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Review

Tailor-made fructan synthesis in plants: A review

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ABSTRACT

Fructan, a fructose polymer, is produced by many bacteria and plants. Fructan is used as carbohydrate reserve, and in bacteria also as protective outside layer. Chicory is a commercial fructan producing crop. The disadvantage of this crop is its fructan breakdown before harvest. Studies using genetically modification showed that fructan biosynthesis is difficult to steer in chicory. Alternatives for production of tailor-made fructan, fructan with a desired polymer length and linkage type, are originally non-fructan-accumulating plants expressing introduced fructosyltransferase genes. The usage of bacterial fructosyltransferases hindered plant performance, whereas plant-derived fructan genes can successfully be used for this purpose. The polymer length distribution and the yield are dependent on the origin of the fructan genes and the availability of sucrose in the host. Limitations seen in chicory for the production of tailor-made fructan are lacking in putative new platform crops like sugar beet and sugarcane and rice.

Contents

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1. Introduction

Fructan is a polymer consisting of fructose units and a terminal glucose residue. Fructan occurs in a wide range of organisms; bacteria, some fungi and in about 15% of the flowering plants. Based on the linkage type fructan can be divided into three groups: (1) levan, with β(2-6) linked fructosyl units; this fructan type mainly occurs in bacteria (Dedonder, Neufeld, & Ginsburg, 1966) and monocotyledonous plants (where it is also called phlein) (Bonnett, Sims, Simpson, & Cairns, 1997), (2) inulin, a β(2-1) linear polymer that is found in dicotyledonous plants (Koops & Jonker, 1996), and (3) fructan neo-series, a mixed type of fructan found in Liliaceae (Pollock, 1986) in which β(2-1) chain elongation occurs on the C1 and the C6 positions of the glucose residue.

Bacteria use fructan as energy storage molecule (Burne, Chen, Wexler, Kuramitsu, & Bowen, 1996) and as a protective layer outside the cell. This fructan layer is used by plant pathogenic bacteria for blocking host–pathogen recognition and against bacteriostatic compounds released by collapsed plant cells (Kasapis, Morris, Gross, & Rudolph, 1994). Streptococci, present in the oral cavity, use the fructan layer as adhesive and as such fructan is important in the formation of dental plaque, which consists for a large part of levan-type fructan (Cote & Ahlgren, 1993). The biosynthesis of levan in bacteria is performed by a single enzyme, levansucrase (E.C. 2.4.1.10).

In plants, fructan serves as a reserve carbohydrate and is stored in stems, tubers or taproots. It has also been suggested that fructan protects the plant against drought and cold stress (Pilon-Smits et al., 1995; Pollock, 1986). The length of plant fructan varies from 10 to approximately 200 fructosyl units. This variation highly depends on the taxonomic diversity of fructan producing plant species. In contrast to bacteria, the biosynthesis of fructan in plants is catalysed by three different classes of enzymes: sucrose:sucrose 1-fructosyltransferase (EC 2.4.1.99) (1-SST), fructan:fructan 1-fructosyltransferase (EC2.4.1.100) (1-FFT) and fructan exohydrolase (EC.3.2.1.153) (1-FEH) (Edelman & Jefford, 1968). 1-SST primarily catalyses the synthesis of the trisaccharide 1-kestose, from two molecules of sucrose. In this reaction glucose is formed in equimolar amounts to 1-kestose. 1-FFT catalyses the transfer of fructosyl units from 1-kestose and any other fructan molecule onto 1-kestose and higher DP fructan molecules. 1-FFT increases the mean degree of polymerisation (mDP) when using 1-kestose, the shortest fructan, as a fructosyl donor because this reaction converts 1-kestose into sucrose, which does not count up as fructan molecule, and a fructosyl unit that is used to elongate a pre-existing fructan molecule. Under 1-kestose limiting conditions, for example when 1-SST activity is low, 1-FFT can also catalyse the transfer of fructosyl units from a fructan molecule onto sucrose (Van den Ende, De Roover, & Van Laere, 1996; Vergauwen, Van Laere, & Van den Ende, 2003), this so-called 'back transfer' reaction results in the decrease of the mDP. The third class of enzymes, 1-FEH, catalyses the degradation of inulin by hydrolysing terminal fructosyl units, which results in the formation of fructose and lower DP inulin (Van den Ende, Michiels, Van Wonterghem, Vergauwen, & Van Laere, 2000).

Plant fructan is used for a range of food and non-food applications (Sévenier, van Arkel, Hakkert, & Koops, 2006) depending on the degree of polymerisation (DP). Short chain inulin is used, for example, for the production of fructose syrup, mainly used for the sweetening of cold drinks. Long chain inulin (mDP $\geq$ 25) is used as fat replacer and foam stabiliser in food products. Long chain inulin is also starting material for the production of carboxymethylinulin, a scavenger of divalent cations in household detergents. The crop that is grown for the production of fructan on a commercial scale is chicory.

Chicory (*Cichorium intybus* L.) is a biennial taproot-bearing crop that is sown in spring and the taproots are harvested in autumn of the same year. Inulin is extracted from the taproot. At harvest, the mean inulin polymer length is 9–10 and the average yield is about 11,000 kg carbohydrate/ha (Wittouck et al., 2002).

One of the most important quality parameters of inulin is the polymer length. For certain applications, like fat replacer which require a minimum DP > 25, the raw inulin extracted from chicory is unsuitable and should be enriched for long molecules, which is a costly process.

Interestingly, it has been observed in chicory, that the polymer length earlier in the growing season is much higher than at harvest. The lower polymer length at harvest is caused by catalytic reactions of 1-FFT and 1-FEH, both enzymes responding to plant and environmental factors.

Plants that naturally accumulate fructan can also degrade the polymer in order to remobilize the stored carbon. This catalytic breakdown of fructan is a major drawback in the production of inulin as is also observed in the crop chicory. Several solutions for this problem of breakdown have been proposed in the past years, including attempts to generate new fructan accumulating plant species by genetic modification of crops that originally did not synthesize (and degrade) fructan.

An additional advantage of using non-fructan-accumulating plant species for the commercial production of fructan is that highly productive crop plants, having well-established husbandry and processing chain, can be chosen. Moreover, the introduction of fructan biosynthesis in non-fructan species renders new types and different sizes of fructan not yet present in natural fructan-producing plants: the tailor-made fructan.

In this paper an overview is given of the research on modification of fructan synthesis in fructan accumulating plants on one hand, and the research on introduction of fructan synthesis in non-fructan-accumulating plants on the other hand. As most of the work on modification of fructan synthesis in fructan accumulation plants is performed on chicory, an introduction to the inulin metabolism of chicory is given. A wide range of bacterial and plant fructosyltransferase genes has been introduced in many different plants varying from monocots, like rice, maize and sugar cane, to dicots, like potato, sugar beet and clover. The effect of the modification or introduction of fructan synthesis is valued on the resulting fructan yield, polymer length and altered linkage type of fructan.

2. Modification of fructan synthesis in fructan-accumulating plants

2.1. Fructan biosynthesis in chicory

Chicory is a biannual crop that is grown for the production of inulin, which is stored in the taproot. The taproot starts to thicken seven weeks after sowing, concomitant with the induced activity of the fructan synthesis enzymes 1-SST and 1-FFT. The activity of 1-SST increases rapidly until it reaches a maximum three weeks later (Druart et al., 2001). Ten weeks after sowing the activity decreases until the end of the growing season in November, when only 10% of the activity is left (Van den Ende, Mintiens, Speleers, Onuoha, & Van Laere, 1996). The activity of 1-FFT follows a different pattern, the activity increases slowly and stabilises after four weeks. The activity of 1-FFT remains stable during the rest of the growing season (Van den Ende, Michiels, De Roover, & Van Laere, 2002). As a result of the fructosyltransferase activities in the first months, mainly short inulin is formed. At nine weeks after sowing inulin molecules with a DP up to 25 are accumulated. The mDP reaches a maximum of about 16 in the beginning of September and then decreases as reported by Druart et al. (2001). This extent of decrease

is dependent on the growing conditions (Van Arkel et al., unpublished results) and on the cultivar (Koch, Andersson, Rydberg, & Åman, 1999). The decrease in mDP is thought to be catalysed by 1-FFT using, in the absence of 1-SST, incoming sucrose as an acceptor for fructosyl units (Van den Ende et al., 2002). The result is a decrease in mDP, but an increase of yield per taproot (Van Arkel et al., unpublished results). Later in the season, the mDP further decreases because of the fructan exohydrolase activity of FEHI, that is induced at low temperatures (mid-October) (Michiels, Van Laere, Van den Ende, & Tucker, 2004). Degradation of inulin is further enhanced by FEHII later in the season, when temperatures of 4 °C and below induce this second exohydrolase (Van den Ende et al., 2002). The degradation catalysed by 1-FFT and 1-FEH occurring in autumn is highly disadvantageous for industrial inulin production, especially for high DP inulin production.

In summary: from the studies on inulin biosynthesis in chicory it could be concluded that the decline in 1-SST activity combined with the increase in 1-FEH activity negatively influences the mDP.

Several groups focussed on the modification of fructan synthesis in chicory in order to produce high DP inulin with the highest possible yield, or fructan with modified linkage type. In our research group, two different approaches to accomplish an increased yield and polymer length have been followed. The first approach to obtain production of inulin with higher mDP was blocking the activity of 1-FEH in the autumn, which might be achieved by down-regulation of FEHI. It was envisioned that the absence of FEHI activity might prevent depolymerisation, although a reduction of mDP by 1-FFT and FEHII might still occur. A second approach to enhance the degree of polymerisation was overexpressing *1-sst* during the growing season. The additional polymerisation activity by the extra 1-SST enzyme activity might maintain a higher concentration of 1-kestose and a lower concentration of sucrose, two factors that might contribute to limiting 1-FFT-mediated depolymerisation.

Other groups focussed on changing the linkage type of fructan in order to modify the fructan produced. Chicory was transformed with genes isolated from species accumulating different types of fructan, such as the *6-sft* from barley (Sprenger, Schellenbaum, van Dun, Boller, & Wiemken, 1997) and *6g-fft* from onion (Vijn et al., 1997).

2.2. Modifying fructan biosynthesis in chicory for a higher mDP and yield

One way of increasing the mDP of chicory inulin, was to prevent the reduction of 1-SST activity at the end of the growing season. Earlier results showed that the endogenous 1-SST enzyme is subjected to a regulatory mechanism that mediates a decrease of the enzyme activity during the growing season (Van Arkel et al., unpublished results). For this purpose, two different *1-sst* genes (*sst-I* and *sst-II*), isolated from *Helianthus tuberosus* (Koops, Sévenier, Van Tunen, & De Leenheer, 2003; Van der Meer, Koops, Hakkert, & van Tunen, 1998) were expressed under control of the CAMV-35S promoter in chicory (unpublished results). Plants harbouring an extra *1-sst* gene were grown under field resembling conditions to study the effect of the genes introduced. The mDP of inulin and the activity of 1-SST, 1-FFT and 1-FEH were monitored from September until November, when normally in wild-type chicory the reduction of 1-SST activity occurs, and the fructosyltransferase activity onto sucrose by 1-FFT takes place. No impairment on growth was observed. Comparison of the control plants with plants harbouring the *H. tuberosus* gene *sst-II* showed that the transgene significantly contributed to the total activity of 1-SST. This additional 1-SST activity resulted in a 20% higher mDP over the first 8 weeks of the experiment. Plants harbouring the *sst-I* gene, however, did not show elevated levels of 1-SST activity, neither showed a significant altered mDP. Low temperatures, however, had a comparable effect on the decrease of mDP in both types of transgenics (containing *sst-I* or *sst-II*) and the wild-type plants. Introduction of an extra *1-sst* gene did not result in longer chain fructan at the end of the growing season, most probably because of the (early) induction of FEH by low temperatures by the end of the experiment.

Another approach tested to enhance the mDP of chicory inulin at harvest was to decrease the 1-FEH activity. A *feh-I* anti-sense fragment driven by the constitutive CaMV 35S promoter was inserted into the genome of chicory. In three independent transgenic lines cold induction of *feh-I* expression could be inhibited by the anti-sense. However this decreased 1-FEH induction had only minor effects on the mDP when the transgenics were subjected to a cold treatment, inducing specifically *feh*-I. A possible explanation could be that the remaining *feh*-I transcript resulted in enough 1-FEH activity to decrease the mDP.

2.3. Modifying fructan biosynthesis in chicory to alter the fructan type

In a study performed by Sprenger et al. (1997) a sucrose:fructan 6-fructosyltransferase (6-SFT) from barley was introduced in chicory. The aim was to produce the mixed type fructan in chicory. Unfortunately the inulin composition in the taproot of the transgenic plants was not changed. However, excised leaves that were placed in sucrose solutions and illuminated continuously accumulated β(2-1) inulin and β(2-6) fructan (kestose and bifurcose), demonstrating the functionality of barley 6-SFT in chicory. After extended illumination most of the fructan in the transgenic leaves was of the inulin type and only a small part consisted of the mixed type fructan. The experiments in leaves showed that most probably 1-FFT out-competes the heterologous 6-SFT for substrate. This competition for substrate could also be the reason for the absence of the mixed type fructan in the taproots and may result from a lower expression of the *6-sft* (not reported) than the endogenous *1-fft* or from a higher affinity of 1-FFT for the substrate.

In another example of modification of the fructan linkage type in chicory the gene encoding onion 6G-FFT was introduced in chicory (Vijn et al., 1997). Although, the authors did not report on the analysis of fructan accumulation in roots, in excised leaves, in which fructan accumulation was induced, fructan of the neo-series could be detected in addition to the native inulin.

In conclusion, the attempts to modify the inulin metabolism in chicory in order to increase the mDP or to alter the accumulated fructan type consisted in the introduction of extra genes and in the knockdown of an exohydrolase gene. Although the introduced genes were functional and the knockdown could be detected in the transgenic plants, only little of the expected effect was observed on the mDP or inulin composition. The reasons for this discrepancies could be: (I) the relative high level of expression of the endogenous inulin biosynthesis genes (unpublished results) compared to the relative low expression level of the introduced transgenes, and (II) the complex mechanism of fructan degradation, which involves three exohydrolase enzymes and 1-FFT.

These aspects could both be addressed. Strong promoters could be used to drive the expression of the transgenes, for instance the *1-fft* promoter from chicory. In order to block all exohydrolase activity, three *feh*-genes (*feh-Ia*, *b* and *feh-II*) may need to be knocked down, preferable by the use of a more effective gene silencing technique like RNAi (manuscript in preparation), or by the use of knock out mutants.

2.4. Modifying fructan biosynthesis in other plants

A few studies on the attempts to modify the fructan composition in other plants than chicory were reported. A study on the

expression of a *Bacillus subtilis sacB* in *Lolium multiflorum* revealed that levan was accumulated in the plant and consequently disturbed the native fructan pattern. Native high DP fructan was depleted and the pattern of lower DP fructan was altered (Ye et al., 2001). The modification of the linkage type negatively affected the fructan yield. The modification also slowed down the plant growth, the flowering plants were stunted, had narrower leaves and poorly developed roots as described by the authors. Contrarily, the expression *6-gfft* or *1-sst* from barley in *Lolium perenne* yielded up to 15% more fructan than in wild-type plants and this increased fructan content did not impair the growth of the plant. Differences on the linkage type or changes in pattern of DP were not reported. The accumulated fructan was shown to have a positive effect on the freezing tolerance, possibly due to increased amounts of fructan, glucose and fructose (Hisano et al., 2004). Rather unexpected, according to Sobolev et al. (2007), transgenic lettuce overexpressing an asparagine synthase from *Escherichia coli* accumulated 30 times more fructan than wild-type plants. The aim of this study was to alter the nitrogen status of the plant and eventually enhance growth. It appeared that the whole metabolism of the plant was enhanced, including the Krebs's cycle and the fructan biosynthesis, although the nitrogen status was not altered. The studies described above show that the native fructan composition can significantly be altered in transgenic natively accumulating plants and that the effect on fructan yield and composition was more pronounced than in the transgenic chicory described early, although those different experiments were not fully comparable.

3. Heterologous production of fructan in non-fructan-accumulating plant species

Alternatives for chicory as an industrial crop for fructan production are other plants genetically modified with fructosyltransferase genes, accumulating fructan as a novel storage polysaccharide. Many studies have been performed to achieve fructan accumulation in other plants. Table 1 summarizes these studies and shows the yield of fructan and the characteristics of the transgenic plants. Examples of plants that have been used are tobacco, potato, maize and sugar beet. Most transgenics producing fructan showed a reduced level of sucrose or starch compared to wild type. Another characteristic was stunted or reduced growth of the plants as seen in several studies. Fructan production seems to go at the cost of the other carbohydrate synthesis pathways, and in some cases have effect on growth.

3.1. The use of bacterial genes in heterologous plant hosts

Many studies focussed on the introduction of bacterial genes in plants to produce high DP fructan in originally non fructan accumulating plants (Table 1). The use of bacterial levansucrases to engineer plants did not prove very successful in terms of fructan yield (Caimi, McCole, Klein, & Hershey, 1997; Ebskamp, van der Meer, Spronk, Weisbeek, & Smeekens, 1994; Pilon-Smits et al., 1995). In some studies, however, high amounts of fructan were shown to be accumulated. Van der Meer et al. found 350 mg/gDW levan in old transgenic potato leaves and 50 mg/gDW in growing microtubers when expressing *sacB* from *B. subtilis* (van der Meer, Ebskamp, Visser, Weisbeek, & Smeekens, 1994). The expression of *lsc* from *Erwinia amylovora* in starch deficient potato yielded 70–120 mg/gDW of fructan in the tubers (Röber, Geider, Müller-Röber, & Willmitzer, 1996). The DP of the *in planta* produced levan exceeded 25,000 in potato, tobacco and white clover (van der Meer et al., 1994; Ebskamp et al., 1994; Jenkins et al., 2002). This DP is comparable to what is found in bacteria (van der Meer et al., 1994). Trujillo et al. (2000) described the first attempt to express a levansucrase in sugarcane, however PCR positive plants were generated but not analysed for fructan accumulation. The disadvantage of the accumulation of levan in plants was that in many cases this led to severe alterations of the plant growth and aberrant phenotype (Table 1). Tobacco and potato plants, expressing the *sacB* gene from *Bacillus subtilus*, had reduced growth (Pilon-Smits et al., 1996; Turk et al., 1997). Potato plants had smaller tubers at harvest, when the fructan concentration was higher than 1% the tubers had a brown phenotype too (Pilon-Smits et al., 1996). Caimi, McCole, Klein, and Kerr (1996) reported on tissue necrosis in tobacco and reduced tuber weight in potato upon expression of *sacB*. Reduced tuber weight in potato was also reported by Röber et al. (1996). As explanation for the phenotype the authors mentioned that the major part of the fructan was not located in the vacuoles of the transgenic plants and that the presence of fructan in other cellular compartments may have been detrimental to cell functioning and normal development. This conclusion was supported by the findings of Caimi et al. (1996), who reported a drastic alteration of the kernel phenotype in *sacB* expressing maize. The dry weight was shown to be 10-fold lower in transgenic seeds compared to wild type seeds. However, when the levansucrase was targeted to the vacuole, using a sweet potato sporamin vacuole targeting sequence, accumulation of fructan in the vacuole had no effect on kernel development (Caimi et al., 1996). Moreover, a study on *L. multiflorum* expressing *B. subtilis sacB* showed that the effects on growth and phenotype also occurs in plants that already accumulate fructan originally (Ye et al., 2001).

The conclusion from the studies on the expression of bacterial genes in non-fructan-accumulating plants is that the accumulation of levan in plants was shown to be possible, but that the plant phenotype and fitness were negatively influenced. The yield remained low in most cases, most probably because the levansucrase was not targeted to the vacuole.

3.2. The use of plant genes in non-fructan-accumulating plant hosts

The expression of fructosyltransferase genes of plant origin in non-fructan-accumulating plant allowed the accumulation of substantial amounts of fructan in the transgenics and did not lead to alteration of the performance of the host plant; see Table 1 for an overview.

3.2.1. Introduction of 1-SST in non-fructan plants

Various *1-sst* genes originating from different fructan accumulating plants were introduced in many different non-fructan-accumulating plant species (Table 1). Some successful studies will be discussed in more detail. The *sst-I* from Jerusalem artichoke was introduced in sugar beet. Carbohydrate analysis of the transgenic plants showed that 90% of the sucrose, normally stored in the taproot of the beet, was converted to fructan. This yielded 110 μmol/gFW fructan, mostly 1-kestose, 1-nystose and Dp4 (Sévenier et al., 1998). The production of the latter two molecules is normally addressed to 1-FFT activity. However it had been shown before that, in vitro, 1-SST alone was able to catalyse the formation of fructan larger than 1-kestose (Koops & Jonker, 1996). The 1-SST from globe artichoke was successfully introduced in potato and sugarcane and showed 1-kestose production in sugarcane under field resembling conditions (Hellwege, Gritscher, Willmitzer, & Heyer, 1997; Nicholson, 2007), the yield however was only 8–112 nmol/gFW. This small amount of fructan did not negatively influence the sucrose concentration: the sucrose levels in the 1-SST plants were even higher than in the wild-type plants. It was stated that the carbon partitioning was changed by the accumulation of 1-kestose. The author showed that invertase was not the cause of this low level of 1-kestose, neither was the

Table 1
Fructan production in genetically engineered originally non-fructan-producing crops.

Crop transformed	Gene introduced	Gene's origin	Enzyme targeting	Tissue analysed	Soluble sugar content of the transformed tissue			Phenotype changes	Article cited
					Sucrose	Fructan	Glucose		
Tobacco	*sacB*	*B. subtilis*	Vacuole	Leaves	0.14 mg/gFW	2.8 mg/gFW	1.5 mg/gFW	No effect on the phenotype	Ebskamp et al. (1994)
Tobacco	*sacB*	*Bacillus subtilis*	Vacuole	Leaves	0.3–0.9 mg/gFW	0.05–0.3 mg/gFW	0.5–0.7 mg/gFW	Enhanced drought stress resistance	Pilon-Smits et al. (1995)
Tobacco	*6-sft*	*Hordeum vulgare*	Vacuole	Leaves	5–10 mg/gDW	0.05–0.3 mg/gDW	N.R.	No effect on the phenotype	Sprenger et al. (1997)
Tobacco	*6-sft*	*Hordeum vulgare*	Vacuole	Roots	50 mg/gDW	0.5–3 mg/gDW	N.R.	No effect on the phenotype	Sprenger et al. (1997)
Tobacco	*sacB*	*B. subtilis*	Vacuole	Leaves	2–16 mg/gFW	6 mg/gFW	2–12 mg/gFW	Stunted growth and bleached leaves	Turk et al. (1997)
Tobacco	*sacB*	*B. amyloliquefaciens*	Cytoplasm (inducible promoter)	Leaves	7 mg/gFW	4 mg/gFW	6 mg/gFW	Necrosis appeared after induction	Caimi et al. (1997)
Tobacco	*6-sft*	*Hordeum vulgare*	Vacuole	Leaves	16 mg/gDW	0.17 mg/gDW	25 mg/gDW	N.R.	Schellenbaum et al. (1999)
Tobacco	*6-sft*	*Hordeum vulgare*	Vacuole	Roots	55 mg/gDW	5.5 mg/gDW	10 mg/gDW	N.R.	Schellenbaum et al. (1999)
Tobacco	*levU*	Z. *mob His*	Cytoplasm	Leaves	N.R.	Visible on TLC	N.R.	Enhanced osmotic stress resistance	Park et al. (1999)
Tobacco	*1-sst*	*Lactuca sativa*	Vacuole	Leaves	0.1–3.8 mg/gFW	40–110 ug/gFW	N.R.	Enhanced freezing tolerance	Li et al. (2007)
Tobacco	*sacB*	*B. subtilis*	plastid	Leaf	N.R.	20 mg/gFW	N.R.	N.R.	Gerrits et al. (2001)
Potato	*sacB*	*B. subtilis*	Vacuole	Leaves (old)	N.R.	350 mg/gDW	N.R.	Reduced starch	van der Meer et al. (1994)
Potato	*sacB*	*B. subtilis*	Vacuole	Microtubers	N.R.	50 mg/gDW	N.R.	Reduced starch	van der Meer et al. (1994)
Potato	*sacB*	*B. subtilis*	Vacuole	Leaves	N.R.	5 mg/gFW	N.R.	Stunted growth	Pilon-Smits et al. (1996)
Potato	*sacB*	*B. subtilis*	Vacuole	Tubers	3–11 mg/gFW	11 mg/gFW	0.5–5 mg/gFW	Reduced starch content and browning	Pilon-Smits et al., 1996
Potato	*sacB*	*B. amyloliquefaciens*	Cytoplasm	Tubers	N.R.	5–50 mg/gDW	N.R.	Reduced starch and tuber DW	Caimi et al. (1997)
Potato	*1-sst*	*Cynara scolymus*	Vacuole	Tubers	14 μmol/gFW	19 μmol/gFW	N.R.	N.R.	Hellwege et al. (1997)
Potato	*1-sst+ 1-fft*	*Cynara scolymus*	Vacuole	Tubers	15 μmol/gFW	50 mg/gDW	3.9 μmol/gFW	No effect on the phenotype	Hellwege, Czapla, Jahnke, Willmitzer, & Heyer, 2000
Potato	*1-sst*	*Helianthus tuberosus*	Vacuole	Tubers	0.08 mg/gFW	1.8 mg/gFW	2.9 mg/gFW	No effect on the phenotype	Stoop et al. (2007)
Potato	*1-sst+ 1-fft*	*Helianthus tuberosus*	Vacuole	Tubers	2.0 mg/gFW	2.6 mg/gFW	3.1 mg/gFW	No effect on the phenotype	Stoop et al. (2007)
Potato – (starch-deficient)	*Isc*	*Erwinia amylovora*	Vacuole	Tubers	N.R.	70–120 mg/gDW	82–201 mg/gDW	No effect on the phenotype	Röber et al., 1996

Potato – (starch-deficient)	*Isc*	*Erwinia amylovora*	Apoplasm	Tubers	N.R.	190 mg/gDW	50 mg/gDW	Reduced tuber FW	Röber et al., 1996
Potato – (starch-deficient)	*Isc*	*Erwinia amylovora*	Cytoplasm	Tubers	N.R.	0 mg/gDW	N.R.	N.R.	Röber et al., 1996
Petunia	*1-sst*	*Helianthus tuberosus*	Vacuole	Leaf	0.41 mg/gFW	0.47 mg/gFW	N.R.	No effect on the phenotype	Van der Meer et al. (1998)
Petunia	*1-fft*	*Helianthus tuberosus*	Vacuole	Leaf	N.R.	0 mg/gFW	N.R.	No effect on the phenotype	Van der Meer et al. (1998)
Maize	*sacB*	*B. amyloliquefaciens*	Vacuole	Seeds	N.R.	10–80 mg/gDW	N.R.	No effect on the phenotype	Caimi et al. (1996)
Maize	*sacB*	*B. amyloliquefaciens*	Cytoplasm	Seeds	N.R.	16–18 mg/gDW	N.R.	Severe reduction of seed DW	Caimi et al. (1996)
Maize	*1-sst*	*Helianthus tuberosus*	Vacuole	Seeds	2.3 mg/gFW	3.2 mg/gFW	27 mg/gFW	N.R.	Stoop et al. (2007)
Maize	*1-sst+ 1-fft*	*Helianthus tuberosus*	Vacuole	Seeds	3.3 mg/gFW	0.6 mg/gFW	10 mg/gFW	N.R.	Stoop et al. (2007)
sugar maize (sh2)	*1-sst*	*Helianthus tuberosus*	Vacuole	Seeds	33 mg/gFW	6 mg/gFW	4 mg/gFW	N.R.	Stoop et al. (2007)
sugar maize (sh2)	*1-sst+ 1-fft*	*Helianthus tuberosus*	Vacuole	Seeds	24 mg/gFW	20 mg/gFW	4 mg/gFW	N.R.	Stoop et al. (2007)
Sugar beet	*1-sst*	*Helianthus tuberosus*	Vacuole	Leaves	1.8 μmol/gFW	0.9 μmol/gFW	4.8 μmol/gFW	No effect on the phenotype	Sévenier et al. (1998)
Sugar beet	*1-sst*	*Helianthus tuberosus*	Vacuole	Roots	23 μmol/gFW	110 μmol/gFW	25 μmol/gFW	No effect on the phenotype	Sévenier et al. (1998)
Sugar beet	*sacB*	*B. subtilis*	Vacuole	roots/shoot	N.R.	0.5 mg/gDW	N.R.	Enhanced drought resistance	Pilon-Smits et al. (1999)
Sugar beet	*1-sst+ 6-fft*	*Allium cepa*	Vacuole	Roots	29 mg/gFW	66.4 mg/gFW	34 mg/gFW	No effect on the phenotype	Weyens et al. (2004)
Rice	*6-sft*	*Triticum spp.*	Vacuole	Leaves	20 mg/gFW	3.7 mg/gFW	1.0 mg/gFW	No effect on the phenotype	Kawakami et al. (2008)
Rice	*1-sst*	*Triticum spp.*	Vacuole	Leaves	24 mg/gFW	16 mg/gFW	2.2 mg/gFW	No effect on the phenotype, increased total carbohydrate	Kawakami et al. (2008)
Rice	*1-sst*	*Smallanthus sonchiflius*	Vacuole	Leaves	N.R.	Visible on TLC	N.R.	N.R.	Pan et al. (2009)
Rice	*1-sst*	*Helianthus tuberosus*	Vacuole	Leaves	N.R.	Visible on TLC	N.R.	N.R.	Pan et al. (2009)
Sweet potato	*IsdA*	*Acetabacter diazotrophicus*	Vacuole	N.R.	N.R.	N.R.	N.R.	N.R.	Trujillo et al. (2000)
Sugarcane	*IsdA*	*Acetabacter diazotrophicus*	Vacuole	N.R.	N.R.	N.R.	N.R.	No effect on the phenotype	Trujillo et al. (2000)
Sugarcane	*1-sst*	*Cynara scolymus*	Vacuole	internodes	470 nmol/gFW	112 nmol/gFW	0.95 nmol/gFW	No change of sucrose pool	Nicholson (2007)
White clover	*ftf*	*Streptococcus salivarius*	Vacuole	Leaves	1.3 mg/gFW	3.1 mg/gFW	0.6 mg/gFW	Reduced growth	Jenkins et al. (2002)

N.R.: not reported.

expression level of the introduced *1-sst*. In rice three different *1-sst* genes were introduced and shown to be fully functional in the leaves resulting in detectable amounts of fructan (Kawakami, Sato, & Yoshida, 2008; Pan et al., 2009). Kawakami also showed that leaves of transgenic rice expressing the *1-sst* from *Triticum* spp. accumulated fructan up to 16 mg/gFW. This accumulation of fructan increased the total water-soluble carbohydrate content of the leaves, while the concentration of sucrose was not altered. Effects of the fructan accumulation on the plant performance or grain yield were not reported (Kawakami et al., 2008).

3.2.2. Introduction of 6-SFT in non-fructan plants

Sucrose:fructan 6-fructosyltransferase (6-SFT) is able to synthesize graminans and phleins. The genes encoding 6-SFT have been isolated from different grasses and introduced in non-fructan plants (Kawakami et al., 2008; Sprenger et al., 1997). Transgenic tobacco and rice plants harbouring *6-sft* accumulated kestose and a series of unbranched fructan of the phlein type (Kawakami et al., 2008; Sprenger et al., 1997). This showed that 6-SFT, in absence of 1-SST could form fructan in plants. However, the yield, for example in rice, was very low (3.7 mg/gFW) (Kawakami et al., 2008). This low yield could relate to the findings of Duchateau who showed that *in vitro* the enzyme exhibits much higher invertase activity than fructosyltransferase activity when incubated with sucrose as the sole substrate (Duchateau, Bortlik, Simmen, Wiemken, & Bancal, 1995). Since wild-type tobacco and rice plants do not accumulate fructan, but do contain sucrose, the 6-SFT's were probably not able to exert sufficient fructosyltransferase activity to accumulate large amounts of fructan in these crops.

3.2.3. Introduction of the complete fructan pathway in non-fructan plants

The introduction of combinations of 1-SST and 1-FFT or 6G-FFT in non fructan accumulating plants demonstrated that the pathway was fully transferable. The transgenic plants containing both genes did not only accumulate fructan with a higher DP than the 1-SST-expressing plants, but also showed inulin chain length distributions comparable to that of the species from which 1-FFT or 6G-FFT originated. Potato, maize and sugar beet transgenic plants, enriched with the fructan biosynthesis pathway from Jerusalem artichoke showed the same chain length distribution as Jerusalem artichoke (Koops et al., 2003; Stoop et al., 2007). These results are supported by the findings of Hellwege, Czapla, Jahnke, Willmitzer, and Heyer (2000) showing that the chain length distribution of inulin from globe artichoke and Jerusalem artichoke was reflected in a transient plant expression system (tobacco protoplasts) using the respective *1-fft* genes. The introduction of *1-sst* and *6g-fft* from onion in sugar beet resulted in the accumulation of a branched fructan with a profile closely resembling that from onion (Weyens et al., 2004), showing that the 6G-FFT-type of FFT also determines the fructan chain length distribution.

A general conclusion of these studies is that the fructan biosynthesis pathway could be transferred in non-fructan-accumulating plants. The genes were fully functional and their expression or the accumulation of fructan did not affect the phenotype of the plant. The chain length distribution of the fructan produced was dependent on the origin of the FFT.

4. Tailor-made fructan

Tailor-made fructan, the synthesis of fructan with the desired chain length and linkage type, largely rely on the choice of the proper genes.

The proof of principal for tailor-made fructan was delivered with transgenic sugar beet as shown by Koops et al. (2003). Different combinations of *1-sst* genes and *1-fft* genes resulted in sugar beet transformants with different inulin profiles. The introduction of *1-sst* and *6g-fft* from onion in sugar beet showed that tailor-made branched fructan could also be made in sugar beet (Weyens et al., 2004). Besides the origin of the genes used for biosynthesis, the availability of substrate and competition with other carbohydrate biosynthesis pathways are important for accumulation of fructan.

4.1. Sucrose availability determines fructan yield

The studies described above taught us that the host crop is very important in determining the yield. When comparing the potato plants described by Stoop et al. (2007) with the sugar beet described by Sévenier et al. (1998), both harbouring the same 1-SST of Jerusalem artichoke, it became clear that a big difference in yield characterized these two production platforms. Sugar beet accumulated 50 times more inulin compared to potato. Similarly, a 60 times higher inulin yield was found in starch deficient maize compared to starch accumulating varieties, both expressing the *1-sst* of Jerusalem artichoke (Stoop et al., 2007). Both sugar beet and the starch deficient maize accumulated high level of sucrose and it can be concluded that sucrose availability appears to be a determining factor for the fructan yield in those transgenic plants.

4.2. Competition of fructan accumulation with starch

Next to sucrose, many plants used as a platform for the production of fructan also accumulate starch, which might be a competing carbohydrate synthesis pathway using the same substrate as the fructan biosynthesis. The competition between the endogenous starch biosynthesis and the fructan biosynthesis appeared clearly when comparing the amounts of fructan accumulated in transgenic starch-deficient maize, storing sucrose, with the amount accumulated in transgenic starch accumulating maize expressing the same fructosyltransferase gene. While the transgenic starch deficient maize accumulated on average 20 mg/gFW fructan in the kernel, the starch accumulating transgenic accumulated 60 times less fructan (Stoop et al., 2007). In studies describing potato as host for the production of fructan, reduced amounts of starch were reported (Caimi et al., 1997; Pilon-Smits et al., 1996; van der Meer et al., 1994). Moreover, severe reduction of seed weight was reported in maize expressing *sacB* and the reduction in starch content was explained by the competition between the sucrose synthase and the SacB protein for sucrose (Caimi et al., 1996). Interestingly, Pilon-Smits et al. showed that when expressing *sacB* in potato the starch content was inversely correlated with the fructan level (Pilon-Smits et al., 1996). The conclusion from these studies could be that when producing fructan in a starch accumulating crop, the competition for sucrose between the starch production and the fructan biosynthesis will affect the synthesis of both or one of the storage carbohydrates, which is not favourable in a platform crop for fructan production.

4.3. Fructan enhances the drought and cold resistance of the platform crop

Fructan has several food and non-food applications and therefore is harvested from plants. But could it also be beneficial for the crop or for the transgenic plant producing fructan? It has been hypothesized that fructan accumulation could be beneficial to a plant because it could protect the plant from drought and cold stress (Pollock, 1986). It was shown in the fructan accumulating perennial ryegrass that the overexpression of *6-sft* or *1-sst* significantly increased the fructan content and, as a consequence, also increased the freezing tolerance (Hisano et al., 2004). Several studies showed that this property is also present in "new fructan accumulating" plants. Transgenic tobacco and sugar beet,

expressing bacterial *sacB*, showed better drought resistance (Pilon-Smits et al., 1995; Pilon-Smits, Terry, Sears, & van Dun, 1999). Tobacco showed also enhanced tolerance to osmotic stress when expressing the *sacB* (Park et al., 1999). The expression of *1-sst* from wheat and *Lactuca sativa* in respectively rice and tobacco enhanced chilling and freezing tolerance (Kawakami et al., 2008; Li, Yang, Zhang, Gao, & Zhang, 2007).

In most cases the fructan increased the total soluble carbohydrate composition and in this way enhanced the osmotic value and resistance to stress (Hisano et al., 2004; Li et al., 2007; Pilon-Smits et al., 1995). Other studies report on an increase of carbohydrate content only during the stress period, contributing to the stress tolerance (Pilon-Smits et al., 1995; Schellenbaum, Sprenger, Schüepp, Wiemken, & Boller, 1999). Schellenbaum proposed that as a consequence of the alteration of carbon partitioning due to the stress more sucrose became available for fructan synthesis. In conclusion, several examples of enhanced tolerance to stress of the transgenic crops producing fructan were reported. This aspect can reveal really advantageous for crops such as sugar beet, potato and rice that are grown in temperate zones where fluctuations in temperature during the growing season can result in drought-, freezing- or chilling stress for the plants.

4.4. Platform crops for tailor-made fructan

A well suited platform crop for fructan production preferably shows a high productivity, possesses a large storage organ, accumulates sucrose and produces only few or no starch. Furthermore, a processing chain for extraction of raw material should be available. Sugar beet is able to accumulate high levels of sucrose (200 mg/gFW), resulting in a yield of 10–14 tons/ha, which most probably explains why sugar beet was also shown to be a successful platform for the production of fructan (Koops et al., 2003; Sévenier et al., 1998; Weyens et al., 2004). A pilot study on the processing of the transgenic sugar beet suggests that the fructan extraction process currently used for chicory inulin is also applicable for fructan-producing sugar beet (Weyens et al., 2004).

Another potentially powerful platform crop for fructan production is sugarcane (*Saccharum* spp. L.) because it has a high content in sucrose, 500 mg/gDW in mature internodes (Glassop, Ryan, Bonnett, & Rae, 2010) and a well-established husbandry and processing chain. The production of sugar by sugarcane (6–14 tons/ha) is comparable to the production by sugar beet. Nicholson (2007) successfully introduced 1-SST from globe artichoke in sugarcane and showed 1-kestose production under field resembling conditions. The yield, however, was at most 112 nmol/gFW, which is 1000 times lower than found in transgenic sugar beet expressing *1-sst*. Additional studies will be necessary to address the full potential of sugar cane as production platform for fructan.

Rice is another interesting platform crop for the production of fructan since it is grown under different environmental conditions and in other "global areas" than the crops mentioned previously. It has been shown that rice was able to express *1-sst* and *6-sft* genes. Transgenic rice expressing *1-sst* accumulated up to 16 mg/gFW of fructan in leaves (Kawakami et al., 2008). Although fructan concentrations were low in the transgenic rice compared to other fructan accumulating crops, an interesting approach might be to produce fructan in the leaves of the rice plants, which are normally a waste product, and in this way add value to a rest stream. However a processing chain should be set up to isolate the fructan from the leaves. In summary, the crops with the highest potential for the production of fructan are sugar beet, sugarcane and, to a lesser extent, rice.

5. Conclusions and outlook

In this paper an overview is given of the research on modification of fructan synthesis in fructan accumulating plants and on the introduction of fructan synthesis in non-fructan-accumulating plants. The effects reported on the endogenous storage carbohydrates and the phenotype are described. The different genes and crops used are evaluated, specially focusing on the fructan yield. To date, mostly chicory is used for the commercial production of fructan. Chicory, however, shows some disadvantages such as the breakdown of inulin in autumn and the difficulty to modify the native inulin biosynthesis pathway via genetic modification, both aspects making this crop less suitable for the synthesis of tailor-made fructan.

The limitations seen in chicory for the production of tailor-made fructan are lacking in the described "new platform crops", although those new crops might not yet compete at the production level with chicory (11 tons inulin/ha). However, they have the advantages of lacking a breakdown mechanism and of providing a clean starting point for the tailor-made fructan production. Sugar beet, sugarcane and rice are the most promising potential production platforms. To date, a wide spectrum of genes has been isolated from different species allowing the fine tuning of the tailor-made fructan production. Dependent on the desired chain length, genes with different affinity for a sub-class of fructan could be combined in a transgenic plant. A way to produce longer inulin would be to combine 1-SST with two different 1-FFT's, one having high affinity for 1-kestose, for the synthesis of short polymers, and the second one having a relative higher affinity for longer polymers as acceptor for fructosyl units for further elongation of the polymers. In practice the genes used could be the 1-SST and 1-FFT from *H. tuberosus* (Koops & Jonker, 1994, 1996) combined with the high DP 1-FFT from *Echinops ritro* (Vergauwen et al., 2003). A way to accumulate longer inulin in naturally fructan accumulating plants can be by down-regulation of *feh1* expression via antisense or RNAi strategies or by (site-directed) mutagenesis of the *feh1* gene, resulting in lower exohydrolase activity in the plant and less depolymerisation of fructan. New mixed-types of fructan with putative interesting properties could be synthesized by combining genes from different classes as was anticipated by Sprenger et al. when expressing the barley 6-SFT in chicory (Sprenger et al., 1997). Moreover, protein engineering of the fructosyltransferase enzymes by modifications of the active site, might allow production of more and longer fructan in plants. Engineering of the chicory 1-FFT enzyme that would lower the affinity for sucrose and fructose as acceptor substrate would prevent the back-transfer of fructosyl units onto these acceptor molecules and thereby the decrease of the mDP. This could be performed by changing critical amino acids near or in the active site of 1-FFT, in a similar way as was performed by Lasseur et al. (2009) when changing a 6G-FFT/1-FFT into an 1-SST. Fructosyltransferase enzyme engineering might allow designing the tools for the synthesis of fructan with desired properties based on linkage type, linkage type combinations and polymer length.

We show that many possibilities exist, and that some already have been proven, for tailor-made fructan synthesis in crops.

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