Genetic manipulation of sucrose-storing tissue to produce alternative products

by

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

9 March 2007

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SUMMARY

The main aim of the work presented in this dissertation was to explore the possibility to genetically manipulate the sucrose storing crops, sugarcane and sweet sorghum, to convert their sucrose reserves into higher-value alternatives. For the purpose of this study we focussed on fructans as alternative sucrose-based high-value carbohydrates, since these fructose polymers are of significant commercial interest. To investigate the technical feasibility of transforming sugarcane and sweet sorghum to produce this novel carbohydrate, we proposed to transfer the fructosyltransferase genes from *Cynara scolymus* into these plants by means of particle bombardment.

In order to apply this technology to sweet sorghum, an *in vitro* culture system suitable for transformation had to be established. For this purpose an extensive screening process with different combinations of variables were conducted. Though the relationships between these variables proved to be complex, it was concluded that immature zygotic embryos could be used to initiate a genotype-independent totipotent regeneration system with a 65% callus induction rate, provided that initiation takes place during summer. Stable transformation and regeneration of these calli were however not successful and will have to be optimised to allow future applications.

By introducing fructosyltransferase genes into sugarcane, we succeeded in transforming sugarcane into a crop that produces a variety of fructans of the inulin-type. Low molecular weight (LMW) inulins were found to accumulate in the mature internodes of 42% of the transgenic sugarcane plants expressing the sucrose:sucrose 1-fructosyltransferase (1-SST) gene, and in 77% of the plants that incorporated both 1-SST and fructan:fructan 1-fructosyltransferase (1-FFT), while only 8% of these plants accumulated high molecular weight (HMW) inulins. Our results demonstrated that sugarcane could be manipulated to synthesise and accumulate fructans without the induction of phenotypical irregularities.

Inulins with a degree of polymerisation up to 60 were found in sugarcane storage tissue. In these HMW inulin-producing plants, up to 78% of the endogenous sucrose in the mature sugarcane culm was converted to inulin. This enabled inulin accumulation up to 165.3 mg g^{-1} fresh weight (FW), which is comparable to that

found in native plants. These transgenic sugarcane plants, therefore exhibit great potential as a future industrial inulin source.

Fructan production was detected in all the sugarcane plant tissue tested, predominantly as 1-kestose. In contrast with the fact that fructan accumulation in leaves did not affect the endogenous sucrose concentrations in these organs, the sucrose content of mature internodes that accumulated high levels of 1-kestose was severely reduced. However, increases in total sugar content, in some instances up to 63% higher than control plants, were observed. This phenomenon was investigated with the use of radio-labelled-isotopes. An increase in the allocation of incoming carbon towards sucrose storage, resulting in higher carbon partitioning into both 1-kestose and sucrose, were detected in the culms of transgenic compared to control lines. This modification therefore established an extra carbohydrate sink in the vacuoles that affected photosynthate partitioning and increased total soluble sugar content. The data suggests that sucrose sensing is the main regulatory mechanism responsible for adapting carbon flow in the cells to maintain sucrose concentration.

OPSOMMING

Die hoofdoelwit van die werk aangebied in hierdie proefskrif was om die moontlikheid te ondersoek om die sukrose-bergings gewasse, suikerriet en soetsorghum, geneties te manipuleer om hul sukrose-reserwes na hoër-waarde alternatiewe om te skakel. Vir die doel van hierdie studie het ons gefokus op fruktane as alternatiewe sukrose-gebaseerde, hoër-waarde koolhidrate, aangesien hierdie fruktose-polimere van beduidende kommersiële belang is. Deur die tegniese uitvoerbaarheid van die transformasie van suikerriet en soetsorghum om dié nuwe koolhidraat te produseer te ondersoek, het ons beplan om die fruktosieltransferasegene van *Cynara scolymus* oor te dra aan dié plante deur partiekelbombardering.

Ten einde hierdie tegnologie op soet-sorghum toe te pas moes 'n geskikte *in vitro*-kultuursisteem vir transformasie daargestel word. Vir dié doel was 'n uitgebreide elimineringsproses, met verskillende kombinasies van veranderlikes, uitgevoer. Alhoewel die verhoudings tussen hierdie veranderlikes geblyk het baie kompleks te wees, was dit duidelik dat onvolwasse sigotiese embrios gebruik kon word om 'n genotipiese-onafhanklike totipotente regenereringssisteem te inisieer met 'n 65% kallus induksiegraad, mits inisiasie tydens die somer geskied. Stabiele transformasie en regenerasie van hierdie kallus was egter nie suksesvol nie, en sal geoptimiseer moet word om toekomstige toepassing toe te laat.

Deur fruktosieltransferasegene in suikerriet uit te druk, het ons daarin geslaag om suikerriet te transformeer na 'n gewas wat 'n variasie van fruktane van die inulien-tipe produseer. Lae molekulêregewig (LMG) inuliene, wat in die volwasse internodes akkumuleer, was gevind in 42% van die transgeniese suikerrietplante wat sukrose:sukrose 1-fruktosieltransferase (1-SST) uitdruk, en in 77% van die plante wat beide 1-SST en fruktaan:fruktaan 1-fruktosieltransferase (1-FFT) geïnkorporeer het, terwyl slegs 8% van hierdie plante hoë molekulêre gewig (HMG) inuliene geakumuleer het. Ons resultate het dus demonstreer dat suikerriet gemanipuleer kan word om fruktane te sintetiseer en te akkumuleer, sonder om fenotipiese onreëlmatighede te veroorsaak.

Inuliene, met ‘n graad van pollimerisasie tot en met 60, was in suikerriet storingsweefsel gevind. In hierdie HMG inulien-produserende plante, was tot 78% van die endogene sukrose in die volwasse suikerrietstingel omgeskakel na inulien. Dit het inulien akkumulasie tot 165.3 mg g^{-1} vars gewig (VG) moontlik gemaak, wat vergelyk kan word met dié gevind in inheemse plante. Hierdie transgeniese suikerrietplante, toon dus groot potentiaal as ‘n toekomstige industriële inulienbron.

Fruktaanproduksie, hoofsaaklik as 1-kestose, was gevind in al die getoetste suikerriet plantweefsel. In teenstelling daarmee dat fruktaan akkumulasie in blare nie die endogene sukrosekonsentrasies in dié weefsel beïnvloed het nie, was die sukroseinhoud van volwasse internodes met hoë vlakke 1-kestose, geweldig verminder. Hierdie verskynsel was ondersoek met die gebruik van radioaktief-gemerkte-isotope. ‘n Toename in die allokasie van inkomende koolstof na sukrose berging, wat gely het tot hoër koolstof verdeling na beide 1-kestose en sucrose, was waargeneem in die stingel. Hierdie modifikasie het dus ‘n ekstra koolhidraat swelgpunt in die vakuole geskep, wat fotosinteseproduk-verdeling beinvloed het, en totale oplosbaresuiker-inhoud verhoog het. Die gegewens dui dus daarop dat sukrosebespeuring die hoof reguleringsmeganisme is wat verantwoordelik is vir die handhawing van sukrosekonsentrasies deur die aanpassing van koolstofvloei in die sel.

Vir my ouers

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ABBREVIATIONS

Bp	base pairs
CaMV-35S	Cauliflower mosaic virus' 35S ribosomal subunit's promoter sequence
2,4-D	2,4-dichlorophenoxyacetic acid
DP	degree of polymerisation
FEH	fructan exo-hydrolases (EC 3.2.1.80)
1-FFT	fructan:fructan 1-fructosyltransferase (EC 2.4.1.100)
FW	fresh weight
G418	Geneticin
6G-FFT	fructan:fructan 6G-fructosyltransferase
GFP	green-fluorescent protein
x g	times gravitational force
HMW	high molecular weight
HPAEC	high-pressure anion exchange chromatographic column
HPLC	
L3	Modified L media
LMW	low molecular weight
MCL-PHAs	medium-chain-length polyhydroxyalkanoates
MS	Murashige and Skoog media
μM	micromolar (10^{-6}M)
mM	milimolar (10^{-3}M)
NPT II	Neomycin phosphotransferase II
PCR	polymerase chain reaction
PHA	polyhydroxyalkanoate
PHB	poly(3-hydroxybutyrate)
P[HB-HV]	poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
Q-RT-PCR	quantitative reverse transcription polymerase chain reaction
RT-PCR	reverse transcription polymerase chain reaction
6-SFT	sucrose:fructan 6-fructosyltransferase (EC 2.4.1.10)
1-SST	sucrose:sucrose 1-fructosyltransferase (EC 2.4.1.99)
SEC	size exclusion chromatography
TC	tissue culture
TLC	thin layer chromatography
Ubi 1	maize polyubiquitin 1 promoter
WT	wild type

CHAPTER 1
General introduction

Sucrose is an important commodity worldwide that is produced in 121 countries, with global production exceeding 120 million tons a year. The sucrose market is, however, highly volatile, due to international and domestic price disputes (2005). Because of this volatility and general long-term decreases in sucrose prices, there is a great interest to add value to the crop through product diversification. Aside from post harvest conversion of sucrose and molasses to alternative products, primarily through fermentation technology; another avenue that warrants further investigation is that of the genetic manipulation of sucrose storing crops to allow for accumulation of alternative products (Godshall 2001).

Sugarcane (*Saccharum* spp. hybrids) and sugar beet (*Beta vulgaris* L) are the only crops commercially utilised for sucrose production. High sucrose concentrations are stored as reserve carbohydrates in the storage organs (i.e. stems and taproots) of these plants. Both these species are highly productive crops, with their carbohydrate production of an average of 10 and 7 ton/hectare/year respectively (2005). Exploiting the nature of these plants by transforming them to convert their sucrose reserves into alternative products could therefore provide highly efficient biofactories for the production of sucrose-based compounds (Börnke et al. 2002). This prospect has the potential to dramatically reshape, expand and diversify the sugar industry (Birch 1996).

An example of such a sucrose based high-value product that is currently receiving much international interest is fructan. Inulin type fructans are of particular importance, as they are considered exceptionally promising functional foods, due to their health-promoting effects (Ritsema & Smeekens 2003). Plant fructans are polymers of fructose that are synthesised from sucrose in the vacuole, and in inulin producing plants this process involves two fructosyltransferases. Like sucrose, fructans are also soluble, stored in the vacuole and function as storage carbohydrates in higher plants (Pollock et al. 1996).

Only a limited number of species in the plant kingdom are able to form fructans, of which merely two crops, Jerusalem artichoke and chicory are suitable for large-scale fructan production. However, both crops are at a production disadvantage relative to more traditional agronomic crops, due to their low yield, poor agronomic performance and the lack of processing technology (Caimi et al. 1996; Pilon-Smits et al. 1996). In an attempt to improve the commercial availability of fructans, a number of projects have been directed at isolating the genes encoding plant fructosyltransferases and introducing them into plants with higher agronomic value (Vijn & Smeekens 1999).

The technical feasibility of transgenic fructan production in non-fructan plants has been demonstrated in petunia, tobacco, potato and sugar beet (Van der Meer et al. 1998; Sprenger et al. 1997; Hellwege et al. 1997; Hellwege et al. 2000; Sévenier et al. 1998; Weyens et al. 2004). However up to now the crop of predominant interest for fructan transformation was sugar beet, since it was the only plant in which high enough fructan levels were obtained that could be compared to natural fructan-accumulators (Cairns 2003). This phenomenon can probably be contributed to the exceptionally high sucrose concentrations (the precursor for fructan) naturally present in sugar beet taproot cell vacuoles (the inherent site for fructan synthesis) (Sévenier et al. 1998; Weyens et al. 2004).

The advantage of using plants that naturally accumulate sucrose, for fructan transformation is therefore evident. And since sugarcane has not been exploited as a transgenic fructan producer, even though it has a higher biomass production and is worldwide more extensively cultivated than sugar beet, we have decided to investigate the possibility of converting sugarcane into a fructan-producing crop. Another plant with an exceptionally high accumulation of sucrose is sweet sorghum (*Sorghum bicolor*). Sweet sorghum also has the added benefit that it is drought tolerant, and can be cultivated in areas where drought and high temperature restrict the use of sugarcane (Hawker 1985; Casas et al. 1997).

Sucrose represents not only the carbohydrate storage form in sugarcane, sugar beet and sweet sorghum plants, but is also the principal photosynthesis product and transport carbohydrate in most higher plants (Caimi et al. 1996). Despite the fact that sucrose reserves are of considerable physiological and economical interest many of

the biochemical processes in sucrose metabolism are not well understood (Rae et al. 2005). Recent advances in molecular approaches, for instance transgenic plants with altered sucrose metabolism provide invaluable tools to elucidate some of these processes. Outputs from such research will add vital detail to our current understanding of the regulation of sucrose metabolism, especially in respect to the role of sucrose in source-sink interaction, and carbon partitioning within tissues and cells (Grof & Campbell 2001).

The focus of the current study was to genetically manipulate sugarcane and sweet sorghum to produce inulin. Theoretically this can be accomplished by the introduction of two fructosyltransferase genes from globe artichoke (*Cynara scolymus*) into these plants. The first gene encoding sucrose:sucrose 1-fructosyltransferase (1-SST) will catalyse the formation of low molecular weight oligofructans from sucrose molecules with release of glucose. Introduction of 1-SST together with a second gene, fructan:fructan 1-fructosyltransferase (1-FFT), will then be responsible for the conversion of 1-SST-derived products into long chain inulins with a high degree of polymerisation (DP) (Vijn et al. 1997).

With this approach the technical feasibility of transforming sugarcane with the fructosyltransferase genes from *Cynara scolymus,* to produce oligomeric and polymeric fructans of the inulin type was investigated (Chapter 4). However, since a tissue culture and transformation system were not yet in place for sweet sorghum, we first had to establishing an efficient and reproducible *in vitro* culture system, suitable for the genetic manipulation of sweet sorghum, before the possibility of transgenic fructan production in this plant could be explored (Chapter 3).

Not only was it important to determine whether transgenic fructans could be produced, but in view of the critical role of sucrose in plant growth and development (Sonnewald et al. 1991; Heineke et al. 1992), it was especially relevant to determine whether this conversion of sucrose into an alternative product might have detrimental effects on tissue development. In addition to gaining valuable insight into the potential for development of alternative sucrose-based products in sugarcane (Chapter 4), the possibility of large-scale cultivation of fructan polymers in sugarcane bio-factories could also be assessed (Chapter 5). For this purpose fructan accumulation in

transgenic sugarcane plants expressing both the 1-SST and 1-FFT genes were characterized and compared to naturally fructan producing plants (Chapter 5).

As the transgene products are targeted to the vacuole (Hellwege et al. 2000) it is safe to assume that this modification will impact on sucrose storage in the transgenic plants. Investigating the implications of this conversion on carbon partitioning and flux will expand and contribute to our understanding of sucrose metabolism in sugarcane (Chapter 5).

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CHAPTER 2

Literature review

Metabolic engineering of plants, the process that involves the redirection of cellular metabolism to create new properties or enhance existing ones through genetic modification, is currently receiving a lot of interest (Jacobsen & Khosla 1998). In this review we discuss the significance of this technology in the present study. Firstly the uses of transgenic plants with altered metabolite fluxes in order to further our understanding of plant sucrose metabolism are discussed. Secondly, recent implementations of this technology to exploit agricultural carbohydrate production for conversion to novel compounds are summarized. Finally, the focus is drawn to one specific possibility of metabolic engineering: the available knowledge concerning fructan synthesis and previous fructan transformation experiments is reviewed and considered in terms of possible sugarcane and sweet sorghum transformation.

Potential for manipulating sucrose metabolism

Plant growth, development and yield are dependent upon the production of carbohydrates and the distribution of these carbohydrates between various parts of a plant, as well as between various biosynthetic pathways. Carbohydrates in the form of sucrose and starch are produced in chloroplasts through the process of photosynthesis, which involves the fixation of carbon dioxide. Mature leaves are the primary sites for photosynthesis and represent net exporters of carbohydrates, thus representing carbohydrate sources. Sucrose is the most important form in which carbohydrates are transported in plants. Whereas starch is formed within chloroplasts and serves as an intermediate deposit for the products of carbon fixation, sucrose is synthesized in the cytosol of source organs, transiently stored in the vacuole, translocated to the phloem and exported via the phloem to photosynthetically inactive parts of the plant (sink organs). In sink organs sucrose is cleaved via sucrose synthase or invertase and utilized in metabolic pathways or deposited as storage carbohydrates in the form of sucrose, triglycerides or carbohydrate-containing polymers such as starch, lipids or fructans (Sonnewald et al. 1993).

The central role that sucrose plays with respect to carbohydrate metabolism in higher plants are therefore apparent; being the principal product of photosynthesis, the

preferred transport carbohydrate, as well as representing a carbohydrate storage form of considerable biochemical, physiological and economical importance (Caimi et al. 1996). The understanding of this primary metabolic pathway can consequently be regarded as very important in order to achieve the optimum utilization of plant carbohydrates in future. However, despite numerous studies, there are still a few critical aspects regarding sucrose metabolism that are poorly understood. In this respect further investigation into the biochemical basis for the regulation of sucrose accumulation and the role of the different compartments in relation to sucrose metabolism are required (Whittaker & Botha 1997; Sonnewald et al. 1993).

The use of transgenic plants with altered metabolite fluxes have recently proved to be a remarkable tool in furthering our understanding of plant carbohydrate metabolism. This method takes advantage of our ability to genetically modify plants to contain foreign genes that will create an interference with the normal biosynthesis, storage and distribution of certain metabolites. The influence of this disturbance on metabolite fluxes can then be studied by analysing these plants with various biochemical and physiological means and by comparing them to wild-type plants (Herbers & Sonnewald 1996; Sonnewald et al. 1991).

The critical role of sucrose in plant growth and development was recently illustrated using transgenic plants, altered in their expression of the genes that code for sucrose-cleaving enzymes. In this way the ability of sink tissues to attract photoassimilates was altered (Stitt & Sonnewald 1995). For the purpose of increasing sucrose hydrolysis, a yeast-derived invertase were over-expressed in different sub-cellular compartments in tobacco and potato plants (Sonnewald et al. 1991; Heineke et al. 1992). All transgenic plants showed stunted growth accompanied by reduced root formation. Starch and soluble sugars accumulated in source leaves indicating that long-distance transport of photoassimilates is dependent on sucrose. As a consequence of sugar accumulation photosynthesis is inhibited, demonstrating sink regulation of photosynthesis (Sonnewald et al. 1993). In addition decreased sucrose utilization was achieved by co-suppression of invertase gene expression in the vacuoles of mature tomato leaves. The reduced vacuolar invertase activity in tomato leaves had no impact on photosynthesis or on shoot growth. Study of the carbohydrate metabolism of these plants supports the involvement of invertase in cycling of sucrose through the vacuole

(Scholes et al. 1996). These results prove the central importance of the compartmentation of sucrose with respect to its biosynthesis, storage and distribution. It also demonstrates that any perturbation of the sucrose metabolism in plants can lead to the development of detrimental phenotypes.

These experiments have illustrated the importance of understanding the regulation of biochemical pathways as a prerequisite for effective metabolic engineering while avoiding undesirable side effects (Herbers & Sonnewald 1996). Realizing the implications of this technique, a wide spectrum of plant science research now utilizes transgenic plants as a means for understanding the synthesis of plant products. Rapid advances in this field are being made, focusing mainly on model systems and major crop species (Knauf 1995).

Although sugarcane produces approximately 70% of the world's sucrose, making it the most important crop species for commercial sugar production, sugarcane research are still lagging behind when compared with the progress made in model plants and major crops. As a result many steps in the models constructed from the available biochemical research for the sucrose transport and accumulation pathway in sugarcane are still unknown. Sugarcane researchers are however beginning to implement the exciting new molecular approaches available to dissect the biochemical processes controlling sucrose accumulation in sugarcane. Recent advances into the isolation of genes encoding the key enzymes and transporters in the sucrose accumulation process will provide valuable tools to assist in defining the sucrose storage process. Once the potential targets for manipulation have been determined, metabolite fluxes can be manipulated to increase the production or yield of sucrose in the stem of the sugarcane plant. Alternatively, novel functions can be introduced in plants to obtain high-value biomaterials, produced in sugarcane as alternative products or as co-products with sucrose (Grof & Campbell 2001; Rae et al. 2005).

Engineering modified carbohydrate metabolic pathways

Carbohydrate metabolism in plants is based on sucrose, the direct product of photosynthesis, and its reversible conversion into storage and structural carbohydrates such as starch and cellulose. Metabolic engineering of carbohydrates therefore refers to the conversion of primary metabolites from sucrose metabolism into particularly

desirable molecules (Capell & Christou 2004). Palatinose, trehalose, cyclodextrins and oligofructans are examples of novel carbohydrates with commercial value, that have already been identified as likely targets for production in transgenic plants (Chen & Murata 2002; Börnke et al. 2002a; Börnke et al. 2002b). The sucrose isomer, trehalose (1-*O*-α-D-glucopyranosyl-D-fructose) has been identified as a potential target for the genetic manipulation of tolerance to abiotic stresses that create water deficit. This notion was confirmed when the drought tolerance of transgenic tobacco and potato plants were found to be significantly enhanced, after being transformed with genes for trehalose synthesis from yeast and *E.coli.* The successful application of this strategy was however hampered by the substantial morphological changes that were exhibited by these transgenic plants (Chen & Murata 2002).

Another sucrose isomer of industrial interest is palatinose (isomaltulose or 6-*O*-α-D-glucopyranosyl-D-fructose), due to its use as a low calorific sucrose substitute in food product. To explore the possibility of palatinose production in transgenic plants, an isolated sucrose isomerase gene from *Erwinia rhapontici* were fused to an apoplasmic signal peptide and expressed in tobacco plants and potato tubers respectively. In the tobacco plant this conversion were found to be harmful to plant development and yield, causing severe growth abnormalities as well as reduced starch and soluble carbohydrate contents, whereas only the soluble carbohydrates were altered within the potato tubers (Börnke et al. 2002a; Börnke et al. 2002b). The true potential of transgenic palatinose production were however only realised in a recent study by Birch and Wu (2005), when a sucrose isomerase gene from the bacteria *Pantoea dispersa* was introduced into sugarcane. The expression of this gene not only resulted in the conversion of stored sucrose into palatinose, but in some instances, it had the added benefit of increasing the amount of sucrose stored in the plant. These remarkable results were attributed to the novel approach followed in this study, which involved combining a highly efficient sucrose isomerase, with the use of a very productive sucrose storing plant species, such as sugarcane. Furthermore it included targeting of the transgene to the sucrose-storing compartment (in this case the vacuole of sucrose storage parenchyma within the mature sugarcane culm) of the chosen plant (Birch & Wu 2005).

On the other hand starch and fructans are both examples of polymeric carbohydrates that are naturally synthesized in plants, for which the biosynthesis is sufficiently understood to allow the bioengineering of their properties, in order to further expand their usefulness as natural biomaterials. As follows, metabolic engineering have been used to modify the properties and uses of starch by changing the relative proportions of its two components, amylose and amylopectin. With the use of antisense technology the synthesis of amylose-free and low-amylose starch has been achieved in potato and rice plants respectively (Visser et al. 1991; Liu et al. 2003). In a similar manner, the isoamylase gene in rice grains has been inhibited to produce a modified amylopectin (Fujita et al. 2003). These structurally modified starches have superior qualities over traditional starches that broaden the scope for industrial applications (Riesmeier et al. 1998).

Novel starches have been produced using bacterial enzymes to change the nature and frequency of branching. The commercial utility of these unusual and novel starches has however not yet been determined (Shewmaker et al. 1994; Kok-Jacon et al. 2003). Conversely, the potential of producing a novel high-value carbohydrate from starch, were identified and illustrated when a bacterial cyclodextrin glycosyltransferase gene from *Klebsiella* were introduced into potatoes and resulted in the conversion of starch to cyclodextrins. Though very little conversion was obtained, this was merely an initial trial experiment, and more data will be needed in future to determine the actual feasibility of this approach (Oakes et al. 1991).

Fructans are another group of storage carbohydrates that are important targets for metabolic engineering, since their normal production is inadequate to supply in the increasing demand for these polysaccharides as functional food ingredients. This strong interest from the food industry in fructans developed and continues to expand due to growing awareness of their health-promoting properties (Heyer et al. 1999). Not only are they low-calorie soluble food fibres that can be used as fat and sugar replacements in a variety of foods, but consumption of this compound will also promote the growth of Bifidobacteria in the gut, resulting in many health benefits (Ritsema & Smeekens 2003a). Fructans are fructose polymers, derived from sucrose that is normally isolated from crop plants with low agronomic value, such as chicory and Jerusalem artichoke. In order to make the production of fructans economically

more feasible, the genes necessary for the synthesis of fructans have been isolated from agronomically unfavourable plant species and introduced into superior crops (Riesmeier et al. 1998). The crop of predominant interest is sugar beet, because the major storage compound of this species is sucrose, which is also the direct precursor for fructan biosynthesis. Constitutive expression of a sucrose:sucrose 1-fructosyltransferase (1-SST) gene from Jerusalem artichoke (*Helianthus tuberosus*) in these plants resulted in a nearly quantitative conversion, as 90% of the taproot vacuolar sucrose was converted into inulin oligomers (short chain fructans of the inulin type) (Sévenier et al. 1998a). A subsequent study focused on producing high molecular weight (HMW) fructans, by introducing a pair of fructosyltransferases from onion (*Allium cepa* L.), namely 1-SST and fructan:fructan 6G-fructosyltransferase (6G-FFT), into sugar beet. This resulted in an efficient conversion of sucrose into complex, onion-type fructans, without a loss in total storage carbohydrate content (Weyens et al. 2004).

The exceptional nature of sucrose for enzymatic synthesis, being hydrolysed by enzymes as well as acting as a donor molecule for transfer reactions, was illustrated in the above-mentioned experiments. New high value products, such as fructan polymers and oligosaccharides as well as palatinose, a non-caloric sweetener, were readily derived from sucrose without any adverse effects (Sévenier et al. 1998; Weyens et al. 2004; Birch & Wu 2005). The two sucrose storing crops, sugarcane and sugar beet, therefore lend themselves to many exciting new areas of metabolic engineering in which sucrose as the carbohydrate source can be converted to novel products. Promising new commodities engineered from sucrose and its co-products with commercial potential include sucrose esters, natural biodegradable plastics, new food products, sweeteners and bio-diesel/ethanol (Godshall 2001).

Fructan production

Fructans, or polyfructosylsucrose, are linear and branched polymers of fructose that are derived from sucrose. Besides being synthesised in plants, fructans are also produced by certain bacteria and several fungi (Riesmeier et al. 1998; Ritsema & Smeekens 2003a).

Bacterial fructan structure and biosynthesis

There are two types of bacterial fructans and bacteria capable of fructan production can be found in a wide range of taxa, including plant pathogens and animal and human microflora. In general, bacteria produce a fructan type known as levan, which mainly consists of β(2-6)-linked fructosyl residues that can occasionally contain β(2-1)-linked branches. Examples of bacterial genera, containing strains capable of levan production, are *Bacillus*, *Streptococcus*, *Pseudomonas*, *Erwinia*, and *Actinomyces*. A few strains of *Streptococcus mutans* and *Bacillus* are conversely known to produce mostly 2,1-linked type fructans called inulin. Bacterial levan and inulin can reach a degree of polymerisation (DP) of more than 100 000 fructose moieties. Synthesis of inulin and levan directly from sucrose, is catalysed each by a single enzyme, known as inulinsucrase and levansucrase (EC 2.4.1.10), respectively. Such fructans are present as part of extracellular polysaccharides. For the degradation of levan, bacteria produces specific enzymes called levanases (Hendry & Wallace 1993; Riesmeier et al. 1998; Pilon-Smits et al. 1996).

Fructans in higher plants

Fructans are used by higher plants as reserve carbohydrate, that are stored if carbon production exceeds demand and are mobilized if energy is required. Although most plants store starch and sucrose as reserve carbohydrate, about 15% of all flowering plant species store fructans. Plants that are able to synthesize fructans are scattered among several families, which include many economically important species, such as cereals (e.g. barley, wheat, and oat), vegetables (e.g. chicory, artichoke, asparagus and onion), ornamentals (e.g. dahlia and tulip) and forage grasses (e.g. *Lolium* and *Festuca*) (Hendry & Wallace 1993).

Structure of plant fructans

Plant fructans are much smaller than bacterial fructans, with DP's of 3 up to 250 fructosyl residues. In contrast with the seemingly uniform structure of bacterial fructans, plant fructans have a far greater structural diversity in which five major classes of fructan molecules can be differentiated: inulin, levan, mixed levan, inulin neoseries, and levan neoseries (Van der Meer et al. 1998).

The simplest fructan is inulin, consisting of a linear chain of fructose molecules connected by β-2,1-linkages and terminated by a glucose unit (G1-2F1-2Fn). Inulins are usually found in plants belonging to the Asterales (e.g. chicory and Jerusalem artichoke). The shortest inulin molecule is the trisaccharide 1-kestose (also called isokestose), the prototype of inulin (Fig. 1a). Similar fructans known as oligofructose, also consisting of β-2,1-linked fructosyl units, but without the glucose unit, have been found in species of the Asteraceae.

Fructan of the levan type, also called phlein type in plants, based on 6-kestose consists of linear (2-6)-linked β-D-fructosyl units (G1-2F6-2Fn) and is found in grasses. Mixed levan is composed of β(2-6)-linked fructose residues, with β(2-1) branches. This type of fructan is characteristic of plant species belonging to the Poales, such as wheat and barley. An example of this type of fructan is the tetrasaccharide bifurcose (Fig. 1b).

Both the inulin neoseries and the levan neoseries is based on neokestose with fructose chains of the inulin and levan type respectively on either sides of the glucose moiety. The structure of the neokestose molecule is shown in Fig. 1c. Fructans of the inulin neoseries is found in plants belonging to the Liliaceae (e.g. onion and asparagus) whereas the levan neoseries is found in some members of the Poales (Pollock et al. 1996; Vijn & Smeekens 1999; Ritsema & Smeekens 2003b).

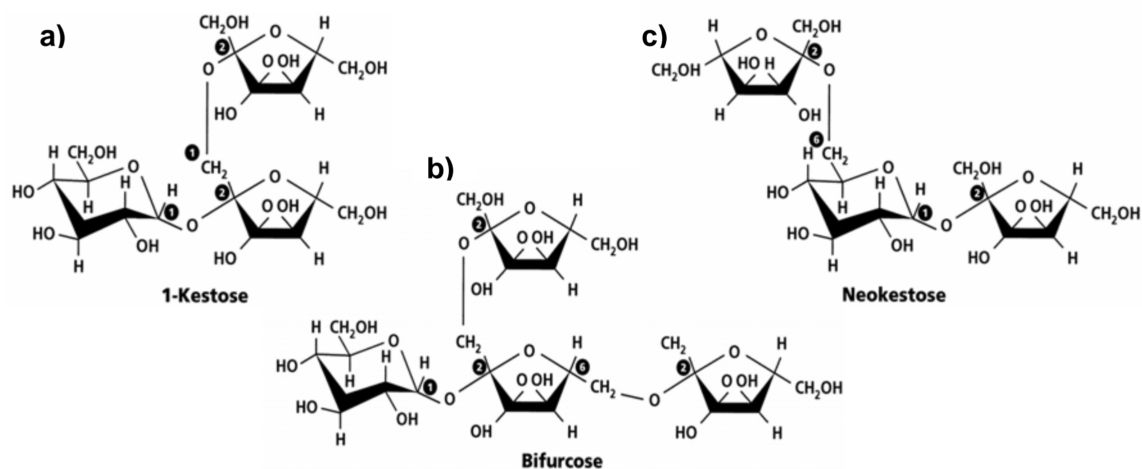

Fig. 1 Schematic representation of structurally different short fructans: a) 1-kestose, b) bifurcose and c) neokestose (Ritsema & Smeekens 2003b).

Plant fructan biosynthesis

In plants, fructans are synthesized and stored in the vacuole. Biosynthesis of fructan starts from two sucrose molecules and involves two or more fructosyl transferases. According to the model of inulin synthesis proposed by Edelman and Jefford in 1968, the first enzyme, sucrose:sucrose 1-fructosyl transferase (1-SST, EC 2.4.1.99) catalyses the transfer of a fructosyl moiety from sucrose to another sucrose molecule. The trisaccharide 1-kestose that is produced by 1-SST serves as donor and acceptor of fructosyl residues for the second enzyme. The second enzyme fructan:fructan 1-fructosyltransferase (1-FFT, EC 2.4.1.100) extends the 1-kestose trisaccharide by transferring fructosyl residues from a fructan molecule with a DP≥3 to another fructan molecule or to sucrose. The joint action of 1-SST and 1-FFT results in the formation of a mixture of fructan molecules with different chain lengths (Edelman & Jefford 1968; Housley & Pollock 1993).

As evidence to sustain the above-described model, both enzymes were purified and, in a mixture with sucrose as the only substrate, demonstrated that these enzymes can catalyse the formation of fructans with DP's up to 20 *in vitro*. The current view that both enzymes are localized in the vacuole was supported by experiments with vacuoles isolated from leaves of *Triticum aestivum* and tubers of *Helianthus tuberosus*. In addition, it was also shown that both 1-SST and 1-FFT are unusual enzymes, since they do not act according to Michaelis-Menten kinetics; their activity depends on both the substrate and the enzyme concentration and is essentially nonsaturable (Koops & Jonker 1996; van der Meer et al. 1998).

Levan production is initiated by the enzyme sucrose:fructan 6-fructosyltransferase (6-SFT, EC 2.4.1.10) that transfers a fructose residue from sucrose to either 1-kestose or sucrose, forming bifurcose and 6-kestose respectively. 6-SFT can further elongate 6-kestose to produce levans. Bifurcose can be elongated by 1-FFT and 6-SFT, resulting in branched, mixed-type levans (Graminan). Fructan:fructan 6G-fructosyltransferase (6G-FFT) is responsible for the forming of neokestose. By using 1-kestose as a fructose donor, the fructose residue is attached to the glucose residue of sucrose via a β(2-6)-linkage. This trisaccharide, neokestose, can then be elongated by either 1-FFT or 6-SFT, resulting in the production of inulin or levan neoseries respectively. The

breakdown of plant fructans is accomplished by two types of fructan exo-hydrolases (FEH, EC 3.2.1.80), with either a β(2-1)-linkage-specific or a β(2-6)-linkage-specific exohydrolytic activity. The current knowledge on the enzymology of fructan synthesis is outlined in the adapted version of the model proposed by Vijn and Smeekens in 1999 (Fig. 2) (Vijn & Smeekens 1999; Heyer et al. 1999).

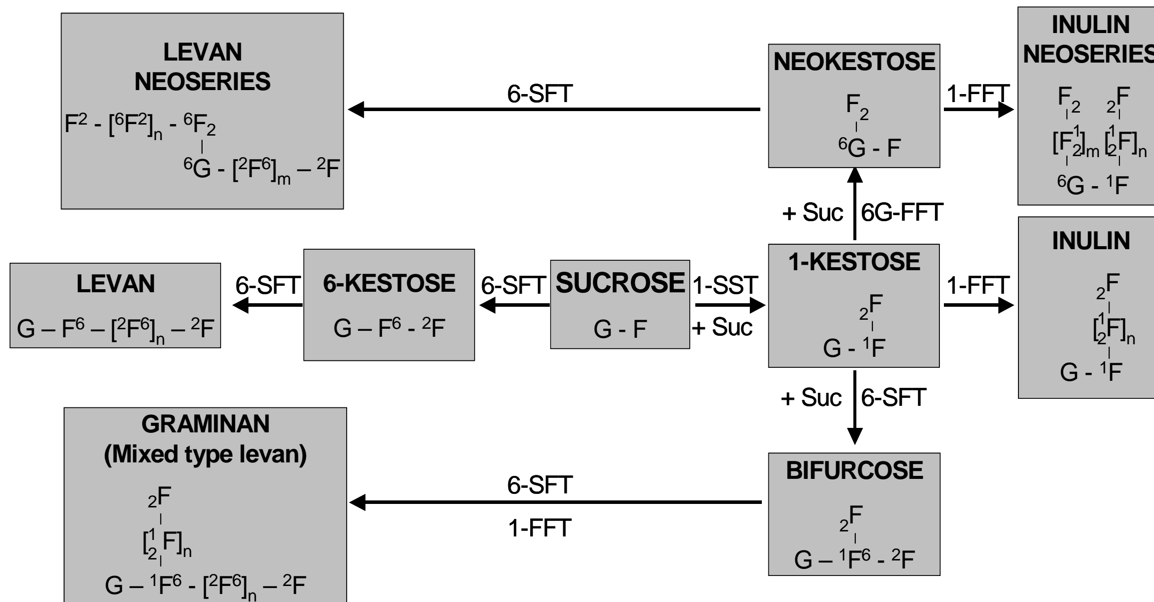

Fig. 2 Model of fructan biosynthesis in plants (Vijn & Smeekens 1999).
F: fructose; G: glucose, $m \geq 1$and $n \geq 1$

Physiological role of fructan in plants

Fructans, have long been known to function as storage carbohydrates in certain plants, and can be utilised as a buffer for maintenance of plant development when photosynthesis is inhibited by adverse conditions (Goggin & Setter 2004). Other functions of fructans in fructan-accumulating plants also include stress protection (both cold and drought) and osmoregulation (Vergauwen et al. 2000). Though the molecular mechanism behind the increased resistance to various stresses is unclear, fructans have been found to play a direct role in conferring tolerance to drought. The interaction of fructans with phospholipid cell layers have been indicated to stabilise plant membranes, thereby preventing membrane damage during drought stress (De Roover et al. 2000; Goggin & Setter 2004).

Osmoregulation has also been suggested as a more indirect way in which fructan metabolism can lead to stress tolerance. Since fructans are water-soluble, they will

contribute to the osmotic potential of the cell. Therefore, by inducing fructan synthesis or by shifting the average length of the fructan pool, natural fructan accumulators can respond to changing conditions by increasing their osmolyte accumulation and thus increasing water uptake. The natural function of fructans therefore has less to do with environmental stress resistance, but more with water uptake, water retention and growth by water driven cell inflation, protecting the plant against water deficit caused by drought and low temperatures (Suzuki 1993; Hendry & Wallace 1993). Osmotic adaptation via the use of fructans, with the rapid hydrolysis of fructans into low-DP products, has also been suggested as a mechanism to facilitate the osmotic driving force involved in the rapid expansion of flowers (Pilon-Smits et al. 1995; Vergauwen et al. 2000).

Fructan synthesis might also control sucrose concentration in the vacuole. Vacuolar fructan production from sucrose should lower the sucrose concentration in the cell and thereby could prevent sugar-induced feedback inhibition of photosynthesis. The vacuolar storage of fructan could therefore facilitate balancing of supply and demand whilst buffering chloroplastic metabolism from changes in the metabolite status caused by fluctuating rates of sucrose export (Pollock 1986). Control of sucrose content has importance because it plays a major role in higher plant carbohydrate partitioning and is believed responsible for changes in fructan metabolic enzyme gene expression (Pollock 1986). The relationships between sucrose and fructan metabolism is illustrated in Fig 3.

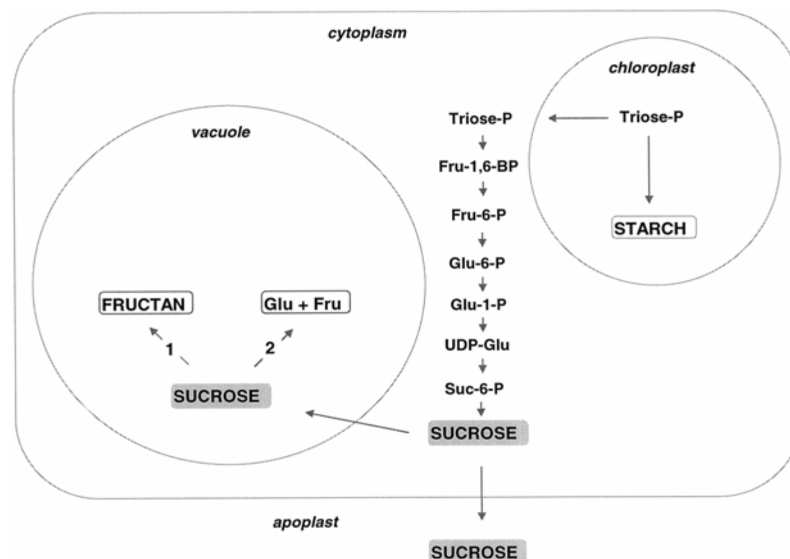

Fig. 3 The relationships between sucrose and fructan metabolism in plants (Vijn & Smeekens 1999).

Fructan production and use

Fructans and especially inulin are of growing interest as functional food ingredient, due to their health-promoting effects. When consumed, they are not hydrolysed into monosaccharide moieties in the upper intestinal tract, since human enzymes cannot digest fructans. Due to this property, fructan consumption does not increase blood sugar or insulin levels and can be classified as low-caloric fibres. Instead of being directly digested they reach the colon where they are fermented by enterobacteria. Fructan-containing diets selectively stimulate the growth of beneficial bacteria, such as bifidobacteria in the colon, and make them the predominant species. This leads to improving mineral absorption and blood lipid composition as well as aiding in the prevention of colon cancer. Fructans therefore have diverse effects in promoting health (Farnworth 1993).

Fructans isolated from plants, that naturally store fructans, have a variety of food applications, where they serve as sugar and fat replacements, for improving taste and texture. Short-chain fructans with DP's of 3 to 6 are sweet tasting and therefore constitutes natural low-caloric sweeteners. Long chain fructans however have a neutral taste and organoleptic properties similar to fat when emulsified with water, and can be used to replace fat in alimentary products. High-DP fructans also hold great promise for a variety of non-food applications (e.g. in the biopolymer industry) (Roberfroid et al. 1998).

Production and manufacture of fructans

Fructans exist naturally in many kinds of plants, but are present in significant quantities in only a few plants including, artichoke, asparagus, salsify, leek, onions, garlic and chicory. Since these plants have a relatively low biomass and often require a complex culture they are not efficient enough to be used as competitive industrial crops. The only two crops currently cultivated for inulin production are *Cichorium intybus* (chicory) and *Helianthus tuberosus* (Jerusalem artichoke). However, for reasons of technical processing and purification, only inulin as stored in the roots of chicory, seem to have a real future as a novel, alternative agricultural raw material with both food and non-food applications (Fuchs 1993).

Inulins extracted from chicory roots have been purified, processed and marketed under a series of trade names by the Belgium Company Orafti. These products, Raftiline® consisting mainly of long chain inulins, Raftilose® composed of short chain inulins ($2 \leq DP \leq 7$) and a fructose syrup Raftisweet® are used as fat and sugar substitutes in a wide variety of foods. However, the function of the fructan isolated from chicory is limited because of the degradation of long fructan chains by fructan exohydrolase upon harvesting (Gibson et al. 1994).

Fructans are also produced enzymatically from sucrose, derived from sugar beet or sugarcane, using a β-fructofuranosidase from a selected *Aspergillus niger* strain. Fructo-oligosaccharides with DP's up to 5 synthesized in this fashion are marketed under the trade name Neosugar®. Production cost is however high because of the use of reactor-based production methods and the requirement for an additional purification step to remove the by-product, glucose (Yang & Wang 1999).

Fructan biotechnology

Fructans are considered the most promising functional food to date, receiving growing industrial interest, mainly because of its health-promoting effect as functional food ingredient (Ritsema & Smeekens 2003a). However, fructan-containing crops are at a production disadvantage relative to more traditional agronomic crops due to their low yield, poor agronomic performance and the lack of processing technology (Caimi et al. 1996). Relatively low molecular weight inulin type fructans with considerable chain length variations, harvested from the roots and tubers of chicory and Jerusalem artichoke are at the moment the only commercially available source of fructan (Turk et al. 1997). Therefore, in order to meet the increase in consumer demand, crops with quantitatively and qualitatively improved fructan sources are needed (Ebskamp et al. 1994). The most promising approach towards agricultural fructan production would thus be to transfer the biochemical capacity for fructan synthesis to crops with superior agronomic performance. In an attempt to understand fructan synthesis, the physiological role of fructan accumulation in plants and to improve the commercial availability of fructans, a number of experiments have been directed at the biosynthesis of fructans in species that do not normally produce fructans.

Since the genes encoding plant fructosyltransferase enzymes were not at first available, initial induction of fructan synthesis in non-fructan-accumulating plants was achieved by expression of microbial levansucrase (Van der Meer et al. 1994). Mainly the SacB gene from Bacillus spp., generally under the control of CaMV 35S were used to transform starch accumulating crops such as potato and maize as well as tobacco. The constitutive expression of this gene resulted in the accumulation of high molecular weight levan-type fructans with a DP of over 25,000 in all the plant organs tested (Cairns 2003). An increase in fructan level with leaf age was reported, coinciding with leaf damage in the older tissue (Gerrits et al. 2001). The diurnal turnover of starch and sucrose was absent in the transgenic plants and total non-structural carbohydrate content was increased in the source leaves. Furthermore, in the sink organs transgenic fructans accumulated at the expense of starch, lead to reduced development and yield. The observed phenotypes are similar to those found in transgenic plants with reduced sucrose transporter activity. Therefore it was suggested that the translocation of carbohydrates from the source to the sink tissue might be blocked in the bacterial-levansucrase plants due to levan production in the phloem tissue (Pilon-Smits et al. 1996; Turk et al. 1997). However, in cases where levansucrase was expressed in the sink tissue only, the same results were obtained. In consequence of these results, it could be reasoned that the diversion of sucrose away from the starch biosynthetic pathway, inhibited the sink strength, resulting in reduced growth and yield of the storage organs. The observed tissue damage in transgenic plants might also be related to the size of the bacterial polymer, since accumulation of very large polymers in relatively small cellular compartments can cause direct physical pressure or altered osmotic potential in the plant cell (Caimi et al. 1997). The necrotic lesions formed, could also be as result of a hypersensitive stress response induced by the bacterial fructans, since fructans with such high DP's are not normally found in plants (Turk et al. 1997; Biggs & Hancock 2001; Cairns 2003). Resulting tissue damage could also be due to a inability of bacterial fructosyltransferase to be targeted to the vacuole even if an appropriate signal is added to the protein (Pilon-Smits et al. 1996; Turk et al. 1997; Ritsema & Smeekens 2003b).

The dramatic phenotypic changes observed in these plants demonstrate the crucial role of the subcellular compartment used for fructan production. These results illustrated the critical function of sucrose in plant growth and development and that

the diversion of sucrose from an existing metabolic pathway may be detrimental to tissue development.

Edelman and Jefford (1968) hypothesised that fructans are synthesised from sucrose in the vacuole of plants by the concerted action of two enzymes. This model was confirmed by the accumulation of high-molecular-weight fructans, when both SST and FFT activities where expressed in transgenic potato and petunia plants (Van der Meer et al. 1998; Hellwege et al. 2000). Although fructans accumulated to significant levels in the potato tubers, these fructans formed at the expense of starch, leaving the total storage capacity of the tubers unchanged (Hellwege et al. 2000). The introduction of 1-SST into sugar beet and potato has shown that large amounts of short-chain fructan molecules are produced, and that despite the storage carbohydrate having been altered, the expression of the 1-SST gene did not have any visible effect on phenotype (Hellwege et al. 1997; Sévenier et al. 1998). In all these studies, changes in fructan levels appear to be closely associated with changes in the translocation of soluble sugars.

Because no aberrant development was observed when plant-derived fructosyltransferase genes were expressed in host plants, the production of plant-plant fructan transformants instead of bacterial-plant transformants seems to be preferable. One likely explanation for this is that plant-derived fructosyltransferase genes encode signal peptides that direct fructosyltransferases to the vacuole (Ritsema & Smeekens 2003b; Hisano et al. 2004).

From these results it is evident that the transfer of this biochemical capacity for the synthesis of fructans to plants with higher agronomic value in order to make the production of fructans economically more feasible is an attainable goal. The advantage of using crops that do not normally produce fructans for fructan accumulation is that they lack fructan-hydrolysing enzymes such as exohydrolase for breaking down the accumulated fructan upon harvesting (Vijn & Smeekens 1999). Another criterion that should receive priority, when selecting a suitable candidate crop for fructan transformation, is that the main storage carbohydrate should rather be sucrose than starch. Since sucrose acts as the sole substrate for SST and the level of fructan synthesis is determined by the concentration of vacuolar sucrose, the

importance of this factor is apparent (Caimi et al. 1996). The observations in the previous studies that fructan production was at the expense of starch in starch-storing sinks can also be interpreted to support this view. Sucrose-synthesizing plants are also more feasible for this purpose, since their total amount of storage carbohydrates is often much higher than starch-accumulating species. This tendency is due to the fact that sucrose is usually stored in the vacuole, a compartment with a much larger storage capacity than plastids since the vacuole constitutes up to 95% of the protoplast volume (Hellwege et al. 1997).

Up to now the crop of predominant interest has been the sugar beet, because it complies to these criteria with the major storage compound of this specie being sucrose, the direct precursor for fructan biosynthesis (Heyer et al. 1999; Vijn & Smeekens 1999). The only plant species commercially used for sucrose production are sugarcane and sugar beet. Since these two species will accumulate up to 50% of their dry weight as sucrose, they are very productive crops (www.sucrose.com/learn.html). From these statistics it is evident that sugarcane shares the same significant characteristics as sugar beet that determines the suitability of this crop as a transgenic fructan producer. It would therefore be very interesting to investigate the possibility of converting sugarcane into a fructan producing crop.

Though sugarcane and sugar beet are the only crops commercially utilized for sucrose production (2005), another plant that accumulates large amounts of harvestable sucrose is sweet sorghum (*Sorghum bicolor* L. Moench). Like sugarcane, sweet sorghum is also a grass species belonging to the Andropogoneae tribe, with similar sucrose accumulation patterns, i.e. a gradient down the culm, with as high as 20% sucrose in the mature internodes (Al-Janabi et al. 1994; Grivet et al. 1994). However, as a prospective crop to be utilized as a fructan biofactory, sweet sorghum has additional advantage over sugarcane and sugar beet, because of its multi-product (grains, sugars and lignocellulosics) usage as food, fibre, feed, and fuel (MacKinnon et al. 1986), and its independency of the sugar industry . It also has other advantages, such as a shorter growth period, high biomass and characteristics including drought resistance, waterlogging tolerance and saline-alkali tolerance, consequently making sweet sorghum widely adaptable to be grown in areas where the cultivation of other crops are restricted (Casas et al. 1993; Casas et al. 1997). And seeing as this plant is

very well adapted to South Africa's harsh climate, rendering it ideal for our needs, the prospects for including sweet sorghum in biotechnology projects aimed at the molecular manipulation of sucrose metabolism to enable fructan production, therefore seems very promising.

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CHAPTER 3

Embryogenesis from immature zygotic embryos of sweet sorghum

This chapter reports on the progress made in our attempt to establish a sweet sorghum *in vitro* culture system suitable for genetic manipulation. However, since the project was terminated before reaching conclusion, the experiments were never finalised. The chapter therefore serves only as a preliminary report, documented for the purpose to assist in the planning of future research on sweet sorghum tissue culture and transformation.

ABSTRACT

Using shoot tips and immature zygotic embryos as explants, four sweet sorghum lines in combination with six different callus induction media were screened for their ability to form regenerable somatic embryogenic calli. Varying degrees of pigment production and vegetative growth observed during callus initiation, in both these cultures, inhibited callus growth and embryogenesis, and were perceived to be influenced by genotype, explant type, media composition as well as season of initiation. Though the relationships between these variables proved to be complex, zygotic embryos were identified as the explant of choice, with 25–75% of the isolated embryos forming regenerable somatic embryogenic calli. It was further concluded that immature zygotic embryos could be used to initiate a genotype-independent totipotent regeneration system with a 65% callus induction rate for all four sweet sorghum genotypes, provided that initiation takes place during summer, on Murashige and Skoog medium supplemented with 2.5 mg l^{-1} 2,4-dichlorophenoxyacetic acid and 0.5 mg l^{-1} kinetin. In subsequent particle bombardment experiments, five times more transient gfp expression was observed on somatic embryogenic calli compared to proembryogenic calli, derived from the same type of immature zygotic embryo explants. Somatic embyogenic calli therefore exhibits a significantly higher potential as suitable target tissue for genetic transformation.

INTRODUCTION

The development of an efficient and reproducible *in vitro*-regeneration technique permitting the production of whole plants from transformed cells is crucial for the successful molecular manipulation of higher plants (Vasil 1987). However, since

biotechnological research on sorghum (*Sorghum bicolor* L. Moench) has far lagged behind that of other major crops, the barrier to efficient genotype-independent plant regeneration and transformation of sorghums has not been overcome (Zhong et al. 1998; Tadesse et al. 2003). Therefore, in order to assess the possibility of applying plant genetic modification technology to sweet sorghum (a type of sorghum), an *in vitro* culture system suitable for transformation via particle bombardment first had to be established.

So far, transgenic sorghum plants have been obtained following microprojectile bombardment of immature zygotic embryos (Casas et al. 1993; Tadesse et al. 2003), immature inflorescences (Casas et al. 1997) and shoot tips (Tadesse et al. 2003). These primary explants of sorghum were selected as principal targets for transformation since they were shown to retain the greatest capacity for expression of morphogenic competence in previous in vitro regeneration studies (MacKinnon et al. 1986; Ma et al. 1987; Bhaskaran & Smith.R.H. 1989; Cai & Butler 1990; Elkonin et al. 1995; Zhong et al. 1998; Oldach et al. 2001; Tadesse et al. 2003). However, the high degree of genetic variation for expression of morphogenic competence, encountered in each explant/genotype combination, substantially limits the application of transformation technology to new genotypic lines (Casas et al. 1997). Because of this variability, our approach in establishing an efficient and reproducible tissue culture system for sweet sorghum, suitable for transformation via particle bombardment, have mainly focused on the screening of genotypes in combination with different explant types and media compositions.

In this paper, we report on the screening of four sweet sorghum genotypes for effective *in vitro* plant regeneration via somatic embryogenesis from shoot tips and immature embryos on six different callus induction media. This resulted in the establishment of a genotype-independent totipotent regeneration system for sweet sorghum from immature zygotic embryos. Furthermore, though the successive transformations of these primary immature zygotic embryo explants as well as somatic embryogenic calli initiated from these explants were unsuccessful, transient green fluorescent protein (GFP) expression observed after bombardment indicated that embryogenic calli is more suitable for this purpose. Stable transformation and

regeneration of these calli will therefore have to be optimised to allow future applications of this technology.

MATERIALS AND METHODS

Sweet sorghum plant material and tissue culture

Four sweet sorghum breeding lines (kindly supplied by Gordon Donaldson), distinguished by their different sugar accumulation levels, referred to as lines B, H, L and O (Table 1), were tested for their capacity to induce callus suitable for transformation and regeneration from shoot tip and immature zygotic embryo.

Table 1 Sugar composition of the four sweet sorghum lines (sugar content as % dry weight)

Lines	Sucrose	Glucose	Fructose
B	40.0	27.6	22.7
H	70.6	11.0	8.4
L	75.3	11.4	7.3
O	49.7	20.1	16.1

Shoot tip cultures

Callus was induced and cultured from shoot tip explants as described by (Tadesse et al. 2003). Surface sterilized seeds were plated on germination medium, with their convex sides in contact with the medium, and incubated for two to three days at ± 25ºC in the dark. The germination medium consisted of half strength MS salts (Murashige & Skoog 1962), with 500 mg l^{-1} myo-inositol, 5 mg l^{-1} thiamine HCl, and 30 g l^{-1} sucrose. The pH of the medium was adjusted to pH 5.8 and 8 g l^{-1} agar was added before autoclaving for 20 minutes at a pressure of 1.5 kg cm^{-1}. Following germination, the emerging shoot tips were excised and plated with the cut ends in contact with callus induction medium, containing MS salts, 1 mg l^{-1} thiamine HCl, 7.5 mg l^{-1} glycine, 100 mg l^{-1} DL-asparagine, 0.2 mg l^{-1} kinetin, 2.5 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g l^{-1} sucrose and 8 g l^{-1} agar (Tadesse et al. 2003).

Immature zygotic embryo cultures

For the isolation and culture of immature zygotic embryos, the procedure as describes by (MacKinnon et al. 1986) was followed. Immature embryos were collected 12-15 days after anthesis from caryopses of greenhouse grown plants, cultivated under natural daylight conditions at 28-30˚C. Embryos ranging in size from 1.0 to 1.5 mm

with their endosperm between milk and dough stage, were isolated from surface sterilized immature seeds and placed on callus induction media with the embryo axis facing the medium. The choice of callus induction medium for immature zygotic embryos was however complicated due to the diverse nature of the media described in the literature. Six different media were therefore selected, based on their performance in previous studies, and screened in combination with the four genotypes. The media was prepared as described in the cited literature, with slight modifications as indicated in Table 2.

Table 2 Composition of different callus induction media (referred to as $I_{1\text{-}6}$) for sweet sorghum immature embryo cultures (all concentrations are given in mg l^{-1})

Chemical components	I_1 MacKinnon *et al.* 1986	I_2 Casas *et al.* 1993	I_3 Casas *et al.* 1997	I_4 Oldach *et al.* 2001	I_5 Tadesse *et al* 2003	I_6 O'Kennedy *et al.* 2004
Inorganic salts (incl. iron-EDTA)	MS	MS	MS	L3	MS	L3
Glycine		2	2		7.5	
Myo-Inositol	100	100	100	100	100	100
Thiamine-HCl	0.4	0.1	0.1	10	1	10
p-Aminobenzoic acid				1		
Pyridoxine-HCl		0.5	0.5	1		1
Nicotinic acid		0.5	0.5	1		1
L-glutamine				750		420
Riboflavin				0.1		
Folic acid				0.2		
Calcium pantothenate				0.5		
Choline chloride				0.5		
Biotin				0.005		
Ascorbic acid				1		
Fumaric acid				10		
Citric acid				10		
Malic acid				10		
Sodium pyruvate				5		
Casein		150				
Proline				150		
Asparagine			150	100	100	
Maltose						30 000
Sucrose	40 000	30 000	60 000	30 000	30 000	
Agar	8 000	8 000	8 000		8 000	
Gelrite				3 000		4 000
Coconut water		10 000				
2,4-D	2	2	2.5	2.5	2.5	2.5
Kinetin	0.5		0.5		0.2	
BAP				0.1		

MS = Murashige and Skoog medium (Murashige & Skoog 1962)
L3 = Modified L medium (Jähne et al. 1991)

Explants were cultured in the dark at 28˚C until the onset of embryogenesis. During this time tissue was sub-cultured every 2 weeks, except when excessive phenolic compound formation necessitated more frequent re-culturing. Somatic embryogenic calli were visually selected and separately sub-cultured. Germination of these somatic embryos with shoot regeneration and eventually root formation was achieved by transferring the respective cultures onto the appropriate shoot and root induction media as described in the corresponding literature and culturing under fluorescent lights with a 16 h light and 8 h dark cycle at 28˚C. Rooted plantlets were transferred to soil and hardened-off in the greenhouse under natural daylight conditions at 28-30˚C (MacKinnon et al. 1986; Casas et al. 1993; Casas et al. 1997; Oldach et al. 2001; Tadesse et al. 2003; O'Kennedy et al. 2004).

Plasmids

A modified version of the green-fluorescent protein (GFP) from the jellyfish *Aequorea victoria* was used as a vital reporter to directly visualize gene expression without tissue destruction. In pGEM.Ubi1-sgfpS65T the modified gfp for improved expression and fluorescence (sgfpS65T) was placed under the control of the maize polyubiquitin 1 promoter (Ubi 1) (Fig. 1) (Elliott et al. 1999). pGEM.Ubi1-sgfpS65T was co-precipitated for transformation purposes with the pEmuKN (LAST et al. 1991) selection plasmid. pEmuKN contains the neomycin phosphotransferase II (nptII) gene coding sequence under control of the engineered Emu promoter (Fig. 1). This selection plasmid was used to confer resistance to G418 (Geneticin, Sigma), based on expression of the aphA gene.

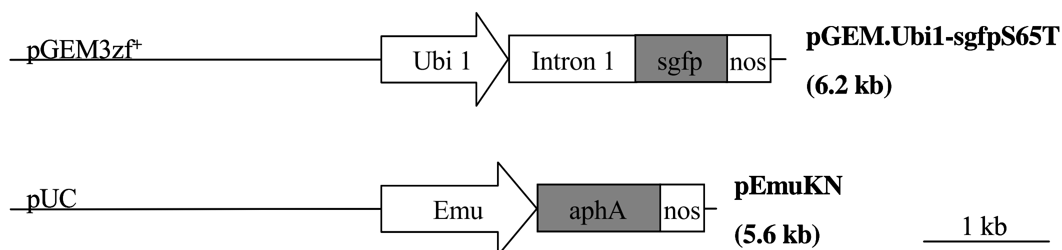

Fig. 1 Plasmids used for transformation. pGEM.Ubi1-sgfpS65T (kindly supplied by CSIRO Tropical Agriculture, St Lucia, Australia) contains a synthetic green fluorescent protein gene (sgfpS65T) driven by the maize polyubiquitin 1 (Ubi 1) promotor (Elliott et al. 1999). pEmuKN carry the geneticin resistance gene (aphA) driven by the Emu promotor (Last et al. 1991). Both genes are followed by a nopaline synthase (nos) terminator sequence. The cloning vectors, pGEM3zf^{+} and pUC, used to construct theses plasmids, are shown as thin lines in the respective constructs.

Sweet sorghum transformation

Transformation of sweet sorghum was conducted by means of microprojectile bombardment of immature zygotic embryos and somatic embryogenic callus.

Tissue preparation

Immature zygotic sweet sorghum embryos from 7-day-old cultures with proembryogenic callus developing on the scutellum side were selected as target tissue. Alternatively a second type of target tissue, i.e. white somatic embryogenic callus segments of 4-8 mm diameter, was selected from ± 6-week-old immature embryo cultures (Tadesse et al. 2003). The two types of target tissue was separately incubated on osmotic medium (callus induction medium supplemented with 0.2 M sorbitol and 0.2 M mannitol), on which it was arranged in a circular target area of 3 cm diameter in the centre of the Petri dish, and incubated for 4 hours prior to bombardment (Gallo-Meagher & Irvine 1996, Bower et al. 1996).

DNA delivery

For the co-precipitation of pGEM.Ubi1-sgfpS65T with pEmuKN, the plasmids were mixed together prior to addition to the precipitation mix, so that each plasmid may be present at 0.5 µg $µl^{-1}$ in the DNA solution. Plasmid DNA (1 µg $µl^{-1}$) was precipitated onto tungsten particles (100 mg ml^{-1}) with $CaCl^2$ (2.5 M) and spermidine (0.1 M) at a ratio of 1:5:5:2 using a method previously described (Franks & Birch 1991). Microprojectile bombardment was performed using a particle inflow gun (Finer et al. 1992) with 1000 kPa gas delivery pressure, 90 kPa chamber vacuum and a 1 ms gas delivery interval. The target tissue was covered with a sterile protective screen and centered 16 cm below the outlet aperture (Bower et al. 1996). Each target was bombarded with 4 µl of the dispersed tungsten-DNA preparation. For control purposes calli were bombarded using only tungsten particles with no plasmids precipitated onto the particles.

Subsequent in vitro proliferation and selection

After bombardment target tissue were incubated on osmotic media for an additional 4 hours before being transferred to callus induction medium for a two-week recovery period. The bombarded calli are then allowed to proliferate on selection medium (induction medium with 30 mg l^{-1} geneticin) in the dark, sub-culturing every 14 days, until somatic embryos form from individual cells that survived selection pressure.

GFP detection

After bombardment, calli were assessed for activity of the introduced sGFP gene 48hr after bombardment, followed by observations every 14 days. Visualization of GFP fluorescence in plant tissue was achieved using a Leica stereomicroscope with GFP Plus filter module (Elliott et al. 1999).

RESULTS

Sweet sorghum callus formation, embryogenesis and regeneration

Formation of phenolic compounds was observed from the earliest stages in the culture period in all four breeding lines regardless of the type of explant used. The amount of phenolic compounds produced varied between lines, and were more pronounced in shoot tip cultures. Despite the frequent transfers of cultures onto fresh medium, phenolic compound formation continued to be a problem, since diffusion of these compounds into the surrounding medium was associated with reduced callus growth, tissue pigmentation and necrosis.

Callus proliferation was observed around the cutting edges of the shoot tips of genotype H. Yet, these calli were very small, hyperhydrisid and never developed into somatic embryogenic calli.

The process of callus initiation from immature zygotic embryos in most of the genotypes was dominated by germination of the immature embryos. The growth of these vegetative parts of the explants seemed to inhibit callus development. When the vegetative extensions were removed during sub-culturing, an action performed to induce callusing, it only stimulated further phenolic compound production. The effect of different genotypes combined with different callus induction media on embryogenic callus formation from immature zygotic embryos, varied from one season to another (results summarised in Table 3).

After subsequent selective sub-culturing of the somatic embryos produced from immature zygotic embryo cultures, these embryos were readily regenerated to obtain plantlets following the respective procedures.

Table 3 Embryogenic calli formation from immature zygotic embryo cultures of four sweet sorghum genotypes on six different induction media (two hundred embryos plated per genotype/medium combination)

Callus induction media	Sweet Sorghum Genotypes	Season of initiation	Callus induction rate[a]
I_1	B, H, L, O	Autumn	0%
I_2	B, H, L, O	Summer	35%
	B, H, L, O	Winter	0%
I_3	B, H, L, O	Summer	65%
	B, H, L, O	Winter	0%
I_4	H	Summer	75%
	H	Autumn	35%
I_5	H	Summer	0%
	B, L, O	Autumn	0%
	H	Autumn	25%
I_6	H	Summer	0%

[a] Percentage of plated embryos forming embryogenic callus

Sweet sorghum transformation

An estimated average of 20 compared to 4 gfp foci, were observed per cm^2 of somatic embryogenic calli and proembryogenic calli respectively, 48 hours after bombardment. In both cases a drastic reduction in gfp expression was observed after this initial transient phase. Stable transformation was not detected in either of the tissue types, with no geneticin resistant callus growth or gfp expressing callus cluster development, regardless of the time spent in culture. During subsequent culturing after bombardment, phenolic production was even more abundant than in initial cultures. Immature zygotic embryos started blackening immediately after bombardment, with most of the tissue black and necrotic two weeks later, even though selection pressure was not yet applied.

DISCUSSION

Optimisation of sweet sorghum tissue culture systems

Phenolic compounds released into the medium by the explant, as well as the growth of vegetative parts on the explants, have been identified in previous studies as factors that appeared to reduce the formation and growth of callus (Cai & Butler 1990). In this study the occurrence of both these phenomena, was also characterized by a

reduction in callus growth. Extreme cases of pigment secretion resulted in tissue necrosis and death of the cultured explants and callus.

A complex and indirect relationship between plant genotype, explant type, and season of initiation, in relation to *in vitro* morphogenic competence has been reported in several species. These discrepancies most probably are related to variations in endogenous hormone levels of the explants (Ma et al. 1987). This effect was evident in the study, in which early summer proved to be the most opportune season for callus initiation. Immature zygotic embryo explants were also identified to surpass shoot tip cultures in their ability to produce embryogenic calli.

Furthermore, significant differences in the ability of immature embryos to produce somatic embryogenic calli were observed when cultivated on different callus induction media. Similar results, with 65% of the isolated embryos forming embryogenic calli, were however achieved with all four genotypes on callus induction medium I_3 described by Casas et al. (1997). This specific explant/media combination therefore rendered a genotype-independent totipotent regeneration system for sweet sorghum. Though a higher efficiency in regenerable calli production (75%) was achieved with one of the sweet sorghum lines on the Oldach et al. (2001) callus induction medium (I_4), further tests with other lines are needed in order to establish the genotype specificity of this system.

From this we conclude that immature embryos from sweet sorghum can be used to initiate a tissue culture system suitable for genetic modification, provided that initiation takes place at the right time of the source plant's growing cycle. The negative effects on callus growth caused by pigment production and vegetative growth of the explant can possibly be reduced by frequent sub-culturing onto callus induction medium containing 0.5 – 1 mg l^{-1} of activated charcoal, and by using younger explant material.

Sweet sorghum transformation proved to be problematic

Immature zygotic embryos appeared to be very sensitive to bombardment shock, with necrosis following directly after bombardment. Unfortunately these initial experiments could not be repeated to test the influence of different bombardment

condition variables such as bombardment pressure, due to a lack in target tissue at the time. This inability to establish a sufficient supply of target tissue for successive bombardment experiments was a direct result of the seasonal nature of callus production (sufficient callus production only possible in summer). At this point in the study the strategic decision was made, to terminate the sweet sorghum project and to rather continue focusing on sugarcane transformation experiments, for a more in depth study of the effect of sucrose conversion to fructans.

From these initial experiments it was however concluded that the bombardment conditions would have to be optimised for future applications. Judging by the amount of transient GFP expression in the different tissues, the potential for transformation is significantly less in the immature zygotic embryos with proembryogenic calli development than in the somatic embryogenic calli derived for the same type of explant. White somatic embryogenic calli, produced from immature embryos, therefore seems to have more potential as suitable target tissue for genetic transformation.

From these results it was clear that there are still major problems that must be resolved before genetic engineering techniques can be readily and effectively applied to sweet sorghum. However, the prospects of using transgenic sweet sorghum as a sucrose-based-product biofactory; or by reason of the close relationship to sugarcane, using the sweet sorghum transformation system as a model system for sugarcane transformation (Ming et al. 1998; Ritter et al. 2004), justifies these efforts. The use of transgenic sweet sorghum as a model system for sugarcane will enable a more rapid assessment (at least 6 months shorter growth cycle than sugarcane) to evaluate gene expression as well as promoter specificity.

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CHAPTER 4

Bioengineering fructan synthesis in sugarcane

ABSTRACT

Sugarcane was successfully transformed to produce fructans, via the introduction and functional expression of the sucrose:sucrose 1-fructosyltransferase (1-SST) and fructan:fructan 1-fructosyltransferase (1-FFT) genes from globe artichoke. Low molecular weight (LMW) inulins were found to accumulate in the mature internodes of 42% of the 1-SST-transgenic sugarcane plants, and in 77% of the plants that incorporated both 1-SST and 1-FFT. In both cases the trisaccharide 1-kestose was the predominant transgenic product, being the only fructan detected in 60% of the lines exhibiting transgene activity. High molecular weight (HMW) inulins, on the other hand only accumulated in sugarcane that incorporated both transgenes, and only in 8% of these plants. Oligomeric-inulin-accumulating plants had unchanged sucrose levels, and slightly raised fructose levels, while a reduction in sucrose levels were apparent in polymeric-inulin-producing plants when compared to controls. The expression of the 1-SST and 1-FFT gene did not have any visible effect on the phenotype of transgenic plants as observed under greenhouse conditions.

INTRODUCTION

Metabolic engineering of sucrose storing plants, in which cellular metabolism is redirected to exploit sucrose as the carbohydrate source for conversion to novel compounds, is currently exhibiting much potential and is hence receiving a lot of interest (Jacobsen & Khosla 1998; Godshall 2001; Capell & Christou 2004). In this fashion, the high value sucrose-based products, fructan and palatinose, have been readily derived from sucrose in transgenic sugar beet (*Beta vulgaris* L) and sugarcane (*Saccharum* spp. hybrids) respectively, without any adverse effects (Sévenier et al. 1998; Weyens et al. 2004; Birch & Wu 2005).

In this study we investigated the possibility of converting the sucrose reserves of sugarcane into oligomeric and polymeric fructans of the inulin type. Theoretically this can be accomplished by the introduction of two fructosyltransferase genes from globe artichoke (*Cynara scolymus*). The first gene encoding sucrose:sucrose 1-fructosyltransferase (1-SST) (EC 2.4.1.99) will catalyse the formation of low

molecular weight (LMW) oligofructans from sucrose molecules with release of glucose. Introduction of 1-SST together with a second gene, fructan:fructan 1-fructosyltransferase (1-FFT) (EC 2.4.1.100), will then be responsible for the conversion of 1-SST-derived products into long chain inulins with a high degree of polymerisation (DP) (Edelman & Jefford 1968; Vijn et al. 1997; Hellwege et al. 1998).

This conversion is therefore expected to have an influence on the normal metabolite composition of the sugarcane plant, with possible decreases in sucrose content and increases in glucose accumulation. Diversion of sucrose from an existing metabolic pathway may however have detrimental effects on sugarcane growth and development (Caimi et al. 1996). From these trials we will thus also be able to determine whether this conversion of sucrose into an alternative carbohydrate, such as fructan will have unwanted effects on sugarcane growth and development.

Yet another factor that might influence the feasibility of transgenic fructan production in sugarcane, is the effect that endogenous sugarcane invertase enzyme activity might have on fructan production. Not only will invertase compete for the same sucrose substrate as 1-SST, but the hydrolysing activity of invertase, breaking down sucrose and oligofructans, may also mask fructosyltransferase activity (Cairns 1993). Polymeric fructans on the other hand can't be hydrolysed by invertase (Van der Meer et al. 1998), and is therefore likely to affect sugarcane metabolism in a different way than transgenic oligofructan production. In order to determine whether sugarcane will respond differently to transgenic LMW inulin, compared to HMW inulin production, we propose to transform sugarcane either with only 1-SST or with 1-SST and 1-FFT combined.

Here we report that transgenic fructan synthesis can be achieved in sugarcane. The expression of 1-SST alone or in combination with 1-FFT resulted in inulin oligomer and polymer production respectively. Moreover the introduction of this new carbohydrate pathway did not have an influence on plant phenotype.

MATERIALS AND METHODS

Callus induction

Callus cultures were initiated from leafroll explants, of the commercial sugarcane variety NCo310, on callus induction medium (without coconut water), under dark conditions at 28˚C, as described by Franks and Birch (1991). Explants were sub-cultured every 14 days until embryogenic callus production became noticeable, subsequently these calli were selectively sub-cultured (Bower et al. 1996).

Plasmids

The plasmids used in this study, pML1 and pML2, were provided by PlantTec. The pML1 chimeric construct contains the purified 1-SST gene (Genbank Y09662) (Hellwege et al. 1997) whereas pML2 contains both the 1-SST and the 1-FFT (Genbank AJ 000481) (Hellwege et al. 2000) genes from *Cynara scolymus*. The cDNAs include endogenous signal sequences for predicted vacuolar localization of the encoded enzymes (Hellwege et al. 2000), and were placed under control of the maize Ubiquitin promoter (Christensen et al. 1992) followed by the nos (nopaline synthase gene) terminator (Fig. 1). pML1 and pML2 were co-precipitated for transformation purposes with the pEmuKN (LAST et al. 1991) selection plasmid. pEmuKN contains the neomycin phosphotransferase II (npt II) gene coding sequence under control of the engineered Emu promoter (Fig. 1). This selection plasmid was used to confer resistance to G418 (Geneticin, Sigma), based on expression of the aphA gene.

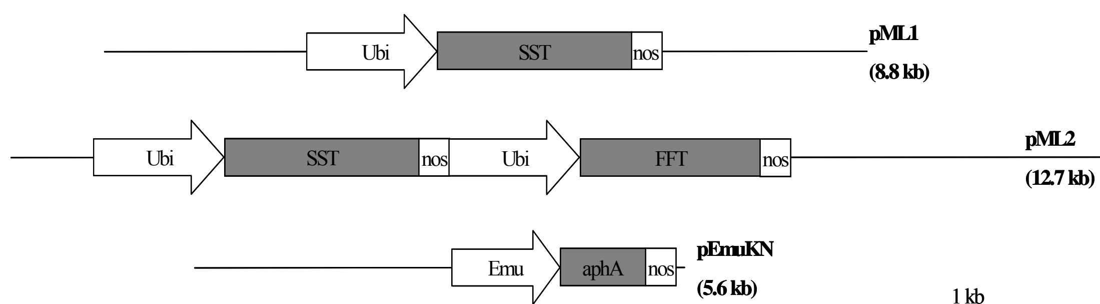

Fig. 1 Plasmids used for transformation. pML1 and pML2 contains the sucrose:sucrose 1-fructosyltransferase (SST) and fructan:fructan 1-fructosyltransferase (FFT) genes driven by the maize polyubiquitin 1 promotor and intron (Ubi). pEmuKN carry the geneticin resistance gene (aphA) driven by the Emu promotor (Last et al. 1991). All three genes are followed by nopaline synthase (nos) terminator sequences.

Microprojectile bombardment and selection of transformants

Tissue preparation

Segments of white embryogenic callus of 4-8 mm diameter were selected from approximately eight-week-old sugarcane leafroll cultures, and subculture separately on induction media for 4 days prior to bombardment. Four hours before bombardment these pre-selected calli were transferred to osmotic medium (callus induction medium supplemented with 0.2 M sorbitol and 0.2 M mannitol) and arranged in the centre of the Petri dish in a circular target area of 3 cm diameter (Gallo-Meagher & Irvine 1996; Bower et al. 1996).

DNA delivery

For the co-precipitation of pML1 or pML2 with pEmuKN, plasmids were mixed together prior to addition to the precipitation mix, so that each plasmid may be present at 0.5 $\mu g\ \mu l^{-1}$ in the DNA solution. Plasmid DNA (1 $\mu g\ \mu l^{-1}$) was precipitated onto tungsten particles (100 mg ml^{-1}) with $CaCl^2$ (2.5 M) and spermidine (0.1 M) at a ratio of 1:5:5:2 using a method previously described (Franks & Birch 1991). Microprojectile bombardment was performed using a particle inflow gun (Finer et al. 1992) with 1000 kPa gas delivery pressure, 90 kPa chamber vacuum and a 1 ms gas delivery interval. The target tissue was covered with a sterile protective screen and centered 16 cm below the outlet aperture (Bower et al. 1996). Each callus target was bombarded with 4 µl of the dispersed tungsten-DNA preparation, containing either pML1 and pEmuKN or pML2 and pEmuKN. For control purposes calli were bombarded using only tungsten particles with no plasmids precipitated onto the particles.

Subsequent in vitro selection

After bombardment, calli were incubated on osmotic media for an additional 4 hours before being transferred to callus induction medium for a two-day recovery period. The bombarded calli were then allowed to proliferate on selection medium (induction medium with 45 mg l^{-1} geneticin) in the dark, sub-culturing every 14 days, so that embryos form from individual cells, which have stably incorporated the introduced genes (i.e. nptII gene). To initiate regeneration, the embryogenic calli that survived selection, were transferred to regeneration media (callus induction media without 2,4-dichlorophenoxyacetic acid (2,4-D) with geneticin (45 mg l^{-1}) and incubated under fluorescent lights with a 16 h light and 8 h dark cycle at 28˚C. Selection was maintained during callus proliferation and regeneration. For control purposes the calli

that received only the tungsten particles were regenerated using the same method, but without including geneticin in the different media (from here on referred to as tissue culture (TC-controls). Shoots were transferred to half strength Murashige and Skoog (MS) media (Murashige & Skoog 1962) for root formation, and when roots were established, plantlets were transferred to pots with soil, to be hardened-off and cultivated in the greenhouse under natural daylight conditions at 28-30˚C (Bower et al. 1996).

DNA isolation and polymerase chain reaction (PCR) analysis

Total genomic DNA was extracted from young leaves of putatively transformed sugarcane plants following the method described by Dellaporte *et al.*, (1983). PCR was carried out in a total volume of 50 µl using 100 ng total DNA as template. To amplify transgenes 1-SST and 1-FFT, two primer pairs specific for the detection of the respective genes were used. Primers specific to the coding region of 1-SST: (SSTf: 5'-AAAGACGTGGAGCTGAAACC -3') combined with (SSTrev: 5'-CCCTATGTCTACATCGTTCG -3') and 1-FFT: (FFTf: 5'-CTAGAGGATGGGCT-ACTATTT-3') together with (FFTrev: 5'-GTTAACTCCGAAAGGGTTCCG-3'), were designed for this purpose. PCR conditions were 94ºC for 2 min with subsequent cycling at 94ºC for 45 sec, 52ºC for 45 sec, and 72ºC for 2 min, these cycles were repeated 35 times, and the reaction mixture was then further incubated at 72ºC for 7 min.

Carbohydrate analyses

Extraction of soluble carbohydrates

Fresh plant material (500 mg) was collected from mature sugarcane internodes (internodes 10 to 20) and powdered in liquid nitrogen. Water-soluble carbohydrates were extracted twice from homogenates in 80% (v/v) ethanol (in 10mM Sodium phosphate pH7.4) at 80°C and twice in 10mM Sodium phosphate pH7.4 at 60°C in portions of 1 ml for 30 min each time. Extracts were centrifuged at 10,000 g at room temperature, and supernatants pooled. The combined supernatants were loaded onto a tandem ion exchange column. The column consisted of a cation exchange column (Supelclean™ SCX tube, Supelco) over an anion exchange column (Supelclean™ SAX tubes, Supelco), which was pre-conditioned according to the manufacturer's specifications. The neutral fraction of each extract was eluted from the tandem

column with 2.5 ml 40% (v/v) ethanol. These fractions were then dried overnight in a vacuum centrifuge at 25˚C and re-dissolved in deionised water (with a ratio of 1 mg fresh plant weight (FW) to 3 µl water) and additionally filtered (0.45 µM sterile syringe filter) before analysis by thin layer chromatography (TLC) and high-performance anion exchange chromatography (HPAEC). These solutions were kept frozen at -20˚C.

TLC analyses

The water-soluble carbohydrate extracts were assayed for fructan production by spotting 2 µl of each extract solution directly onto silica-gel 60 TLC foils (Merck). The foils were developed twice in a solvent mixture of propanol:butanol:water (12:3:4, v/v/v) and allowed to air dry after each run. Fructose-containing sugars were detected by staining with a urea-phosphoric acid spray, followed by incubation of the thin-layer foils at 80˚C for 15 min (Wise et al. 1955). 2 µl of each of the 10 mg ml^{-1} fructose, sucrose, 1-kestose, nystose and fructosyl-nystose standards were run simultaneously with the samples. This particular system easily resolved fructose, sucrose, 1-kestose, nystose and f-nystose with Rf values of 0.29, 0.23, 0.13, 0.09 and 0.06, respectively.

HPLC analyses

The deionised water-soluble carbohydrate extracts were analysed by high-performance anion exchange chromatography (HPAEC) using a CarboPac PA-100 guard and separation column on the Dionex DX-500 gradient chromatography system (Dionex, Sunnyvale, CA, USA) coupled with pulsed amperometric detection by a gold electrode. The detector settings were: T_1 = 0.48 sec; T_2 = 0.12 sec; T_3 = 0.12 sec; E_1 = 0.05 V; E_2 = 0.65 V; E_3 = -0.96 V; sensitivity range = 0.1 µC; integration range = 0.28-0.48 sec; solvent A = 0.15 M NaOH; solvent B = 1 M NaAc in 0.15 M NaOH; conditions 5 min 100% A; 65 min linear change from 100% A to 45% B; 5 min linear change from 55% A to 100% B; 5 min 100% B; 5 min linear change from 100% B to 100% A. The sugars were eluted at a constant flow rate of 1 ml min^{-1}. Fructose, glucose, sucrose, 1-kestose, nystose and fructosyl-nystose were identified by comparison of their retention times with those of their respective purified standards.

RESULTS

Recovery of 1-SST- and 1-FFT- transgenic sugarcane

Somatic embryogenic sugarcane calli were co-transformed by means of microprojectile bombardment with either pML1 (containing the 1-SST gene) or pML2 (with both the 1-SST and 1-FFT genes) combined with the selection plasmid pEmuKN (containing the NPTII gene). Potential transformants were selected on geneticin containing media. A total of 14 putative transformants were selected from the pML1 (referred to as the SST 1-14 series in this study) and 30 for the pML2 (referred to as the SFT 1-30 series) based transformation constructs.

The transgenic nature of the recovered plants was confirmed by demonstrating the presence of either or both the 1-SST and 1-FFT sequences using PCR analyses (Table 1). The results indicated a high co-transformation frequency with 71% and 90% of putative transgenic pML1 and pML2 lines testing positive respectively for the presence of their particular transgenes (Table 1).

Performance evaluation of the transgenic plants in comparison with TC-controls and wild type plants were based on the general phenotypical appearances of the plants under greenhouse conditions, characterised by plant height, stalk thickness, colour, shape and amount of leaves. Precise measurements were however not recorded since greenhouse conditions were not standerdised. Transgenic plants showed no visual phenotypic differences when compared to TC-control sugarcane plants. Furthermore, the comparison between transgenic and wild type sugarcane plants were complicated, since the plants grown directly from tissue culture showed epigenetic effects when compared to wild type plants which were vegetatively propagated. This aspect of the transformation process will therefore only really be assessable in succeeding trails in which both the wild type plants and transgenic plants are propagated in the same way.

New sugars produced in transgenic sugarcane

In order to identify in which of these transgenic plants the expression of the fructosyl-transferase genes resulted in fructan production; the sugar composition in the water-soluble-carbohydrate extracts from mature internodes of these plants were investigated by thin layer chromatography (TLC). In this system, sugars present in the neutral fraction of the extracts, were separated primarily according to molecular size,

and the fructose containing sugars subsequently visualized. An example with representative samples of the different results obtained is shown in Fig. 2.

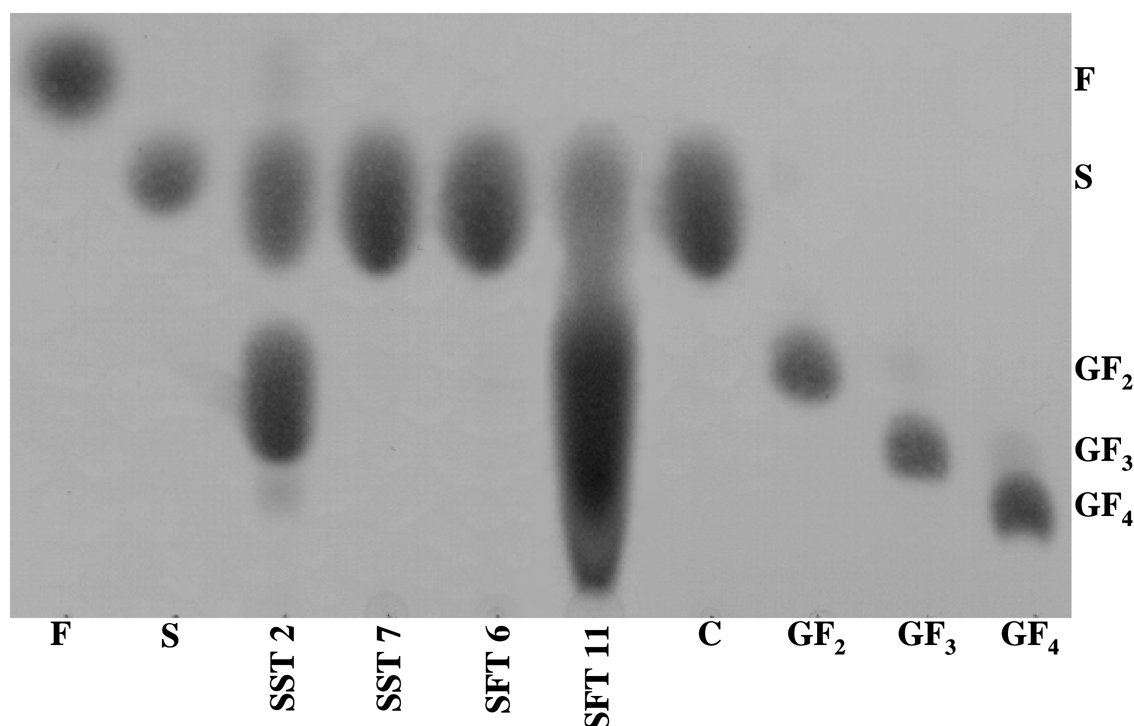

Fig. 2 A typical TLC analyses of the water-soluble carbohydrate extracts of mature internodes (internodes 15 – 20) of sugarcane plants transformed with pML1 (SST 2 and SST 7) and pML2 (SFT 6 and SFT 11), as well as an TC-control (C). F: fructose; S: sucrose; GF_2: 1-kestose; GF_3: nystose; GF_4: fructosyl-nystose.

The TLC analyses indicated that only one fructose containing sugar is present in the control plants (lane 7, Fig. 2), with an Rf value of 0.23. This is identical to that of authentic sucrose (S) (lane 2, Fig. 2). The apparent absence of fructose in the control plants (lane 7, Fig. 2) is indicative of the low concentration of free hexoses in the mature internodes of the sugarcane culm. Except for sucrose that was prevalent in all the transgenic plants, a series of "new" fructose containing sugars with higher molecular weights than sucrose were also detected in the transgenic clones (e.g. in SST 2/lane 3 and in SFT 11/lane 6, Fig. 2). The Rf values (0.13, 0.09, and 0.06) of the first three sugars in this series corresponded to that of the authentic oligofructan standards: 1-kestose, nystose and fructosyl-nystose (lane 8, 9 and 10, Fig.2). These inulin type oligofructans represents subsequent steps of polymerisations, consisting of a linear chain of two, three and four fructose molecules (F) respectively, connected by β-2,1-linkages and terminated by a glucose unit (G) (i.e. 1-kestose = GF2 with a DP

of 3; nystose = GF3 with a DP of 4; and fructosyl-nystose = GF4 with a DP of 5). A variety of fructose containing sugars was detected in the pML1 and pML2 transgenic lines (Table 1). Furthermore, traces of fructose were observed in mature internodes of a number of transgenic oligofructan producing plants (Table 1).

Table 1. PCR and TLC results of: a) pML1 and b) pML2 transgenic lines. 2µl of each standard (F: fructose; S: sucrose; GF2: 1-kestose; GF3: nystose; GF4: fructosyl-nystose) and each neutral sugar solution (1mg FW: 3 µl H2O) extracted from mature stalk sections (internodes no. 10 – 12) of transgenic and control lines were spotted onto TLC's. The presence (+) or absence (-) of the transgenes and fructose containing sugars, as observed in the PCR and TLC analyses are indicated in the table. The types of sugars present in the transgenic lines were identified by comparison with standards and the amount of sucrose observed in each line were visually characterised as approximately the same (++) or less (+) than the amount of sucrose detected in the control plants.

	PCR Results		**TLC results**					
a) pML1 transgenic lines	**1-SST /**	**1-FFT**	**F**	**S**	**GF2**	**GF3**	**GF4**	**>GF4**
SST 6; 7; 10; 11; 12; 13; 14	+	–	–	++	–	–	–	–
SST 5	+	–	+	++	?	–	–	–
SST 1	+	–	+	++	+	–	–	–
SST 2	+	–	–	++	+	+	–	–
SST 4	–	–	+	++	?	–	–	–
SST 3; 8; 9; Control	–	–	–	++	–	–	–	–
	PCR Results		**TLC results**					
b) pML2 transgenic lines	**1-SST /**	**1-FFT**	**F**	**S**	**GF2**	**GF3**	**GF4**	**>GF4**
SFT 3; 5; 6; 9; 16; 28	+	+	–	++	–	–	–	–
SFT 8; 14; 15; 22; 25; 26	+	+	–	++	+	–	–	–
SFT 1; 12; 20, 23	+	+	+	++	+	–	–	–
SFT 2; 4; 7; 13; 18	+	+	–	++	+	+	–	–
SFT 17; 19; 29	+	+	+	++	+	+	–	–
SFT 10; 11	+	+	–	+	+	+	+	+
SFT 21; 24, 30	+	–	–	++	+	–	–	–
Control	–	–	–	++	–	–	–	–

In the pML1 transformants, fructan production was only clearly identifiable in two of the transgenic lines, SST 1 and SST 2. With fructan production limited to 1-kestose in SST 1, while oligofructans up to DP4 were detected in SST 2. The presences of oligofructans (2 < DP ≤ 5) were evident in most (more than 65%) of the pML2 transformants. Yet, only two transgenic pML2 plants were identified to produce long chain fructans with DP's > 5. In all these transgenic plants, 1-kestose was the major

transgenic product, being the only fructan detected in 14 of the 23 lines exhibiting oligofructan production (Table 1).

Confirming and identifying the type of sugars present

To confirm the identity of the products that were detected during the TLC screening analyses, the soluble carbohydrate compositions of selected samples were also established by means of HPAEC (Fig. 3 shows representative examples). In the HPAEC analyses, the observed oligosaccharides (DP3-5) were identified as 1-kestose, nystose and fructosyl-nystose, by comparison with the relevant standards also included in the HPAEC run. Similarly the polysaccharides observed in two of the SFT transformants could be recognized as inulin, by comparison with chicory inulin (Fig. 3).

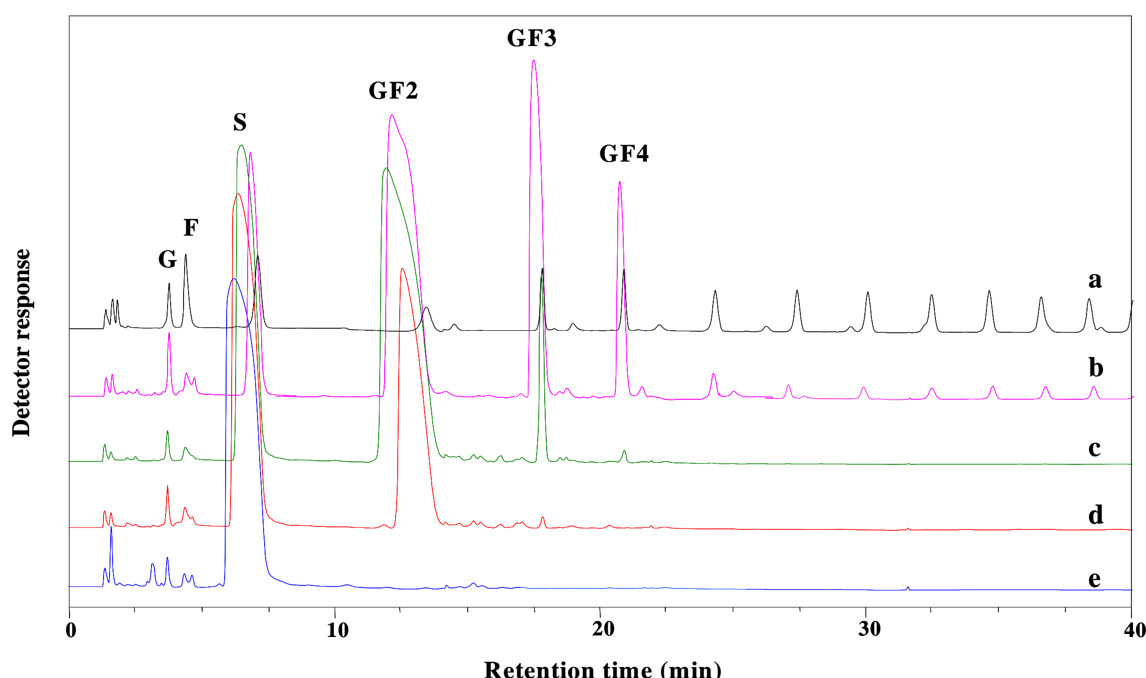

Fig. 3 HPAEC analyses of water soluble carbohydrate extracts from mature internodes of transgenic sugarcane lines b) SFT 11, c) SFT 18 and d) SFT 8 as well as e) an untransformed control sugarcane plant. Chromatogram a) represents an extract from Chicory containing inulin-type fructans. The peaks with retention times that correspond with known standards are indicated with the following abbreviations: G = glucose; F = fructose; S = sucrose; GF2 = 1-kestose; GF3 = nystose and GF4 = fructosyl-nystose.

Additionally, to confirm the identity of the trisaccharide detected with TLC, the hexose composition of the trisaccharide was determined. For this purpose the trisaccharide was isolated from the TLC plates and then hydrolysed enzymatically. The end products of this reaction were subsequently characterized and found to be

fructose and glucose in a 2:1 ratio. The trisaccharide was therefore positively identified as 1-kestose. The identity of the trisaccharide was also confirmed as 1-kestose by means of a GCMS analyses. The observed polymers were also re-established as inulin by proof of their degradability by inulinase (results not shown).

DISCUSSION

The expected products from the functional expression of 1-SST or 1-SST combined with 1-FFT, in cells with sufficient sucrose substrate, is a series of inulin-type fructans ranging from short oligomeric to long chain fructans (Vijn et al. 1997). Since it is known that 1-SST/1-FFT enzymes produce fructans from sucrose in plant vacuoles, and storage of significant amounts of sucrose occurs in the vacuoles of mature internodes of the sugarcane stalks, production of detectable amounts of these fructans is most likely to be achieved in the mature internodes of the transgenic sugarcane stalks (Hawker 1985). Screening for fructan production was therefore conducted on this tissue type.

The results obtained from 1-SST-transgenic sugarcane plants were in agreement with observations made for 1-SST expressing potato (Hellwege et al. 1997) and sugar beet plants (Sévenier et al. 1998), with oligofructans being the transgenic products. The detection of nystose in addition to 1-kestose, in 1-SST-transgenic sugarcane plants, supports the theory that 1-SST can produce longer oligofructans than 1-kestose (Cairns 1995). Furthermore the fact that 1-kestose was the major transgenic product in all the oligofructan producing plants, can be interpreted as confirmation of the proposed cascade-like fashion of fructan synthesis; where a threshold concentration for a molecule of a given chain length must be reached before the next higher homolog is synthesized (Koops & Jonker 1996).On the other hand, since long chain inulins were only detected in sugarcane plants transformed with both 1-SST and 1-FFT; HMW inulin production, as observed in SFT 10 and SFT 11, verifies the crucial role of the 1-FFT enzyme in elongating 1-SST products as proposed in the Edelman and Jefford inulin model (Edelman & Jefford 1968).

Since oligofructan production in the mature internodes were not associated with a distinctive decrease in sucrose content as initially anticipated, it suggests that oligofructan production was occurring in addition to sucrose production rather than as

an alternative to sucrose production. On the other hand, the noticeable decrease in sucrose associated with HMW inulin production, signified that polymeric fructans are produced at the expense of sucrose. One likely explanation for this discrepancy is the difference in ability of invertase to hydrolyse oligofructans compared to HMW fructans (Van der Meer et al. 1998). Hydrolysis of oligofructans by invertase, establishes carbon flow between the hexose's, sucrose and oligofructan pools, incorporating oligofructans into the existing metabolic pathway. On the other hand, HMW inulins cannot be hydrolysed by invertase, resulting in a separate carbohydrate pool without any carbon flow back into the existing pools. The different mechanisms involved in carbon sensing in these two situations, can ultimately affect the normal mechanisms responsible for the regulation of carbon partitioning, flow and source:sink relationship in the plant (Turk et al. 1997). The detection of fructose in a number of oligofructan producing sugarcane could also possibly be attributed to invertase activity in these internodes. These results suggest that oligofructans are fairly sensitive to invertase working.

Though these findings were only based on observations made in preliminary TLC screenings, and did not include concentration determinations; the estimated sucrose contents provided us with a good indication of the expected influence of fructan production on endogenous sucrose content. The intriguing nature of these results, indicating that fructan production will not necessarily take place at the expense of sucrose, justifies further research into this phenomenon. Transgenic fructan producing sugarcane plants can therefore be used in the future as a tool to investigate the mechanisms regulating sucrose accumulation.

The lack of a distinct phenotype, in these fructan producing transgenic plants, could be interpreted to signify that transgenic 1-SST and 1-FFT expression did not have far reaching effects on plant metabolism. It could mean that sucrose conversion was a gradual process, and that the observed fructan accumulation took place over a long period of time. It could also indicate that the plant metabolism was able to sufficiently compensate for the change, or that by targeting the reaction to the vacuole we succeeded in isolating the reaction from affecting the rest of the cell metabolism. The fact that this conversion of sucrose into fructan in transgenic sugarcane was

accomplished without interfering with plant growth and development makes the idea of future application of this technology very appealing.

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CHAPTER 5

Converting sugarcane into a fructan biofactory

ABSTRACT

The previous chapter describe the successful genetic manipulation of sugarcane to enable fructan production. In the transgenic sugarcane lines fructan production of the inulin type was detected in all the sugarcane plant tissue tested, with exceptionally high-level inulin accumulation in mature internodes. Although the full spectrum of inulin molecules as present in artichoke roots was not achieved, inulins with a degree of polymerisation up to 60 were found in sugarcane storage tissue. Up to 78% of the endogenous sucrose in the mature sugarcane culm was converted to inulin. This enabled inulin accumulation up to 165.3 mg g^{-1} fresh weight. This conversion was associated with an increase in total carbohydrate yield. These transgenic sugarcane plants, therefore exhibit great potential as a future industrial inulin source.

INTRODUCTION

Demand for the polymeric carbohydrate known as fructan is rapidly increasing due to its beneficial effects on human health (Weyens et al. 2004). However crop plants that naturally accumulate high concentrations of fructans, are at a production disadvantage relative to more traditional agronomic crops due to their low yield and poor agronomic performance (Caimi et al. 1996). Therefore, in order to meet the increase in consumer demand, crops with quantitatively and qualitatively improved fructan sources are needed (Ebskamp et al. 1994).

In an attempt to make the production of fructans economically more feasible, the potential of transferring the biochemical capacity for the synthesis of fructans to plants with higher agronomic value was explored (Heyer et al. 1999). Fructosyltransferases were cloned from their native plant sources and introduced into productive carbohydrate storing crops, such as sugar beet and potato, that do not make fructans and therefore have the advantage that they lack fructan-hydrolysing enzymes (Turk et al. 1997).

Thus the technical feasibility of transgenic fructan production in non-fructan crops has been demonstrated in tobacco, potato and sugar beet (Vijn & Smeekens 1999;

Hellwege et al. 2000; Weyens et al. 2004). However in very few cases have these discoveries been put into commercial use, since transgenic fructan concentrations were generally to low. Sugar beet was the only plant in which high enough fructan levels were obtained to justify the processing costs (Cairns 2003). This phenomenon can probably be contributed to the exceptionally high sucrose concentrations (the precursor for fructan) naturally present in sugar beet taproot cell vacuoles (the inherent site for fructan synthesis) (Vijn & Smeekens 1999).

The only other agricultural crop with reserve sucrose concentrations as high, and depending on the cultivars, even higher than those found in sugar beet is sugarcane. The expression of fructosyltransferase activities in sugarcane could therefore lead to exceedingly high transgenic fructan production. Since sugarcane is a C_4 species, second only to Napier grass in primary productivity and the most efficient crop species in water use efficiency, it could potentially be the ideal vehicle for large-scale transgenic fructan production. The fact that sugarcane also produces a higher sucrose yield per hectare, at a much lower production cost than sugar beet, adds to the attractiveness of sugarcane as an ideal option (en.wikipedia.org/wiki/sugar and www.sucrose.com/learn.html).

In order to assess the potential of transgenic sugarcane as a large-scale fructan (of defined structure and size) producer, fructan accumulation was characterized in sugarcane plants transformed with both 1-SST and 1-FFT genes from globe artichoke (*Cynara scolymus*).

Here we report that fructan was produced in all the transgenic sugarcane tissues analysed, with exceptionally high-level inulin accumulation in mature internodes. Fructan concentration in the transgenic sugarcane storage tissue was comparable to those found in native plants. Transgenic sugarcane therefore exhibits great potential as a future industrial inulin source.

MATERIALS AND METHODS

Sugarcane transformation

Sugarcane variety NCo310, were co-transformed with pML2 and pEmuKN (both plasmids described in Chapter 4) according to the procedure (including tissue culture

practises, target tissue preparation, microprojectile bombardment and subsequent regeneration) described in Chapter 4.

DNA isolation and polymerase chain reaction (PCR) analysis

DNA was extracted and PCRs for the detection of transgenes 1-SST and 1-FFT were conducted according to the methods described in Chapter 4.

Carbohydrate analyses

Water-soluble carbohydrates were extracted from different tissue types and assayed for fructan production by means of thin layer chromatography (TLC), and high-performance anion exchange chromatography (HPAEC) (see Chapter 4 for description of methods).

Size Exclusion Chromatography (SEC) on Inulin Extracts

The molecular mass distribution of the inulin in different stalk sections of transgenic sugarcane line SFT 11 (internodes 19 – 21, 10 – 12, 1 – 3 and leaf roll), was determined by SEC with dimethyl sulfoxide as eluent. The SEC system consisted of a Dionex P580 pump module, Dionex AS50 Autosampler, column compartment, Dionex model 585 column oven and a Shodex R171 detector. The columns were the PSS GRAM 10 μ guard column, and the PSS GRAM 3000 10 μ and PSS GRAM 100 10 μ, separation columns. The elution of samples was carried out with dimethyl sulfoxide containing 90 mM $NaNO_3$ at a flow rate of 0.5 ml min^{-1} for 60 min at a temperature of 60°C (Heyer & Wendenburg 2001). The system was calibrated with Dextran standards to allow an approximate determination of inulin DP. Data was evaluated via PSS WinGPC compact V.6.20.

RNA extraction and analysis

Northern blot analysis

Total RNA was isolated from young mature leaves using the Cesium Chloride method (Woodhead et al. 1997). Total RNA (12 μg) was size fractionated on formaldehyde agarose gels and blotted onto a positively charged nylon membrane and ultraviolet cross-linked. Membranes were prehybridised in UltraHibe-buffer before hybridised with labelled fragments of the fructosyltransferases encoding 1-SST and 1-FFT-specific cDNA probes: a 264bp EcoRI/BamHI fragment of pML2 (1-SST), and a

351bp XbaI/HincII fragment of pML2. Hybridise overnight at 42ºC in hybridisation buffer containing radioactively labelled probe. Membranes were washed to a final stringency of 0.1× SSC and 0.1× SDS at 65ºC (2× 15 min), and finally exposed to Super Sensitive Phosphor Screen for 4 hours before scanned and visualized with Phosphor Imager.

Quantitative reverse transcription polymerase chain reaction (Q-RT-PCR)

Total RNA was extracted from leaves of transgenic sugarcane plants expressing transgene activity with the RNeasy Mini Kit (QIAGEN). DNA was removed by treatment with the DNAfree Kit (Ambion). RT-PCR analysis was carried out using a Perkin-Elmer ABI PRISM 7700 sequence detection system (Applied Biosystems). The following standard thermal profile was used for all PCR reactions: 30 min at 60°C; 2 min at 95°C; followed by 40 cycles of 15 sec at 94°C, and 1 min at 60°C. For 1-SST detection the program was however slightly modified by extending the time period at 95°C to 15 min. RT-PCR was performed using either 350 ng (for 1-SST and 1-FFT detection) or 3.5 ng (for 18S detection) of total RNA as template. The TaqMan system with the Access RT Kit (Promega) and 500nM of the forward primer FFT-F2: 5'-TCCTGTACCAACACGATTCC-3' and 300 nM of the reverse primer FFT-R2: 5'-TGTTCTCTCCCATCTCAGGC-3' were used for 1-FFT detection, while 200 nM of both 18S-F1: 5'-GTCATCAGCTCGCGTTGACT-3' and 18S-R1: 5'-TCAATCGGT-AGGAGCGACG-3' were used for 18S detection. The two fluorescence-labelled Probes: FFT-FAM: 5'-ACACCGATGATAATTCAGCACCGT-3' and 18S-VIC: 5'-ACGTCCCTGCCCTTTGTACACACCGC-3' were used to quantify the mRNA expression levels of the 1-FFT and 18S genes respectively. Alternatively the One Step RT-PCR Kit (QIAGEN) with 500 nM of each primer, SST-F1: 5'-AATG-GTAATGGGGTCCAAGC-3' and SST-R2: 5'-CGTGAAGCACCTCTTCCTTG-3', and 10^{-5} diluted SYBR-Green double-stranded-DNA specific dye, were used for detection and quantification of the 1-SST transcript. Relative expression was calculated using the comparative cycle threshold method with normalization of data to the geometric average of the internal control gene 18S (Pfaffl 2001).

RESULTS

Analysing transgene expression

To further investigate the expression of the introduced genes, the pML2-transgenic sugarcane plants (described in the previous chapter), containing both genes (as confirmed by PCR analysis, Table 1, Chapter 4) and expressing transgene activity (fructan production whether short or long chain), were analysed by northern blot hybridisation for the expression of the introduced genes. This analysis failed to reveal detectable levels of either 1-SST or 1-FFT transcripts. Therefore, the more sensitive reverse transcription-polymerase chain reaction (RT-PCR) method was used for this purpose. The expected amplification products corresponding to gene fragments at the 5' end of the mRNA of both the 1-SST and 1-FFT genes were detected in most of the transgenic plants analysed. However in terms of relative expression of these genes, two plants (SFT 10 and SFT 11) exhibited significantly higher levels of 1-SST gene expression in addition to relatively high 1-FFT expression (Fig. 1).

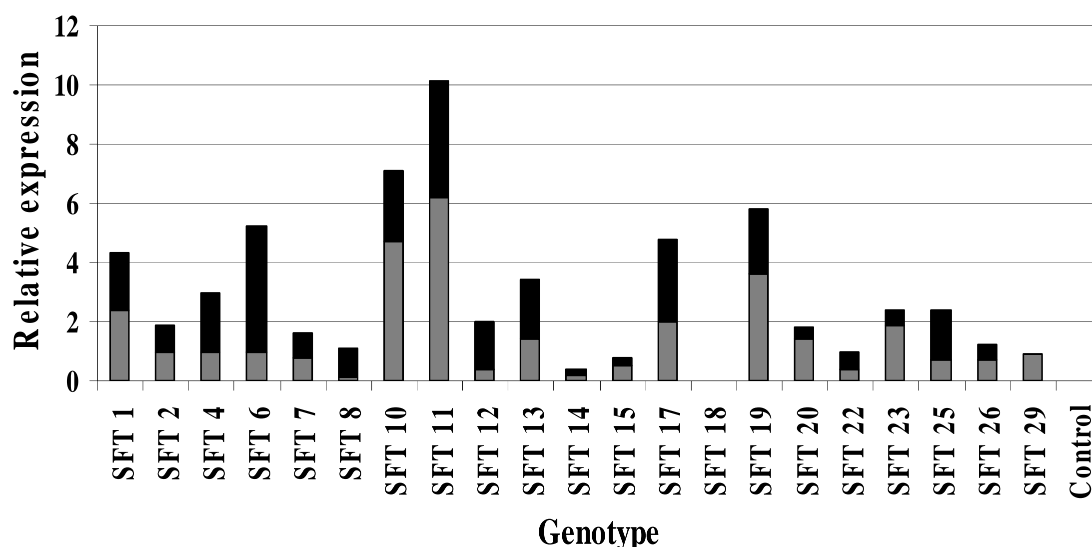

Fig. 1 Quantitative RT-PCR analysis of transgenic sugarcane plants transformed with pML2 (SFT 1 - 30 series) that tested positive for the presence of 1-SST (-■ -) and 1-FFT (-■ -)as well as expressing transgene activity (fructan production) together with an untransformed control. The mRNA levels of the genes were quantified relative to the ribosomal subunit 18S.

These two transgenic lines with combined higher relative expression of the transgenes were also identified by TLC screening (Table 1, Chapter 4) as the only two lines, from a total of 26 transgenic lines that incorporated both fructosyltransferases, in which HMW inulin production was observed.

Characterising fructan accumulation in transgenic sugarcane plant

High-pressure anion exchange chromatography (HPAEC) was used to study fructan production in different types of tissue of the SFT 11 line, with the highest 1-SST and 1-FFT gene expression of all the transformants. All the tissues analysed of this transformant contained inulin-type fructans. The fructan content was significantly lower in leaf tissue compared to the other organs, and only low molecular weight inulin, predominantly 1-kestose, was detected in the leaves. The carbohydrate composition of different sections of the SFT 11 stalk is demonstrated in Fig. 2. A clear pattern, illustrating the relationship between inulin production and internode age, could be identified. This data suggest that the amount of fructan accumulated increase with stalk maturity and that the DP also increase. Moreover, while only 1-kestose and minimal levels of nystose were detected in the leafroll, HMW inulins could be detected in mature internodes (Fig. 2).

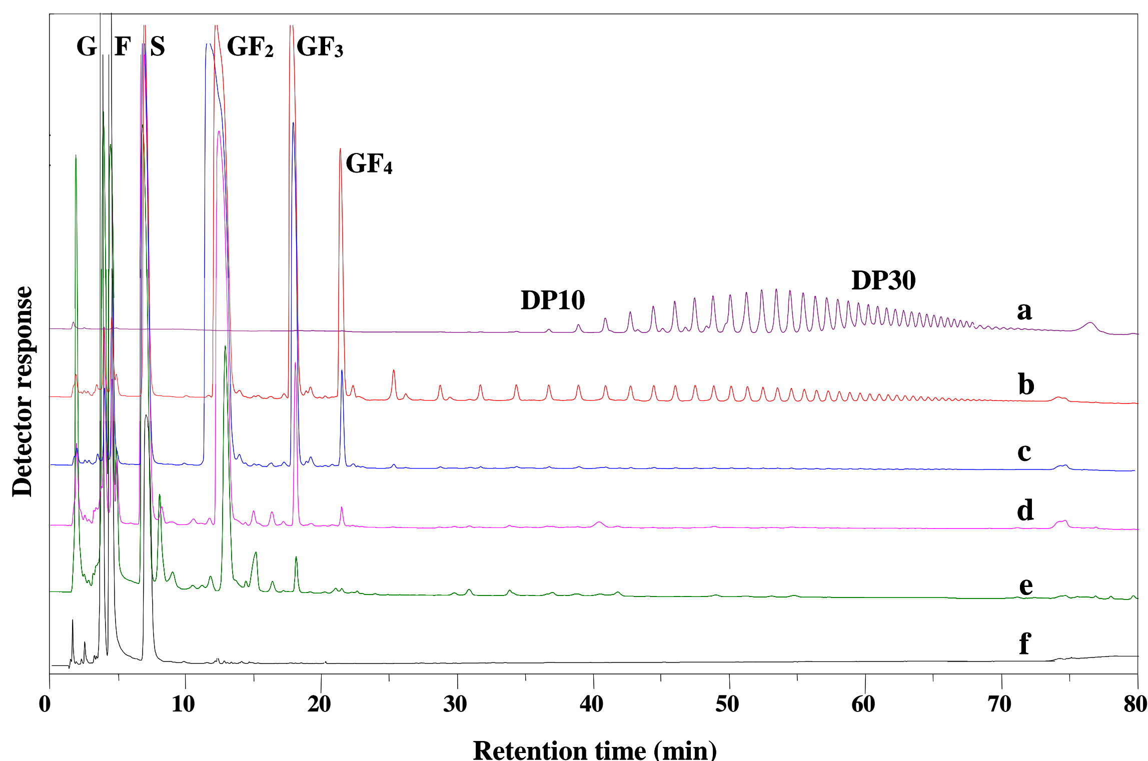

Fig. 2 HPAEC analyses of fructans in different stalk sections of transgenic sugarcane line SFT 11; b) internodes 19 - 21, c) internodes 10 – 12, d) internodes 1 – 3 and e) leaf roll. Chromatogram f) represents an extract from internodes 19 – 21 of a control plant and a) represents a Chicory root extract. Peaks at retention times of known standards are indicated: G = glucose, F = fructose, S = sucrose, GF2 = 1-kestose, GF3 = nystose, GF4 = fructosyl-nystose.

The observed oligofructans (DP3-5) were identified as 1-kestose, nystose and fructosyl-nystose, by comparison with the relevant standards also included in the HPAEC analysis (Fig. 2 b, c, d and e). Whereas the elution pattern of the polysaccharide peaks extracted from the transgenic mature internodes, closely resembled that of chicory, which was indicative of the presence of HMW inulin type fructans in the transformant (Fig. 2 b, a). Polymers up to a molecular weight of 10,000 g mol^{-1} were detected via GPC analyses, which respond to a maximal DP of about 60 (Fig. 3). The analysis of the extracts via SEC showed that with increasing maturity of the tissue, the DP of the inulin polymers also increase (Fig. 3). No fructan molecules could be detected in the control plants (Fig. 2 f).

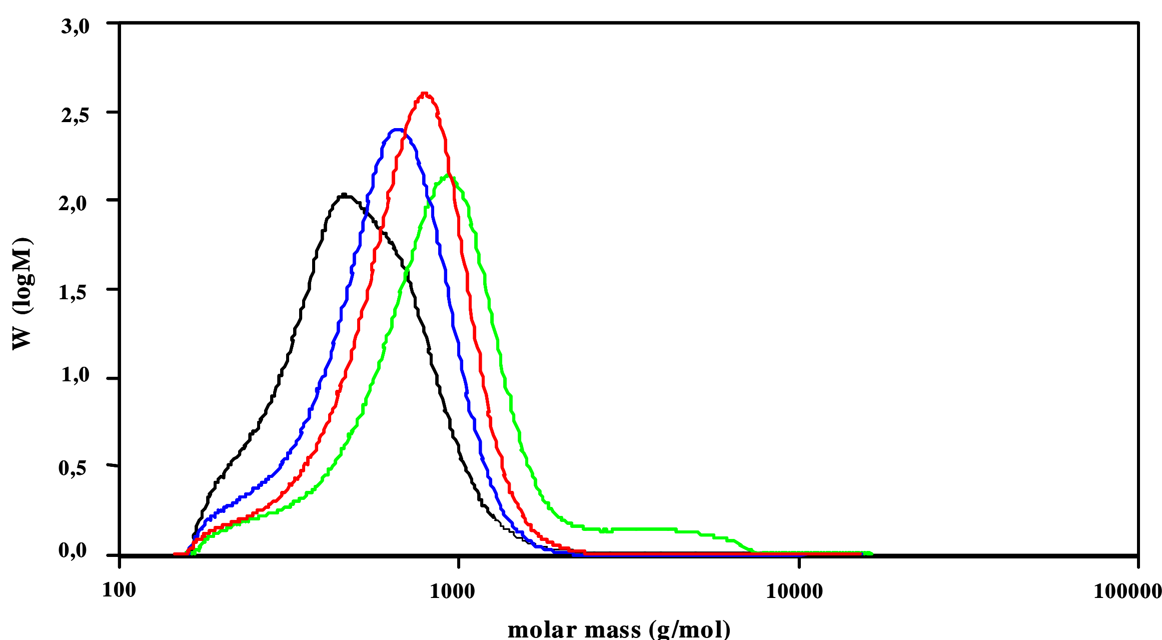

Fig. 3 SEC chromatograms of soluble carbohydrate extracts from different stalk sections; internodes 19 - 21 (—), internodes 10 – 12 (—), internodes 1 – 3 (—) and leaf roll (—); of transgenic sugarcane line SFT 11. The graph shows molecular mass distribution between 300 g mol^{-1} and 10 000 g mol^{-1}.

For a quantitative impression of the productivity of the two transgenic sugarcane lines, SFT 10 and SFT 11, both substrate (sucrose) and product (fructan) levels in their respective storage tissues were determined by HPAEC. When these storage carbohydrate levels were compared to that of the wild type, a drastic reduction in sucrose was observed (Fig. 4). This indicates that large amounts of fructans are produced at the expense of sucrose, the normal storage product of sugarcane. Thus the sucrose concentration in control stalks (internodes 16 to18) was 160.2 mg g^{-1} fresh

weight (FW), whereas it was only 84.8 and 35 mg g^{-1} FW in the corresponding tissues of SFT 10 and SFT 11. Fructans alternatively accumulated to 89.2 and 165.3 mg g^{-1} FW in the respective tissues. Since there were only 53 and 22 % sucrose left of the natural sucrose storing potential of these internodes, it indicated that nearly 50 and 80% of the stored sucrose was converted into fructan.

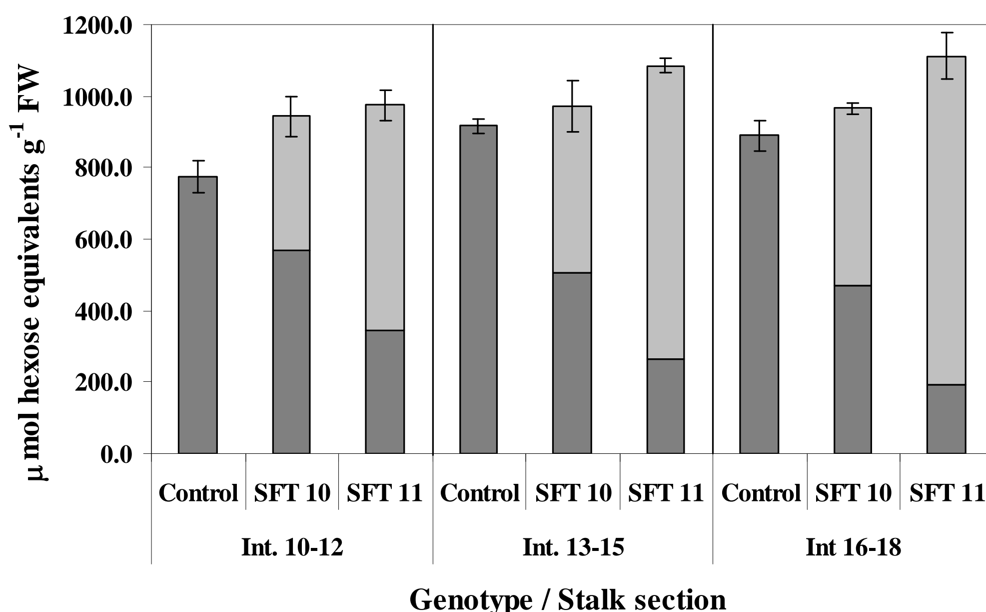

Fig. 4 Sucrose (-■-) and fructan (-□-) contents of selected mature sugarcane stalk sections (consisting of internodes 10 to 12; 13 to 15 and 16 to 18) of pML2 transgenic sugarcane lines (SFT 10 and SFT 11) and a control (untransformed sugarcane). Sucrose and fructan content is expressed in μmol hexose equivalents per g fresh weight (FW) and collective standard deviations are indicated on the combined yields (n = 3).

A comparison of the fructan concentrations in the specified lines at different stages of maturity indicated that fructan content increased over time (Fig. 4). As demonstrated in Fig 4, transgenic fructosyltransferase activity resulted in a significant increase in total soluble storage carbohydrate content (combined sucrose and fructan content). In comparison with the control, the total storage carbohydrate yield was increased with an average of about 101 and 198 μmol hexose equivalents g^{-1} FW in each of the selected internode sections of SFT 10 and SFT 11 respectively.

DISCUSSION

Quality and quantity of inulin is determined by enzyme concentration and substrate availability

Both 1-SST and 1-FFT are unusual enzymes in that they do not show simple Michaelis-Menten kinetics; their activity depends on both the substrate and the enzyme concentration and is essentially nonsaturable (Vijn & Smeekens 1999). This suggests that the variation in chain length of the inulin molecules observed in the different transgenic sugarcane lines could be due to different expression rates of the transgenes.

Mature sugarcane internodes, having an abundance of sucrose, low invertase activity and lacking fructan-hydrolysing enzymes, meet all the requirements identified in previous transgenic studies for high-level fructan production (Hawker 1985; Ritsema & Smeekens 2003). Expression of the 1-SST and 1-FFT genes from globe artichoke, in sugarcane tissue with sufficient sucrose substrate, is expected to lead to the production of HMW inulin with maximum chain lengths between DP 60 and 200, since the source plant is renowned for the production of these exceptionally long chain plant inulins (Hellwege et al. 1998). Therefore after sugarcane was transformed with both these transgenes, screening was conducted on the mature internodes, identified in accordance with these criteria, as the tissue type most suited for transgenic high-level, long chain inulin production.

Consequently, when only two of the 26 transgenic lines that incorporated both transgenes were identified to produce HMW inulin in their mature internodes, unsuccessful transgene expression and/or insufficient enzyme activity were suspected. Though low-level expression of the mRNA's was demonstrated in most of the transformed sugarcane, by the sensitive RT-PCR method, two lines stood out with significantly higher 1-SST transcript levels (Fig. 2).

The correlation between the RT-PCR and TLC-screening results, indicating the same lines with higher 1-SST expression as the lines with HMW inulin production, suggests that the induction of inulin synthesis seems to be controlled by 1-SST activity. In previous related studies a direct association between the enzyme concentration and the nature of the product was observed, when an increase in 1-SST enzyme concentration

led to the synthesis of longer oligofructans (higher DP than 1-kestose) (Ritsema & Smeekens 2003). If then also the theory proposed by Hellwege et al (1998) is correct; suggesting that the 1-FFT enzyme of globe artichoke have a higher affinity to longer oligofructans as fructosyl acceptors; it will mean that only lines with high enough 1-SST enzyme concentrations, producing oligofructans of adequate length, will have the suitable substrate required for subsequent 1-FFT activity and ultimately HMW inulin production. It is therefore reasonable to conclude that the primary reason for the low incidence of HMW inulin accumulation in the mature internodes of the different transformants were insufficient 1-SST message expression, resulting in inadequate 1-SST enzyme quantity.

Complete opposite conditions exist in the autotrophic and metabolically active tissue of sugarcane, with little sucrose accumulation and high endogenous soluble acid invertase activity (Moore & Maretzki 1996). Not only does invertase and fructosyl transferase compete for the same substrate, but invertase is also capable of hydrolysing oligofructans. For these reasons, despite the fact that we used the constitutive maize ubiquitin promoter, capable of expression in any cell type, and regardless of the high transgene expression rates in SFT11, we expect very little or no inulin production in leaves and young internodes of this plant, due to limited sucrose availability in these organs (Hawker 1985).

The HPAEC results of leaf and stalk tissue (Fig. 2) from the transgenic sugarcane line SFT11, is in agreement with what we know about sucrose concentration in sugarcane and its expected influence on transgenic inulin production. Inherently sucrose concentrations are very low in leaves and young internodes, increasing from the top (young internodes) to the bottom of the stalk (old internodes) where large quantities are accumulated. The level of inulin accumulation and the DP of the synthesized inulin increased as sucrose content increased, reflecting the differences in sucrose concentration in each tissue type.

The quantitative analysis of the sucrose and inulin levels in the two HMW inulin producing transgenic sugarcane lines, revealed a substantially higher sucrose conversion with consequentially more inulin production and ultimately higher inulin accumulation in SFT11 compared to SFT10. The differences observed between the

inulin levels of the two lines, reflected the differences in their transgene expression levels as illustrated in Fig. 2.

Taken together, the evidence suggests that transgenic enzyme concentration and source plant sucrose substrate availability have a direct effect on the quality (with reference to average molecular weight) and quantity of the inulin produced.

Exceptionally high fructan concentrations obtained in transgenic sugarcane

Transgenic fructan levels attained in previous studies, in which plant fructosyltransferases were expressed in non-fructan plants, were generally very low, with concentrations below 9 mg g^{-1} FW, with the exception of transformed sugar beet (Cairns 2003). Sugar beet transformed with 1-SST from Jerusalem artichoke and onion, at two separate occasions, resulted in oligofructan production with maximums of 62.3 mg g^{-1} FW and 104.4 mg g^{-1} FW respectively (Sévenier et al. 1998; Weyens et al. 2004). Also when both 1-SST and 6G-FFT from onion were expressed in sugar beet, fructans of the inulin neo-series were produced at a high of 87.7 mg g^{-1} FW (Weyens et al. 2004).

Transgenic sugar beets have therefore been the only plants produced thus far, that could compete with endogenous reserve accumulation of between 60 and 150 mg g^{-1} FW as found in Jerusalem artichoke and chicory (Cairns 2003). However when the productivity of the transgenic sugarcane line SFT 11, with the highest fructan production attained in this study, were compared to that of the transgenic sugar beet plants with the highest fructan levels achieved thus far; it was found that even though the transgenic sugarcane displayed a similar sucrose conversion rate to that of the transgenic sugar beets (78 % in SFT 11 compared to 91 % by Sévenier et al. (1998) and 72 and 67 % by Weyens et al. (2004)), the endpoint fructan concentration obtained (165.3 mg g^{-1} FW in SFT 11 storage tissue), surpasses all the yields previously reported.

Although subsequent field trials with these plants are still required before the true potential of sugarcane as a fructan-biofactory can be assessed, the preliminary results seem to satisfy all the demands for commercial production. These results clearly show the potential of the sugarcane plant as a bioreactor to efficiently produce fructans. In

future additional engineering to define the structure and size fructan produced can be considered.

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CHAPTER 6

Fructan accumulation and sucrose metabolism in transgenic sugarcane

ABSTRACT

Vacuolar constitutive expression of the sucrose:sucrose 1-fructosyltransferase gene in sugarcane resulted in fructan production, predominantly as 1-kestose, throughout the plant. Though very little 1-kestose was detected in immature leaves and internodes, these levels increased as the organs matured, reaching up to 37 and 830 µmol hexose equivalents per gram fresh weight in mature leaves and internodes respectively. Fructan accumulation in leaves did not affect endogenous sucrose concentrations, resulting in an 84% increase in total sugar content. In contrast with this, the sucrose content of mature internodes that accumulated high levels of 1-kestose was severely reduced. However, increases in total sugar content, in some instances up to 63% higher than control plants, were observed. This phenomenon was investigated with the use of radio-labelled-isotopes. An increase in the allocation of incoming carbon towards sucrose storage, resulting in higher carbon partitioning into both 1-kestose and sucrose, were detected in the culms of transgenic compared to control lines. This modification therefore established an extra carbohydrate sink in the vacuoles of the various organs of the transgenic plants that affected photosynthate partitioning and increased total soluble sugar content. The data suggests that sucrose sensing is the main regulatory mechanism responsible for adapting carbon flow in the cells to maintain sucrose concentration.

INTRODUCTION

Plant growth, development and yield, are dependent upon the efficiency of carbon fixation and partitioning of the reduced carbon between various biosynthetic pathways. Ultimately the production of carbohydrates, distribution of these carbohydrates between various parts of a plant and carbon loss due to respiration determines yield (Moore & Maretzki 1996).

Carbon dioxide is reduced in the chloroplasts of the leaf (source) cells through the process of photosynthesis and this result in the production of carbohydrates. In most plants the primary product of carbon fixation is sucrose. Sucrose is also the dominant carbohydrate exported via the phloem system to heterotrophic tissues (sinks) where it

is either utilised for growth or deposited as storage carbohydrates (ap Rees 1984). Sucrose is therefore involved in the fundamental processes of photoassimilate transport, partitioning, and accumulation, which form the basis for the productivity and success of any plant (Moore 1995b).

The critical roles of sucrose in plant growth and development have been demonstrated in transgenic C_3 plant systems by altering the expression of sucrose-cleaving enzymes. Examples are the expressing of a yeast-derived invertase in different sub-cellular compartments of tobacco, Arabidopsis, tomato and potato (Sonnewald et al. 1991; Heineke et al. 1992) and decreased expression of endogenous invertase activity in tomato (Scholes et al. 1996). These genetic modifications induced severe phenotypical changes and demonstrated the central importance of compartmentation of sucrose with respect to its biosynthesis, storage and distribution (Herbers & Sonnewald 1996).

Sucrose accumulation has been studied more in sugarcane than in any other plant, due to its unique ability to accumulate significant quantities of sucrose in its culm (Moore 1995b). Sugarcane (*Saccharum* spp.) is a robust grass species belonging to the group of plants, which are characteristically the world's most productive, utilizing the C_4 mechanism of CO_2 fixation (Grof 2001). Similar strategies that involve genetic manipulating of key enzymes in the sucrose metabolic pathway were also implemented in sugarcane to investigate the role of invertase in sucrose accumulation. In a study by Ma *et al.* (2000), a yeast invertase was targeted to the cytoplasm, apoplast and vacuole of sugarcane callus. This intervention in the primary metabolic pathway of sugarcane resulted in changes in carbon partitioning and sugar composition, emphasizing the significance of the invertase enzyme in the sucrose accumulation process (Ma et al. 2000). In contrast with these findings, no significant change in sucrose yield or purity was observed when acid invertase activity was reduced with 70% in transgenic sugarcane plants. This consistency in sugar levels demonstrated the substantial internal buffering that exists in the sugarcane metabolic network, through which carbon flow could be adapted to restore flux equilibrium (Botha et al. 2001).

An interesting alternative method to manipulate sucrose metabolism in transgenic plants is the introduction of foreign genes causing consumption of sucrose, by converting sucrose into a new carbohydrate that does not ordinarily exist in the plant (Herbers & Sonnewald 1996). Fructans (oligo- and poly- fructosyl sucrose) and palatinose (a sucrose isomer) are examples of carbohydrates that have been produced in this manner in a number of crop plants. Due to the distinct nature of both, fructan and palatinose, transgenic plants producing these compounds provide a novel and powerful tool to address a variety of physiological questions in order to gain useful insight into the general metabolism of sugars in higher plants (Sonnewald et al. 1994). For instance, in contrast with sucrose, palatinose and polymeric fructans, can't be hydrolysed into their constituent hexoses in these transgenic plants, since they lack the necessary native hydrolysing enzymes. Thus, because signalling and control mechanisms that operate on plant metabolism are incompletely understood, and based on considerations of the differential capacity of plants to hydrolyse and to sense sucrose versus related compounds, transgenic plants can be used to investigate metabolite-specific signal perception and transduction (Börnke et al. 2002; Birch & Wu 2005). Using appropriate promoter elements and targeting signals, transgenic expression and synthesis of new sugars can be directed to specific compartments and organs. Accordingly, sink-source relationships can be altered to study sink development, sink regulation of photosynthesis and factors that might limit sink strength and source capacity. Consequential changes in carbon availability and carbohydrate accumulation, also offers a way in which to study co-ordination of metabolism, transport of sucrose and osmoregulation (Sonnewald et al. 1991).

A highly efficient sucrose isomerase was targeted to the vacuoles, the sucrose storage compartment, within the mature stalk internodes of the sucrose storing plant sugarcane. With this approach an adequate supply of sucrose, the substrate for palatinose production, was ensured at the site of enzyme activity. Expression of the introduced gene in sugarcane resulted in the partial conversion of sucrose into palatinose, a substance that is not perceived in an equivalent manner as sucrose within the plant. The effect was that source-sink signalling and consequently metabolic flows were altered, resulting in the accumulation of higher yields of the endogenous sugars and much higher total soluble sugar content (Birch & Wu 2005).

The purpose of this study was to alter the normal biosynthesis, storage and distribution of sucrose in sugarcane by establishing transgenic fructan production; and to determine what effect this would have on carbohydrate metabolism and partitioning. Transgenic fructan production can be initiated in plants that do not ordinarily accumulate fructans, by the introduction of fructosyltransferase genes as demonstrated in several previous studies (see Cairns 2003) for review on latest developments in this field). Here we report on the changes in metabolism of sugarcane plants in response to the constitutive expression of the *Cynara scolymus* sucrose:sucrose 1-fructosyltransferase (1-SST) gene by means of comparison with near-isogenic wild-type plants.

The conversion of sucrose into fructan in the vacuole of various organs of sugarcane could potentially affect the capacity of the plant organs to allocate carbohydrates, resulting in a disturbance of the normal source-sink interaction, which could have four consequences; (i) it could established an extra carbohydrate sink in the source leaves resulting in a decrease in carbohydrate export causing a depletion of the carbohydrate source for sink development (Börnke et al. 2002) (ii) or change sugar signalling for example 'feedback' control of sucrose on source metabolism, leading to a change in photoassimilate production and consequently net biomass accumulation (Van der Meer et al. 1994) (iii) it is expected to have a major effect on carbohydrate composition of the internode and this could in turn alter the regulation of carbon partitioning and growth (Sévenier et al. 1998) (iv) conversion of sucrose to fructans could alter the water potential balance and subsequently effect water movement, the main driver in growth of the internode (Cairns 2003). Analysis of the resulting effects on photosynthesis, carbohydrate partitioning and sucrose metabolism will expectantly increase our understanding of the regulation of sucrose contents of different plant organs (Hellwege et al. 2000).

Here we report that the expression of 1-SST in sugarcane resulted in the conversion of sucrose into predominantly 1-kestose. It is then proposed that through the consequential change in sucrose signalling, metabolic flows were altered, resulting in an increased flux into sucrose storage, ultimately leading to the accumulation of a higher total soluble sugar content.

METHOD AND MATERIALS

Sugarcane transformation

Sugarcane variety NCo310, were co-transformed with pML1 and pEmuKN (both plasmids described in Chapter 4) according to the procedure (including tissue culture practises, target tissue preparation, microprojectile bombardment and subsequent regeneration) described in Chapter 4.

Plant material

Transgenic and control sugarcane plants were grown to maturity in the greenhouse under natural daylight conditions at 28-30˚C before sampled for subsequent experiments. Stalks from separate plants of two selected individually transformed lines and three different control plants were harvested in the morning. The leaf with the first exposed dewlap was defined as number 1, and the same number designated the attached internode. Internodes and their attached leaves were numbered sequentially down the stalk.

HPLC analyses of sugars

Leaf number 1 and 5 and stalk sections comprised of internodes 1 to 3 and 13 to 15 were selected to represent immature and mature tissue respectively of both tissue types. Samples from the two tissue types, at the specified stages of development, were taken from the stalks identified for harvesting (see plant material description above). The collected fresh plant material (50 mg fresh weight) were powdered in liquid nitrogen and extracted in 1 ml 10mM sodium phosphate buffer pH 7.4 at 95°C for an hour, followed by two additional extractions for 30 min each in 0.5ml buffer. Extracts were centrifuged at 10,000 g, and supernatants pooled. The combined supernatants were then dried under vacuum and re-dissolved in deionised water (with a ratio of 1 mg fresh plant weight (FW) to 10 µl water). These extracts were filtered (0.45 µM sterile syringe filter) and further diluted in order to achieve linear calibration when the samples were analysed by high-pressure anion exchange chromatography (HPAEC). The HPAEC analyses of these samples were conducted according to the method described in Chapter 4.

Radiolabelling of internodal tissue discs

Internode sections were collected and longitudinal cores (9 mm diameter) were excised mid-way between the core and periphery of the internodes with a cork borer. Transverse sections (1 mm) were cut from these cores using a hand microtome and immediately placed in 30 ml wash buffer containing 25 mM K-Mes (pH 5.7), 250 mM mannitol and 1 mM $CaCl_2$ (Lingle 1989) and kept on ice for at least 15 min (Bindon & Botha 2001). Three repetitions of each sample (consisting of 6 tissue discs each taken from the core of the internodes and equally divided between repetitions) were taken from young stalk sections (comprising of internodes 4 and 5) as well as from mature stalk sections (comprising of internodes 11 and 12) of each of the harvested stalks.

Excess washing buffer was blotted from tissue discs before being transferred to 250 ml Erlenmeyer flasks (6 discs per flask) containing 1 ml buffer made up of 25 mM K-MES (pH 5.7), 250 mM mannitol, 5 mM fructose, 5 mM glucose, and 7.5 µCi [U-^{14}C]glucose (55.2 MBq mmol^{-1}) (Vorster & Botha 1999). Discs were vacuum-infiltrated for approximately 5 seconds and the flasks sealed with rubber stoppers. Samples were incubated for 5.5 h on a rotary shaker at room temperature. $^{14}CO_2$ released over the incubation period was collected hourly in vials containing 500 µl 12% (w/v) KOH. For CO_2 determination, 4 ml scintillation cocktail (Ultima Flo™ M, Packard) was added to the KOH vials and counted for 15 min in a Beckman LS 1801 scintillation counter to determine released radioactivity. After incubation discs were rinsed with ice-cold ddH_2O and washed for 10 min in 30 ml wash buffer. Thereafter excess buffer was removed and tissues flash frozen until extraction (Bindon & Botha 2001).

Fractionation of cellular constituents

Discs were weighed and chopped-up with a scalpel before it was transferred to eppindorf tubes with 1 ml extraction buffer containing 50 mM Tris (pH 7) and 70% (v/v) ethanol. The tissue was then crushed with an eppi-pestle in the buffer and incubated overnight at 75ºC. Extracts were centrifuged at 12,000 g for 10 min at 25˚C and the supernatant removed and transferred to a new tube. The remaining pellet was extracted for a second time with 1 ml extraction buffer for 15 min at 75ºC, centrifuged as before, and supernatants pooled to render the total water-soluble fraction in a

volume of 2 ml. 40 µl of the total extract was added to 4 ml scintillation cocktail (Ultima Flo™ M, Packard) and counted for 15 min in a Beckman LS 1801 scintillation counter to determine radioactivity in the total water-soluble fraction. The pellets with 500 µl added Soluene®-350 (Packard), were incubated in the dark at room temperature until the tissue was dissolved. In order to determine the radioactivity in the total insoluble fraction, the dissolved pellet was added to 4 ml scintillation cocktail (Ultima Flo™ M) and counted for 15 min in a Beckman LS 1801 scintillation counter.

Water-soluble components were fractionated, by passing 1ml of the total extract through cation and anion exchange columns in tandem (Dowex 50W–X8 and 1W-X8 resin in the H^+ and formate form respectively, packed in 1ml columns) (Dickson 1979). The yielded neutral fraction of the extract, i.e. the sugars, was eluted with 10 ml 70% EtOH into containers with 300 µl 1M Tris pH8. Subsequently the acid (consisting of amino acids) and basic (consisting of organic acids and sugar-phosphates) fractions were eluted with 10 ml 6 M NH_4OH and 10 ml 6 M formic acid respectively. The different fractions were reduced completely, and dried samples resuspended in 600 µl ddH_2O. Aliquots of each of the fractions were added to 4 ml Ultima Flo™ M scintillation cocktail and radioactivity measured for 15 min in a Beckman LS 1801 scintillation counter. The readings obtained for the last two fractions were added together to represent a combined Acidic/Basic fraction. Estimated recovery of all three water-soluble fractions was 98%.

TLC analyses of water-soluble carbohydrates

Water-soluble carbohydrates present in the total water-soluble fraction were separated by means of TLC. TLC analyses were performed on silica gel 60 foils (Merck) on which 1µl of each extract was spotted per lane, with two lanes per sample. 10 mg ml^{-1} glucose, fructose, sucrose, 1-kestose and nystose combined with 7.4 kBq ml^{-1} [U-^{14}C]glucose, [U-^{14}C]fructose and [U-^{14}C]sucrose were used as standards. The experiment was done in duplicate and both TLC foil replicas for each set of samples were developed twice to the top of the foil in ethylacetate:propanol:water (6:3:1, v/v/v) (Lenkey et al. 1986). One TLC foil of each set of samples was stained with a fructose specific urea phosphoric acid spray (Wise et al. 1955), while the other one was stained with a 5% sulphuric acid spray (Lenkey et al. 1986).

The different sugar fractions were identified by comparison with the standards, and the concentrations of these sugars were determined by densitometry. The radioactivity in the different water-soluble carbohydrates was visualized on phospho-screens, which were exposed to the fructose specific stained TLC's, and scanned with a phospho-imager. The amount of radioactivity in each of the different sugars identified on the TLC's was determined by means of densitometry.

RESULTS

Soluble carbohydrate composition of 1-SST transgenic sugarcane plants

Sugarcane calli were transformed with a chimeric construct containing the 1-SST gene comprising a vacuolar targeting signal, under control of the constitutive maize ubiquitin promoter. Among the geneticin-resistant primary transformants, which contained the 1-SST construct, two individually transformed lines were selected on basis of their TLC screening results. The presence of 1-kestose in the mature internodes of these transgenic lines demonstrated the expression of 1-SST activity in these plants.

Phenotypically the 1-SST transformants did not exhibit any visual amendments when compared to untransformed control plants regenerated and cultivated under the same conditions. Different parts of these plants at different stages of development were further analysed at the product level by comparison with corresponding tissues from control plants.

High-pressure anion exchange chromatography (HPAEC) was used to analyse both immature actively growing and mature, leaf (leaf 1 and 5) and stalk tissue (internode sections consisting of internodes 1-3 and 13-15) of the transgenic sugarcane lines for the presence of 1-kestose and other oligofructans. Of the two lines expressing 1-SST gene activity, line 2 accumulated 1-kestose in all the tissues tested (shown in Fig. 1).

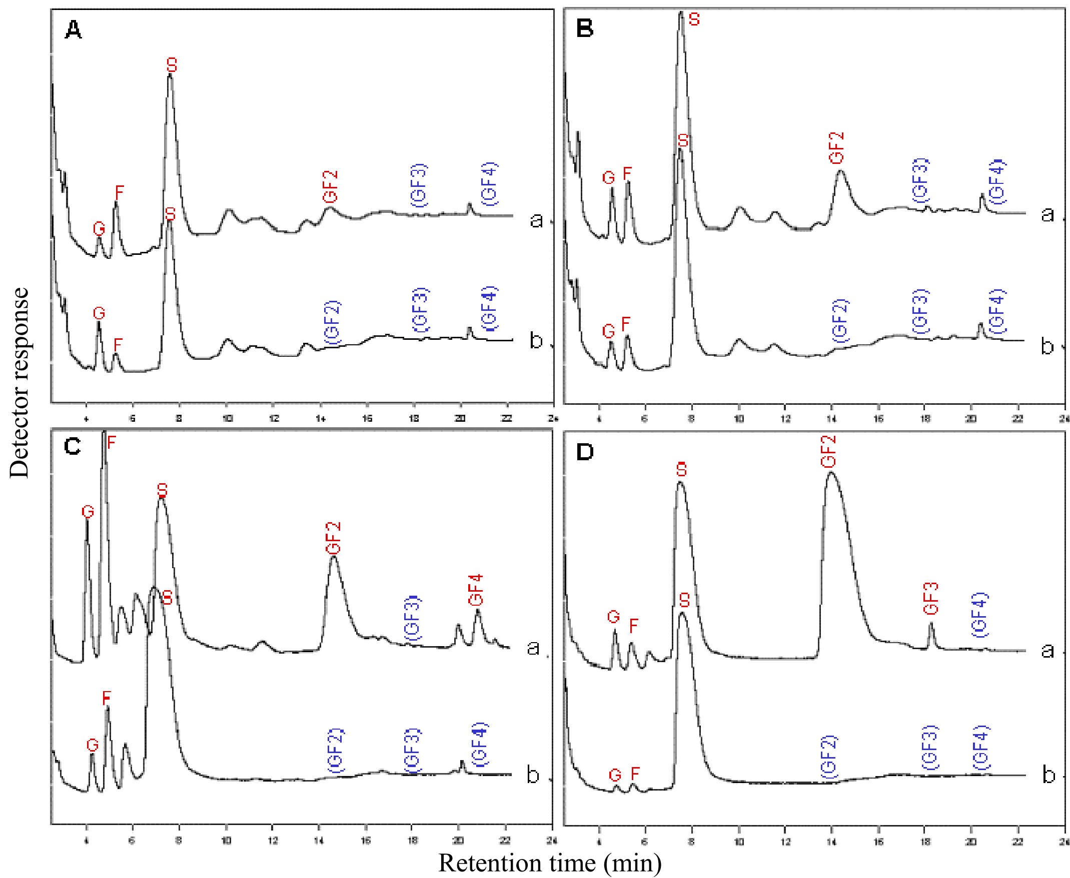

Fig. 1 HPAEC analyses of water-soluble carbohydrates in young leaves (A), mature leaves (B), immature internodes (C) and mature internodes (D) of transgenic sugarcane line 2 (a) and an untransformed control (b). F: Fructose; G: Glucose; S: Sucrose; GF_2: 1-Ketose; GF_3: Nystose; GF_4: Fructosyl-nystose.

1-Kestose was the predominant fructan produced in all the plant organs. However, besides 1-kestose, traces of oligofructans with higher degrees of polymerisation could also be detected in transgenic sugarcane stalks, with fructosyl-nystose (GF_4) in immature internodes and nystose (GF_3) production in mature internodes. All types of fructan molecules were absent in the control plants (Fig. 1).

In order to investigate the effect of fructan production on carbohydrate composition, the soluble carbohydrate content separated by HPAEC, were determined based on standardization with pure substances. In this manner carbohydrate composition and total yield were determined for untransformed control plants and the two different transgenic lines grown under greenhouse conditions (Fig. 2).

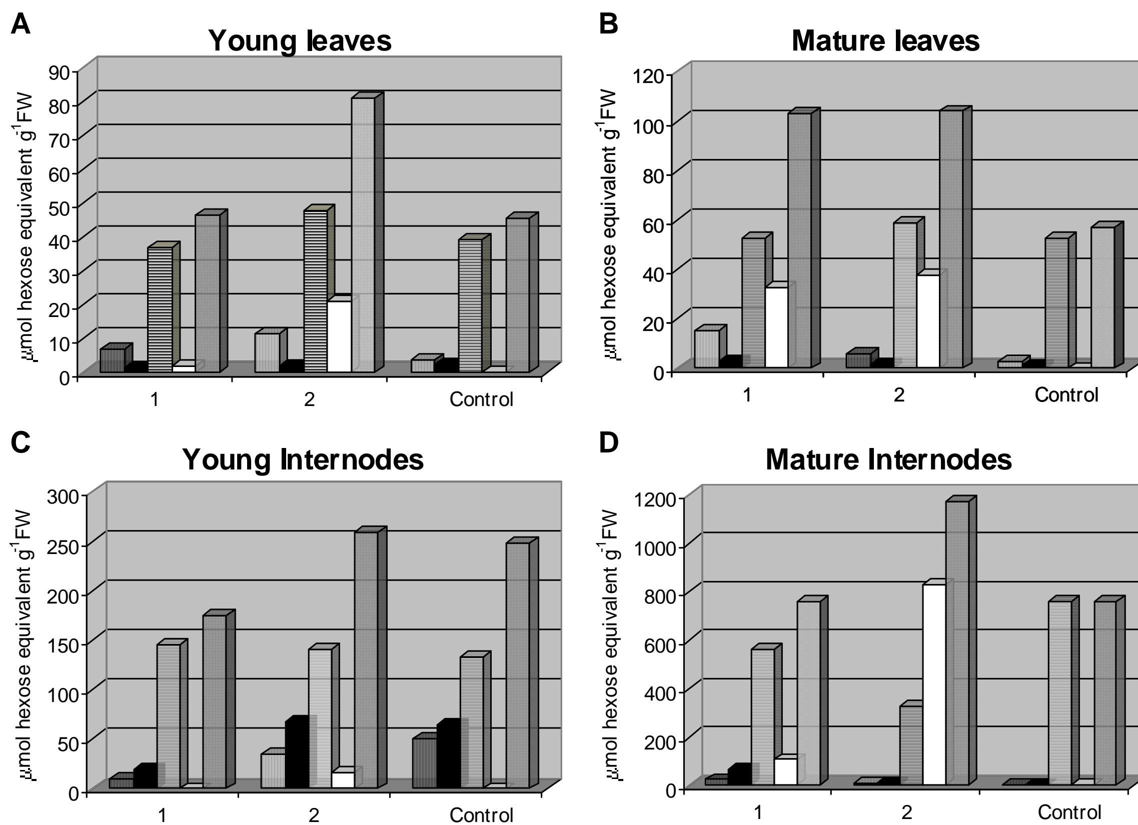

Fig. 2. Soluble carbohydrate levels: 1-kestose (-□-), sucrose (-▤-), glucose (-▥-), fructose (-■-), and total (-▨-), in young leaves (A), mature leaves (B), immature internodes 1–3 (C) and mature internodes 13-15 (D) of 1-SST-transgenic sugarcane lines 1 and 2 and an untransformed control.

Transgenic sugarcane line 2 exhibited higher 1-kestose accumulation throughout the plant, in all the different tissue types, when compared to line 1. Fructan production in young and mature leaves was coupled to a significant increase in total soluble sugar concentration. Glucose and fructose levels did not contribute much to the total soluble carbohydrate concentrations of leaves, however a slight increase in glucose content was detected in both types of transgenic leaves. Very little 1-kestose was detected in immature internodes and this production had no visible impact on the other sugar concentrations. The sucrose content of mature internodes that accumulated high levels of 1-kestose were severely reduced (Fig. 2).

Fructan synthesis in these internodes seems to be occurring at the expense of some sucrose accumulation. However, there was evidently not a 1:1 stoichiometry between the decrease in sucrose and increase in the new product, as the total sugar content increased in line 2. 1-Kestose levels in mature internodes were approximately 3 times

higher in line 1, and 22 times higher in line 2 when compared to 1-kestose levels in the mature leaves. In line 2 the amount of trisaccharide exceeded that of sucrose (Fig. 2).

With respect to the hexose levels in these plants, significant changes in glucose and fructose concentrations were observed in line 1 when compared to line 2 and control plants. In the young internodes, where hexoses are expected to contribute significantly to the total sugar content, as seen in line 2 and control plants, the glucose and fructose concentrations of line 1 were significantly lower, resulting also in significantly lower total sugar concentrations. In contrast with this, significantly higher hexose concentrations were observed in mature internodes of line 1 (Fig 2). The steady-state levels of oligofructans with DP's > 3 were insignificant and thus for the purpose of this study excluded from the data set.

[U-^{14}C]glucose labelling studies in 1-SST transgenic sugarcane plants

To analyse the effect of transgenic fructan production on carbohydrate metabolism in sinks, uniformly labelled glucose was supplied externally to internodal tissue discs and the extent of carbon partitioning and flux within sinks were analysed. This was done to allow a comparison of carbon partitioning into different cellular constituents, and to enable calculation of metabolic flux in internodal tissues of 1-SST-transgenic and control sugarcane plants.

Table 1 Sugar content in young and old internode sections, separated by TLC and determined with spot densitometry in 1-SST-transgenic sugarcane lines 1 and 2 and an untransformed control. Each value is the mean ± SD of 3 separate samples, with 3 repetitions per sample.

		Content (μmol hexose equivalents g^{-1} FW)				
Event	**Internode no.**	**Glucose**	**Fructose**	**Sucrose**	**1-Kestose**	**Total**
Line 1	4 + 5	10.2 ± 6.1	12.3 ± 7.4	340.1 ± 146.5	11.0 ± 6.0	373.6
Line 2	4 + 5	27.5 ± 17.9	8.7 ± 3.6	339.3 ± 120.0	80.0 ± 96.5	455.6
Control	4 + 5	13.9 ± 9.6	15.9 ± 4.4	272.5 ± 32.7	0.0	302.2
Line 1	11 + 12	4.3 ± 1.2	4.0 ± 1.1	600.1 ± 122.9	15.8 ± 5.4	624.1
Line 2	11 + 12	4.9 ± 0.7	7.6 ± 2.2	434.7 ± 53.2	250.6 ± 76.8	697.8
Control	11 + 12	3.3 ± 0.4	4.0 ± 1.7	419.6 ± 30.3	0.0	426.8

The sugar content in the control plants and two transgenic lines differed significantly, with additional 1-kestose accumulations and higher sucrose levels resulting in significantly higher total sugar concentrations in both internode sections of the transgenic lines (Table 1). In line 1 the production of kestose in the mature internodes (11 and 12) was not accompanied by a decrease in the sucrose concentration as observed in internodes 13 to 15 (Fig. 2); instead an increase in sucrose was detected in line 1. A tendency towards higher sucrose accumulation in internodes 4 and 5, associated with 1-kestose accumulations, ultimately leading to an increase in total carbohydrate content was also observed. When the two sets of data was compared (Table 1 and Fig. 2), a drastic increase in 1-kestose concentrations (×6.8 in line 1; ×3.3 in line 2) together with a decrease in sucrose levels (×1.07 in line 1; ×1.33 in line 2) were observed in the successive mature internode sections (from internodes 11 and 12 to internodes 13 to 15). As established in the HPAEC analyses of these plants, the production of oligofructans other than 1-kestose was insignificant and thus excluded.

Partitioning of label in internodal tissue

After an incubation period of 5.5 hours with uniformly labelled glucose, the amount of label released as $^{14}CO_2$ during incubation and incorporated by the internodal cells were determined. Internodal discs were homogenised and water-soluble carbohydrates were extracted, separating the soluble fraction from the insoluble (pellet) fraction. The radioactive readings in CO_2 together with the readings in the water-soluble and insoluble cellular constituents resembled the total amount of metabolised label. To determine whether fructan production had an effect on label distribution, the percentage of label incorporated into each of the fractions were calculated and the incorporation patterns of transgenic lines were compared to that of the control (Fig. 3).

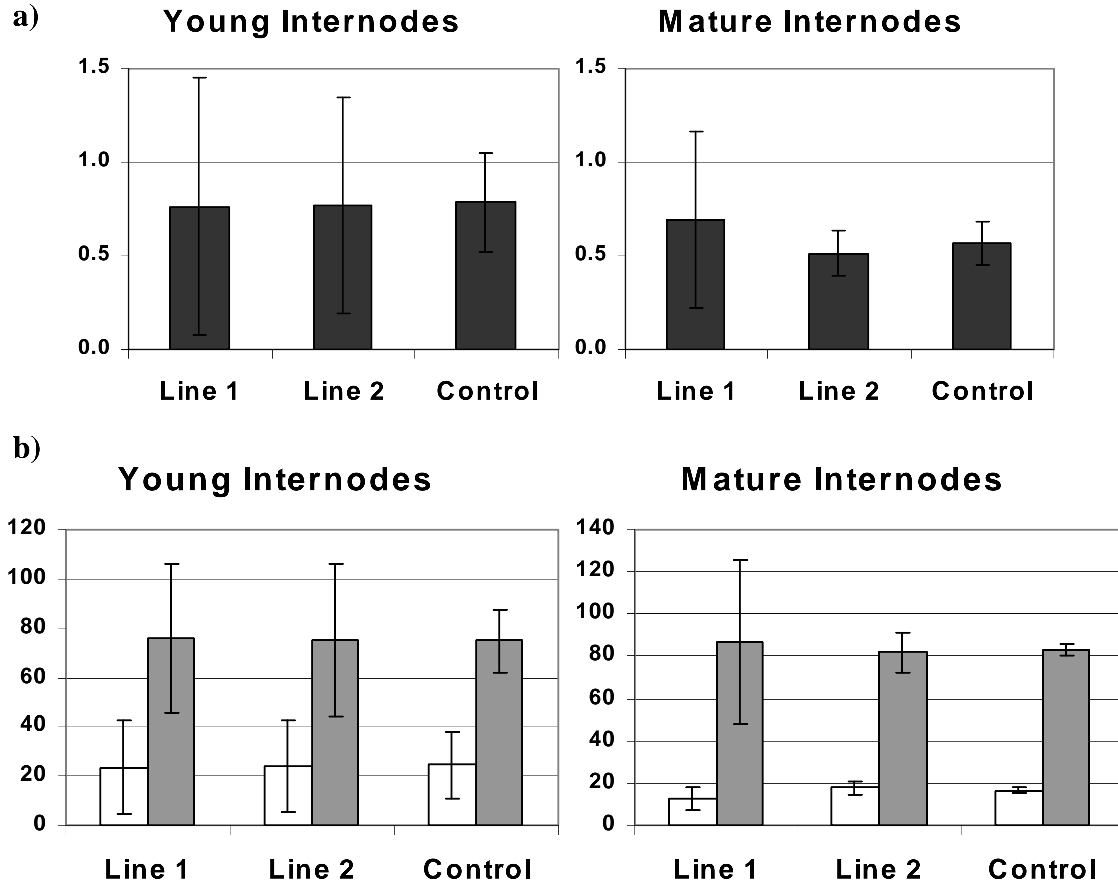

Fig. 3 Percentage distribution of ^{14}C incorporated into a) CO_2 (-■-), and b) water-insoluble (-□-) and water–soluble (-■-) fractions of young (internodes nos. 4 and 5 combined) and mature (internodes nos. 11 and 12 combined) internodal tissue discs, from two 1-SST-transgenic lines and an untransformed control, supplied with [U-^{14}C]glucose for 5.5 h. (Each plotted value is the mean ± SD of 3 separate samples)

A trend in label incorporation into the three primary constituents could be identified, with similar patterns of distribution observed in transgenic as well as control plants. The distribution of label into the CO_2, water-insoluble and water-soluble fractions respectively were as follows: ±0.8%;24%;75% in young internodes and ±0.6%;16%;84% in mature internodes (Fig. 3).

With no apparent effect on carbon partitioning, observed within these primary constituents, we proceeded to investigate the composition of the soluble fraction. This fraction was separated into a neutral component consisting of sugars and an ionic component comprising amino acids, organic acids, and phosphorylated sugars. When the percentages of label incorporated into these two components in the transgenic plants were compared to the controls, a distinctive change in partitioning was observed. A trend, characterised by an increase of carbon allocation towards the

neutral sugar component coinciding with a decrease into the ionic component, was apparent in both internodal sections in both transgenic lines (Fig. 4).

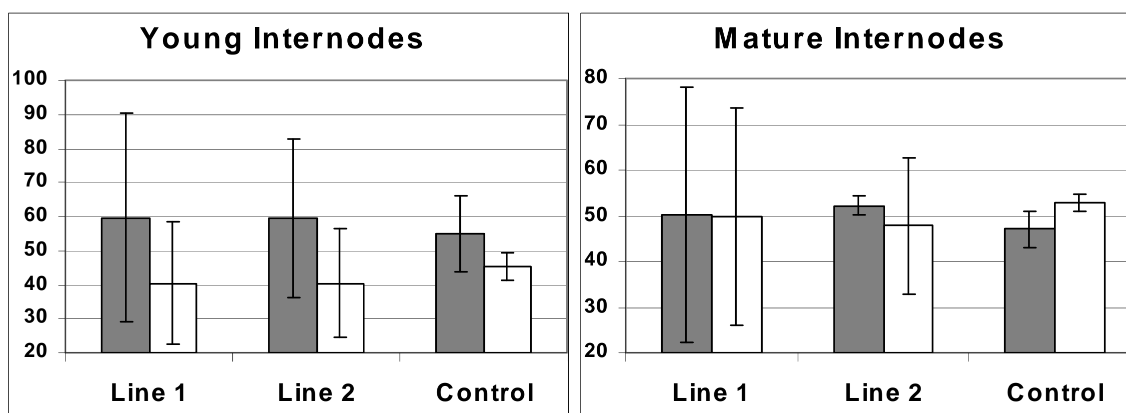

Fig. 4 Percentage distribution of ^{14}C incorporated into the neutral (-■-) and ionic (-□-) components of the water–soluble fraction in young (internodes nos. 4 and 5 combined) and mature (internodes nos. 11 and 12 combined) internodal tissue discs, from two 1-SST-transgenic lines and an untransformed control, supplied with [U-^{14}C]glucose for 5.5 h. (Each plotted value is the mean ± SD of 3 separate samples)

Subsequent analyses of the carbohydrates in the soluble fraction, entailed separating the different components present in this solution by means of TLC. After these TLC's were exposed to phospho-screens and the radioactivity was visualized with a phospho-imager, it was possible to identify the different neutral sugars by comparison with the appropriate standards and to determine the amount of radioactivity in each sugar with spot densitometry. With the sum total of the sugars representing the neutral component of the water-soluble fraction, the amounts of label incorporated into the neutral sugars were calculated as fractions of the percentages of carbon allocated to the neutral component as established in Fig. 4. This analysis revealed that sucrose was the main metabolite in this pathway, with 93%, 93.7% and 96.8% of the total carbon allocated to this pathway, directed into sucrose, in young internodal tissue and with 93.2%, 89.8% and 94% in mature internodal tissue of line 1, line 2 and WT respectively. When the patterns of ^{14}C distribution between the different sugars were compared, an average increase of 2.5% into sucrose, along with partitioning into 1-Kestose, was observed in transgenic lines (Fig. 5).

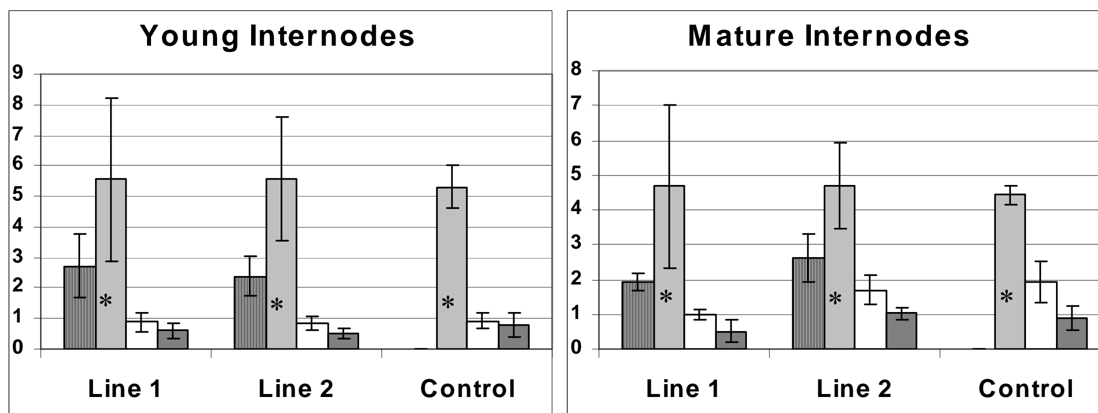

Fig. 5 Percentage distribution of ^{14}C incorporated into 1-kestose (-▥-), sucrose (-▢-), glucose (-□-) and fructose (-■-), interpreted as a fraction of the total percentage ^{14}C allocated to the neutral components in young (internodes nos. 4 and 5 combined) and mature (internodes nos. 11 and 12 combined) internodal tissue discs, from two 1-SST-transgenic lines and an untransformed control, supplied with [U-^{14}C]glucose for 5.5 h. (Each plotted value is the mean ± SD of 3 separate samples)
* sucrose levels indicated on graph = % ^{14}C incorporated into sucrose divided by 10

Interestingly, when the sugar content of the soluble fraction was analysed, radioactivity was also detected in the control plant in the same region on the TLC plate where 1-kestose was located. The extracts spotted onto these TLC's were however not deionised prior to analysis, and seeing as no trace of 1-kestose was detected in the previous more sensitive HPLC analysis conducted on deionised control extracts (example in Fig. 1); the additional radioactivity detected here could have likely been caused by a sugar-phosphate or any one of the number of components also still present in the extracts. Furthermore, though oligofructan production by invertase, at high sucrose concentration, has been proposed (Cairns 1993), the presence of oligofructans in sugarcane has never been documented, not even in the highest sucrose accumulating cultivars. Thus far sequencing analysis has failed to find the SST sequence in sugarcane. Hence there is currently no reason to believe that the radioactivity observed in the control plants were due to 1-kestose production. Since the reading was always less than 1.7% and due to uncertainty as to the identity of this component, this value was ignored in ^{14}C labelling calculations.

Estimated metabolic flux

Flux into the system was calculated using the specific activity of the supplied glucose (55.24 kBq µmol^{-1}) and the total amount of radioactivity incorporate into the different metabolic components. As previously shown, isotopic equilibrium between the external glucose and cytosolic glucose is reached within three hours in this system

(Bindon & Botha 2001). To investigate the flow of ^{14}C through the plant system, the fluxes into different competitive metabolic pathways in the transgenic and control lines were therefore calculated using the specific activity of the supplied glucose. As follows the flux into the sugar storage pathway was calculated using the total amount of label incorporated into both sucrose and 1-kestose, since 1-kestose production forms an extension of this pathway. On the other hand, flux into 1-kestose from sucrose was calculated by subtracting the flux from glucose into sucrose from the total flux into the sugar storage pathway. Due to complications, no attempt was made to calculate further flux into fructose.

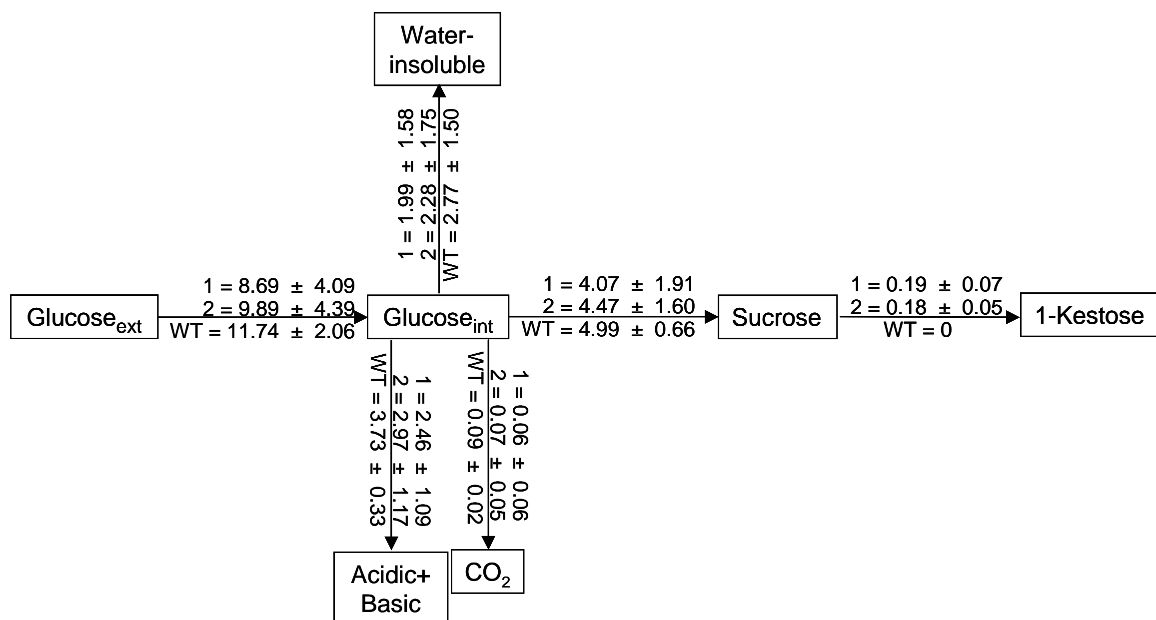

Fig. 6 Calculated metabolic flux (nmol min^{-1} g^{-1} FW) in young internodal tissue (internodes 4 and 5 combined), from two 1-SST-transgenic lines and an untransformed control. Each value is the mean ± SD of three separate samples.

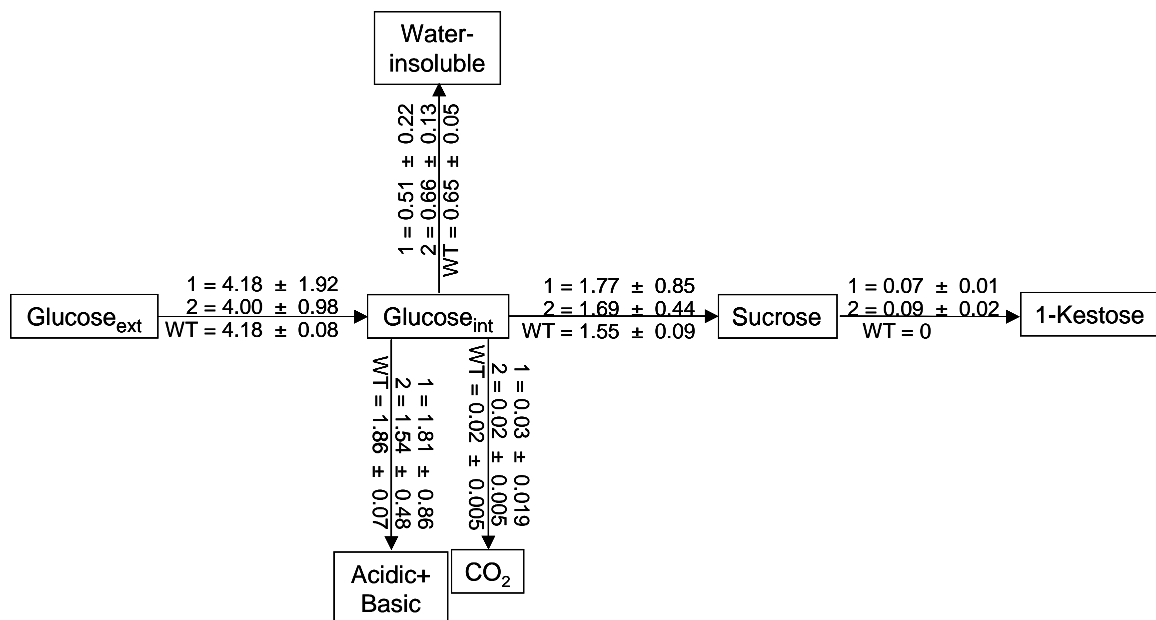

Fig. 7 Calculated metabolic flux (nmol min^{-1} g^{-1} FW) in mature internodal tissue (internodes 11 and 12 combined), from two 1-SST-transgenic lines and an untransformed control. Each value is the mean ± SD of at least 6 separate samples

The high SD's again complicated interpretation of the data as noted in the previous analyses. Nonetheless, based on averages, certain trends in carbon flow could be identified when the fluxes in transgenic plants were compared to that of control plants. Accordingly lower flux into the system, as well as into each of the separate metabolic pathways were evident in young transgenic internodes (Fig. 6). While changes in carbon uptake was not observed in the mature internodes of transgenic lines, increased flux into sucrose storage, and decreases in flux into the amino- and organic- acid synthesis could be identified (Fig. 7).

DISCUSSION

Effects of transgenic fructan production on sugar concentrations and composition

In order to investigate the resulting effects of transgenic fructan production on sugarcane metabolism, metabolite concentrations and composition in tissues representing different 'metabolic states' were determined and compared to that of control plants. For this purpose mature leaves functioning as source tissue in the sugarcane plant, young developing leaves and immature stalk internodes representing growth sinks, and the storage sink i.e. mature stalk internodes, were selected and analysed (Moore 1995b; Moore & Maretzki 1996).

Transgenic fructan production emulates the natural sucrose gradient in sugarcane

The nature of the products formed as a result of transgenic 1-SST expression, are determined by both enzyme and substrate concentrations (Cairns 1995). These characteristics associated with the 1-SST enzyme were reflected in the type and amount of fructans produced in the different tissue types of 1-SST-transgenic sugarcane. Since sucrose acts as the sole substrate for 1-SST (Caimi et al. 1997), and seeing as sucrose availability in storage tissue is much higher than in the rest of the sugarcane plant (Glasziou & Gayler 1972); 1-kestose (GF_2) concentrations in leaves and young internodes were considerably lower compared to levels in mature sucrose storing internodes (Fig. 2). Transgenic 1-kestose productions were therefore seen emulating the natural sucrose gradient in sugarcane plants. The lower 1-kestose accumulation in the tissues of line 1, compared to consistently higher levels in all the tissue types in line 2, could on the other hand possibly be attributed to lower 1-SST concentrations (Fig. 2).

Despite the predominant production of 1-kestose in the mature internodes of line 2, traces of nystose (GF_3) were also detected (Fig. 1). The production of this additional oligofructan in these tissues, can be explained by referring to the theory, that fructan synthesis proceeds in a cascade-like fashion, where a threshold concentration for a molecule of a given chain length must be reached before the next higher homolog is synthesized (Hellwege et al. 2000). The higher sucrose concentrations in these internodes could therefore also account for additional nystose production, with adequate substrate availability for sufficient 1-kestose production, in order to exceed the threshold and proceed with nystose synthesis.

The same pattern of oligofructan production was however not found in young internodes (Fig. 1). According to the theory presented above, the 1-kestose concentrations found in these internodes is too low to allow further oligofructan production. Furthermore, you would not only expect high GF_2 concentrations, but also high GF_3 concentrations associated with additional fructosyl-nystose (GF_4) productions. The fact that GF_4 accumulation was the only oligofructan in addition to low GF_2 accumulation, detected in young internodes, was therefore puzzling. This inconsistency could possibly be due to factors like sucrose availability and competing

enzymes, e.g. invertases. Invertase is able to degrade low molecular weight fructans and may be responsible for masking the activity of 1-SST in young internodes. Seeing as the ability of invertase to degrade oligofructans decrease with an increase in DP (Van der Meer et al. 1998), it could explain the low 1-kestose concentration in the tissue and why only fructosyl-nystose and not nystose was detected. This indicates higher vacuolar invertase activity in young internodes compared to mature internodes preventing the build-up of high concentrations of oligofructans. Judging by the unaffected concentrations of the other sugars in the young internodes, it also suggests a higher rate of cycling between hexose, sucrose, 1-kestose, nystose, and even some fructosyl-nystose. These findings correlates with those of previous studies in which the rapid cycling of sucrose in young internodes were found to result in much shorter turnover-times for the sucrose pool compared to mature internodes (Glasziou 1961; Vorster & Botha 1999; Bindon & Botha 2002).

1-Kestose accumulated in addition to sucrose up to internode 12

Sugarcane stalk development is a continuous process resulting in increasing sucrose accumulation down the stalk to a point where full maturity and a stable, high sucrose concentration are reached (Moore 1995a). The increasing sucrose concentrations observed in the successive internodal sections analysed in this study is characteristic of this maturation process. Though this increase in sucrose is seen to continue in the control internodes, this pattern is interrupted after internodes 11 and 12 in the transgenic plants, when a sudden decrease in sucrose was observed. Associated with this decrease was also a dramatic increase in 1-kestose concentrations. Yet the loss in sucrose did not fully compensate for the gain in 1-kestose (Fig. 2 and Table 1).

Up to internode 12, 1-kestose production seem to be occurring in addition to sucrose, since increasing sucrose concentrations comparable and even higher than that of controls were observed together with 1-kestose accumulation. Internode 12 therefore seems to represent some kind of “cut of point” after which oligofructan production continues partially at the expense of sucrose. Since sucrose accumulation rate typically decreases after the initial increase in rate observed in the top 10 internodes (Whittaker & Botha 1997; Botha & Black 2000), it is possible that internode 12 represents the specific internode in the culm after which sucrose accumulation

becomes to low to supply sufficient carbon flow into both the sucrose and 1-kestose pools.

Transgenic 1-kestose accumulation, associated with increased total yield

Sucrose produced as a result of photosynthesis in source leaves is either exported from the leaf to sink tissue or enters the vacuole where it is transiently stored (Hawker 1985). Since sucrose concentrations stayed constant, and total soluble carbohydrate content was increased in 1-kestose accumulating source leaves (Fig. 2), the carbohydrate accumulation capacity of these leaves seems to have increased. Furthermore, seeing as the total sugar contents were not altered in young internodes and were increased in the other two sinks (young leaves and mature internodes) (Fig. 2), and since no phenotypical effect was observed, export capacity of these leaves also seemed to have increased.

The data therefore suggests that by introduction of the foreign gene, 1-SST in sugarcane, an extra carbohydrate sink was established in the vacuoles of the various organs of the transgenic plants. This modification increased the capacity of the plant organs to allocate carbohydrates, resulting in higher total sugar contents in the different organs that are expected to change source-sink interaction.

Investigating the effect of 1-SST expression on sink metabolism

From the concentrations of the soluble sugars in the mature internodes it is not possible to establish whether the increased carbohydrate content was the result of an increase in sink strength resulting in higher carbon uptake from phloem, or whether it was due to a change in balance and partitioning of the endogenous carbon contents. In order to establish the mechanism behind this, radio-labelled-isotopes were used to investigate carbon partitioning and flux within internodal sinks.

Incorporation of radio labelled carbons was characterized in tissue discs of internodes at different stages of development. Stalk sections comprised of internodes 4 and 5, representing young internodes rapidly accumulating sucrose, and internodes 11 and 12, representing older internodes with high sucrose content, were selected for this purpose (Moore 1995b). In order to investigate the effect that transgenic fructan production had on photosynthate partitioning and flux, the allocation of ^{14}C to the

primary pathways competing for incoming carbon in the sugarcane system, namely: fibre synthesis, respiration and sucrose storage were determined (Bindon & Botha 2002). For the purpose of this study, we have simplified carbohydrate fractionation by defining the respiratory pathway by the sum of ^{14}C recovered in CO_2 (catabolic respiration), and the amino- and organic- acids (anabolic respiration) (Whittaker & Botha 1997). Furthermore, though the insoluble component consists of fibre, starch and protein (Bindon & Botha 2002), this fraction was viewed to primarily represent fibre. The interpretation of the data obtained, when the sugar concentrations (Table 1) and carbon partitioning (Fig. 3, 4 and 5) in transgenic tissue samples were compared to controls in this ^{14}C study, was however obscured in certain instances, especially in young internodes, due to high standard deviations (SD).

Since sucrose as well as 1-kestose can be hydrolysed by invertase (van der Meer et al. 1998), part of the label incorporated into these sugars will eventually return to the fructose pool. While 1-kestose degradation is limited to the vacuole (foreign sugar probably limited to vacuole), sucrose hydrolyses takes place in both the vacuole and cytosol in the sugarcane cell (Hawker 1985). The flux into fructose in this system therefore involves three different reactions taking place in two different compartments. Due to this complicated nature of flux into fructose, calculating this flux was not possible at this level of analysis.

1-Kestose synthesis coincides with a repartitioning of carbon from anabolic respiration towards sucrose and 1-kestose storage

The ^{14}C distribution pattern between CO_2, water-soluble and -insoluble matter, found in both young and old internodes of transgenic and control lines (Fig. 3), indicated that 1-kestose synthesis did not influence carbon partitioning into catabolic respiration and fibre synthesis. Irregularities in carbon partitioning in transgenic versus control plant internodes did however become apparent when the soluble fraction was further separated into its components. In both the young and mature internode sections investigated, an increase in allocation of carbon to sucrose and 1-kestose coincided with a decreased allocation into the ionic fraction (consisting of amino acids, organic acid and sugar phosphates) (Fig. 4 and 5).

A change in source-sink interaction was apparent in the young transgenic internodes where lower metabolic flows were observed (Fig.6). Despite the decreases in overall flux observed in these internodes, flux into the sugar storage pathway showed a marked increase proportionally to the flux in the control. Even higher increases in flux towards sugar storage when compared to control plants were detected in mature internodes (Fig. 7). 1-Kestose accumulation together with increased sucrose concentrations in transgenic plants, were thus associated with increased allocation of incoming carbon towards sucrose storage, resulting in higher carbon partitioning into both 1-kestose and sucrose.

Since no increase in flux into either the young or mature internode systems were observed when transgenic lines were compared to control plants (Fig. 6 and 7); the possibility of higher carbon uptake from phloem, as the reason for increased carbohydrate content, was ruled out. Furthermore, the increase in flux towards sugar storage in transgenic lines was also associated with a decrease, though less pronounced, in flux towards amino acids and organic acids (values in Fig. 6. viewed proportionally to controls and Fig. 7). It could therefore be concluded that the observed increase in total sugar content in transgenic plants, was solely due to repartitioning of incoming carbon away from the anabolic respiration pathway towards the sucrose storage pathway.

In view of the fact that carbon partitioning and flux into 1-kestose was similar in lines 1 and 2 in both young and mature internodes (Fig. 5, 6 and 7), the data suggests that 1-SST activity in these two transgenic lines is also similar. Moreover, the amounts of radiolabel found in fructose following feeding with glucose (Fig. 5), and the concentrations of the hexose pools in both stages of internode maturation (Table 1), indicated that sugar (whether sucrose or 1-kestose) breakdown occurs to more or less the same degree in the two transgenic lines and control plants. The consistent significant differences in 1-kestose concentrations between lines 1 and 2 (Fig. 2 and Table 1) could therefore not have been due to differences in 1-SST and invertase activity as previously speculated and needs to be further investigated.

By comparison with natural fluxes in the internodal cells, the fluxes into 1-kestose, and the diversion in fluxes associated with 1-kestose syntheses were relatively small

(Fig. 6 and 7). We are therefore led to believe that 1-kestose accumulation in transgenic sugarcane is a gradual process, occurring over the entire growth period. The nature of these changes could therefore explain why phenotypical changes were not observed in the transgenic plants, even though changes in carbon partitioning and carbohydrate composition is expected to have major effects on plant growth (Van der Meer et al. 1994, Sévenier et al. 1998a).

CONCLUSION

In transgenic sugarcane the expression of 1-SST established an extra carbohydrate sink in the vacuoles of all the organs, resulting in a continual flow of carbon from the sucrose pool into 1-kestose. This removal and conversion of sucrose did not however result in a depletion of the sucrose pool, but instead it stimulated an increased flux into this pathway, increasing the non-structural carbohydrate content of the plant. A plausible explanation for this phenomenon is that the system responds to sucrose, and by adapting carbon flow in the cell it is attempting to maintain a constant sucrose concentration in the cells. The rate of sucrose synthesis in different plant organs is therefore regulated by the sucrose contents of that organ. The existence of similar sucrose-specific regulatory pathways has been shown in a number of higher plants (Chiou & Bush 1998).

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CHAPTER 7

General discussion and conclusions

By the introduction and expression of fructosyltransferase genes from globe artichoke (*Cynara scolymus*) in sugarcane, we have succeeded in producing novel products, i.e. inulin-type fructans in all parts of the sugarcane plant. With this strategy we managed to address a variety of questions concerning sucrose metabolism in and metabolic engineering of sugarcane. The work demonstrates the feasibility of diverting sucrose from its usual pathways without causing undesirable side effects (Chapter 4). The results demonstrated the potential to convert sugarcane into an efficient biofactory for fructan production (Chapter 5). Moreover, except for the practical applications of this research, the investigation of the implications of this conversion on carbon partitioning and flux, fundamentally contributed to our understanding of sucrose metabolism in sugarcane, offering a solution for increasing yield (Chapter 6).

Hypothetically the conversion of sucrose to fructan in the vacuole should have a major effect on carbon partitioning in sugarcane. Most likely this redistribution of carbon should lead to a decrease in sucrose content and increases in glucose content. Such an altered carbon partitioning could affect sugar-sensing, that in turn can disrupt the normal sugar-mediated gene regulation as well as source-sink interactions and sugar signalling, such as 'feedback' control of sucrose on source metabolism. Collectively all these perturbations might have a major impact on photoassimilation and the capacity of the plant organs to allocate carbohydrates as described by others (Van der Meer et al. 1994; Röber et al. 1996; Sévenier et al. 1998). The diversion of sucrose from an existing metabolic pathway are therefore expected to change the normal mechanisms responsible for the regulation of carbon partitioning and growth (Turk et al. 1997; Sévenier et al. 1998), that can ultimately induce severe phenotypical changes.

The lack of distinct phenotypical changes in the transgenic sugarcane lines therefore indicates that this metabolic alteration did not seriously affect the cellular constituents required for plant growth and survival. This was demonstrated in the carbohydrate levels, in particular sucrose content, which were not significantly altered within tissues undergoing cell division and/or cell expansion; as observed in source tissue

(mature leaves) and growth sinks (young developing leaves and immature stalk internodes) of the transgenic plants (Fig.2, Chapter 6). Furthermore, although the introduction of the fructan pathway in sugarcane established an extra carbohydrate sink in the vacuoles of the various organs, leading to increased total carbohydrate levels in the different organs tested (Fig.2 and Table 1, Chapter 6), the impact on source:sink interaction and consequently growth, were probably minimised by the metabolic buffering ability of the system. In the internodes these adjustments in cell metabolism were observed as modest changes in carbon partitioning (Fig. 4 and 5, Chapter 6) and carbon flux (Fig. 6 and 7, Chapter 6). Through these metabolic adjustments, small fraction of incoming carbon was repartitioned away from competing pathways towards the sugar storage pathway, ultimately leading to increased sucrose content and fructan accumulation. Judging by the subtle nature of these changes, fructan accumulation in transgenic sugarcane appeared to be a gradual process, occurring over the entire growth period. The increase in fructan content from young leaves to mature leaves (Fig. 2, Chapter 6) and from young internodes to mature internodes (Fig. 2 and Table 1, Chapter 6), can also be interpreted to verify this nature of transgenic fructan production: With a slow but continuous accumulation process, resulting in the build-up of fructan levels in a tissue type.

The fact that sucrose content were not reduced in the leaves and young stalk internodes (from internode 1 up to internode 12) contributing to plant growth, despite the additional pressure of an extra carbon sink on this metabolite pool in oligofructan producing plants (Table 1, Chapter 4; Fig.2 and Table 1, Chapter 6); suggests that a distinct sucrose-sensing pathway exists in sugarcane to maintain sucrose concentrations. Sucrose sensing is therefore expected to be the main regulatory mechanism responsible for adapting carbon flow and flux through the sucrose synthesis and degradation pathways in the cells to insure adequate metabolite levels, as substrates for metabolic activity and growth. These findings agrees with recent experimental evidence, indicating that sucrose-specific regulatory pathways in plants initiates changes in gene expression in order for individual cells to respond to changes in the internal carbohydrate environment of the plant, thus allowing coordinated long-term adjustment for the benefit of the whole (Coruzzi & Zhou 2001; Koch 1996).

We therefore must conclude that sucrose is not only the major form of translocated carbon and the primary storage substrate in sugarcane, but was also shown to be an important signal metabolite, responsible for controlling its own synthesis in order to regulate the sucrose contents of different plant organs. The ability to measure sucrose content enables the plant to adapt and regulate the flow of carbon into the sugar storage pathway in order to maintain sucrose levels in the organs where it is needed for growth and development. Thus, allowing the plant to produce transgenic fructans without lowering the overall yield of sucrose, or causing detrimental effects, resulting in higher total soluble sugar content in the transgenic sugarcane lines. However, the nature of this cross talk between sucrose and metabolism still have to be investigated and the transgenic plants still have to undergo field trials to establish the full impact of the transformation on agronomic factors, such as yield and disease resistance.

Based on these results and similar research done in the past on the two cultivated sucrose-storing plants, sugarcane and sugar beet; we now know that it is possible to manipulate the sucrose metabolic pathway in naturally sucrose-storing plants in order to create new carbohydrate sinks, without causing detrimental effects to the plants(Sévenier et al. 1998b; Weyens et al. 2004; Birch & Wu 2005). However, our sweet sorghum research (documented in Chapter 3) showed that in order to expand the application of this technology to an alternative sucrose-storing crop, such as sweet sorghum, we first have to establish efficient tissue culture transformation practises for the crop. Our research therefore stresses the importance of investing resources in the development of reliable tissue culture systems, as a prerequisite for successful transformation.

Metabolic engineering have also proofed to be a useful molecular tool to help dissect the intricacies of plant metabolism in a variety of other crops. The understanding of the control of partitioning between different metabolic pathways is regarded as very important for effective metabolic engineering in order to achieve the ultimate aim of optimum utilization of plant carbohydrates in future. Once the appropriate processes have been determined, metabolite fluxes can be manipulated to increase the yield of commercially important metabolites (Herbers & Sonnewald 1996). Alternatively, novel functions can be introduced in plants to obtain high-value biomaterials (Grof & Campbell 2001; Rae et al. 2005).

So as to maximize the size of the sucrose pool in sugarcane, partitioning of photoassimilates between competing sinks of vegetative growth, fibre, and stored sucrose, have in the past been partially manipulated by conventional breeding (Moore & Maretzki 1996). Increased sucrose content through traditional plant breeding have however reached an apparent threshold (Grof & Campbell 2001), despite the fact that a significantly higher biophysical sucrose storing capacity has been assessed in the sugarcane stem (Moore & Maretzki 1996). To overcome this productivity barrier, research is currently focussing on metabolic engineering strategies to improve sucrose yield (Grof & Campbell 2001).

Up to date, genetic manipulation strategies (all of the studies done in the past with the exception of the latest research by Birch & Wu, 2005) aimed at increasing stem sucrose content, have focussed on increasing the amount of photoassimilate directed towards stored sucrose, or a reduction in sucrose turnover. These strategies entailed manipulating key enzymes and transporters involved in the sucrose accumulation process (Grof & Campbell 2001). However, despite these numerous studies, increasing the concentration of sucrose in the stem remains a great challenge due to the complexity of the system, which makes it virtually impossible to identify good target reactions to modify (Rohwer & Botha FC. 2001). In this respect is was concluded that further investigation into the biochemical basis for where and how sucrose storage is regulated in the sugarcane plant are required for developing strategies for genetic regulation of partitioning (Ritter et al. 2004).

Although this research was primarily directed at the biosynthesis of fructans in sugarcane, the results obtained also provided a tool that shed light on basic questions concerning sucrose metabolism. Not only did these fructan-producing sugarcane plants increase our understanding of the regulation of sucrose synthesis, but most importantly they exhibited much potential as a means to increase yield. This was evident from the increase in total soluble sugar content, with up to 63% increase in mature internodes of transformed plants when compared to control plants (Fig.4, Chapter 5; Fig.2 and Table 1, Chapter 6). These results illustrated that the flux into the sucrose-storing pathway can be enhanced by the introduction of an extra carbohydrate sink in the vacuoles of the plants. This modification led to the continuous removal of

endogenous sucrose, in order for the sucrose to be converted to fructans. The system then compensates for this loss, by repartitioning more of the incoming photoassimilates towards the sucrose-storing pathway, thereby increasing the total flux of carbon through this pathway.

This strategy for increasing yield, also proofed successful in a recent study by Birch & Wu (2005), in which sugarcane lines were engineered for expression of a sucrose isomerase directed to the vacuole. With this approach partial conversion of sucrose into isomaltulose (palatinose), and much higher total soluble sugar content was achieved (Birch & Wu 2005).

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