratory by borohydride reduction of the corresponding D-glucopyranosyl-D-glucose. There are eleven glucose disaccharides that are either reducing or non-reducing depending upon the nature of their linkage. Three lack a reducing end and therefore cannot be reduced (α,α-trehalose, α,β-trehalose and β,β-trehalose). The remaining eight (2-, 3-, 4-, 6-O-α- and
2-, 3-, 4-, 6-O-β-D-gluco.pyranosyl-D-glucose) are reducing sugars and therefore can be converted into the corresponding eight isomeric variants of D-gluco.pyranosyl-D-glucitol (2-, 3-, 4-, 6-O-α- and 2-, 3-, 4-, 6-O-β-D-gluco.pyranosyl-D-glucitol). It was expected that MST52 corresponded to one of these eight compounds. To verify the anomeric configuration predicted by $^{13}$C NMR, these anomeric pairs of D-gluco.pyranosyl-D-glucitols were compared to MST52 by ligand exchange chromatography.

The retention characteristics of sugar molecules on metal-form ion-exchange columns is determined by the formation of stable complexes between metal ions and the axial and equatorial pairs of hydroxyls on adjacent carbon atoms (Goulding, 1975). This stereochemical dependence permits the separation of α- and β-anomers of certain monosaccharides. Therefore, it may be possible to discriminate between anomeric pairs of D-gluco.pyranosyl-D-glucitols by comparing their retention times on this type of column. The result obtained using the BioRad Aminex HPX-87P column (with lead as the counterion) demonstrated that the β-anomer of each D-gluco.pyranosyl-D-glucitol anomeric pair migrated faster through the column than the corresponding α-anomer (Supplementary table 3). Anomeric selectivity was highest for the 1,6-linked anomers and the lowest for the 1,3-linked anomers. Peak resolution factors indicated that the resolution was superior between the 1,6-linked anomeric pair. The retention time of MST52 matched 6-O-β-D-gluco.pyranosyl-D-glucitol thus validating the $^{13}$C NMR prediction. This sugar alcohol is commonly known as gentiobiitol.

### 2.5. Options for industrial production of gentiobiitol
Functional assessment of gentiobiitol has revealed that it possesses health-promoting qualities (Dr Anne Rae, personal communication). Manufacturing commercial quantities of gentiobiitol could be challenging. Complexity and cost prohibit the use of organic synthesis methods (Kuszmann et al., 2004). It could be produced by chemical or enzymatic reduction of gentiobiose. For example, the *Gluconobacter suboxidans* sorbitol dehydrogenase, can selectively reduce isomaltulose to isomaltitol (Kunz et al., 2004). However, the availability of gentiobiose is limited. The compound can be produced in sugarcane as demonstrated by the present study. The viability of this approach relies upon accumulating high levels of gentiobiitol in the culm. However, gentiobiitol has not been detected in sorbitolcane culm tissue (Fong Chong et al., 2007).

Direct enzymatic synthesis from glucose and sorbitol may be the method of choice as these substrates are abundant and relatively inexpensive. There are several examples where bacterial enzymes have been used to synthesize D-glucopyranosyl-D-glucitols. *Bacillus macerans* cyclodextrin glucanotransferases are capable of catalyzing the formation of a range of α-1,4-linked glucosylated sugar alcohols using sorbitol or maltitol as the aglycone and starch as the glycosyl donor (Kim et al., 1997). Likewise, α-1,6-linked transglycosylation products can be obtained by using *Bacillus stearothermophilus* maltogenic amylase with a mixture of maltotriose and sorbitol (Kim et al., 2002). One of the key steps towards the development of an enzymatic method for the manufacture of gentiobiitol will be to identify an enzyme possessing the requisite β-1,6-stereospecificity.
3. Conclusion

Leaf metabolite profiles from sorbitol-producing sugarcane were examined. A salient feature revealed by the analysis was the occurrence of two β-1,6-linked carbohydrates. One was the disaccharide gentiobiose and the other was a novel glucoside derivative of sorbitol that was identified as gentiobiitol. To our knowledge, there are no prior reports of gentiobiitol biosynthesis in plants.

Acknowledgements

We are grateful to Niall Masel (BSES Limited) for assistance with purification and chromatography analysis and to Dr Tri Le (School of Chemistry and Molecular Biosciences, The University of Queensland) for performing the $^{13}$C NMR measurements. This work was funded by the Cooperative Research Centre for Sugar Industry Innovation through Biotechnology.
4. Experimental

4.1. Plant material

Embryogenic callus was generated from the sugarcane variety Q117 and biolistically transformed with the *Malus domestica* sorbitol-6-phosphate dehydrogenase gene, *Zymomonas mobilis* glucokinase gene, and neomycin phosphotransferase gene as outlined previously (Fong Chong et al., 2007). The leaf tissue analysed in this study was collected from mature transformed sugarcane plants that were propagated in a glasshouse under conditions described previously (Fong Chong et al., 2007).

4.2. Metabolite extraction and GC-MS measurement

The last fully expanded leaf was harvested from six-month-old plants at midday on the same day under full sunlight and immediately quenched in liquid nitrogen. One gram of lamina from each leaf was homogenized in liquid nitrogen and approximately 70 mg of the homogenate was collected and stored at −80°C for metabolite profiling. Extraction and GC-MS analysis of metabolites from sugarcane tissue has been described previously (Glassop et al., 2007). Briefly, metabolites were extracted from the homogenized samples into methanol containing the internal standard ribitol. The methanol extract was extracted with chloroform and derivatized with *N*-methyl-*N*-(trimethylsilyl) trifluoracetamide and analyzed by GC-MS. The identity of the metabolites resolved were assigned by comparing their mass spectrum against the commercial mass spectra library at NIST (www.nist.gov), the public domain mass spectra library at the Max Planck Institute of Molecular Plant Physiology, Golm,
Germany (http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html), and the library constructed at the Australian Centre for Plant Functional Genomics, University of Melbourne, Australia.

4.3. GC-MS data pre-processing and analysis

PCA was used to resolve the underlying structure in the metabolite dataset. A series of manipulations were applied to the dataset prior to PCA. Metabolite response values (peak areas) were converted into relative response values by dividing with the response value of the internal standard (ribitol) and the fresh weight of the leaf sample extracted. The relative response values for all metabolites in each sample were then normalized to a constant sum (i.e. the sum of all relative response values in each sample totalled 100%). This normalized data was then log$_{10}$(1+x) transformed, where x was the normalized metabolite responses. Subsequently, the transformed values were mean-centred. PCA was performed with the software Sirius 7.1 (Pattern Recognition Systems AS, Bergen, Norway).

ANOVA and Pearson’s correlation coefficients were calculated in Statistix 8 for Windows (Analytical Software, Tallahassee, Florida, USA) using log$_{10}$(1+x) transformed data, where x was the metabolite relative response values.

4.4. Sugar extraction and HPLC measurements

Leaf tissue was vacuum dried and then homogenized in a FP120 FastPrep cell disruptor (Savant Instruments, Inc., Holbrook, NY, USA). 6.25 ml of 0.02% w/v
sodium azide was added to each gram of dry leaf powder extracted. After three hours
incubation at 80°C, the supernatant was recovered and the extraction step was
repeated on the pellet. The pooled extracts were filtered through a 0.2 μm syringe
filter to remove particulates.

Sugars in the extract were analysed using a liquid chromatography system comprising
a LC-10AT VP pump (Shimadzu Corp., Kyoto, Japan), DGU-12A inline degasser
(Shimadzu), Sugar-Pak Guard-Pak guard column (Waters, Milford, MA, USA), a 7.8
× 300 mm Aminex HPX-87P analytical column (Bio-Rad, Hercules, CA, USA), and a
RID-10A VP refractive index detector (Shimadzu). The column was maintained at
80°C in a CTO-10A VP column oven (Shimadzu). The aqueous mobile phase was
pumped at a constant flow rate of 0.8 ml/min and 20 μl of sample was injected per run
(SIL-10AD VP autosampler, Shimadzu).

4.5. Composition analysis by acid hydrolysis

Approximately 15 ml of leaf extract was collected as described for sugar extraction.
The extract was passed through a 3 ml, 500 mg strong anion exchange (SAX) column
(Isolute, International Sorbent Technology, Hengoed, Mid Glamorgan, UK). The
solution eluting from the column was freeze-dried, redissolved in 1 ml of water and
then filtered through a 0.2 μm syringe filter to remove suspended matter. 100 μl
aliquots of the concentrate were injected into the liquid chromatography system
described previously and the peak corresponding to the unknown compound was
manually collected as it eluted from the system. The dilute fractions were pooled
together and freeze-dried. A 10 g/L stock was prepared from the desiccated material by adding the appropriate volume of water.

50 μL of the 10 g/L stock was evaporated in a rotary drier. The desiccated material was then hydrolysed by dissolving it in 50 μL of 2 M trifluoroacetic acid and incubating at 100°C for 4 h. The trifluoroacetic acid was removed from the reaction products by evaporation in a rotary drier. The reaction products were then resuspended in 100 μL of water and analyzed by HPLC.

4.6. $^{13}$C NMR measurement

Approximately 25 mg of the target compound was purified from leaf tissue as described earlier and dissolved in 1 ml of D$_2$O. The $^{13}$C NMR spectrum of the compound was acquired on a Bruker Avance 400 spectrometer (Bruker Biospin Pty Ltd, Alexandria, NSW, Australia) equipped with a 5 mm BBO (broadband observe) probe operating at room temperature. Chemical shifts were reported in ppm relative to TMS. Fully proton-decoupled and off-resonance decoupled $^{13}$C and DEPT spectra were obtained at a base frequency of 400 MHz. The pulse sequence employed a 2 second delay and 2 second acquisition time and a spectral width of 92 kHz. The DEPT spectrum was obtained at $\theta = 135^\circ$.

4.7. Synthesis of D-glucopyranosyl-D-glucitols by chemical reduction

Of the eight D-glucopyranosyl-D-glucitols used to determine the anomeric configuration of the unknown compound, three were commercially available and the
remaining five were synthesized by reduction of the progenitor D-glucopyranosyl-D-glucose. Maltitol, celliobiitol, gentiobiose, kojibiose, and sophorose were obtained from Sigma-Aldrich Pty Ltd (Castle Hill, NSW, Australia), isomaltitol and nigerose was obtained from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan), and laminaribiose was obtained from Megazyme International Ireland Ltd (Bray, Wicklow, Ireland).

Gentiobiitol, kojibiitol, laminaribiitol, nigeritol, and sophoritol were synthesized by combining equal volumes of 6 mM gentiobiose, kojibiose, laminaribiose, nigerose, and sophorose, respectively, and 6 mM sodium borohydride (Sigma-Aldrich Pty Ltd). The reactions were incubated at room temperature for at least 12 h. After incubation, the reactions produced approximately 1 mg/ml of D-glucopyranosyl-D-glucitol. 20 ul of each reaction was analysed by HPLC.

4.8. Supplementary material

Supplementary material cited in this article is available to online subscribers at the journal website.
References


Figure captions

Fig. 1 Principal component analysis of the 86 metabolites detected in leaf extracts from 36 sorbitolcane and control plants (six biological replicates of six lines). The principal component scores for each sample are plotted in (A) (filled circles, C-4; empty circles, C-17; filled triangles, S-34; empty triangles, S-76; filled squares, GS-4; empty squares, GS-90) and the principal component loadings for each metabolite are plotted in (B).

Fig. 2 Correlations between sorbitol and (A) gentiobiose, \( y = 0.812x - 0.394, P < 0.0001, r^2 = 0.81 \) and (B) MST52, \( y = 1.191x - 0.797, P < 0.0001, r^2 = 0.83 \) calculated from logarithmically transformed relative response values. The samples were derived from six biological replicates from six lines (filled circles, C-4; empty circles, C-17; filled triangles, S-34; empty triangles, S-76; filled squares, GS-4; empty squares, GS-90). The MST52 best-fit line was only fitted to the sorbitolcane data points.

Fig. 3 HPLC profiles of the soluble sugars detected in leaf extracts from control sugarcane (A) and sorbitolcane (B). Fru, fructose; Glc, glucose; Sor, sorbitol; Suc, sucrose; MST52, 6-\( \text{O-}\beta-\text{D-glucopyranosyl-D-glucitol} \).

Fig. 4 The \(^{13}\text{C} \) NMR spectrum of MST52 (6-\( \text{O-}\beta-\text{D-glucopyranosyl-D-glucitol} \)). (A) is the fully decoupled spectrum indicating carbon assignments (which are also shown on the structural formula of the compound) and (B) is the DEPT spectrum at 135°. The bottom left inset shows the location of C-1 in the fully decoupled spectrum. A
similar peak due to C-1 was also observed in the DEPT spectrum (not shown). The peak at 66.5 ppm is the internal control dioxane.
Fig 1B

A principal component analysis plot showing the distribution of various compounds.

- **Isocitric acid**
- **Galactinol**
- **Sorbitol**
- **MST52**
- **Gentiobiose**

The plot highlights the relationship between principal component 1 (53%) and principal component 2 (13%). The data points are clustered to show the variance in the dataset.
Supplementary table 2

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Supplementary table 3
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