ORIGINAL ARTICLE

Reuteran and levan as carbohydrate sinks in transgenic sugarcane

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Abstract The present study reports the effect of high molecular weight bacterial fructan (levan) and glucan (reuteran) on growth and carbohydrate partitioning in transgenic sugarcane plants. These biopolymers are products of bacterial glycosyltransferases, enzymes that catalyze the polymerization of glucose or fructose residues from sucrose. Constructs, targeted to different subcellular compartments (cell wall and cytosol) and driven by the Cauliflower mosaic virus-35S: maize-ubiquitin promoter, were introduced into sugarcane by biolistic transformation. Polysaccharide accumulation severely affected growth of callus suspension cultures. Regeneration of embryonic callus tissue into plants proved problematic for cell wall-targeted lines. When targeted to the cytosol, only plants with relative low levels of biopolymer accumulation survived. In internodal stalk tissue that accumulate reuteran (max 0.03 mg/g FW), sucrose content (ca 60 mg/g FW) was not affected, while starch content (<0.4 mg/g FW) was increased up to four times. Total carbohydrate content was not significantly altered. On the other hand, starch and sucrose levels were significantly reduced in plants accumulating levan (max 0.01 mg/g FW). Heterologous expression resulted in a reduction in total carbohydrate assimilation rather than a simple diversion by competition for substrate.

Abbreviations

FW	Fresh weight
DP	Degree of polymerization
HoPS	Homopolysaccharide
FTF	Fructosyltransferase
GTF	Glucosyltransferase
PAS	Periodic acid-Schiff
MS	Murashige–Skoog
WT	Wild type

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Introduction

The market for liquid biofuel through fermentation of starch and sugars has expanded many-fold in the last few years. This demand has renewed research and speculation into factors that determine yield in plants (Smith 2008).

Sugarcane is a C4 grass crop (Moore and Maretzki 1996) that accumulates sucrose in the internodal tissue of the stalk to levels of up to 20 % of fresh weight, or exceeding 50 % of dry weight (Komor et al. 1981; Rae et al. 1996). The plant is unusual in its ability to store sucrose in relatively mature sink tissue, compared to growth apices and developing organs such as tubers. Mechanisms involved in accumulation of sucrose in crops like sugarcane are not well understood and little is known with regard to factors that control the extent of sucrose storage in stalks. Advances made with routine transformation methodology and increasing availability of genomic tools allow for new experimentation and assessment of hypotheses (Rossouw et al. 2007; Wu and Birch 2007). Recent studies have been aimed at the development of sugarcane as a vehicle for production of sugar-derived biomaterials (Basnayake et al. 2012; Hamerli and Birch 2011). Several examples of the creation of transgenic plants expressing heterologous enzymes converting sucrose into non-metabolizable glucans (Zhang et al. 2007) or fructans (Caimi et al. 1996; Sévenier et al. 1998; Hellwege et al. 2000; Weyens et al. 2004) have been reported. No convincing evidence was thus far presented showing that total carbohydrate content was significantly increased in any of these plants.

Fructan accumulates naturally as the primary reserve carbohydrate in 12–15 % of the higher plants (Hendry and Wallace 1993; Cairns 2003). Synthesis from sucrose is extrachloroplastic, without the involvement of phosphorylated sugars or nucleotide co-factors, and it accumulates in the vacuole of both photosynthetic and storage cells. Plant derived fructans have a low degree of polymerization (DP) that seldom exceeds 100 fructose units, due to the presence of fructan-degrading enzymes. Short chain products harvested from plants limit technical application of this polymer (Pilon-Smits et al. 1996). This hurdle may be overcome by the production of microbial polysaccharides in plants lacking degrading enzymes. The molecular weight of microbial polysaccharides exceeds those of plant polymers by at least two orders of magnitude, while the DP can exceed 100,000 monomer units.

Bacterial homopolysaccharides (HoPSs) are synthesized from sucrose through the action of only one enzyme belonging to the group glycosyltransferases, also termed glycansucrases (Bauer et al. 2009). Group members involved in the biosythesis of fructans and glucans are termed fructosyltransferases (FTFs; EC 2.4.1.10) and glucosyltransferases (GTFs; EC 2.4.1.5), respectively. Levan and reuteran are examples of bacterial HoPS that may be exploited commercially, in particularly by the food industry (Roberfroid 1999; Bauer et al. 2009). Levan is composed of β-2,6 linkages and several related FTF-encoding genes have been identified from lactic acid bacteria (Korakli and Vogel 2006). Reuteran is a highly branched

glucan with predominately α-(1 → 4) glucosidic bonds. Two strains of *L. reuteri* (121 and ATCC 55730) have been implicated in reuteran production and the corresponding genes identified (Kralj et al. 2002, 2005).

We incorporated bacterial glycansucrases genes, encoding enzymes for levan and reuteran production, into sugarcane. No published paper has yet described the exploitation of sugarcane as vehicle for transgenic biopolymer production. Sugarcane lacks the enzymes for fructan production as well as enzymes for hydrolysis of bacterial HoPSs, while high levels of sucrose, the sole substrate for bacterial glycansucrases, accumulate. Objectives for this study include (i) co-production of sucrose and potentially valuable biopolymers such as fructan and glucan and (ii) establishment of plants with altered source–sink balance for the study of carbon partitioning.

Materials and methods

Bacterial strains, DNA methodology and construction of glycosyltransferase coding sequences

Routine procedures were used for DNA isolation, subcloning and restriction analysis (Ausubel et al. 1994). For cytosol targeting, two glycosyltransferase open reading frames (ORFs), truncated to exclude native secretion peptides, were constructed (Fig. 1) based on the fructansucrase gene (*ftfA*) from *Lactobacillus sanfranciscensis* TMW 1.392 (Tieking et al. 2005) and the glucansucrase gene (*ftfO*) from *L. reuteri* ATCC 55730 (Kralj et al. 2005). A third construct included a bacterial secretion signal, assembled with an ORF encompassing the entire
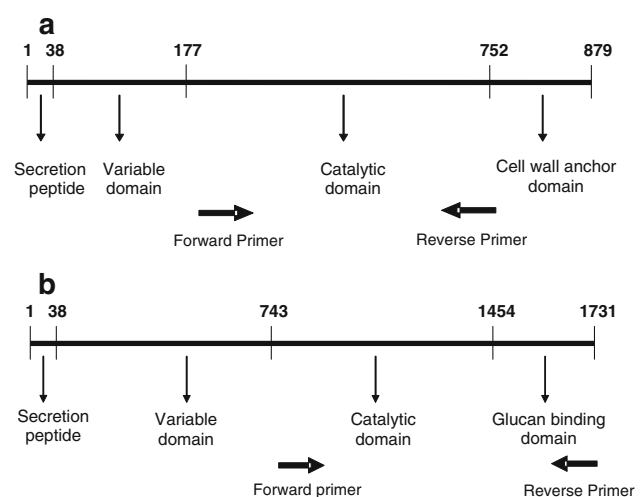
Fig. 1 Truncated glycosyltransferase genes. **a** Fructansucrase (SL) from *Lactobacillus sanfranciscensis* TMW 1.392. **b** Glucansucrase (RR) from *Lactobacillus reuteri* ATCC 55730

Table 1 PCR primer pairs

Gene and Producer Strain	Forward Primer (5' – 3')	Reverse Primer (5' – 3)	Product	Product Size (bp)
A				
Levansucrase (*ftfA*) *L. reuteri* LTH5448	CC<u>GGATCC</u>**ATG**CTAGAACGCAAGGAACAT *Bam*HI	GG<u>CCCGGG</u>TTAGTTAAA**TC**GACGTTTGTT *Sma*I　　　　　　　　　**st**	RL* ORF	2389
	ATACGCTATTTATTTGCTTGG	GTTAGCTGGTGCAGCTTTTTG	PCR fragment	453
	TGGGCAGTCGTTACACTCTCT	GTTAGCTGGTGCAGCTTTTTG	RT-PCR fragment	384
B				
Levansucrase (*ftfA*) *L. sanfranciscensis* TMW 1.392	CC<u>GGATCC</u>**ATG**GATACTAAAACTGCTGTTG *Bam*HI	CC<u>GGATCC</u>**TTA**CTTTAAGCCTAAGCCAGA *Bam*HI　**st**	SL* ORF	1722
	ATACGCTATTTATTTGCTTGG	GGAATACCCATCATCGCAATA	PCR fragment	509
	CAGCTAAAGCCGTTCAAAATG	GGAATACCCATCATCGCAATA	RT-PCR fragment	349
C				
Glucansucrase (*gtfO*) *L. reuteri* ATCC 55730	CC<u>GGATCC</u>**ATG**CAGAAGAAGGATGGCAAG *Bam*HI	CC<u>GGATCC</u>**TGA**TTGAATTCTCTCATCAGCC *Bam*HI　**st**	RR* ORF	3173
	ATACGCTATTTATTTGCTTGG	ACGGCGTTAATTTCGAGTTTT	PCR fragment	661
	GGGCCGATTCTAAGGATACTG	ACGGCGTTAATTTCGAGTTTT	RT-PCR fragment	348

[a] Name designated to transgenic sugarcane lines expressing open reading frame (ORF)

fructansucrase gene (*ftfA*) of *L. reuteri* LTH5448 (Schwab and Gänzle 2006). Appropriate primer pairs used to construct ORFs of the native genes are given in Table 1. To determine whether mutations had been introduced by PCR, the ORFs were cloned into the *E. coli* pGEM®-T Easy Vector System (Promega) and verified by nucleotide sequencing as described elsewhere (Bauer et al. 2005). ORFs were subsequently excised and placed under control of a strong, constitutive, tandem Cauliflower mosaic virus (CaMV)-35S: maize polyubiquitin (*UBI-1*) promoter, which drives stable, high-level gene expression in sugarcane (Wei et al. 2003). Table 1 also lists gene- and vector sequence-specific PCR primer pairs used to verify the correct orientation of cloned products, and sequence specific primer pairs for confirmation of transcription with RT-PCR.

Plant tissue and transformation

Growth of sugarcane embryogenic callus for biolistic transformation was initiated from immature leaf roll tissue harvested from the commercial variety NCo310 (Taylor et al. 1992; Snyman et al. 1996). Constructs were co-transformed with the pEmuKN expression vector containing the npt-II selectable marker (Bower and Birch 1992). Selection was done on geneticin-containing medium and putative transgenic plants from independent transformation events were regenerated and hardened off based on standard protocols (Snyman et al. 1996). Plants were grown in a greenhouse for at least 12 months before characterization. Callus and cell suspension cultures were grown on modified Murashige–Skoog (MS) medium (Murashige and Skoog 1962) containing 58 mM sucrose, 0.5 g/L casein and 13.6 μM 2,4 dichlorophenoxyacetic acid.

Cell suspension cultures

Suspension cultures were initiated from embryonic callus (1–2 g) inoculated into 50 mL MS medium. Cell viability was dependent on a gradually increase in culture volume (up to 150 mL). Cultures were replenished with 75 mL fresh MS medium on a weekly basis after removing exactly half of the previous week's growth medium from settled cultures. Clumping callus material was removed when cells in suspension reached an OD_{600} of 0.3. Due to pronounce differences in growth speed of individual transgenic lines, growth could not be synchronized. All transgenic lines representative of an individual construct were subjected to the same period of growth and analyzed as a group when the slowest member reached a minimum OD_{600} of 0.6. Cells were washed three times with 25 mM sodium acetate buffer (pH 5.5) containing 1 mM $CaCl_2$, and collected by centrifugation. Both fresh and dry weights were noted.

DNA extraction and PCR analysis

Genomic DNA was extracted from plant material using a Nucleon PhytoPure DNA extraction kit (Tepnel Life

Sciences, Manchester, UK). Lines containing ORFs of interest were selected with vector- and gene-specific PCR primer pairs (Table 1).

RNA extraction and RT-PCR analysis

Plant material was ground in liquid nitrogen and total RNA extracted from ca. 0.25 g tissue according to the method of Chang et al. (2003). Ten micrograms of total RNA were treated with Turbo DNA-free DNase I (Ambion Inc., Austin, TX, USA) and purified by RNeasy MinElute columns (Qiagen Inc., Valencia, CA, USA). The presence of transcripts was confirmed with RT-PCR. RT reactions were performed using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA), and PCR experiments contained 200 nmoles each of gene-specific primer (Table 1). Products were sequenced to verify correct amplification.

Protein extraction

Callus and suspension-cultured cells were ground in liquid nitrogen (ca. 1 g fresh weight) in the presence of 5 mL Tris–HCl buffer (50 mM, pH 8) containing 1 mM EDTA, 1 mM $CaCl_2$, 5 mM DTT and 2 % (m/v) PVPP. Insoluble residues were removed by centrifugation at 10,000g for 5 min. Protein was precipitated from supernatant by dropwise adding 80 % ice-cold acetone, while stirring (Klibanov 2003; Abdel-Fattah et al. 2005; Esawy et al. 2008), and collected by centrifugation (13,000g for 5 min). Protein concentration was determined by the method of Bradford (1976) using the BioRad microassay (Hercules, CA, USA) with BSA as a standard.

Extraction of metabolites and water-soluble polysaccharides

Buffer for extracting sugars from callus and suspension cultures contained 30 mM Hepes (pH 7.8), 6 mM $MgCl_2$ and 80 % ethanol (v/v). Sugar extraction from suspension-cultured cells were performed in 10 volumes (m:v) buffer. Ground callus tissue or plant material (0.5–1.0 g) was homogenized in 20 volumes extraction buffer (m:v). Sugars were extracted for 2 h at 80 °C and the liquid fraction removed by centrifugation. Sugar extraction was repeated and clarified samples pooled. Raw material remaining after extraction was washed twice with 70 % ethanol, dried and polysaccharides extracted twice with 5 volumes water (15 h at 37 °C, followed by 1 h at 50 °C). Aqueous extracts were clarified by centrifugation and polysaccharides precipitated overnight at −20 °C with 5 volumes ethanol, washed three times with 70 % ethanol and vacuum dried.

Sugar analysis with $NADP^+$-coupled assay

Sugar (glucose, fructose and sucrose) concentrations were measured by means of a $NADP^+$-coupled assay (Jones et al. 1977) with modifications (Basson et al. 2010). The reaction buffer (200 µL) contained 158 mM Tris–HCl (pH 7), 3.3 mM ATP, 0.8 mM $NADP^+$ and 5.27 mM $MgCl_2$. Phosphorylation of glucose was monitored by following the reduction of $NADP^+$ to NADPH after the simultaneous addition of hexokinase (1.5 U) and glucose-6-phosphate dehydrogenase (1.0 U). Fructose concentrations were measured in the same reaction mixture after the addition of phosphoglucoisomerase (4.0 U). Finally, hydrolysis of sucrose was measured after the addition of invertase (60 U). Assays were monitored at 340 nm on a Powerwave X Microplate Spectrophotometer with KC4 Kineticalc for Windows Version 2.7 (Bio-Tek Instruments, Winooski, VT, USA).

Monosaccharide analysis

Polysaccharides were hydrolyzed in 5 M trifluoro acetic acid at 100 °C for 2 h, vacuum dried and washed twice with 100 % methanol. Glucose and fructose concentrations were determined with the $NADP^+$-coupled assay as described above.

Sucrase activity assays

Sucrase activity was determined by measuring enzymatic release of glucose and fructose from sucrose. Protein extracts were incubated at 35 °C in 25 mM sodium acetate buffer (pH 4.7) containing 1 mM $CaCl_2$ and 50 mM sucrose. Aliquots were withdrawn at 30-min intervals over a period of 3 h and reactions stopped with 2 M NaOH (5:1). Glucose and fructose releases were quantified enzymatically with the $NADP^+$-coupled system (described above).

Radiolabeling experiments

Washed suspension-cultured cells (1.0–1.5 g fresh weight) were resuspended in 2 mL sodium acetate labeling buffer (25 mM, pH 5.5) containing 1 mM $CaCl_2$ and 50 mM sucrose. After the addition of 0.06 MBq of ^{14}C-labeled sucrose, samples were incubated for 20 h at 30 °C and unincorporated label removed by five consecutive washes in ice-cold buffer (6000×g for 1 min). Metabolites and water-soluble polysaccharides were extracted as described above, and incorporated label counted.

Aliquots of protein (20 µL), extracted from washed suspension-cultured cells (1 g fresh weight), were incubated for 20 h at 30 °C in 120 uL labeling buffer

containing 0.04 MBq of ^{14}C-labeled sucrose. Products were precipitated with 5 volumes ethanol, washed eight times with 70 % ethanol, and incorporated label counted. Radioactivity was determined with a Beckman LS 1801 scintillation counter in 1 mL Ultima Flo M scintillation cocktail containing 50 µL extracts.

Histological study

Callus cells were fixed for 12 h with 2 % paraformaldehyde and 4 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). Glutaraldehyde was washed out from the fixing material by placing specimens in phosphate buffer. Fixed tissue was dehydrated in a graded ethanol series and imbedded in Kulzer 7100 resin (Labonord, Templemars, France) or paraffin wax. Histological sections (3–5 µm) were attached to glass slides using Haupt's solution and rinsed with xylene to remove wax. Specimens were treated with periodic acid-Schiff (PAS) reagent that stains selectively for polysaccharide compounds, and post-stained with hematoxylin. After removing excess stain, the sections were dehydrated in an ethanol series for a few seconds, embedded in euparel, covered with glass and dried at room temperature. Specimens were viewed and photographed under light microscope. Cell sections from the following lines were studied: SL 1.3, 4.3, 8.1 and 8.2; RL 1.3, 2.2 and 3.2; RR 1.2, 1.3, 1.4 and 3.1.

Determination of starch and soluble sugars content

Plant material (100–300 mg of leaf or internodal tissue) was extracted three times with 80 % ethanol at 80 °C, extracts filtered through a 0.22-µm membrane filter, and sucrose, glucose and fructose contents determined. Sugars were quantified with NADP-coupled assays as described above. Starch was extracted by incubating the pellet with 20 mM NaOH for 1 h at 80 °C. After neutralization with 1 M acetic acid, the extracts were incubated in 20 mM sodium acetate (pH 4.6) with 5 U of amyloglucosidase at 37 °C overnight. The amount of glucose released was determined with NAD-coupled assays.

Sampling of internodal tissue from mature plants

Four stalks of each clone (3 clones per line) were randomly selected totaling 12 stalks per line or transformation event. Internodes were numbered from the top of the culm, and stalks separated into internodal sections. The 'first internode' is defined as the node immediately below the node to which the last fully expanded leaf subtends (Moore 1987). The leaves and leaf sheath were removed from the stalks. Internodal sections (e.g. 9 and 10) were pooled, cut into small pieces, finely homogenized in liquid nitrogen and the

fresh weight measured. Subsamples were dried to a constant weight at 80 °C and dry weight noted.

Statistical analyses

Quantitative analyses are presented as mean values and significant differences were analyzed by Student's t-test. Differences reaching a minimal confidence level of 95 % were considered as being statistically significant.

Results

Transgenic sugarcane callus accumulating fructans and glucans

Three bacterial glycansucrase ORFs were introduced into sugarcane to analyze whether fructan and glucan accumulation can be induced. Lines containing the fructansucrase coding sequences of *L. sanfranciscensis* (strain TMW 1.392) and *L. reuteri* (strain LTH5448) were designated SL and RL, respectively (Table 1). Those containing the glucansucrase coding region of *L. reuteri* ATCC 55730 were designated RR.

Microbial EPS are water soluble, unlike the majority of plant polysaccharides. This presented us with a convenient way to selectively screen for polymer accumulation in transformants. As starch loses crystallinity when heated above 60 °C, care was taken not to exceed this temperature when extracting biopolymers from plant tissue. Transformants screening positive for the accumulation of water-soluble polysaccharides in callus tissue are presented in Supplementary Figs. S1–S3. Callus tissue was not of the same age (1–3 months old) when harvested for analysis, since tissue of individual lines from the same construct differed in rates of biomass production (growth). As a consequence, interpretations of data presented in Supplementary Figures were only applied as a means of screening for positive transformants. Even so, reuteran accumulated to levels 1–2 order of a magnitude higher than levan (Supplementary Figs. S1–S3). Where levan was targeted to the cell wall, on average, 5× higher levels were accumulated compared to lines putatively expressing levan in the cytoplasm.

Subcellular targeting of transgene products

Cellular location of transgenic polymer was investigated by conducting histological studies with callus cells. During the fixation and embedding procedures, care was taken to retain the water-soluble polysaccharides. Thin sections of embedded callus material were stained specifically for polysaccharide-based compounds. Compared to the WT

Fig. 2 Periodic acid-Schiff (PAS) histochemistry (carbohydrate) of the developing callus tissue in sugar cane. *Intense violet/magneta* staining confirms carbohydrate presence. **a** Wild type showing moderate staining in cytoplasm and cell membrane. **b** Putative cytosolic β-2,6 fructan (SL) line showing intense staining of the cytosol (400×). **c** Putative cytosolic α-1,4-α-1,6-glucan (RR) line. Developing callus cells show the augmented presence of cytoplasmic granules. **d** Cell wall-targeted β-2,6 fructan (RL) line showing the intense PAS staining of the outer perimeter. The *inset* is a magnification of mature callus cells showing intense PAS staining of the cell wall
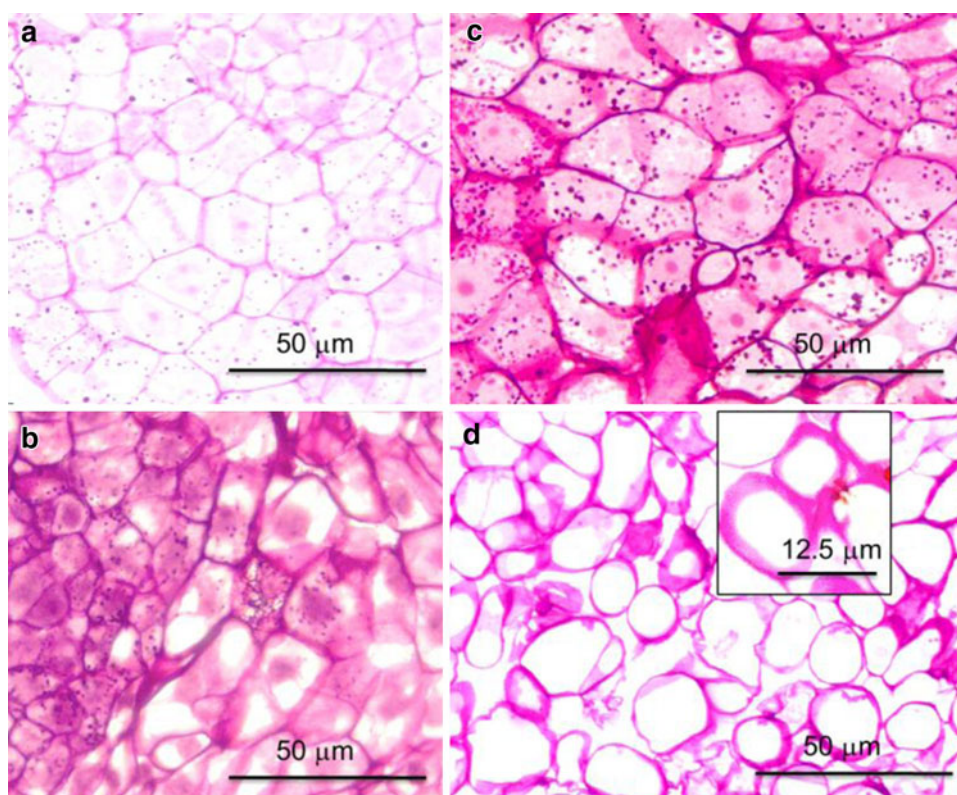

(Fig. 2a), the cytoplasm of SL and RR cells stained darker in all cases (Fig. 2b). In sections from RR lines 1.2 and 3.1, granule formation was more marked (Fig. 2c). Cell wall-targeted levan (RL) lines showed striking coloration along the cell rims (Fig. 2d).

Glycansucrase activity in callus paralleled with growth rate in suspension cultures

Northern blots could not detect mRNA for the glycosyltransferases. To confirm expression of the bacterial genes, total RNA was extracted from callus tissue and purified preparations subjected to the more sensitive technique, RT-PCR. Single amplification products of the expected size (depicted in Table 1) were reproducibly obtained. As sucrose activity in plant tissue could not be detected by conventional enzyme assays, activity was quantified with sensitive radiochemical detection using ^{14}C-labeled sucrose (Figs. 3a, 4a, 5a). Specific activity estimates were expressed on a cellular dry weight rather than a total protein basis to avoid distortion by the accumulation of storage proteins. As only trace amounts were synthesized, the structure of the radiolabeled enzyme products was not determined. Among highest levan accumulators, specific activity in cell wall-targeted RL 3.3 tissue (Fig. 3a) was approximately seven times higher than in putative cytoplasmic SL 4.3 (Fig. 4a). Specific activity displayed by the most productive reuteran line RR 1.2 (Fig. 5a) was two

times higher than the highest levan producer (RL 3.3). While pronounced differences in polysaccharide accumulation between individual lines originating from the same construct were evident, activity ranges were clearly construct dependent. Incorporation of radioactivity in glucan lines was higher than in levan lines, among which cell wall-targeted lines displayed the highest specific activity. The same trend was observed when screening callus tissue for polysaccharide accumulation (Supplementary Figs. S1–S3). Intracellularly produced radiolabeled product, measured over a period of 20 h, was quantifiable only for SL 8.2 and SL 8.1, both putative cytosolic expressed levan lines (Fig. 4c). Not surprisingly these lines were the highest accumulators in callus tissue (Supplementary Fig. S2).

An inverse correlation between specific activity and growth rate among the cell lines from all three constructs was observed (Figs. 3b, 4b, 5b). Growth of suspension cultures representative of lines where levan was targeted to the cell wall (RL) was more inhibited compared to putative cytosolic targeting (SL). Levels of bacterial polysaccharide accumulation in lines RL 1.1 and RL 3.2 (Fig. 3a) were similar to levels in lines SL 6.1 and SL 4.3 (Fig. 4a). However, suspension cultures took 30 days (Fig. 3b) to reach optical densities ($OD_{600} \sim 0.1$), similar to those of 9-day-old SL lines (Fig. 4b). Growth of suspension cultures putatively accumulating reuteran in the cytoplasm (RR lines) was most inhibited, reaching cellular densities not higher than 0.2 in 60 days.
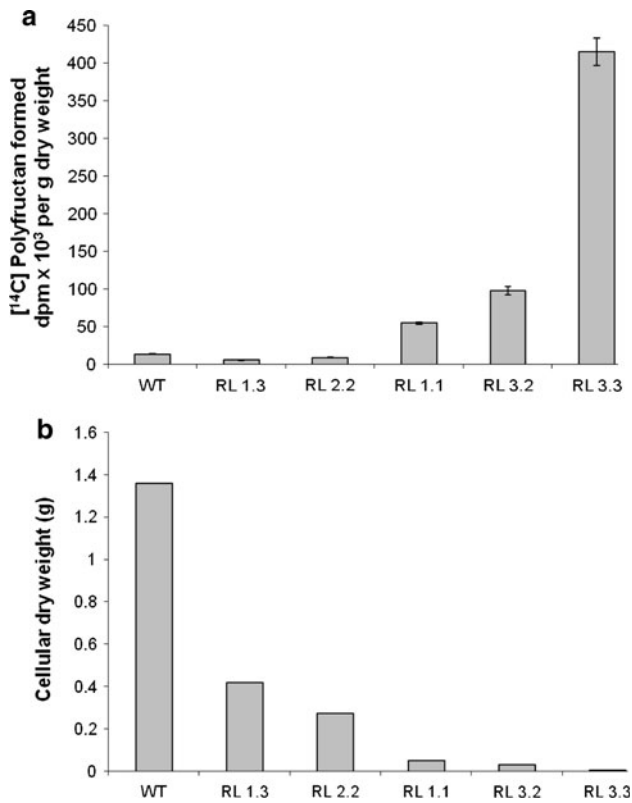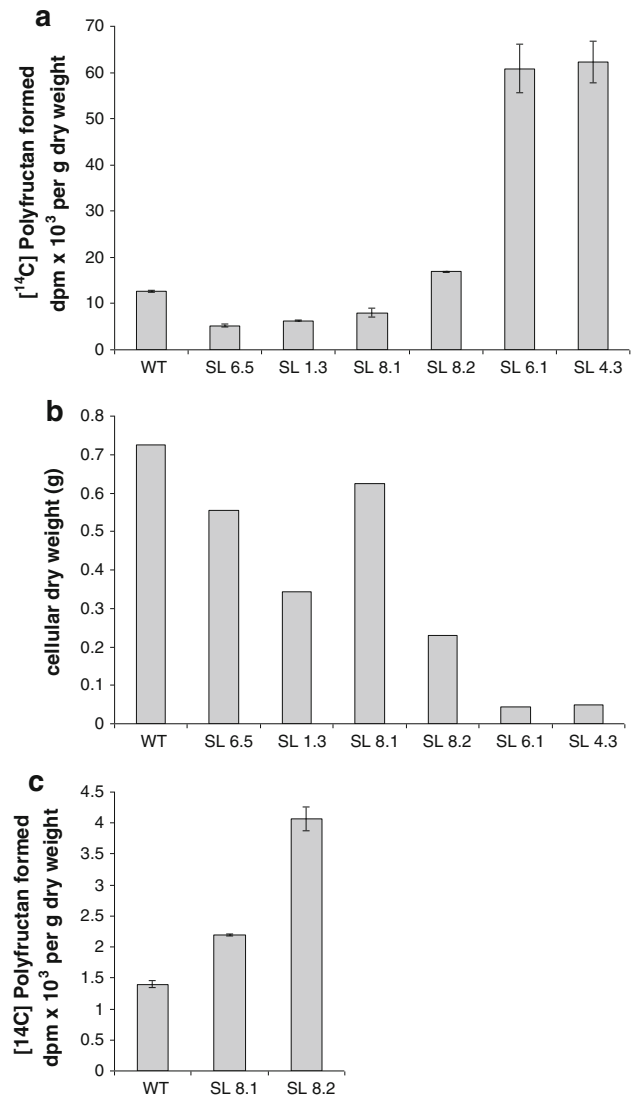
Fig. 3 Sugarcane lines accumulating cell wall-targeted fructan (RL) compared with wild type (WT). **a** Formation of radiolabeled water-soluble polysaccharide from [^{14}C] sucrose in the presence of total proteins extracted from suspension cultures. Values shown are means with standard errors indicated by *bars* ($n = 5$). **b** Growth of suspension cultures was maintained for 30 days

Regeneration of callus tissue into transgenic plants and plant development

The presence of transcripts in mature plant tissue was confirmed with RT-PCR (results not presented). Mature SL and RR plant lines positive for HoPSs production were indistinguishable from wild-type plants based on morphology, however, growth rate was visibly retarded. Regeneration of embryonic callus tissue into plants proved problematic for cell wall-targeted levan lines (13 RL lines in total, Supplementary Fig. S1). Only two lines, RL1.3 and RL2.2, could be regenerated into plants. These lines accumulated the lowest amount of levan as measured in callus tissue (Fig. 3a), no bacterial polymer could be detected in internodal tissue of mature plants (data not shown), and plants were indistinguishable from wild type in morphology and growth rate.

Carbohydrate content in transgenic sugarcane producing levan and reuteran

The starch and low molecular mass neutral sugar (sucrose, glucose, and fructose) contents in internodal tissue of

Fig. 4 Sugarcane lines accumulating putatively cytosolic fructan (SL) compared with wild type (WT). **a** Formation of radiolabeled water-soluble polysaccharide from [^{14}C] sucrose in the presence of total proteins extracted from suspension cultures. **b** Growth of suspension cultures was maintained for 9 days. **c** Formation of radiolabeled water-soluble polysaccharide from [^{14}C] sucrose by cultures in cell suspension. Values shown in **a** and **c** are mean ± SE ($n = 5$)

control plants and transformants were compared and related to the water-soluble fructan/glucan content. Metabolite content was expressed in terms of fresh weight to facilitate comparisons with previously published data. Trends remained the same when data were expressed in dry weight (data not shown).
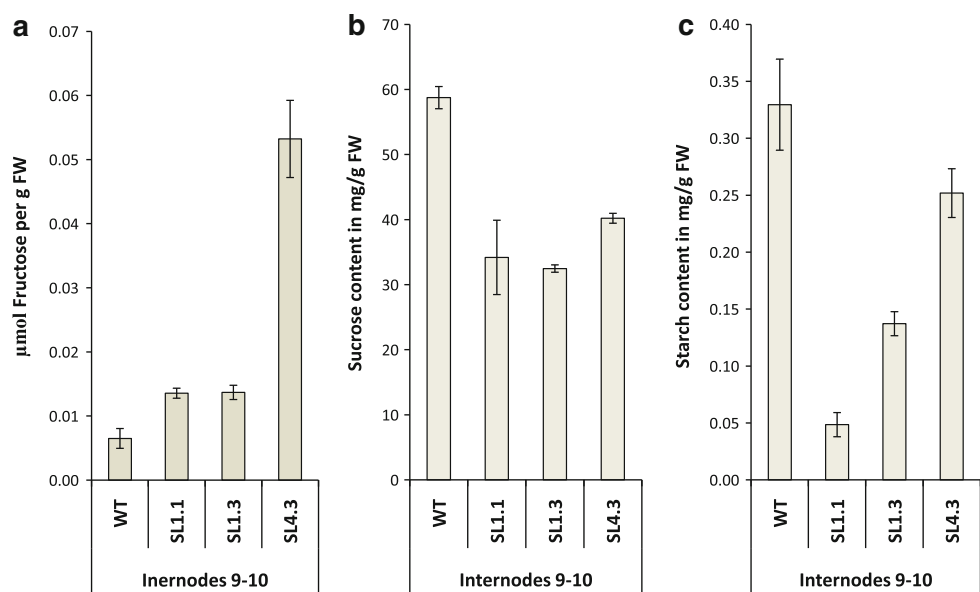
Levan lines SL1.1, SL1.3 and SL4.3 accumulated significant ($P < 0.05$) levels of fructan (Fig. 6a). The sucrose content in internodal tissue of stalks harvested from these lines was also significantly reduced, with amounts being only about two-third of the level observed in control plants
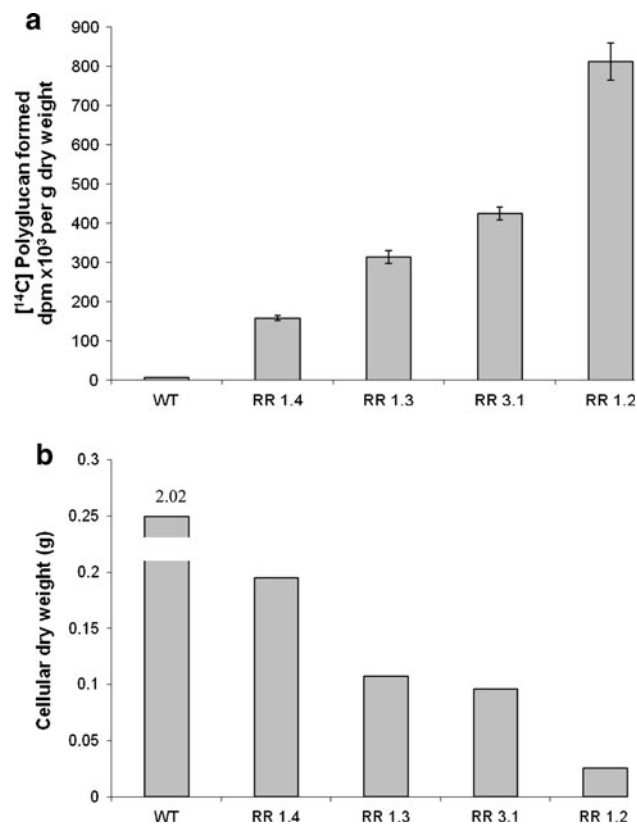
Fig. 5 Sugarcane lines accumulating putatively cytosolic polyglucan (RR) compared with wild type (WT). **a** Formation of radiolabeled water-soluble polysaccharide from [^{14}C] sucrose in the presence of total proteins extracted from suspension cultures. Values shown are means with standard errors indicated by *bars* ($n = 5$). **b** Growth of suspension cultures was maintained for 60 days

higher in the WT (Fig. 6c). Ambiguously, reduction in starch was least pronounced in the highest fructan accumulator (SL4.3).

Only two RR lines were successfully regenerated from callus tissue and significantly increased amounts of water-soluble polysaccharides were detected when compared to wild-type controls (Fig. 7a). Sucrose, glucose and fructose contents remained unchanged (data not shown). Starch content was significantly increased, up to four times in internodes 9 and 10 (Fig. 7b). These results spurred analyses of the starch content over the length of the stalks. In all samples, starch content was increased and remained stable, even in older internodes.

Discussion

Sugarcane is an economically important plant that lacks glycansucrases, but accumulates high levels of the enzyme's substrate, sucrose, in internodal tissue of the stalk. We introduced a pair of bacterial FTFs and a GTF into sugarcane. This study investigated the effects of heterologous accumulation of the gene products, levan (fructan) and reuteran (glucan), on plant development and carbon partitioning.

Sugarcane and sucrose accumulation

The biology of sucrose accumulation in sugarcane provides an interesting physiological system for the study of carbohydrate partitioning. Figure 8 depicts the range of sucrose concentrations as measured on internodal tissue of various sugarcane sources. Data assembly from literature and statistical analysis are shown in Supplement

(Fig. 6b). No significant effect on glucose and fructose contents was observed (data not shown), while starch content was significantly reduced, being up to six times

Fig. 6 Metabolite content as measured in internodal tissue (9 and 10) of mature sugarcane lines accumulating fructan (SL; putative cytosolic) compared with wild type (WT). **a** Fructose levels in hydrolyzed water-soluble polysaccharides. **b** Sucrose content. **c** Starch content. Values shown are mean ± SE ($n = 3$)

Fig. 7 Polysaccharide content of mature sugarcane lines accumulating polyglucan (RR) compared with wild type (WT). **a** Glucose levels in hydrolyzed water-soluble polysaccharides extracted from internodes 9 and 10. **b** Starch content in sugarcane internodal tissue. Sucrose content in transgenic plants was not significantly different from WT. Values shown are mean $\pm$ SE ($n = 3$)
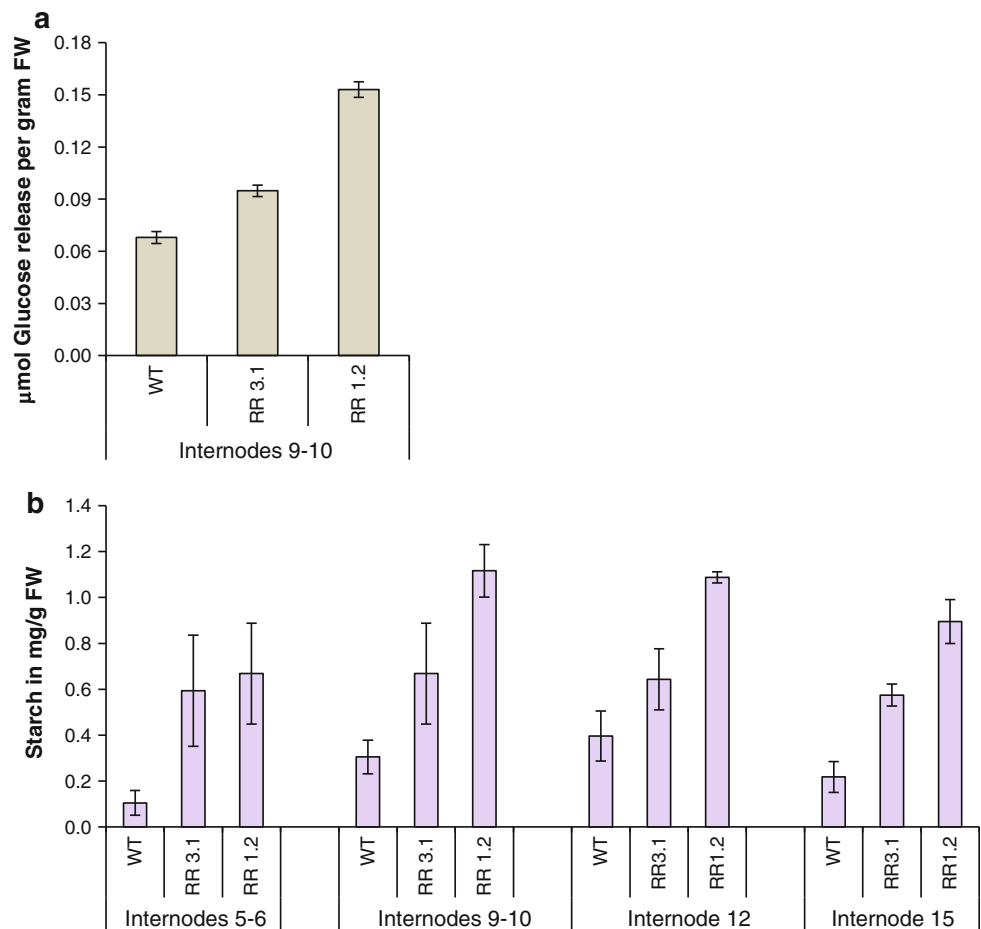
(Uys 2006, 2009). Internodal tissue matures progressively toward the base of the stalk with a corresponding increase in sucrose concentration up to an astonishingly high concentration of 0.5 M. The plant is unusual in its ability to store sucrose in relatively mature sink tissue, compared to growth apices and developing organs such as tubers. Accumulation of bacterial polysaccharides and the effect on carbohydrate assimilation were therefore studied by measuring target metabolites in internodal tissue.

Growth inhibition in levan and reuteran transformants

Several publications noted that plant transformants expressing fructansucrase exhibit aberrant phenotypes and/ or growth appear to be inhibited (Pilon-Smits et al. 1996; Cairns 2003). Tissue damage by transgenic levan accumulation has been suggested and possible mechanisms have been reviewed, however, the query remains unresolved (Cairns 2003). With the aid of suspension cultures, this study convincingly demonstrated that heterologous accumulation of bacterial HoPSs, levan (fructan) and glucan (glucan), in transgenic sugarcane leads to growth inhibition. Suspension cultures are ready for the analysis

within months, while it may take up to 2 years to generate mature sugarcane plants from embryonic callus tissue. Control and transgenic lines can be grown in a uniform environment and at a fraction of the costs compared to greenhouse- and field-grown plants. Suspension cultures furthermore allow for the creation of homogenous replicates for rapid and reproducible sampling.

Low rates of product accumulation in levan and reuteran transformants

Callus tissue as well as internodal tissue harvested from mature plants accumulated HoPSs at low levels, in accordance with most other studies where plants express transgenic fructansucrase (Cairns 2003). Low rates were accompanied with low message expression and low enzyme quantities. Not only growth rate but also survival of plants regenerated from callus tissue was severely impaired. This study supports the notion that biopolymer accumulation is a function of lethal negative selection of transformants displaying high levels of expression. The variation of expression levels between transgenic tissue containing the same construct is not correlated with the
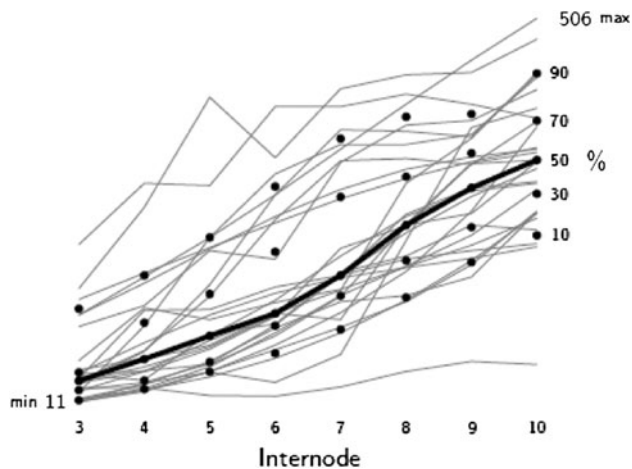
Fig. 8 Sucrose concentrations (converted to mM) as measured for internodes 3–10 (216 points in total) from 27 different sugarcane sources. *Lines* connecting a series of points represent a single set of measurements. The *thick line* depicts the sample median (50 percentile). Data were collected from the literature and collated as shown in Supplement. *Min* minimum, *max* maximum

number of inserted transgenes, but appears to be caused by plant sequences in the proximity of the insertion site (Weising et al. 1988; van der Meer et al. 1994). The mechanism of HoPS-mediated developmental interferences is unclear. Considering that the 35S promoter is active in phloem tissue, developmental aberrations have been blamed on high MW HoPSs disturbing the source to sink transport (Pilon-Smits et al. 1996). This problem was not overcome by targeting levan to the cell perimeter (RL lines, this study).

Subcellular targeting of transgene products

Differential localization of the gene product may modulate the detrimental effects reported for FTF (fructan) expression. Fluorescence experiments have however shown the miss-localization of levan regardless of the putative specificity of the targeting sequences (for review see Cairns 2003). For example, the sporamin vacuolar targeting sequence is known to localize levansucrase into the endomembrane system rather than the vacuole (Turk et al. 1997). To circumvent ambiguous targeting, this study avoided the addition of foreign targeting sequences to the plant expression constructs. In the absence of targeting sequences, transgene products have been established to accumulate in the cytosol.

Thin cross-sections of resin embedded callus cells were selectively stained for polysaccharide-based compounds. Transgenic cells, expressing reuteransucrase from *L. reuteri* (RR) and fructansucrases from *L. sanfranciscensis* (SL), stained positive for increased polysaccharide accumulation in the cytosol. The expression of levan in the

cytoplasm may disrupt cellular spatial organization so severely that the cell dies (Cairns 2003). Expression of reuteran in sugarcane (this study) resulted in plants with increased starch content. Glucan (starch and reuteran) accumulation in reuteran lines RR 1.2 and RR3.1 was furthermore correlated with augmented granule formation. Glucan appears to precipitate in the cytosol above a certain threshold level. The 35S promoter is active in phloem tissue and high DP HoPSs may perturb metabolite transport for instance by precipitation, as previously suggested (Pilon-Smits et al. 1996). Among RR lines, growth in suspension of lines 1.2 and 3.1 were most severely inhibited. Surprisingly, these were the only RR lines that could be generated into mature plants. Soluble glucans occupy much larger volumes than granules and have potentially serious impacts on the osmotic and volume regulation of cells (Raven 2005). Formation of granules, rather than soluble glucan, in the cytosol might have allowed these plants to survive. Cell rims of RL plants displayed marked coloration, a clear indication that the gene product (levan) is localized at the cell perimeter. The FTF gene of *L. reuteri* was not truncated and contains a native bacterial secretion sequence, explaining its defaults to the extracellular compartment in plants. While targeting to the cell perimeter may avoid direct effects on cytoplasmic organization, growth of RL suspension cultures was more inhibited than with putative cytosolic targeting (SL). Absence of granules in tissue culture cells of RL lines could not be explained. Plants with measurable levels of fructan could not be generated from RL callus tissue. This study highlights the complexities and far reaching consequences arising from introducing polymers with high DP in plants.

Carbon partitioning

Endogenous sugars are subject to hourly transport and turnover, while HoPSs accumulate over months. Instantaneous concentrations of endogenous carbohydrates such as sucrose, starch, and glucose, cannot assess the extent of HoPS accumulation as a proportion of primary metabolism. We therefore compared accumulation of transgenic polymer with the flux of sugar through photoassimilation as suggested by Cairns (2003). Table 2 summarizes parameters associated with transgenic levan/reuteran accumulation and compares values with results from other studies. Estimate of flux into HoPSs was, in all cases, less than 0.1 % of primary metabolism (also see Cairns 2003), suggesting that pilfering sucrose for transgenic biopolymer production is unlikely to impact on the steady-state sucrose pool or fluxes of photoassimilate.

Starch content in levan (fructan) accumulators (SL lines) was significantly reduced relative to controls. A number of

Table 2 Accumulation rates (net flux) of carbohydrate (nkat/g) (Adapted from Cairns 2003)

Host	Tissue	Accumulation period (d)	Endpoint (mg/g FW)	Calculated rate (nkat/g)[f]	Reference
Potato[a]	Tuber	135	66	0.03	Gerrits et al. (2001)
Potato[b]	Tuber	70	9.8	0.01	Caimi et al. (1997)
Potato[c]	Tuber	56	17.5	0.02	Röber et al. (1996)
Potato[a]	Leaf	49	5.0	0.007	Pilon-Smits et al. (1996)
Maize[a]	Kernel	30	18	0.03	Caimi et al. (1996)
Maize[a]	Kernel	55	7.5	0.01	Caimi et al. (1996)
Tobacco[a]	Leaf	77	6	0.006	Turk et al. (1997)
Tobacco[a]	Leaf	28	0.35	0.0009	Pilon-Smits et al. (1995)
Sugar beet[a]	Leaf	101	1	0.0007	Pilon-Smits et al. (1995)
Sugarcane[d]	Stalk	350	0.01	0.000002	This study
Sugarcane[e]	Stalk	520	0.03	0.000004	This study

[a] Fructansucrase gene (SacB) from *Bacillus subtilis*, constitutive promoter

[b] Fructansucrase gene from *Bacillus amyloloquifaciens*, patatin promoter

[c] Fructansucrase gene from *Bacillus amyloloquifaciens*, SSU/2-2 promoter

[d] Fructansucrase ORF from *Lactobacillus sanfranciscensis*, constitutive promoter

[e] Glucansucrase ORF from *Lactobacillus reuteri*, constitutive promotor

[f] Net flux of carbohydrate determined using the conventional enzymological unit nkat (nanomoles/s) for reserve polymer synthesis. Given that fructan polysaccharides are of disperse Mr, molar fluxes were estimate from mass data by dividing by the Mr of anhydrohexose (162 Da)

studies reported on lowered starch concentrations when compared to WT or empty vector controls. To our knowledge, only two studies reported on an increase in starch (Turk et al. 1997; Jenkins et al. 2002), associated with a decreased translocation of photoassimilates from leaves (Turk et al. 1997). Reduced starch concentration has been considered due to a competition for carbon by fructansucrase and a resulting diversion of carbon flow from primary metabolism (van der Meer et al. 1994). Considering the low rate of levan synthesis in transgenic plants (see Table 2), metabolic competition is, however, an unlikely cause for reduced starch accumulation. Total reduction in carbohydrate accumulation is furthermore non-stoichiometric; the sum of fructan, starch and sucrose concentrations in transformants is significantly lower than in WT or empty vector controls. Heterologous expression clearly results in a reduction in total assimilation rather than a simple diversion by competition.

GTF enzymes of LAB generally share a common structure composed of four distinct domains: a signal peptide, a variable domain, a catalytic domain, and a glucan-binding domain (Fig. 1). A truncated version of the reuteransucrase gene, *gtfA*, encompassing the catalytic and glucan-binding domains was expressed in sugarcane (RR lines). These two domains have been shown to be required for functional expression in *Escherichia coli* (Kralj et al. 2002, 2004). Unlike with levan, putative cytoplasmic expression of reuteran in sugarcane was positively correlated with starch content, a minor storage carbohydrate in sugarcane. No significant changes in low molecular mass neutral sugar (sucrose, glucose, and fructose) contents were

observed. Cytoplasmic accumulation of reuteran may interfere with synthesis/breakdown metabolic cycles controlling starch formation. It is also conceivable that reuteran binds to starch and decreases the susceptibility of starch to hydrolysis. Little is unfortunately known about the processes involved in starch degradation outside of cereal endosperm (Kossmann and Lloyd 2000).

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