Fructan biosynthesis in crop plants

The molecular regulation of fructan biosynthesis in chicory (*Cichorium intybus* L.)

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Chapter 1

General introduction

Introduction

Fructan is a polymer of fructose produced by plants and microorganisms. Within the plant kingdom, about 45,000 species, accounting for about 15% of the flowering plants, accumulate fructan as storage carbohydrate in addition to or instead of starch. Fructan accumulating species are mainly found in temperate and sub-tropical regions with seasonal or sporadic rainfall. Fructan is thought to be one of the factors that helps plants maintaining growth during periods of limited water availability [1]. Many microorganisms, some algae and some liverworts can also synthesize fructan, although the role of fructan in these organisms is essentially unclear. During the last decades, the use of fructan in the food industry has rapidly evolved, because of its health promoting characteristics and interesting functional properties. Nowadays, the types of fructan used in food are inulin, fructooligosaccharides (FOS), and graminan. Inulin is industrially extracted from chicory taproots while FOS are either derived from inulin through partial hydrolysis or prepared from sucrose with microbial enzymes [2, 3]. The scope of this introduction is to introduce the different aspects of fructan, its biosynthesis, its industrial applications, and its nutritional and medical significance, as well as the potential of genetic engineering to improve fructan quality and production.

Structure, biosynthesis and role of fructan in plants

Fructan is a polydisperse fructose polymer, of which biosynthesis uses sucrose as the starter molecule to which fructosyl units (obtained from sucrose) are added for further elongation (Fig. 1). While bacterial fructan has a very high degree of polymerization (DP) up to 100.000 fructose units, the DP of plant-derived fructan does generally not exceed DP100. Different classes of fructan can be distinguished depending on the glycosidic linkage between the fructose moieties. The most occurring fructans are described.

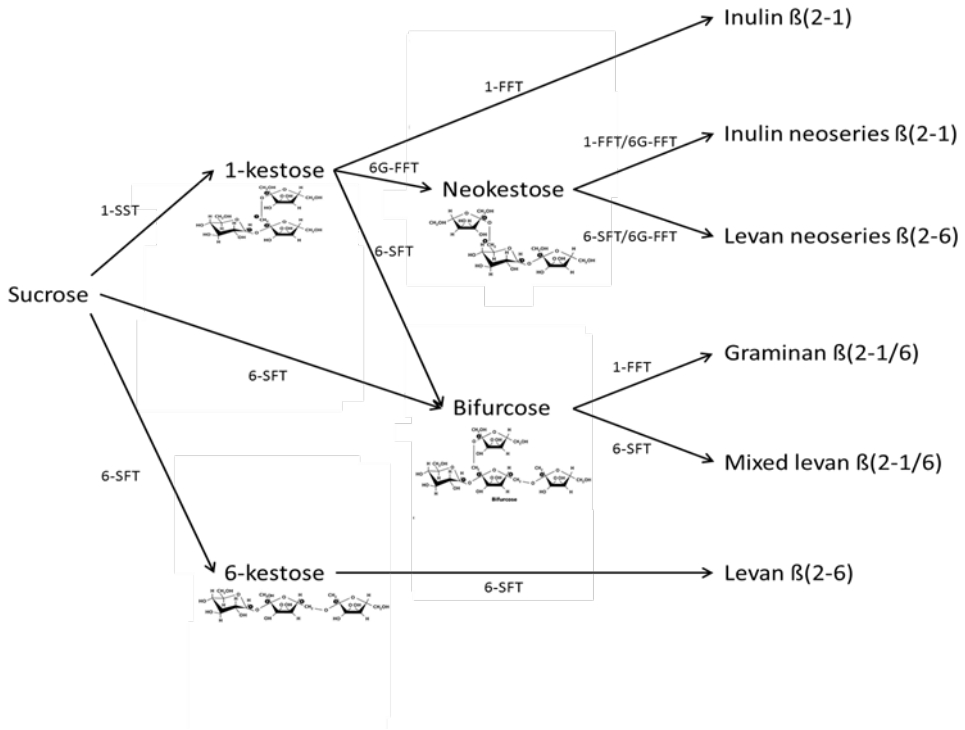

Figure 1. Schematic representation of enzymatic reaction of the mayor fructans biosynthesis in plants

ß(2-1) linkage – inulin and inulin neo-serie

Fructan with ß(2-1) glycosidic linkages is called inulin (Fig. 1 and 2). Inulin *sensu stricto* results from the elongation of the fructan chain starting from the C1 of the fructose moiety of the initial sucrose. The inulin neo-series comprises molecules for which elongation has started from the C1 of the fructose moiety as well as from the C6 of the glucose moiety of the starter sucrose (Fig. 1).The first description of inulin dates from the year 1804. Thomson named this fructan inulin because it was first extracted from the Asteraceaous *Inula helenium* [4]. Inulin is typical for the Asteraceae. Since the introduction

of chicory for the production of industrial fructan, the use of inulin has strongly increased. In 2000, about 20,000 ha of chicory were cultivated, mainly in The Netherlands, Belgium and the northern part of France, for the extraction of 120.000 tons inulin [3]. Some bacteria, such as Streptococcus and Bacillus species, and fungi, such as Penicillium and Aspergillus species, also synthesize inulin.

ß(2-6) linkage – levan/phlein

Fructan of the levan type, also called phlein in plants, are produced by some bacteria and grasses. In levan, the fructosyl groups are linked through ß(2-6) linkages (fig. 1). Analogous to the biosynthesis of inulin, the elongation starts at the C6 of the fructosyl group of the initial sucrose (levan) or from the C6 of the glucose group (levan neo-series).

Mixed type

Mixed type fructan, such as graminan and mixed levan, consists of fructose molecules linked through the two types of linkages leading to the formation of branched fructan molecules (Fig. 1). This kind of fructan is found in oat [5] and several other Graminaea. A mixed type of fructan was also found in a eudicot, *Pachysandra terminalis* with ß(2-6) as dominant linkage [6].

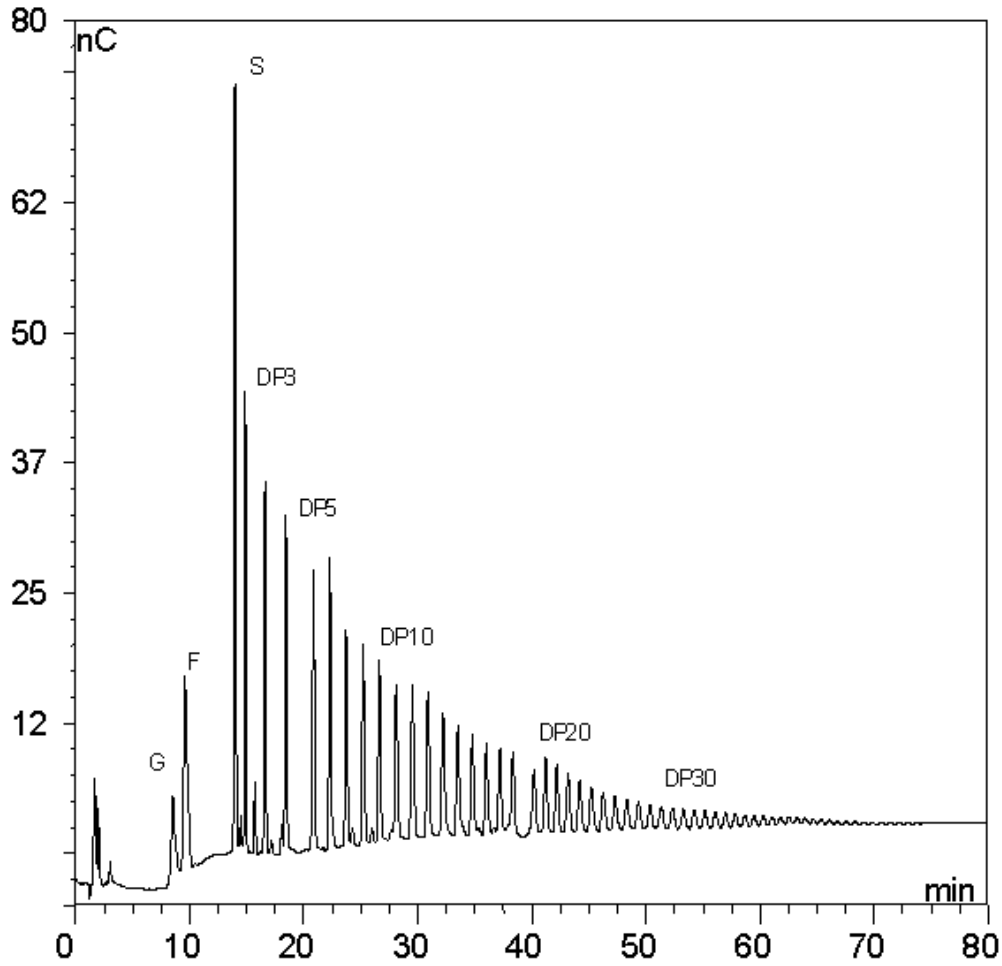

Figure 2. High Performance Anion Exchange Chromatography-Pulsed Amperometric Detection (HPAEC-PAD, Dionex) analysis of chicory inulin. The elution positions of glucose (G), fructose (F), sucrose (S), 1-kestose (DP3), DP5, DP10, DP20 and DP30 are indicated.

Nutritional and medical significance

Occurrence in the diet

Fructan is present in several food plants and is part of the daily diet of humans and animals [7]. It has been estimated that the daily intake by U.S. consumers is on average 1 to 4 g, while Europeans consume 3 to 11 g [8, 9]. Studies on the maximal acceptable daily dose of fructan showed that an intake of 20 g/day is well tolerated by healthy adult humans

[10]. About 70% and 25% of the intake of fructan can be explained by consumption of wheat- and onion- derived products, respectively. The remainder comes from several other sources such as garlic, leek, banana and other fruits [9, 11].

Nutritional aspects

The ß(2-1) or ß(2-6) linkages in fructan confer resistance towards human digestive enzymes that are involved in starch and sucrose degradation (ß-glucosidase, maltase-isomaltase, sucrase). Therefore, fructan is not digested in the upper part of the digestive track, and hence 85% of the ingested fructan reaches the colon, where it is fermented, mainly into short chain fatty acids (SCFA), by the endogenous microflora [12, 13]. Some studies showed that soluble fiber intake reduces serum glucose by reducing the postprandial glucose response. However, a meta-study performed by Bonsu *et al*. revealed no significant effect of inulin-type fructan and the authors suggested that more research is needed to determine whether the inulin-type fructans, inulin and oligofructose, have effects on blood glucose in humans [14]. A limited number of bacterial species, such as bifidobacteria and lactobacilli, have the ability to use fructan as a carbon source. Kolida and colleagues reported that out of 20 studies on the prebiotic effect of oligofructose and inulin, 18 reported selective fermentation by beneficial flora, bifidobacteria and to a lesser extent lactobacilli [15]. For a number of reasons these bacteria are considered to be beneficial to humans [16-18]. Because fructan promotes the growth of bifidobacteria over other non-beneficial bacteria it is considered a prebiotic [19-22]. The promotion of bifidobacteria can already be achieved with ingestion of as little as 5 g fructan/day which is in the range of the estimated average daily intake of fructan by Europeans [23]. A study in rats showed that the DP of the fructan that was fed affects the prebiotic effectiveness, with the fructooligosaccharides, oligofructose and medium DP (DP≥9) fructan promoting bifidiobacterial growth most. A positive effect of high DP fructan (DP ≥22) seemed to depend on the presence of other bacteria that initiate the degradation, followed by the possible subsequent stimulation of bifidobacterium growth [24]. Colonic fermentation of fructan produces short chain fatty acids (SCFA), lactate and gases. In addition, fructan increases the volume of microbial biomass in the colon, with each gram of ingested fructan increasing the fecal wet weight by 1.5 – 2 g. This bulking effect results in increased stool frequency and relief of constipation [12]. Several studies were performed on how prebiotic feeding with inulin contributes to animal well-being and technical performance of livestock animals as reviewed by van Loo [25]. Interestingly it was shown that fructan strengthens the colonization resistance against invading pathogens and suppresses parasites in pigs. The effect of application of chicory to the diet of pigs showed a positive correlation between the molar proportion that was added to the diet and the amount of butyrate-producing bacteria in the colon [26].

Claimed health promoting effects

Immunomodulation

Through the modification of the intestinal microbiota, β2-1 fructan may have beneficial effects on the immune function, and inflammatory processes and conditions. Findings are sometimes inconsistent most probably because human studies are difficult to carry out. However, results indicate that fructan is able to modulate some aspects of the immune function, to improve the host's ability to respond to several intestinal infections, and to modify some inflammatory conditions as stated by Lomax *et al*. [27]. In humans [28] and in rats the intake of FOS led to a significant reduction of the proinflammatory cytokine IL-1β in macrophage cultures and elevation of the levels of fecal IgA. According to the authors that would imply that a daily intake of fructan would help to preserve an anti-inflammatory state in phagocytic cells, and improve mucosal immunity, possibly preventing the risks associated with autoimmune and metabolic diseases [29]. Recently was shown that fructan possess direct signaling capacity on human immune cells when human peripheral blood mononuclear cells were stimulated *in vitro* with inulin [123]. In 2006 a first small open label trial was performed to investigate the effect of fructan on the chronic inflammation of the mucous membranes of the gastrointestinal tract, an effect of Crohn's disease. Inulin supplementation increased the fecal bifidobacteria concentration, reduced the disease activity and modified mucosal dendritic cell function [30]. Recently a double-blinded randomized controlled trial studying the impact of oligofructose combined with inulin in Crohn's disease patients showed that the composition of the intestinal microbiota was changed in a beneficial way, like was shown by Lindsay *et al*. The increased level of fecal butyrate that was measured probably has immunomodulatory and anti-inflammatory properties and could be therapeutic for Crohn's disease patients [31].

Cancer prevention

Several studies focused on the potential cancer preventing effect of fructan [20, 32]. Rats fed with fructan, together with carcinogenic compounds, developed less neoplastic tissue than the control rats. One hypothesis for this protective effect is that bifidobacteria, which are preferentially promoted by fructan, contain fewer enzymes converting precancerous molecules into carcinogenic compounds than other gut micro-organisms. Another health promoting factor of fructan may be its bulking effect. By accelerating the intestinal transit, the exposure time of the gut epithelium to potential carcinogens is reduced. Fructan has also been shown to lower the blood glucose and fatty acid levels, while high levels of these compounds stimulate the development of cancerous cells [33]. Several studies (reviewed in Taper (2002)) showed that inulin or oligofructose can be used to improve cancer chemotherapy treatments, with inulin showing additive or, in some cases, even synergistic effect with the drug used [33].

Osteoporosis prevention

Ingestion of fructan may also have a beneficial effect on mineral absorption [34]. The most frequently documented effect is an improved absorption of calcium [35], but also magnesium and iron uptake have been reported to be improved [36]. The effect of fructan on calcium uptake is principally occurring in the colon [37]. The improvement of mineral uptake can most probably be attributed to the increased bacterial production of SCFA and lactate, which is promoted by the greater supply of substrate, fructan. These acids reduce the cecal pH, thereby improving solubility and uptake of minerals [32]. In addition, proliferation of enterocytes is stimulated by lactate and butyrate (both increased by the increased bacterial growth) which enlarges the absorption surface [38].

Lipid metabolism improvement

In addition to total cholesterol, also triacylglycerol (TAG) has been identified as a risk factor for the development of coronary heart diseases. Several animal studies showed that inulin and oligofructose modified hepatic metabolism of lipids [39, 40]. The presence of fructan in the animal diet lowered blood TAG and phospholipid levels, most probably by the inhibition of liver lipogenic enzymes [41]. The bulking effect of fructan could lower the absorption of nutrients, such as carbohydrates, which would modify the glycemic/insulemic response. The increased release of peptides from endocrine cells of the intestinal mucosa could influence glucose and lipid metabolism in the adipose tissue [39]. However, the effects of fructan in humans are less clear, according to Delzenne *et al.* [42]. This could be the result of differences in background diet or in the experimental food used. Only the effect of inulin intake on circulating TAG and cholesterol levels appears to be consistent across many conditions [42, 43]. Hepatic fatty acid biosynthesis, which is found to be reduced by fructan in animals, is relatively inactive in humans. Indeed in a study on the effect of inulin on human body weight, hepatic and renal function parameters were not changed [44]. Nevertheless, a high-level glucose-based carbohydrate diet can activate this pathway in humans, which could explain why inulin showed a positive effect in certain studies.

Food and non-food applications of fructan

Food applications

Because of its unique properties, fructan use has rapidly increased during the last two decades, although the applications are still mainly limited to food products.

Low calorie sweetener

Although native chicory inulin, with an average DP of 13, is only slightly sweet (one tenth of the sweetness of sucrose), short chain fructan is much sweeter. Fructan with a DP of 3, 4, and 5 have a relative sweetness of 31, 22 and 16% of that of sucrose, respectively [2]. Therefore, short chain fructan can be used as a low calorie sweetener, alone or in combination with more intense sweeteners (aspartame, acesulfame K), which gives a synergistic sweetening effect [3]. The end products of fructan fermentation are SCFA, lactate, bacterial biomass and gasses, and only SCFA and lactate contribute to the host's energy metabolism resulting in a caloric value for inulin of 1 – 1.5 $kcal.g^{-1}$. The caloric value of a fructosyl unit of fructan is 30 – 40% of that of a digested fructose molecule [16].

Fat replacer

At concentrations above 15% inulin has gelling properties and forms a gel network after shearing [3]. The white creamy gel obtained can be incorporated in food to replace fat (up to 100%) while lowering the caloric value without compromising taste and texture [3]. Inulin has a synergistic effect with most other gelling agents and improves the stability of foams and emulsions. It can replace stabilizers in food [45]. Inulin is used in several food products such as dairy products, frozen desert, table spreads, salad dressings, meat products, chocolate and bread [3, 46].

Alcohol production

Plant material containing fructan can also be used to produce alcohol. The most noted example is the production of tequila, the most consumed Mexican liquor worldwide. Tequila is the product of fermentation of the stems and leave bases of *Agave tequilana* [47]. This plant accumulates a complex mixture of fructans containing principally ß(2-1) linkages but also ß(2-6) linked and branched moieties [48]. During tequila production the Agave plant material is cooked for at least 32 h at 100°C, after which the extract obtained is fermented and double distilled [49]. During the cooking the fructan is hydrolyzed into free fructose, which can readily be fermented.

Fructose syrup

Inulin is also used for the production of concentrated fructose syrup – which is mainly used for the sweetening of cold drinks - by hydrolysis [50, 51]. It is a possible alternative to high fructose corn syrup that is produced from corn starch. In contrast to the production of fructose from inulin, the production of fructose from starch is a multi-step

process in which starch is first hydrolyzed into glucose before being converted to fructose by glucose isomerase and further enriched by chromatographic methods.

Non-food applications

In contrast to the applications for food which require native, enriched or only hydrolysed fructan, non-food applications mostly require chemical modification of the fructose moieties. These modifications widen the spectrum of physiochemical properties and the number of possible non-food applications. More than 15 possible different chemical modifications of inulin leading to a wide range of potential applications were reviewed by Stevens [52, 53]. For example, carboxymethylinulin (CMI) and carboxyethylinulin inhibit calcium carbonate crystal formation. CMI is industrially produced by the Dutch Cosun/Sensus in collaboration with Solutia. CMI is effective even at very low concentrations and is reasonably biodegradable. CMI is added to cleaning agents to better remove stains [54]. The industrial applications of CMI consist in the treatment of industrial wastewater, process water and cooling water where it avoids scale formation in piping and heat exchangers. New applications for the use of CMI, e.g. in oil drilling operations, are under development. Fructan can also be converted into furan, a chemical building block for polymers, lacquers, thiophene, insecticides and pharmaceuticals [55, 56]. Esterified inulin and inulin carbamate possess surfactant properties and can be used as emulsifiers in detergents, cosmetics and pharmaceuticals. Polycarboxylate inulin and oxidized inulin are good sequestering agents for calcium ions and can be used in detergent formulations. Etherified inulin can be used as carrier for water-insoluble molecules in cosmetics or pharmaceuticals. Within the medical field, inulin can be coupled to active molecules in order to slow down their release in the body. Cross-linked inulin forms a hydrogel that can be used to make capsules for colon-specific drug delivery [57].

Biosynthesis of fructan

In plants, fructan biosynthesis is believed to occur in the vacuole. This hypothesis is supported by a number of observations. Fructan biosynthesis has been demonstrated with isolated vacuoles, which were only supplied with sucrose [58, 59]. Moreover, it was shown that kestose was not imported through the tonoplast, which excludes that small fructan molecules are being synthesized in the cytosol before being transported into the vacuole for further elongation [60]. Indeed, all fructosyl transferases cloned from plants contain a putative vacuolar targeting signal. Inulin biosynthesis has been investigated in a number of Asteraceae species, including Jerusalem artichoke and chicory [61, 62]. According to the model of Edelman and Jefford (1968), two enzymes, glycoproteins, are required for the synthesis of inulin [63]. The first enzyme, sucrose: sucrose 1-fructosyltransferase (1-SST) uses sucrose as a substrate and catalyzes the transfer of a fructosyl group from a donor sucrose onto a starter sucrose molecule (Fig. 1). The products

of this reaction are 1-kestose, the shortest member of the inulin series, and glucose. Further elongation of the fructose chain can be catalyzed to some extent by 1-SST, but only up to DP6. The synthesis of longer inulin molecules generally requires the action of fructan: fructan 1-fructosyltransferase (1-FFT) (Fig. 1). 1-FFT catalyzes the transfer of a fructosyl moiety from one molecule of fructan onto another. Only by removing sucrose from the inulin pool, FFT can mediate a net chain elongation of the inulin molecule. The upper limit in the DP, which is generally species specific, depends most probably on the catalytic properties of the 1-FFT, as was shown with *in vitro* studies [64, 65]. Synthesis of the neo-series of inulin in Liliaceae requires the presence of a specific fructan: fructan 6G-fructosyltransferase (6G-FFT), which catalyzes the transfer of a fructose group from 1-kestose onto the C6 of the glucosyl group of the starter sucrose (via a ß(2-6) linkage), yielding the trisaccharide neo-kestose (Fig. 1). Neo-kestose can be elongated on both terminal fructosyl groups with ß(2-1) linked fructoses through reactions catalyzed by a 1-FFT or 6G-FFT [66]. Several members of the Poaceae accumulate a mixed type of fructan comprising ß(2-1) and ß(2-6) linkages, graminan. Synthesis of graminan requires the action of at least two enzymes. In barley, 1-SST catalyzes the synthesis of 1-kestose while sucrose: fructan 6-fructosyltransferase (6-SFT) uses this kestose or sucrose to synthesize bifurcose (1-kestose with an additional fructose on the C6 of the fructose from the original sucrose) (Fig. 1). Further elongation of the fructan chain is most probably catalyzed by 6-SFT [67]. In contrast to plants, in microorganisms only a single enzyme called fructosyltransferase (FTF) or levansucrase (SacB) catalyzes fructan biosynthesis, producing levan. Some classes of bacteria produce inulin-type fructan by an enzyme called inulosucrase. In plants, fructan exohydrolase (FEH) catalyzes the catabolism of fructan [68]. FEH catalyzes the hydrolysis of the terminal fructose from fructan resulting in a shorter fructan molecule and free fructose. The breakdown of fructan is promoted in order to release the stored carbohydrates necessary in periods of stress, like cold or drought periods or flowering. It is generally accepted that 1-FEH in fructan accumulating plants evolved from cell wall invertase [124]. Evidence for this evolution comes from the fact that inactive cell wall invertases occur in non-fructan plants. Recently is described that these cell wall invertases have been mutated during evolution [125]. Further evolution may result in a 1-FEH. Indeed, FEH enzymes occur in non-fructan plants, like sugarbeet [126]. Giving evidence for the idea that FEH is evolved from cell wall invertase.

Role of fructan in plants

In plants, fructan acts as a storage carbohydrate. Biennial species, such as chicory, rely on the inulin stored during the first year of growth to initiate re-growth during the second year of their life cycle. Other plant species, such as cereals, accumulate both fructan and starch. Fructan temporarily accumulates in the stem and is mobilized after the onset of starch accumulation in the grain. Fructan can accumulate to up to 70% of the dry weight

without inhibiting photosynthesis [67]. While starch biosynthesis decreases dramatically when the temperature decreases below 10°C, fructan biosynthesis still proceeds even at low temperatures [67]. Chicory and Jerusalem artichoke 1-SST and 1-FFT retained about 50% of their activity at 5°C compared with their activity at optimal temperature (about 25°C) [61, 69-71]. Therefore, species that need to be photosynthetically active during winter or early spring may benefit from carbon storage as fructan rather than starch [67]. Fructan may also play a role in the protection against different environmental stresses as was shown for many fructan accumulating plants. *Bromus pictus*, a grass species that is well adapted to cold and drought, constitutively produces fructan, while *Bromus auleticus*, adapted to a warmer climate, only accumulates fructan under cold stress [72].Furthermore, tobacco and sugar beet that were genetically engineered to accumulate levan or inulin, were more tolerant to drought [73, 74] and frost [75, 76] than control plants. Based on studies in tall fescue and some other plant species, it has been suggested that hexose from hydrolyzed fructan lowers the water potential of intracellular liquid and allows continued leaf expansion during drought [77-79]. Hexose from hydrolyzed fructan, released into the apoplast, was shown to affect the freezing tolerance in some species while in others no correlation was found[77]. This contradiction shows that the role of sugars and fructan in protection from abiotic stress is complex as also concluded by Livingston *et al*. [80]. Improved tolerance to stresses could also be related to the ability of fructan to interact with membranes [81]. At room temperature, fructan stabilizes model membranes upon drying, most probably through lowering of the gel to liquid-crystalline phase transition temperature [82, 83]. Inulin showed a more profound interaction with membranes than levan, which could be explained by the larger flexibility of the inulin chain [84]. The interaction was shown to take place at the level of the phospholipid head groups, thus creating a protective layer of fructan at the surface of the membrane [80, 85, 86]. Some studies showed the presence of fructan near the cell membrane in the apoplast, although normally present only in the vacuole. In oat this is shown to be caused by membrane disruption during cold acclimation [87] and in Agave by phloem transport [88]. The presence of fructan in the apoplast supports the idea that fructan may protect tissues from freezing/dehydration injury in a direct way [80], rather than only indirectly via the release of hexoses. However, proof for this is still missing. The ability of fructan to scavenge radicles was recently studied [89]. Inulin turned out to be a better scavenger of both hydroxyl and superoxide radicals than mannitol and sucrose. Hydroxyl radicle scavenging is hypothesized to result in splicing of the fructan as a result of hydrogen transfer from the fructan to the radicle [90]. Also *in planta,* fructan could play a role in antioxidant defense. The addition of fructan to food with the goal to elevate the antioxidant properties of the food is not widely used, however. One study on food products with respect to antioxidant capacity of fructan derived products showed that agave syrup, a breakdown product of agave fructan, had no significant higher antioxidant capacity than table sugar [91].

Molecular biology and genetic engineering of fructan biosynthesis in plants

Structural genes

Several genes encoding fructan biosynthetic enzymes have been cloned from species from different plant families. A phylogenetic analysis of the amino acid sequences of fructosyltransferases shows three clusters; the biosynthesis enzymes from dicots, the biosynthesis enzymes from monocots and a cluster of FEH enzymes. Within the dicot biosynthesis enzymes cluster two distinguished classes are formed, based on enzymes catalyzing the same reaction, 1-SST and 1-FFT (Fig. 3). The monocot cluster forms one group of enzymes, however the 1-FFT from *Agave tequilana*, a Liliaceae and the 6-SST/6-SFT from *Pachysandra terminalis*, a buxaceae are more apart. The FEHs of all species cluster in a separate group. However, also within the FEH cluster a species dependent separation could be observed. The enzymes involved in fructan metabolism are believed to originate from invertases, a class of enzymes that catalyze the hydrolysis of sucrose and that share sequence homology with fructosyl transferases [92, 93]. While fructan biosynthetic enzymes are most probably derived from a vacuolar-type invertase, FEHs seem to originate from a cell-wall- type invertase. Fructosyltransferases likely evolved separately in dicot and monocot species [94] (Fig. 2).

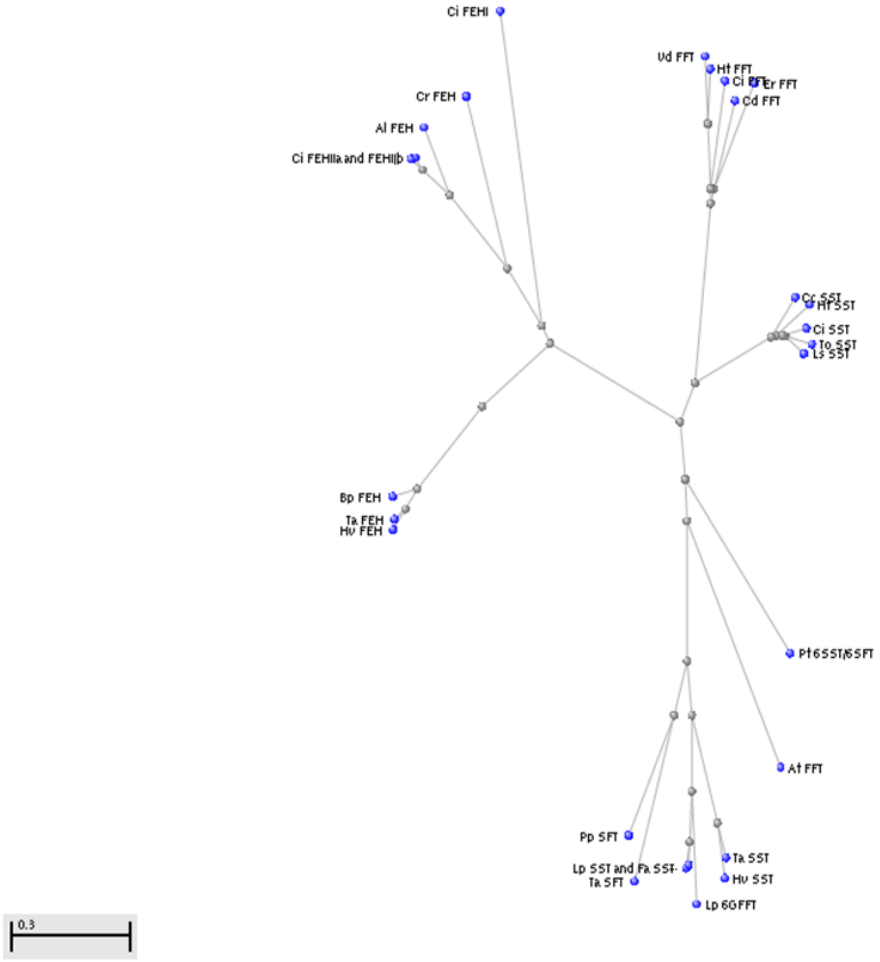

Figure 3. Phylogenetic tree of fructosyltransferases and fructan exo-hydrolases. The tree was made using COBALT, with settings; fast minimum evolution; Gishin distance and a max. sequence difference of 0.65 [95].
Abbreviations: Al FEH: *Arctium lappa*, FEH [BAL73222.1]; At FFT: *Agave tequilana*, FFT [ABS72186.1]; Bp FEH: *Bromus pictus*, FEH [ACZ65470.1]; Cc SST: *Cynara cardunculus var. scolymus*, SST [CAA70855.1]; Cc FFT: *Cynara cardunculus var. scolymus*, FFT [CAA04120.2[; Ci FEH I: *Cichorium intybus*, FEH I [CAC19366.1]; Ci FEH IIa: *Cichorium intybus*, FEH IIa [CAC37922.1]; Ci FEH IIB: *Cichorium intybus*, FEH IIb [CAC37923.1]; Ci FFT: *Cichorium intybus*, FFT [AAD00558.1]; Ci SST: *Cichorium intybus*, SST [AAB58909.1]; Cr FEH: *Campanula rapunculoides*, FEH [CAD49079.1]; Er FFT: *Echinops ritro*, FFT [CAH18891.1]; Fa SST: *Festuca arundinacea*, SST [CAC05261.1]; Ht FFT: *Helianthus tuberosus*, FFT [CAA08811.1]; Ht SST: *Helianthus tuberosus*, SST [CAA08812.1]; Hv FEH: *Hordeum vulgare*, FEH [CAE53426.1]; Hv SST: *Hordeum vulgare subsp. vulgare*, SST [CAD98793.2[; Lp 6GFFT: *Lolium perenne*, SFT [AAM13671.1]; Lp SST: *Lolium perenne*, SST [CAL51273.1]; Ls SST: *Lactuca sativa*, SST [ABX90019.1]; Pp SFT: *Phleum pratense*, SFT [BAH30252.1]; Pt 6SST/6SFT: *Pachysandra terminalis*, 6SST/6SFT [CBM41476.2[; Ta FEH: *Triticum aestivum*, FEH [ACI16115.1]; Ta FFT: *Triticum aestivum*, FFT [ACH73191.1]; Ta SFT: *Triticum aestivum*, SFT [AFK29573.1]; Ta SST: *Triticum aestivum*, SST [BAD72792.1]; To SST: *Taraxacum officinale*, SST [CAB60153.1]; Vd FFT: *Viguiera discolor*, FFT [CAH18892.1]
Numbers in brackets are gene bank accession numbers.

Regulation of fructan biosynthesis

Fructan biosynthesis in plants is regulated spatially as well as developmentally and the function of fructan in the species determines how. In grasses, for example, fructan plays an important role in re-growth after grazing or in response to changing environmental conditions [96, 97]. In cereal stems, fructan accumulates during growth and flowering, serving as temporal carbon storage, but disappears during grain filling. In the grain, fructan content is maximal at the initial stages of filling, but decreases when starch accumulation proceeds [122]. In the dicot chicory, only the taproot accumulates fructan. Fructan accumulation begins when the root starts thickening (about one month after sowing) and proceeds until late fall. The activity of FEH is relevant in relation to industrial processing, because it affects the quality (decreases the DP) and yield of inulin [68].

Carbohydrate-mediated regulation of fructan biosynthesis

The molecular basis of the regulation of fructan biosynthesis has been the subject of several studies but is still poorly understood. Excised leaves showed to be a powerful model to study the regulation of fructan biosynthesis in grasses [98-100] and in dicots as *Echinops ritro* [101] and chicory [102]. In excised wheat leaves *6-SFT* could be induced by several carbohydrates such as sucrose, glucose, fructose and maltose, while non-metabolisable sugars failed to promote induction [103]. Based on the use of different effectors these authors concluded that sugar sensing and induction of fructan biosynthesis proceed via a hexokinase- independent pathway and that protein kinases and protein phosphatases mediate the induction of fructan biosynthesis by mono- and disaccharides. Interestingly, trehalose, a dissacharide structurally related to sucrose, could replace sucrose as a regulatory compound in detached barley leaves [104]. The relationship between fructan biosynthesis and nitrogen was studied in chicory. When grown in the presence of a high nitrogen concentration, chicory exhibited decreased 1-SST activity and fructan content [105]. Kusch *et al*. [106] studied the regulation of 1-SST and 1-FFT activity in relation to the nitrogen availability in hairy roots of chicory. When the hairy roots were transferred from a normal medium to a medium with high-carbon and low-nitrogen, expression of 1-SST and 1-FFT was strongly induced and inulin accumulated. The same effects were observed when excised barley leaves were depleted of nitrate, while the addition of trehalose inhibited this effect of nitrate starvation [107]. In barley, fructan biosynthesis is primarily controlled via the regulation of the activity of the first committed enzyme. 1-SST mRNA and enzyme activity quickly responded to stimuli and were subject to rapid turnover, while 6-SFT mRNA and enzyme levels were much more stable. From these observations it was concluded that 1-SST plays the role of pacemaker in fructan biosynthesis in barley leaves [108]. In 2001, the promoter sequence of the barley *6-SFT* was isolated [109]. When fused to a reporter gene, this promoter showed induction by light and sucrose. Recently a transcription factor, TaMYB13 from *Triticum aestivum,* was shown to be positively regulating fructan biosynthesis in temperate grasses [110]. Mutagenesis of the TaMYB13-bindingsite in the promoters of the fructan biosynthesis-genes reduced gene transactivation.

Genetic engineering

A number of fructosyltransferase genes were cloned from several plant species and functionally tested in transgenic plants. In general two strategies were followed, expression of fructosyltransferases in a natural fructan accumulating plant species in order to modify the endogenous fructan profile, or expression in a non fructan accumulating plant species to introduce a whole new pathway.

Modification of endogenous fructan

Several attempts have been made to modify the endogenous type of fructan. Chicory naturally accumulates fructan of the inulin type. The introduction of the 6-SFT from barley, under control of the 35S promoter, into chicory did not result in the modification of the inulin profile of the roots, however, after induction of fructan biosynthesis in excised leaves of the transgenic plants, new fructan molecules, derived from 1-kestose, with a branched structure could be detected [111]. More effective was the introduction of the onion 6G-FFT into chicory which resulted in the accumulation of inulin from the neoseries in addition to the endogenous inulin [112]. Lolium normally accumulates levan of DP5-60. The introduction of the bacterial *SacB* gene resulted in the depletion of the normally present high molecular weight fraction (DP>50) and in the accumulation of small amounts of bacterial levan [113]. The transformed Lolium plants were stunted, had narrower leaves and poorly developed roots, especially during the onset of flowering.

Introduction of fructosyltransferase genes in non- fructan accumulating plant species

Several plant species that do not naturally accumulate fructan have been genetically engineered with genes encoding fructan biosynthesis enzymes. Both bacterial and plant derived genes were used. The use of bacterial genes to engineer plants never proved very successful in terms of fructan yield. Moreover, expression of bacterial fructosyltransferases generally induced severe alterations in the growth of the plant. These phenotypes were in some cases the consequence of improper targeting of the fructosyltransferase enzyme. The use of fructosyltransferases from plant origins did not result in growth abnormalities of the host plant. Furthermore, the use of plant genes allowed the accumulation of substantial amounts of fructan in transgenic plants. To date, the highest levels of accumulation were obtained with sugar beet transformed with the 1-SST from Jerusalem artichoke [114] or with the 1-SST and 6G-FFT from onion [115]. In sugar beet, up to 90% of the sucrose normally stored in the taproot was converted into fructan resulting in the accumulation of 65 mg.gFW^{-1} of short chain fructan [114].

Production of tailor-made fructan in plants

The structure of the fructan chain is an important quality determinant: different fructans have different food or non-food applications. The advantage of the transgenic fructan producing sugar beet introduced above over chicory or grasses is the high yield

potential, the presence of a well-organized production chain and the absence of fructan degrading enzymes which could compromise yield and quality. By expressing the Jerusalem artichoke *1-SST*, beet could be induced to produce DP3 and DP4 [114]. The advantage of these fructans is that they are non-digestible but still sweet. With native fructan this specific advantage can only be exploited after separation of the low molecular weight fructan from the higher DP fraction (>DP5). Such a costly process is not necessary for the fructan isolated from the *1-SST* overexpressing sugar beet. Using different combinations of different 1-SSTs and 1-FFTs it was possible to produce different inulin profiles [116]. The different transgenic sugar beet lines produced different inulin profiles. Another fructosyltransferase combination in sugar beet, 1-SST and 6G-FFT of onion, resulted in the production of branched fructan [115].

Another frequently used engineering concept is the modification of existing genes to manipulate the catalytic properties of the encoded enzymes. To this end, the use of a heterologous expression system to rapidly test the properties of the modified gene is extremely useful. Heterologous expression of plant fructosyltransferase in *E. coli* or in *Saccharomyces cerevisiae* was not successful so far, most probably because of improper processing of the expressed protein (cleavage, glycosylation). However, expression in *Pichia pastoris* proved successful [117]. In several labs this yeast system is nowadays used to test the functionality of newly isolated plant fructosyltransferase genes. Using this expression system, it was shown that the catalytic specificity of barley 6-SFT and tall fescue 1-SST are controlled by the large sub-unit of these enzymes [118]. More recent work on the effect of amino acid substitutions and the influence of it on the substrate specificities [119, 120, 121] gave valuable information for the better understanding of the catalytic activity of plant fructosyltransferases, a prerequisite for the assembly of chimeric enzymes with defined properties for industrial applications.

Outline of this thesis

During the last years, the use of food with specific nutritional or physiological properties, functional foods, has increased continuously. Fructan is an interesting compound since it has, based on its chemical properties, various applications in food, medicine and non-food products. At the moment, chicory is still the main source of inulin, one of the most important fructans, with a steadily increasing acreage. Genetically engineered plant species, platform crops, could present an alternative to chicory, allowing both an increase of total production and the design of tailor-made fructan best suited for industrial applications. High yielding crops, such as sugar beet or sugar cane, can be engineered to produce fructan, and could provide additional and advantageous new sources of fructan in the future. To achieve an increase of total fructan production and the tailor-made production of different fructan types in chicory or other (platform) crops, more knowledge about inulin biosynthesis is required. Therefore, in this thesis I investigate inulin accumulation and biosynthesis, and its regulation.

In **Chapter 2** I study the mechanism behind the changes in the pattern of inulin composition during the growing season and describe the changes observed in expression and activity of the fructosyltransferase enzymes and environmental factors in relation to the changes in carbohydrate composition. Three different phases in the growing season were defined based on product formation, enzyme activity and gene expression. The results were validated by performing experiments under controlled conditions in climate rooms. Transgenic chicory plants were generated aiming at improved inulin biosynthesis. This study provides background information on the regulation and how to improve inulin yield and quality in chicory.

In **Chapter 3** I want to get a better understanding of the regulation of the genes involved in inulin biosynthesis to be able to change the expression of genes involved. The chapter describes the regulation of the fructan metabolic genes, *1-SST* and *1-FFT* in chicory. The use of computational analysis on putative cis-acting elements in combination with feeding studies in chicory, potato and Arabidopsis transgenics reveals valuable information about the regulation of inulin biosynthesis in chicory.

In **Chapter 4** the ectopic expression of the complete fructan biosynthetic pathway in maize and potato is pursued, aiming at the production of inulin and generating more knowledge on the developmental modulation and stability of tailor-made inulin in these crops. The type of organs for storage of fructan is discussed as well as the availability of sugar precursors, important for high levels of accumulation of inulin.

In **Chapter 5** I discuss the main findings from this thesis and place them in the frame of the recent advantages in fructan research. Important factors for tailor-made fructan biosynthesis and putative platform crops are evaluated. The chapter is finalized with future perspectives on fructan research and tailor-made fructan production.

References

[1] G.A.F. Hendry, Evolutionary origins and natural functions of fructans – a climatological, biogeographic and mechanistic appraisal, New Phytologist, 123 (1993) 3-14.

[2] J.W. Yun, Fructooligosaccharides—Occurrence, preparation, and application, Enzyme and Microbial Technology, 19 (1996) 107-117.

[3] A. Franck, L. De Leenheer, Inulin, in: E.J. Vandamme, S.D. Baets, A. Steinbüchel (Eds.) Biopolymers Polysaccharides II: Polysaccharides from Eukaryotes, John Wiley & Sons, Inc., 2002, pp. 439-479.

[4] T. Thomson, T. Cooper, A system of chemistry: in four volumes, Printed and published by Abraham Small, No. 112, Chesnut Street, 1818.

[5] D.P. Livingston, D.P. Knievel, F.E. Gildow, Fructan synthesis in oat, New Phytologist, 127 (1994) 27-36.

[6] W. Van den Ende, M. Coopman, S. Clerens, R. Vergauwen, K. Le Roy, W. Lammens, A. Van Laere, Unexpected Presence of Graminan- and Levan-Type Fructans in the Evergreen Frost-Hardy Eudicot *Pachysandra terminalis* (Buxaceae): Purification, Cloning, and Functional Analysis of a 6-SST/6-SFT Enzyme, Plant Physiology, 155 (2011) 603-614.

[7] L. Incoll, G. Bonnett, The occurrence of fructan in food plants, in: A. Fuchs (Ed.) Inulin and inulin- containing crops, Elsevier Science Publishers B.V., Amsterdam, 1993, pp. 309-322.

[8] J. Van Loo, P. Coussement, L. De Leenheer, H. Hoebregs, G. Smits, On the presence of inulin and oligofructose as natural ingredients in the western diet, Critical Reviews in Food Science & Nutrition, 35 (1995) 525-552.

[9] A.J. Moshfegh, J.E. Friday, J.P. Goldman, J.K.C. Ahuja, Presence of inulin and oligofructose in the diets of Americans, The Journal of nutrition, 129 (1999) 1407S-1411s.

[10] J. Bruhwyler, F. Carreer, E. Demanet, H. Jacobs, Digestive tolerance of inulin-type fructans: a double-blind, placebo-controlled, cross-over, dose-ranging, randomized study in healthy volunteers, International Journal of Food Sciences and Nutrition, 60 (2009) 165-175.

[11] C. L'homme, J. Peschet, A. Puigserver, A. Biagini, Evaluation of fructans in various fresh and stewed fruits by high-performance anion-exchange chromatography with pulsed amperometric detection, Journal of chromatography A, 920 (2001) 291-297.

[12] G. Flamm, W. Glinsmann, D. Kritchevsky, L. Prosky, M. Roberfroid, Inulin and oligofructose as dietary fiber: a review of the evidence, Critical Reviews in Food Science and Nutrition, 41 (2001) 353-362.

[13] M. Roberfroid, N. Delzenne, Dietary fructans, Annual review of nutrition, 18 (1998) 117-143.

[14] N.K.A. Bonsu, C.S. Johnson, K.M. McLeod, Can dietary fructans lower serum glucose?, Journal of Diabetes, 3 (2011) 58-66.

[15] S. Kolida, K. Tuohy, G. Gibson, Prebiotic effects of inulin and oligofructose, British Journal of Nutrition, 87 (2002) S193-S197.

[16] M. Roberfroid, Dietary fiber, inulin, and oligofructose: a review comparing their physiological effects, Critical Reviews in Food Science & Nutrition, 33 (1993) 103-148.

[17] V. De Preter, H.M. Hamer, K. Windey, K. Verbeke, The impact of pre- and/or probiotics on human colonic metabolism: Does it affect human health?, Molecular nutrition & food research, 55 (2011) 46-57.
[18] F. Miremadi, N. Shah, Applications of inulin and probiotics in health and nutrition, International, Food Research Journal 19 (2012): 1337-1350
[19] G.R. Gibson, M.B. Roberfroid, Dietary modulation of the human colonie microbiota: introducing the concept of prebiotics, Journal of Nutrition, 125 (1995) 1401-1412.
[20] B.L. Pool-Zobel, J. Sauer, Overview of experimental data on reduction of colorectal cancer risk by inulin-type fructans, The Journal of nutrition, 137 (2007) 2580-2584.
[21] S. Kolida, D. Meyer, G.R. Gibson, A double-blind placebo-controlled study to establish the bifidogenic dose of inulin in healthy humans, 61 (2007) 1189-1195.
[22] C. Ramirez-Farias, K. Slezak, Z. Fuller, A. Duncan, G. Holtrop, P. Louis, Effect of inulin on the human gut microbiota: stimulation of *Bifidobacterium adolescentis* and *Faecalibacterium prausnitzii*, British Journal of Nutrition, 101 (2009) 541-550.
[23] V.A. Rao, The prebiotic properties of oligofructose at low intake levels, Nutrition Research, 21 (2001) 843-848.
[24] E. Biedrzycka, M. Bielecka, Prebiotic effectiveness of fructans of different degrees of polymerization, Trends in Food Science & Technology, 15 (2004) 170-175.
[25] J. Van Loo, How chicory fructans contribute to zootechnical performance and well-being in livestock and companion animals, The Journal of nutrition, 137 (2007) 2594S-2597S.
[26] H. Liu, E. Ivarsson, J. Dicksved, T. Lundh, J.E. Lindberg, Inclusion of Chicory (*Cichorium intybus* L.) in Pigs' Diets Affects the Intestinal Microenvironment and the Gut Microbiota, Applied and environmental microbiology, 78 (2012) 4102-4109.
[27] A.R. Lomax, P.C. Calder, Prebiotics, immune function, infection and inflammation: a review of the evidence, British Journal of Nutrition, 101 (2009) 633-658.
[28] G.T.C. Delgado, W.M.S.C. Tamashiro, G.M. Pastore, Immunomodulatory effects of fructans, Food Research International, 43 (2010) 1231-1236.
[29] G.T. Choque Delgado, R. Thomé, D.L. Gabriel, W.M.S.C. Tamashiro, G.M. Pastore, Yacon (*Smallanthus sonchifolius*)-derived fructooligosaccharides improves the immune parameters in the mouse, Nutrition Research, 32 (2012) 884-892.
[30] J.O. Lindsay, K. Whelan, A.J. Stagg, P. Gobin, H.O. Al-Hassi, N. Rayment, M. Kamm, S.C. Knight, A. Forbes, Clinical, microbiological, and immunological effects of fructo-oligosaccharide in patients with Crohn's disease, Gut, 55 (2006) 348-355.
[31] V. De Preter, M. Joossens, V. Ballet, Z. Shkedy, P. Rutgeerts, S. Vermeire, K. Verbeke, Metabolic Profiling of the Impact of Oligofructose-Enriched Inulin in Crohn's Disease Patients: A Double-Blinded Randomized Controlled Trial, Clinical and translational gastroenterology, 4 (2013) e30.
[32] J.E. Teitelbaum, W.A. Walker, Nutritional Impact of Pre-and Probiotics as Protective Gastrointestinal Organisms*, Annual review of nutrition, 22 (2002) 107-138.
[33] H. Taper, M. Roberfroid, Inulin/oligofructose and anticancer therapy, British Journal of Nutrition, 87 (2002) S283-S286.
[34] W. Caers, The role of prebiotic fibres in the process of calcium absorption, in: J.W. Van der Kamp, N.-G. Asp, J. Miller Jones, G. Schaafsma (Eds.) Dietary fibre: bio-active

carbohydrates for food and feed, Wageningen Academic Publishers, Wageningen, 2004, pp. 255-264.
[35] V. Coxam, Current Data with Inulin-Type Fructans and Calcium, Targeting Bone Health in Adults, The Journal of nutrition, 137 (2007) 2527S-2533S.
[36] C. Coudray, J.C. Tressol, E. Gueux, Y. Rayssiguier, Effects of inulin-type fructans of different chain length and type of branching on intestinal absorption and balance of calcium and magnesium in rats, European journal of nutrition, 42 (2003) 91-98.
[37] S.A. Abrams, K.M. Hawthorne, O. Aliu, P.D. Hicks, Z. Chen, I.J. Griffin, An Inulin-Type Fructan Enhances Calcium Absorption Primarily via an Effect on Colonic Absorption in Humans, The Journal of nutrition, 137 (2007) 2208-2212.
[38] K.E. Scholz-Ahrens, P. Ade, B. Marten, P. Weber, W. Timm, Y. Açil, C.-C. Glüer, J. Schrezenmeir, Prebiotics, Probiotics, and Synbiotics Affect Mineral Absorption, Bone Mineral Content, and Bone Structure, The Journal of nutrition, 137 (2007) 838S-846S.
[39] N. Delzenne, C. Daubioul, A. Neyrinck, M. Lasa, H. Taper, Inulin and oligofructose modulate lipid metabolism in animals: review of biochemical events and future prospects, British Journal of Nutrition, 87 (2002) 255-260.
[40] G.R.J. Taylor, C.M. Williams, Effects of probiotics and prebiotics on blood lipids, British Journal of Nutrition, 80 (1998) S225-S230.
[41] N.M. Delzenne, N.N. Kok, Biochemical basis of oligofructose-induced hypolipidemia in animal models, The Journal of nutrition, 129 (1999) 1467S-1470s.
[42] N. Delzenne, P.D. Cani, A. Neyrinck, J. Versalovic, M. Wilson, Prebiotics and lipid metabolism, Therapeutic microbiology: Probiotics and related strategies, ASM Press, Washington, DC, (2008) 183-192.
[43] F. Brighenti, Dietary fructans and serum triacylglycerols: a meta-analysis of randomized controlled trials, The Journal of nutrition, 137 (2007) 2552S-2556S.
[44] H.-Y. Yang, S.-C. Yang, J.C.-J. Chao, J.-R. Chen, Beneficial effects of catechin-rich green tea and inulin on the body composition of overweight adults, British Journal of Nutrition, 107 (2012) 749-754.
[45] P. Kip, D. Meyer, R.H. Jellema, Inulins improve sensoric and textural properties of low-fat yoghurts, International Dairy Journal, 16 (2006) 1098-1103.
[46] C. Morris, G.A. Morris, The effect of inulin and fructo-oligosaccharide supplementation on the textural, rheological and sensory properties of bread and their role in weight management: A review, Food Chemistry, 133 (2012) 237-248.
[47] M.C. Cedeño, Tequila production, Critical reviews in biotechnology, 15 (1995) 1-11.
[48] M.G. Lopez, N.A. Mancilla-Margalli, G. Mendoza-Diaz, Molecular structures of fructans from *Agave tequilana* Weber var. azul, Journal of Agricultural and Food Chemistry, 51 (2003) 7835-7840.
[49] N.A. Mancilla-Margalli, M.G. López, Generation of Maillard compounds from inulin during the thermal processing of *Agave tequilana Weber* var. azul, Journal of Agricultural and Food Chemistry, 50 (2002) 806-812.
[50] J. Barta, A. Fuchs, Jerusalem artichoke as a multipurpose raw material for food products of high fructose or inulin content, in: A. Fuchs (Ed.) Inulin and Inulin Containing Crops, Elsevier Science Publishers, 1991, pp. 323-339.
[51] A.K. Gupta, N. Kaur, Fructan storing plants: a potential source of high fructose syrups, Journal of scientific & industrial research, 56 (1997) 447-452.

[52] C.V. Stevens, A. Meriggi, K. Booten, Chemical modification of inulin, a valuable renewable resource, and its industrial applications, Biomacromolecules, 2 (2001) 1-16.
[53] H. Van Bekkum, D.L. Verraest, New Starch and Inulin derived Products with Potential Applications, in: Perspektiven nachwachsender Rohstoffe in der Chemie, John Wiley & Sons, Inc., 2009, pp. 191-203.
[54] I.L.S.S. Leonard, BE), Kochowski, Valerie (Rhode - St-Genese, BE), Bonnechere-delstanche, Genevieve (Awans, BE), Henry, Olivier (Nil-St-Vincent, BE), Cleaning composition with improved stain removal, in, DEQUEST AG (Zug, CH), United States, 2013.
[55] A. Gandini, Furans as offspring of sugars and polysaccharides and progenitors of a family of remarkable polymers: a review of recent progress, Polymer Chemistry, 1 (2010) 245-251.
[56] A. Gandini, M.N. Belgacem, Furans in polymer chemistry, PROG POLYM SCI(OXFORD), 22 (1997) 1203-1379.
[57] T. Barclay, M. Ginic-Markovic, P. Cooper, N. Petrovsky, Inulin: A versatile polysaccharide with multiple pharmaceutical and food chemical uses, J Excipients Food Chem, 1 (2010) 27-50.
[58] C.W.E. Darwen, P. John, Localization of the enzymes of fructan metabolism in vacuoles isolated by a mechanical method from tubers of Jerusalem artichoke (*Helianthus tuberosus* L.), Plant Physiology, 89 (1989) 658-663.
[59] M. Frehner, F. Keller, A. Wiemken, Localization of Fructan Metabolism in the Vacuoles Isolated from Protoplasts of Jerusalem Artichoke Tubers (*Helianthus tuberosus* L.), Journal of Plant Physiology, 116 (1984) 197-208.
[60] H.G. Pontis, P. González, E. Etxeberria, Transport of 1-kestose across the tonoplast of Jerusalem artichoke tubers, Phytochemistry, 59 (2002) 241-247.
[61] A.J. Koops, H.H. Jonker, Purification and Characterization of the Enzymes of Fructan Biosynthesis in Tubers of *Helianthus tuberosus Colombia* (II. Purification of Sucrose:Sucrose 1-Fructosyltransferase and Reconstitution of Fructan Synthesis in Vitro with Purified Sucrose:Sucrose 1-Fructosyltransferase and Fructan:Fructan 1-Fructosyltransferase), Plant Physiology, 110 (1996) 1167-1175.
[62] W. Van den Ende, A. Van Laere, De-novo synthesis of fructans from sucrose in vitro by a combination of two purified enzymes (sucrose: sucrose 1-fructosyl transferase and fructan: fructan 1-fructosyl transferase) from chicory roots (*Cichorium intybus* L.), Planta, 200 (1996) 335-342.
[63] J. Edelman, T. Jefford, The mechanisim of fructosan metabolism in higher plants as exemplified in *Helianthus tuberosus*, New Phytologist, 67 (1968) 517-531.
[64] E.M. Hellwege, M. Raap, D. Gritscher, L. Willmitzer, A.G. Heyer, Differences in chain length distribution of inulin from *Cynara scolymus* and *Helianthus tuberosus* are reflected in a transient plant expression system using the respective 1-FFT cDNAs, FEBS Letters, 427 (1998) 25-28.
[65] R. Vergauwen, A. Van Laere, W. Van den Ende, Properties of Fructan:Fructan 1-Fructosyltransferases from Chicory and Globe Thistle, Two Asteracean Plants Storing Greatly Different Types of Inulin, Plant Physiology, 133 (2003) 391-401.
[66] T. Ritsema, J. Joling, S. Smeekens, Patterns of fructan synthesized by onion fructan:fructan 6G-fructosyltransferase expressed in tobacco BY2 cells is

fructan:fructan 1-fructosyltransferase needed in onion?, New Phytologist, 160 (2003) 61-67.
[67] I. Vijn, S. Smeekens, Fructan: More Than a Reserve Carbohydrate?, Plant Physiology, 120 (1999) 351-360.
[68] A. Van Laere, W. Van den Ende, Inulin metabolism in dicots: chicory as a model system, Plant, Cell & Environment, 25 (2002) 803-813.
[69] A.J. Koops, H.H. Jonker, Purification and characterization of the enzymes of fructan biosynthesis in tubers of *Helianthus tuberosus* 'Colombia': I. Fructan: fructan fructosyl transferase, Journal of Experimental Botany, 45 (1994) 1623-1631.
[70] W. Van den Ende, D. Van Wonterghem, E. Dewil, P. Verhaert, A. De Loof, A. Van Laere, Purification and characterization of 1-SST, the key enzyme initiating fructan biosynthesis in young chicory roots (*Cichorium intybus*), Physiologia Plantarum, 98 (1996) 455-466.
[71] W. Van den Ende, D. Van Wonterghem, P. Verhaert, E. Dewil, A. Van Laere, Purification and characterization of fructan: fructan fructosyl transferase from chicory (*Cichorium intybus* L.) roots, Planta, 199 (1996) 493-502.
[72] A.F. Puebla, G.L. Salerno, H.G. Pontis, Fructan metabolism in two species of Bromus subjected to chilling and water stress, New Phytologist, 136 (1997) 123-129.
[73] E. Pilon-Smits, M. Ebskamp, M.J. Paul, M. Jeuken, P.J. Weisbeek, S. Smeekens, Improved Performance of Transgenic Fructan-Accumulating Tobacco under Drought Stress, Plant Physiology, 107 (1995) 125-130.
[74] E.A.H. Pilon-Smits, N. Terry, T. Sears, K. van Dun, Enhanced drought resistance in fructan-producing sugar beet, Plant Physiology and Biochemistry, 37 (1999) 313-317.
[75] T. Konstantinova, D. Parvanova, A. Atanassov, D. Djilianov, Freezing tolerant tobacco, transformed to accumulate osmoprotectants, Plant Science, 163 (2002) 157-164.
[76] H.-J. Li, A.-F. Yang, X.-C. Zhang, F. Gao, J.-R. Zhang, Improving freezing tolerance of transgenic tobacco expressing sucrose: sucrose 1-fructosyltransferase gene from *Lactuca sativa*., Plant Cell, Tissue and Organ Culture, 89 (2007) 37-48.
[77] L. Hoffman, M. DaCosta, J.S. Ebdon, J. Zhao, Effects of drought preconditioning on freezing tolerance of perennial ryegrass, Environmental and Experimental Botany, 79 (2012) 11-20.
[78] W.G. Spollen, C.J. Nelson, Response of fructan to water deficit in growing leaves of tall fescue, Plant Physiology, 106 (1994) 329-336.
[79] B. Darbyshire, R. Henry, The distribution of fructans in onions, New Phytologist, 81 (1978) 29-34.
[80] D.P. Livingston, D.K. Hincha, A.G. Heyer, Fructan and its relationship to abiotic stress tolerance in plants, Cellular and molecular life sciences, 66 (2009) 2007-2023.
[81] R. Demel, E. Dorrepaal, M. Ebskamp, J. Smeekens, B. De Kruijff, Fructans interact strongly with model membranes, Biochimica et Biophysica Acta (BBA)-Biomembranes, 1375 (1998) 36-42.
[82] D.K. Hincha, E.M. Hellwege, A.G. Heyer, J.H. Crowe, Plant fructans stabilize phosphatidylcholine liposomes during freeze-drying, European Journal of Biochemistry, 267 (2001) 535-540.

[83] D.K. Hincha, E. Zuther, E.M. Hellwege, A.G. Heyer, Specific effects of fructo-and gluco-oligosaccharides in the preservation of liposomes during drying, Glycobiology, 12 (2002) 103-110.
[84] I.J. Vereyken, J. Albert van Kuik, T.H. Evers, P.J. Rijken, B. De Kruijff, Structural requirements of the fructan-lipid interaction, Biophysical journal, 84 (2003) 3147-3154.
[85] I.J. Vereyken, V. Chupin, R.A. Demel, S. Smeekens, B. De Kruijff, Fructans insert between the headgroups of phospholipids, Biochimica et Biophysica Acta (BBA)-Biomembranes, 1510 (2001) 307-320.
[86] I.J. Vereyken, V. Chupin, F.A. Hoekstra, S. Smeekens, B. De Kruijff, The effect of fructan on membrane lipid organization and dynamics in the dry state, Biophysical journal, 84 (2003) 3759-3766.
[87] D.P. Livingston III, C.A. Henson, Apoplastic sugars, fructans, fructan exohydrolase, and invertase in winter oat: responses to second-phase cold hardening, Plant Physiology, 116 (1998) 403-408.
[88] N. Wang, P.S. Nobel, Phloem transport of fructans in the crassulacean acid metabolism species Agave deserti, Plant Physiology, 116 (1998) 709-714.
[89] D. Peshev, R. Vergauwen, A. Moglia, E. Hideg, W. Van den Ende, Towards understanding vacuolar antioxidant mechanisms: a role for fructans? Journal of Experimental Botany, 64 (2013), 1025-1038.
[90] E. Hernandez-Marin, A. Martínez, Carbohydrates and Their Free Radical Scavenging Capability: A Theoretical Study, The Journal of Physical Chemistry B, (2012).
[91] K.M. Phillips, M.H. Carlsen, R. Blomhoff, Total Antioxidant Content of Alternatives to Refined Sugar, Journal of the American Dietetic Association, 109 (2009) 64-71.
[92] W. Van den Ende, A. Michiels, J. De Roover, A. Van Laere, Fructan biosynthetic and breakdown enzymes in dicots evolved from different invertases. Expression of fructan genes throughout chicory development, TheScientificWorldJournal-[electronic-resource], 2 (2002) 1281-1295.
[93] I.M. Van der Meer, A.J. Koops, J.C. Hakkert, A.J. van Tunen, Cloning of the fructan biosynthesis pathway of Jerusalem artichoke, The Plant Journal, 15 (1998) 489-500.
[94] J.Z. Wei, N.J. Chatterton, Fructan biosynthesis and fructosyltransferase evolution: Expression of the 6-SFT (sucrose: fructan 6-fructosyltransferase) gene in crested wheatgrass (*Agropyron cristatum*), Journal of Plant Physiology, 158 (2001) 1203-1213.
[95] J.S. Papadopoulos, R. Agarwala, COBALT: constraint-based alignment tool for multiple protein sequences, Bioinformatics, 23 (2007) 1073-1079.
[96] V. Amiard, A. Morvan-Bertrand, J.P. Billard, C. Huault, F. Keller, M.P. Prud'homme, Fructans, but not the sucrosyl-galactosides, raffinose and loliose, are affected by drought stress in perennial ryegrass, Plant Physiology, 132 (2003) 2218-2229.
[97] A. Morvan-Bertrand, J. Boucaud, J. Le Saos, M.P. Prud'homme, Roles of the fructans from leaf sheaths and from the elongating leaf bases in the regrowth following defoliation of *Lolium perenne* L., Planta, 213 (2001) 109-120.
[98] D.M. Obenland, U. Simmen, T. Boller, A. Wiemken, Regulation of Sucrose-Sucrose-Fructosyltransferase in Barley Leaves, Plant Physiology, 97 (1991) 811-813.

[99] A.L. Winters, J.H.H. Williams, D.S. Thomas, C.J. Pollock, Changes in gene-expression in response to sucrose accumulation in leaf tissue of *Lolium temulentum* L, New Phytologist, 128 (1994) 591-600.

[100] K. Maleux, W. Van den Ende, Levans in Excised Leaves of *Dactylis glomerata* : Effects of Light, Sugars, Temperature and Senescence, Journal of Plant Biology, 50 (2007) 671-680.

[101] W. Van den Ende, S. Clerens, R. Vergauwen, D. Boogaerts, K. Le Roy, L. Arckens, A. Van Laere, Cloning and functional analysis of a high DP fructan: fructan 1-fructosyl transferase from *Echinops ritro* (Asteraceae): comparison of the native and recombinant enzymes, Journal of Experimental Botany, 57 (2006) 775-789.

[102] R. Améziane, A. Limami, J.-F. Morot gaudry, Fructan biosynthesis and SST activity in excised leaves of *Cichorium intybus* L., in: U. Rennes (Ed.) Plant Sciences 1994, Saint malo (France), 1994, pp. 123.

[103] G.M. Noël, J.A. Tognetti, H.G. Pontis, Protein kinase and phosphatase activities are involved in fructan synthesis initiation mediated by sugars, Planta, 213 (2001) 640-646.

[104] J. Müller, R.A. Aeschbacher, N. Sprenger, T. Boller, A. Wiemken, Disaccharide-mediated regulation of sucrose: fructan-6-fructosyltransferase, a key enzyme of fructan synthesis in barley leaves, Plant Physiology, 123 (2000) 265-274.

[105] W. Van den Ende, J. De Roover, A. Van Laere, Effect of nitrogen concentration on fructan and fructan metabolizing enzymes in young chicory plants (*Cichorium intybus*), Physiologia Plantarum, 105 (1999) 2-8.

[106] U. Kusch, S. Greiner, H. Steininger, A.D. Meyer, H. Corbière-Divialle, K. Harms, T. Rausch, Dissecting the regulation of fructan metabolism in chicory (*Cichorium intybus L.*) hairy roots, New Phytologist, 184 (2009) 127-140.

[107] R. Morcuende, S. Kostadinova, P. Pérez, D. Molino, I. Martín, R. Martínez-Carrasco, Nitrate is a negative signal for fructan synthesis, and the fructosyltransferase-inducing trehalose inhibits nitrogen and carbon assimilation in excised barley leaves, New Phytologist, 161 (2004) 749-759.

[108] V.J. Nagaraj, D. Altenbach, V. Galati, M. Lüscher, A.D. Meyer, T. Boller, A. Wiemken, Distinct regulation of sucrose: sucrose-1-fructosyltransferase (1-SST) and sucrose: fructan-6-fructosyltransferase (6-SFT), the key enzymes of fructan synthesis in barley leaves: 1-SST as the pacemaker, New Phytologist, 161 (2004) 735-748.

[109] V.J. Nagaraj, R. Riedl, T. Boller, A. Wiemken, A.D. Meyer, Light and sugar regulation of the barley sucrose: fructan 6-fructosyltransferase promoter, Journal of Plant Physiology, 158 (2001) 1601-1607.

[110] G.-P. Xue, M. Kooiker, J. Drenth, C.L. McIntyre, TaMYB13 is a transcriptional activator of fructosyltransferase genes involved in β-2,6-linked fructan synthesis in wheat, The Plant Journal, 68 (2011) 857-870.

[111] N. Sprenger, L. Schellenbaum, K. van Dun, T. Boller, A. Wiemken, Fructan synthesis in transgenic tobacco and chicory plants expressing barley sucrose:fructan 6-fructosyltransferase, FEBS Letters, 400 (1997) 355-358.

[112] I. Vijn, A. Van Dijken, N. Sprenger, K. Van Dun, P. Weisbeek, A. Wiemken, S. Smeekens, Fructan of the inulin neoseries is synthesized in transgenic chicory plants

(*Cichorium intybus L.*) harbouring onion (*Allium cepa L.*) fructan:fructan 6G-fructosyltransferase, The Plant Journal, 11 (1997) 387-398.

[113] X. Ye, X. Wu, H. Zhao, M. Frehner, J. Nösberger, I. Potrykus, G. Spangenberg, Altered fructan accumulation in transgenic *Lolium multiflorum* plants expressing a *Bacillus subtilis* sacB gene, Plant Cell Reports, 20 (2001) 205-212.

[114] R.E. Sévenier, R.D. Hall, I.M. van der Meer, H.J.C. Hakkert, A.J. van Tunen, A.J. Koops, High level fructan accumulation in a transgenic sugar beet, Nature Biotech, 16 (1998) 843-846.

[115] G. Weyens, T. Ritsema, K. Van Dun, D. Meyer, M. Lommel, J. Lathouwers, I. Rosquin, P. Denys, A. Tossens, M. Nijs, S. Turk, N. Gerrits, S. Bink, B. Walraven, M. Lefèbvre, S. Smeekens, Production of tailor-made fructans in sugar beet by expression of onion fructosyltransferase genes, Plant Biotechnology Journal, 2 (2004) 321-327.

[116] A.J. Koops, R.E. Sévenier, A.J. Van Tunen, L. De Leenheer, Transgenic plants presenting a modified inulin producing profile, in, United states of America, 2003.

[117] U. Hochstrasser, M. Lüscher, C. De Virgilio, T. Boller, A. Wiemken, Expression of a functional barley sucrose-fructan 6-fructosyltransferase in the methylotrophic yeast *Pichia pastoris*, FEBS Letters, 440 (1998) 356-360.

[118] D. Altenbach, E. Nüesch, A.D. Meyer, T. Boller, A. Wiemken, The large subunit determines catalytic specificity of barley sucrose: fructan 6-fructosyltransferase and fescue sucrose: sucrose 1-fructosyltransferase, FEBS Letters, 567 (2004) 214-218.

[119] B. Lasseur, L. Schroeven, W. Lammens, K. Le Roy, G. Spangenberg, H. Manduzio, R. Vergauwen, J. Lothier, M. Prud'homme, W. Van den Ende. Transforming a Fructan: Fructan 6G-Fructosyltransferase from Perennial Ryegrass into a Sucrose: Sucrose 1-Fructosyltransferase. Plant Physiology, 149 (2009) 327-339.

[120] L. Schroeven, W. Lammens, A. Kawakami, M. Yoshida, A. Van Laere, W. Van den Ende. Creating S-type characteristics in the F-type enzyme fructan:fructan 1-fructosyltransferase of *Triticum aestivum* L. Journal of Experimental Botany, 60 (2009), 3687-3696.

[121] L. Schroeven, W. Lammens, A. Van Laere, W. Van den Ende, Transforming wheat vacuolar invertase into a high affinity sucrose:sucrose 1-fructosyltransferase. The New Phytologist, 180 (2008) 822-831.

[122] H. Schyder, , The role of carbohydrate storage and redistribution in the source-sink relations of wheat and barley during grain filling — a review. New Phytologist, 123 (1993) 233–245

[123] L. Vogt, U. Ramasamy, D. Meyer, G. Pullens, K. Venema, M.M. Faas, H.A. Schols, P. de Vos. Immune Modulation by Different Types of β2→ 1-Fructans Is Toll-Like Receptor Dependent. PloS one, 8 (2013), e68367.

[124] W. Van den Ende, A. Michiels, J. De Roover, P. Verhaert, A. Van Laere. Cloning and functional analysis of chicory root fructan 1-exohydrolase I (1-FEH I): a vacuolar enzyme derived from a cell wall invertase ancestor? Mass fingerprint of the enzyme. Plant Journal, 23 (2000) 447–456.

[125] K. Le Roy, R. Vergauwen, T. Struyf, S. Yuan, W. Lammens, J. Matrai, M. De Maeyer, W. Van den Ende. (2013). Understanding the role of defective invertases in plants: tobacco Nin88 fails to degrade sucrose. Plant Physiology, 161 (2013) 1670-1681.

[126] W. Van den Ende, B. De Coninck, S. Clerens, R. Vergauwen, A. Van Laere, Unexpected presence of fructan 6-exohydrolases (6-FEHs) in non-fructan plants: characterization, cloning, mass mapping and functional analysis of a novel 'cell-wall invertase-like'specific 6-FEH from sugar beet (*Beta vulgaris* L.), The Plant Journal, 36 (2003) 697-710.

Chapter 2

Sink filling, inulin metabolizing enzymes and carbohydrate status in field grown chicory (Cichorium intybus L.*)*

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Summary

Inulin is a fructose-based polymer that is isolated from chicory (*Cichorium intybus* L.) taproots. The degree of polymerization (DP) determines its application and hence the value of the crop. The DP is highly dependent on the field conditions and harvest time. Therefore, the present study was carried out with the objective to understand the regulation of inulin metabolism and the process that determines DP and inulin yield throughout the whole growing season. Metabolic aspects of inulin production and degradation in chicory were monitored in the field and under controlled conditions. The following characteristics were determined in taproots: concentrations of glucose, fructose and sucrose, the inulin mean degree of polymerisation (mDP), yield, gene expression and activity of enzymes involved in inulin metabolism. Inulin synthesis, catalyzed by sucrose: sucrose 1-fructosyltransferase (EC 2.4.1.99) (1-SST) and fructan: fructan 1-fructosyltransferase (EC 2.4.1.100) (1-FFT), started at the onset of taproot development. Inulin yield as a function of time followed a sigmoid curve reaching a maximum in November. Inulin reached a maximum mDP of about 15 in September, than gradually decreased. Based on the changes observed in the pattern of inulin accumulation, we defined three different phases in the growing season and analysed product formation, enzyme activity and gene expression in these defined periods. The results were validated by performing experiments under controlled conditions in climate rooms.

Our results show that the decrease in 1-SST that starts in June is not regulated by day length and temperature. From mid-September onwards, the mean degree of polymerisation (mDP) decreased gradually although inulin yield still increased. The decrease in mDP combined with increased yield results from fructan exohydrolase activity, induced by low temperature, and the back transfer activity of 1-FFT. Based on the observations, performed during the field experiments, transgenic chicory plants were generated aiming at a higher mDP inulin at harvest. Overall, this study provides background information on how to improve inulin yield and quality in chicory.

Key words

1-FEH; 1-FFT; 1-SST; chicory; fructan; yield

Abbreviations

DP, degree of polymerization; mDP, mean degree of polymerization; FW, fresh weight; SEM, standard error of means; WAS, weeks after sowing.

Introduction

In many Asteraceae, inulin, a ß(2,1) linked fructose polymer with a terminal glucose residue, serves as a reserve carbohydrate in stems, tubers and taproots. Chicory (*Cichorium intybus* L.) is a biennial taproot-bearing crop species from this family that is grown for the production of inulin on an industrial scale. Chicory is sown in spring and the taproots are harvested in autumn of the same year. At harvest the mean degree of polymerisation is 9-10 and the average yield is about 11,000 kg carbohydrate per hectare [1]. Inulin is isolated and used for a wide range of food and non-food applications [2] depending on the degree of polymerization (DP). Short chain inulin is used, for example, for the production of fructose syrup, which is mainly used for the sweetening of cold drinks. Long chain inulin (mean DP of 25) is used as fat replacer and foam stabilizer in food products. Long chain inulin is also starting material for the production of carboxymethylinulin, a scavenger of divalent cations in household detergents.

The biosynthesis of inulin is induced when the radial root growth starts, and is catalyzed by the activity of three different classes of enzymes: sucrose: sucrose 1-fructosyl transferase (EC 2.4.1.99) (1-SST), fructan: fructan 1-fructosyl transferase (EC2.4.1.100) (1-FFT) and fructan exohydrolase (EC.3.2.1.153) (1-FEH) [3]. 1-SST primarily catalyses the synthesis of 1-kestose from two molecules of sucrose (Fig. 1A). 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 inulin molecule onto 1-kestose and higher DP inulin molecules (Fig. 1A). 1-FFT increases the mean degree of polymerization (mDP) when using 1-kestose as a fructosyl donor because the reaction degrades the shortest inulin, 1-kestose, into sucrose, which is not accounted in the inulin pool, and a fructosyl unit that is used to elongate a pre-existing inulin 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 an inulin molecule onto sucrose, resulting in a decrease in mDP and an increase of the number of inulin molecules [4, 5], the so-called 'back transfer' reaction (Fig. 1B). A third reaction catalyzed by 1-FFT is the synthesis of inulo-*n*-oses, polymers of only fructose moieties, by transfer of fructosyl units from inulin onto free fructose (Fig. 1C) [4]. 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 [6] (Fig. 1D).

Fructan biosynthesis has been studied in the past decades in a number of plant species, with emphasis on grasses [7]. The older studies dealt with the identification and quantification of fructan molecules and the elucidation of their biosynthesis [3, 8, 9]. In later studies, enzymes involved in inulin biosynthesis were purified and characterised [10-12], and more recently several genes encoding these biosynthetic enzymes were cloned from different plant species, functionally characterised [13-17] and ectopically expressed [13, 18]. The kinetic specificities of the transferases and hydrolases were studied in vitro and in vivo [19-21]. Studies on field grown plants in which the mDP and sugar content were measured have been performed in order to determine the right harvesting period [9, 22,

23]. Hence, all the individual components of fructan biosynthesis such as substrates, intermediates, enzymes, and genes are largely known. However, the relationship between these components has not yet been fully studied in a commercial crop under field conditions.

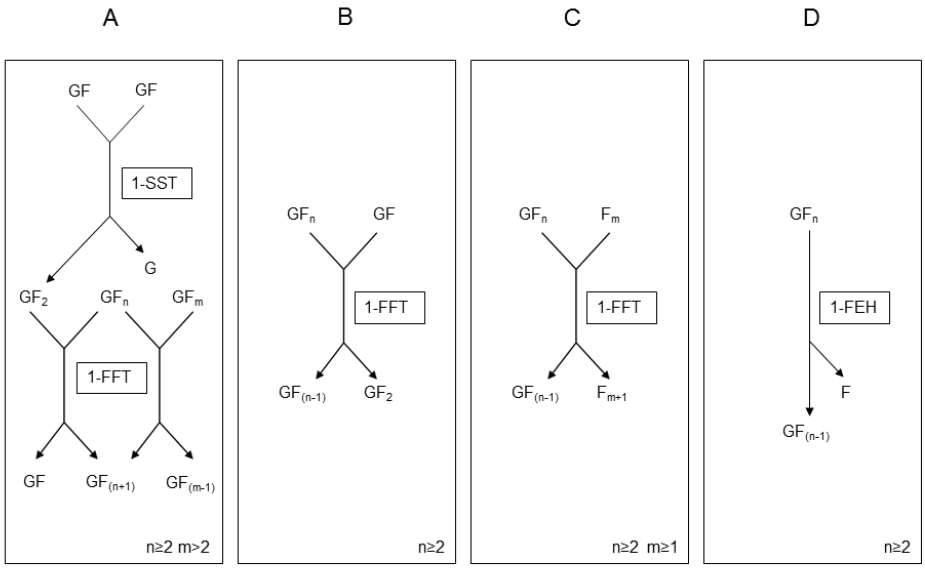

Figure 1. The reactions of fructan biosynthesis in chicory. Fructan polymerization by 1-SST and 1-FFT (A), back transfer by 1-FFT (B), inulo-*n*-ose production by 1-FFT (C), fructan exohydrolysis by 1-FEH (D) (GF= sucrose, F=fructose, G=glucose, GF_2= 1-kestose, $GF_{n/m}$ = inulin, F_m= inulo-*n*-ose).

The present study was carried out with the objective to understand the regulation of inulin metabolism and the processes that determine the chain length and inulin yield throughout the growing season. Experiments under controlled conditions were subsequently used to confirm hypotheses. The relationships between carbohydrate content, mDP, yield, gene expression and enzyme activities related to inulin metabolism were analysed. Insight in the regulation of the DP by internal and external factors may lead to agricultural practices that can contribute to the improvement of the quality of inulin.

Material and Methods

Plant material

Cichorium intybus L. (cv. Orchies) was grown in experimental fields in Belgium and The Netherlands during four different years (1998, 1999, 2003, and 2004). Plants were sown in the beginning of May and crop husbandry was performed comparable to commercial fields in all experiments. To allow detailed study on the beginning of the growing season, taproot samples were taken weekly in 1998 from June until September from a commercial field in Boutersem, Belgium. The USDA soil classification was Alfisol. To allow more detailed analysis on the later part of the growing season plants were harvested in the growing season of 1999 and 2004. In 1999 plants were grown on a field in Ottenburg, Belgium with a soil classified as Alfisol (USDA soil taxonomy). Samples were taken weekly from September until December. In 2004 taproot samples were taken twice a week from September until December from a field in Melderslo (The Netherlands). Taproot samples were harvested according to a randomized block design with three replicates. The USDA soil classification was Alfisol and the temperature was logged at ground level (Hanna Instruments, USA). In 2003, samples were harvested every two weeks from July until December from a field in Wageningen, The Netherlands, with a soil classified as Entosol (USDA soil taxonomy). For the growing seasons 2003 and 2004 the temperatures were logged at a weather station in Wageningen (Haarweg weather station). During the growing season of 1998 and 1999 the temperatures were logged at the KMI, Belgium (Ukkel weather station). The cumulative precipitation deficit (KNMI, De Bilt) was used to determine putative effects of drought during the study.

Experiments with chicory plants grown under controlled environmental conditions were performed in Weiss cabinets nr. 1700sp (Weiss Technik, Belgium) equipped with Philips TL5 XTRA 54W840 lamps. The plants were sown in DeschC 17cm H pots (three plants per pot) filled with potting soil (Lentse potgrond, The Netherlands). Temperature and day length was set per experiment.

In order to analyse the combined effect of day length and temperature on the activity of 1-SST in growing chicory, plants were grown at 16h of light with photosynthetically active radiation (PAR) of 200 $\mu mol.m^{-2}.s^{-1}$ at 22°C and 8h of darkness at 15°C. Two independent experiments were performed. Sampling started at 8 weeks after sowing (WAS). The first experiment ended at 26 WAS. The second ended at 21 WAS.

To analyse the combined effect of temperature and day length on 1-FEH induction, plants were grown for 3-4 months at 16h of light with photosynthetically active radiation (PAR) of 200 $\mu mol.m^{-2}.s^{-1}$ at 22°C and 8h of darkness at 15°C. For the induction of 1-FEH the conditions were changed as follows. In the first two experiments the conditions were changed to 12h of light at 13°C and 12h of darkness at 3.5°C, the light intensity remained unchanged. The variation applied to the third experiment compared with the earlier experiments was a night temperature set on 6°C instead of 3.5°C. Control plants were kept at the light and temperature regime used for raising the chicory plants. In all

experiments using chicory plants three taproots were collected and pooled as one sample. For the field experiment performed in 2003 five taproots were collected for each sample. After harvest, the taproots were rinsed with tap water, weighed, peeled and cut into two longitudinal halves. One half was stored for maximally one hour on ice and then used for sugar and enzyme analysis. The other half was cut into pieces of 1 cm^3, immediately frozen in liquid nitrogen and used for RNA extraction and expression analyses.

Carbohydrate and protein extraction

Thirty gram of fresh plant material was homogenised with a Waring blender for 40 sec in 30 mL ice-cold 50 mM sodium acetate buffer, pH 5, containing 100 mM mannitol, 10 mM $NaHSO_3$, 0.1% Polyclar® (Serva, Heidelberg, Germany), 1 mM β-mercaptoethanol, 1 mM phenylmethylsulphonylfluoride and 0.02% sodium azide. The extracts were used for sugar and enzyme analyses. Data presented are average values of two extracts from each sample; variation between the duplicate measurements was always less than 10%.

Carbohydrate analysis

Ten gram of taproot homogenate was incubated in a water bath at 90°C for 30 min. After cooling to room temperature, the extract was passed through cheesecloth. Of the filtered juice, 1.5 mL was centrifuged at 10,000 x g. A 200 µL sample of the supernatant was loaded on top of a 0.5 mL bed volume of Dowex®-50 H^+ which was loaded on top of a 0.5 mL bed volume of Dowex®-1-acetate. The resins were rinsed 6 times with 200 µL distilled water [12]. From this neutral fraction a 30-fold dilution was made and 25 µL thereof (corresponding to 60 µg FW of the taproot material) was analysed by HPLC (Dionex, Sunnyvale, CA, USA) equipped with a Carbopac™ PA-100 guard column, a Carbopac™ PA-100 (4x250) column and an ED40 Electrochemical detector. The columns were equilibrated with 50 mM NaOH for 24 min. Sugars were eluted from the column using 50mM NaOH supplemented with increasing concentrations of sodium acetate (NaAc) using the following gradient: 0-6 min, 10 mM; 6-16 min, 10-100 mM; 16-26 min, 100-175 mM, 26-36 min, 175-230 mM; 36-61 min, 230-315 mM; 61-86 min, 315-360 mM; 86-125 min, 360-400 mM. Regeneration was done by rinsing the column with 500 mM sodium acetate for 5 minutes and 1 M NaOH for 10 minutes. Mannitol was used as an internal standard. Calibration curves for glucose, fructose, sucrose, 1-kestose and 1-nystose were obtained by using pure compounds in a wide range of concentrations. Concentration of oligo fructan > DP4 was estimated using the response coefficients [24, 25] as described earlier [16]. The mDP of inulin was estimated based on the ratio between glucose and fructose, formed after mild hydrolysis in 100 mM HCl at 60°C for two hours. The inulin concentration is represented by the concentration of glucose that is formed after hydrolysis. The amount of mol inulin per taproot was calculated by multiplying the inulin concentration by the taproot mass. This amount was converted to gram inulin per taproot by using the molar mass of the mean polymer per sample.

Enzyme assays

The taproot homogenate was centrifuged for 5 min at 10.000 x g in a 1.5 mL reaction vial. An aliquot (300 µL) of the supernatant was carefully mixed with 1.2 mL of a saturated ammonium sulfate solution. After 45 min at 0°C, the precipitate was collected by centrifugation at 10,000 x g for 5 min. To eliminate the residual sugars, the protein pellets were washed in 600 µL of 80% ammonium sulphate. After a second centrifugation at 10,000 x g for 5 min, the precipitate was dissolved in 300µL of 50 mM sodium acetate buffer, pH 5, containing 0.02% sodium azide. To remove the $(NH_4)_2SO_4$ from the protein extract, 0.6 mL of the protein extract was loaded on top of a 2.5 mL Sephadex G-25 column. The proteins were eluted from the column by centrifugation at 3,000 x g. The activities of 1-SST, 1-FFT and 1-FEH were measured according to the procedures described by Van den Ende *et al*. [12]. Data presented are average values of triplicate measurements; variation between these was always less than 10%.

RNA isolation

Total RNA was isolated from chicory taproot material using TRI reagent (Sigma) using the protocol supplied with the TRI reagent. The RNeasy Mini Kit (Qiagen) was used to purify the isolated RNA. The RNA quality and quantity was determined spectrophotometrically.

Quantitative real time RT-PCR

From each RNA sample 1 µg was treated with DNAse (Invitrogen). Subsequently the RNA was reverse transcribed to cDNA, using TaqMan reverse transcription reagents (Applied Biosystems) and random hexamer primers using conditions recommended by the supplier. Specific mRNAs steady state levels were quantified using quantitative real-time PCR. The reaction was performed using a Sequence Detector System (ABI Prism 7900 Sequence Detection System and software; Applied Biosystems). Amplification was performed in a final volume of 20 µL, containing 40 ng of cDNA from the reversed transcribed reaction, 0.3 µM of both sense and antisense primers and 2 µL of 10x Sybr Green PCR Master Mix (Applied Biosystems). The standard amplification program recommended by the supplier was used. The mRNA levels of the studied genes were normalized using the comparative C_T method [26], using the expression of a homologue of ribosomal protein L19 as internal standard. The L19 homologue from chicory has shown to be an effective normaliser, as it is stably expressed during the growing season (see Appendix B in Supplementary Data). The mean values of triplicate measurements are presented in the figures. The oligonucleotide primers were designed using Primer Express1.5 software (Applied Biosystems). The primers are shown in Table 1.

Table 1: Primers used in the quantitative PCR experiments

Gene	Accession no.	Primer	Sequence (5'> 3')
Ribosomal protein L19	-	*Sense*	*CTGCCAGCGTCCTCAAGTG*
Ribosomal protein L19	-	*Antisense*	*CATTGGGATCAAGCCAAACCT*
1-FEH I	CAC19366	*Sense*	*GGAGGATTGTGATCGGAAGTG*
1-FEH I	*CAC19366*	*Antisense*	*ACAAAAGTGCGGTTCCATGAC*
1-FEH IIa and b[a]	CAC37922, CAC37923	*Sense*	*GCGTGGGTTCCTGAAACTGAT*
1-FEH IIa and b[a]	CAC37922, CAC37923	*Antisense*	*ACCAGCCCATCCTTTTTCAA*
1-SST	U81520	*Sense*	*CCAACAACCATCAGGGAGAAG*
1-SST	*U81520*	*Antisense*	*AGCAACGGAGCTGTGAACGT*
1-FFT	U84398	Sense	*GAACCAGCAAGATTCCACGAA*
1-FFT	*U84398*	*Antisense*	*TTCTCCGGCAGTGCTAAATTG*

[a]*FEH IIb* has a G to T substitution at the position of the third nucleotide from the 5' end of the sense primer and a T to C substitution at the position of the second nucleotide from the 3'end of the antisense primer compared to *FEH IIa*.

Data presentation and curve fitting

All graphs present the data collected in 2003 as a representative sample of the four years, unless stated otherwise. The dotted vertical lines in the graphs indicate the different phases of the growing season 2003 described in this paper, the phases are indicated by roman numbers I, II and III, the name of the month on the x-axis is placed at the beginning of each month.

Data on the years 1998, 1999 and 2004 are given in the supplementary data (see Appendices C and D in Supplementary Data). Data plotting curve fitting and calculation of mean and Standard Error of Mean (SEM) were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego CA USA). Student's t-test was used to test significance of differences.

Results and discussion

Development of taproot mass, inulin yield and mDP

To analyse the development of taproot mass, inulin yield and mDP, and to study the influence of different field conditions on inulin biosynthesis, plants were grown under field conditions during four growing seasons (1998, 1999, 2003 and 2004). After sowing at the beginning of May, taproot growth started in June, and from July until September taproot mass increased over 5-fold, from about 100 g fresh weight (FW) per plant at the beginning of July to 600 g FW at the end of September (see Appendix A in Supplementary Data). After September, the taproot mass further increased by 25-100% depending on the year. The taproot grew only a little after the first night frost. The lower plant density used in the experiment of 2003 resulted in a higher plant weight compared with the other experiments (see Appendix A in Supplementary Data). The inulin concentration (mM) in the taproot increased until mid-July and remained more or less constant during the rest of the growing season and was remarkably similar over the years, except in 1999 when the concentration was higher (Fig. 2A). The inulin yield (gram inulin/taproot) of the taproots increased almost linearly throughout the growing season until October (Fig. 2B). The mDP increased from 6 in June to a maximum value of about 14 at the beginning of September, the same pattern was observed over the four years, (Fig. 2A). After the maximum value was reached, the mDP decreased to values below DP10 in December.

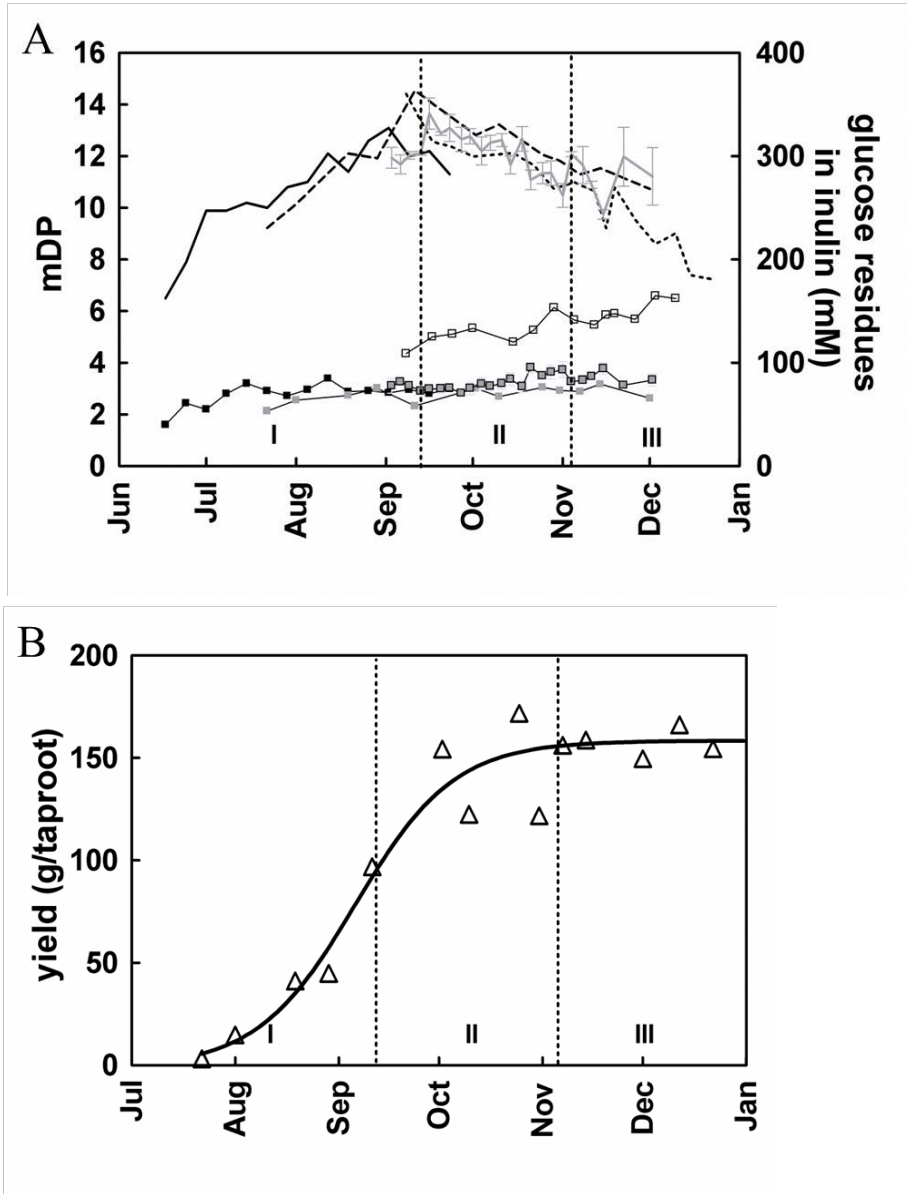

Figure 2. Panel A: Changes in inulin mDP and concentration in chicory taproots.
The mDP (lines) during four growing seasons, 1998 (line), 1999 (dotted line), 2003 (striped line) and 2004 (gray line) is presented on the left y-axis. The inulin concentration in mM, represented by the concentration of the glucose residues in inulin (squares) during the four seasons, 1998 (closed black squares), 1999 (open squares), 2003 (closed gray squares) and 2004 (open gray squares) is presented on the right y-axis. The error bars on the data collected in 2004 show the SEM of three independent experiments. The error bars may be smaller than the symbol, and not visible. Panel B: Changes in inulin yield of chicory taproots in 2003. The inulin yield (gram/taproot) and its best fitted sigmoid curve are presented. The dotted vertical lines bound the different phases of the growing season 2003 described in this paper.

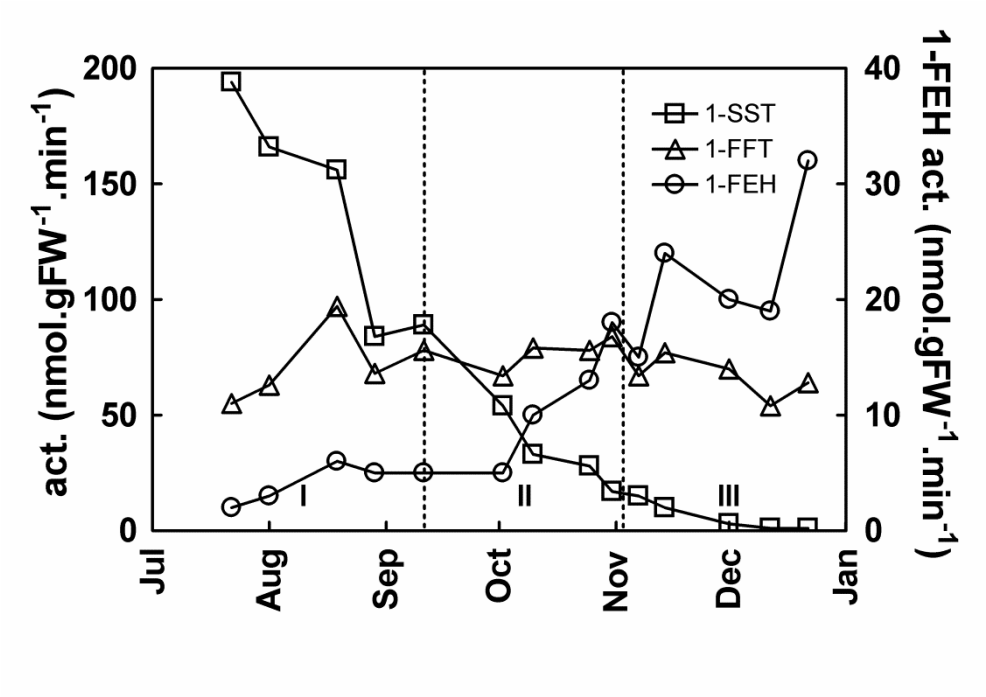

Figure 3. Enzyme activity of the different inulin biosynthesis enzymes.
The graph shows enzyme activities in chicory taproot grown in the growing season 2003 of 1-SST (squares), 1-FFT (triangles) and 1-FEH (circles). 1-SST and 1-FFT are expressed as nmol 1-kestose.gFW^{-1} min^{-1}. 1-FEH is expressed as nmol fructose.gFW^{-1} min^{-1}.
The dotted vertical lines bound the different phases of the growing season 2003 described in this paper.

Three phases in inulin metabolism

To facilitate the description and discussion of the processes involved in inulin polymerisation, we defined three successive phases in the growing season based on changes observed in the pattern of inulin accumulation (Fig. 2A and B). This classification was used earlier by Edelman for the inulin metabolism of *Helianthus tuberosus L.* [3]. The first phase started with the onset of inulin biosynthesis and was characterised by a steady increase of the inulin mDP (Fig. 2A) and yield (Fig. 2B). In the second phase, the mDP decreased, while the inulin yield still increased. The third phase started while the mDP continued to decrease, the yield became constant and the taproot growth slowed down. For each of these phases the observed inulin profile is discussed in relation to the changes in the activity of the enzymes involved.

Phase 1, the onset of inulin biosynthesis

The first phase started about one month after sowing, with the onset of root thickening and inulin biosynthesis. In the growing season of 2003 the mDP gradually increased to a maximum of 14 around the second week of September (Fig. 2A). The maximum mDP was reached in the beginning of September in the other years as well (Fig.

2A). Although the enzymes 1-SST and 1-FFT appeared to be activated at the same time, as shown by Druart [27], the activity profiles of both enzymes strongly differed (Fig. 3). 1-SST already reached its maximum activity in June (data not shown) and then decreased in a linear way from July until December (Fig. 3). A Student's t-test on the slopes of the 1-SST activity, over the four years, showed that this decrease in activity was significant with a 95% confidence interval. 1-FFT followed an almost reciprocal pattern: its activity increased until August and then remained constant (Fig. 3). The activity of 1- FEH was low during phase 1 (Fig. 3).

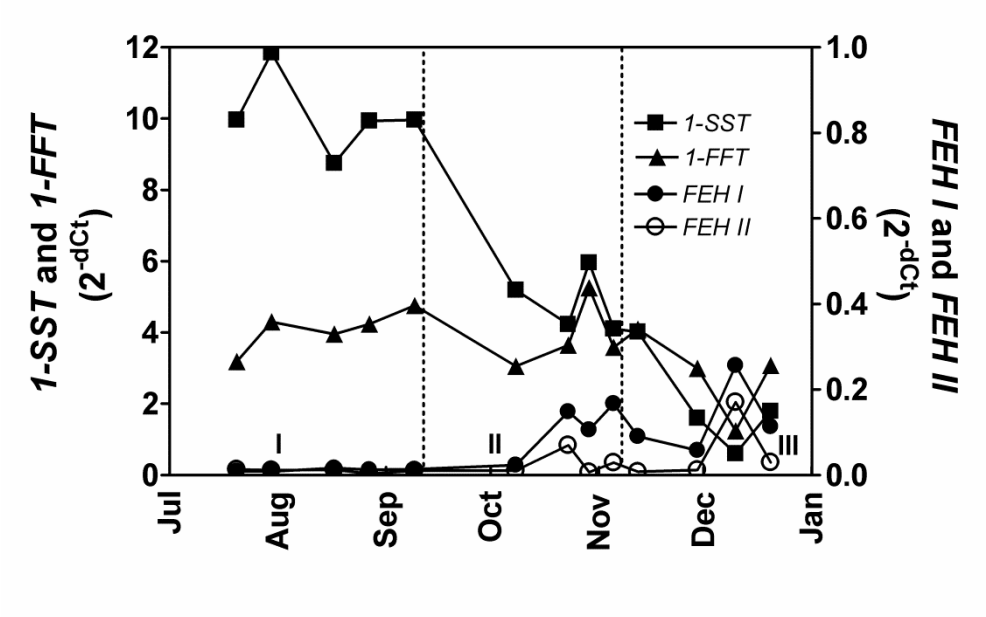

Figure 4. Relative gene expression of the different inulin biosynthesis genes in chicory taproot. The graph shows the relative gene expressions of *1-SST* (squares), *1-FFT* (triangles) and *1-FEH I* and *1-FEH II* (closed and open circles resp.) in chicory taproot grown in the growing season of 2003. The relative expression of *1-SST* and *1-FFT* is expressed on the left y-axis and for both *1-FEH* genes it is expressed on the right y-axis.
The dotted vertical lines bound the different phases of the growing season 2003 described in this paper.

The strong decrease in 1-SST activity did not correlate with changes in *1-SST* expression (Fig. 4). The expression of *1-SST* was relatively constant during phase 1, whereas 1-SST enzyme activity was already decreasing (Fig. 3). An earlier field experiment showed that 1-SST activity did correlate with the protein level (data not shown). The discrepancy between constant mRNA levels and decreasing activity in this phase suggests a regulation of 1-SST at the post-transcriptional level rather than on the transcriptional level, the same was suggested by Van Laere and Van den Ende when comparing the relatively high *1-SST* transcript levels with the low 1-SST activity at the end of the growing season [28]. Several post-transcriptional mechanisms could be involved, including inactivation by specific inhibitor proteins, like invertase inhibitors as found for invertases [29]. As fructosyltransferases are thought to be evolved from invertases, this type of regulation

mechanism could also have been evolved for *1-SST* genes. However, known inhibitors of plant invertases do not affect the enzymes of fructan metabolism [30].

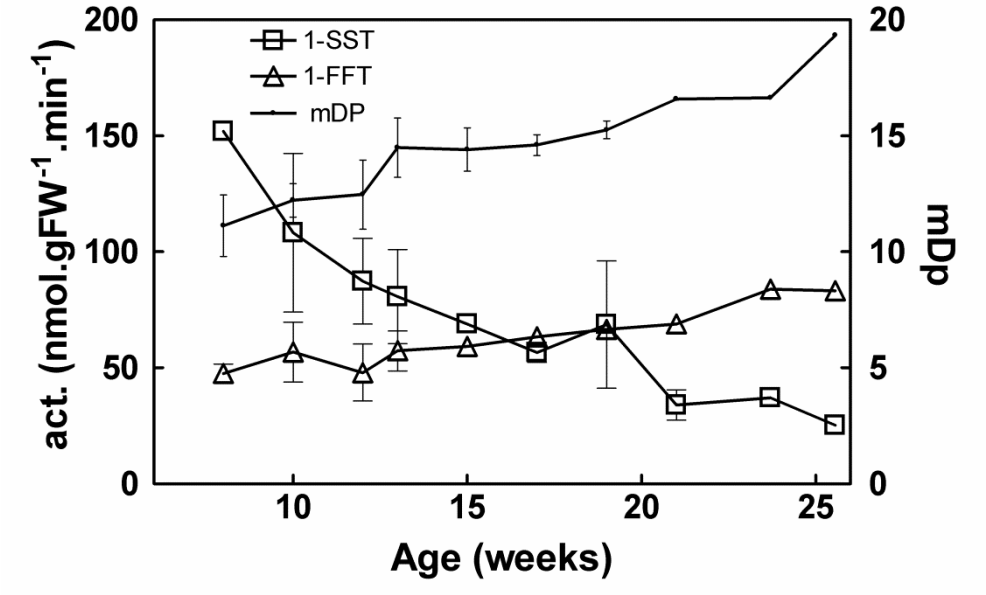

Figure 5. Inulin mDP and inulin biosynthesis enzymes in chicory grown under controlled climatic conditions.
Inulin mDP (small dots) and enzyme activity of 1-SST (open squares), 1-FFT (open triangles) were measured in taproots of plants grown under controlled climatic conditions in growth chambers. The error bars show the SEM of the two replicate experiments. The age of the plants is expressed in weeks after sowing (WAS), 1-SST and 1-FFT activities are expressed as nmol 1-kestose.gFW^{-1} min^{-1}. The inulin mDP is plotted on the right y-axis, the enzyme activities are plotted on the left y-axis.

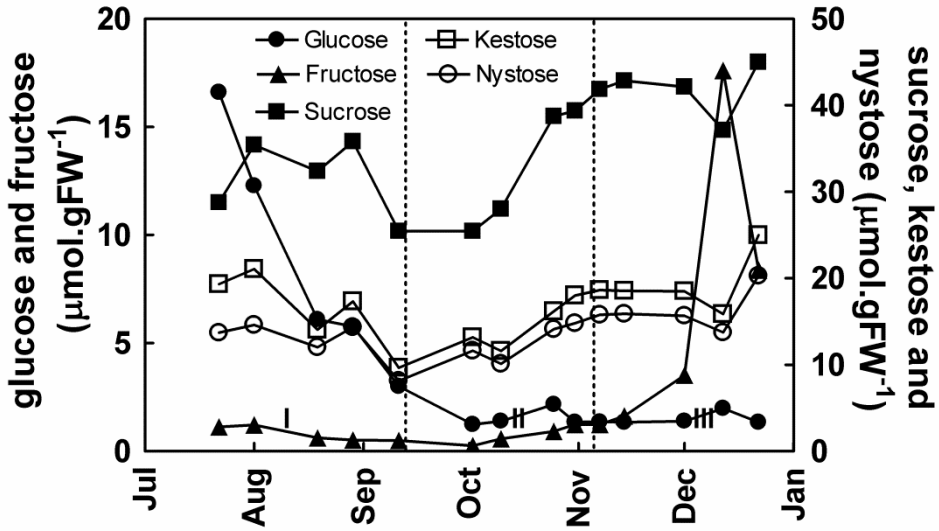

Figure 6. Glucose, fructose, sucrose, 1-kestose and 1-nystose in chicory taproots during the growing season of 2003. Concentration is shown in µmol.gFW^{-1} of glucose (closed circles), fructose (closed triangles), sucrose (closed squares), 1-kestose (open squares), and 1-nystose (open circles) in chicory taproot.

Another mechanisms could be inhibition of translation by micro RNA's, which inhibition mechanism was shown to be wide spread in plants [31]. To analyse if 1-SST activity is controlled by environmental factors, plants were grown for several months under a constant day length and temperature regime in growth chambers. The 1-SST and 1-FFT activity patterns observed under controlled constant conditions were comparable to those observed in the field (Fig. 3 and 5). From the onset of inulin synthesis the 1-SST activity increased, reaching a maximum 7 weeks after sowing (WAS), after which its activity decreased gradually to reach about 20% of its maximum activity at 22 WAS (Fig. 5). This shows that the decrease in 1-SST enzyme activity as observed in the field can also be observed under constant conditions and therefore is not induced by changes in temperature and day length. The gene expression pattern of *1-SST* under constant photoperiod and temperature (data not shown) was also comparable to that of plants grown in the field: the expression level stayed constant throughout the period tested. As drought can influence the inulin metabolism in chicory [32] the water gift was kept constant during the experiment. The decrease in 1-SST activity found under these constant conditions, and in the different field experiments with different cumulative precipitation deficit values, were comparable, showing that the decrease in activity of 1-SST was most probably not caused by a possible cumulative precipitation deficit or drought during the growing season. A reduced influx of sucrose could also be the cause of the reduction of 1-SST as suggested earlier for the reduced activity during phase 2 [33]. When field grown

plants were defoliated in September, the influx of sucrose to the root decreased drastically and the activity of 1-SST dropped. However, our experiments showed that 1-SST activity decreases already during phase 1 when sucrose concentrations were still constant (Fig. 6). The stress reaction of the plant upon defoliation, as observed by Van den Ende and Van Laere [33] could well be differentially regulated from the reduction of 1-SST observed under normal field conditions. The decrease of 1-SST activity observed in the tap root during that period could be linked to the level of nitrate accumulated in the root. It has been shown previously that reduced amounts of nitrate have a positive effect on the activity of 1-SST in fructan-accumulating plants [34-36]. Nitrate accumulates during the growing season in the taproot of chicory [34], which could negatively regulate 1-SST activity. Recently it was proposed that the ratio between carbohydrate and nitrate is the decisive factor for *1-SST* and *1-FFT* expression in an artificial hairy root system [37]. In future experiments, this aspect could be further investigated by analyzing the effect of the nitrate fertilization on the 1-SST activity in tap root of chicory grown in the field.

Under the controlled conditions 1-FEH enzyme activity remained very low throughout the whole experiment and was comparable to the activity measured in the field during phase 1 (data not shown). Interestingly, under controlled constant conditions, the mDP continued to increase, even after 25 WAS, whereas under field conditions the mDP started to decrease at approximately 18 WAS (Fig. 2A and 5). Apparently, when chicory plants were raised under climatic conditions similar to the ones observed during phase 1 (climate room experiment with temperature always above 10°C) a continued elongation of the inulin was observed and a mDP of about 20 was reached at the end of the experiment. This observation is supported by the findings of Bhatia, who reported that chicory, grown in warm climates, accumulates inulin until it starts to bolt [38]. This shows that the remaining 1-SST activity was enough to efficiently channel sucrose into the inulin pool and that the decrease of mDP observed normally in field-grown chicory at the end of the growing season must be caused by other enzymatic activities, such as 1-FFT and/or 1-FEH. In conclusion, phase 1 is characterised by inulin anabolism, in which the reduction in activity of 1-SST seems to be regulated at the post-transcriptional level and is not controlled by day length and temperature.

Phase 2, the production of low DP inulin

Phase 2 is characterised by a decrease in mDP while inulin yield still increases, revealing active inulin biosynthesis. In 2003 this phase started around the second week of September and ended at the beginning of November (Fig. 2A). In the other years the phase started at about the same time as 2003, but lasted longer, until mid-November when the growth stopped due to night frost. During phase 2, the taproot mass increased substantially, in some years even doubled, while the concentration of inulin remained constant (Fig. 2A). This implies that inulin biosynthesis kept pace with taproot growth. This also holds true for the year 1999 although the inulin concentration was higher compared to the other years (Fig 2A). The higher concentration of inulin observed in 1999 coincides with

a lower mass of the taproots (see Appendix A in Supplementary Data). In phase 2, inulin biosynthesis probably proceeds through a slightly different mechanism than in phase 1, as substantiated by a number of observations done in all the four years. The 1-SST activity had already decreased considerably at the start of phase 2 (Fig. 3). This low 1-SST activity was also reflected by the low glucose level and the relatively low concentrations of 1-kestose and 1-nystose at the start of phase 2 (Fig. 6). However, despite a further decrease in 1-SST activity during phase 2, 1-kestose and 1-nystose concentrations continued to increase. The sucrose concentration also increased gradually throughout phase 2 (Fig. 6). While 1-SST activity decreased continuously during phase 2, a Student's t-test on the slopes of 1-FFT activity curves over three years showed that 1-FFT activity remained constant (with a 95% confidence interval) from September until December. It has been reported before that 1-FFT has a higher affinity for sucrose as a fructosyl unit acceptor [4] than for 1-kestose and 1-nystose [5, 10]. Based on these data (increased sucrose concentration and high affinity of 1-FFT for sucrose), it can be envisioned that in phase 2 inulin biosynthesis might have proceeded via the so-called 1-FFT back transfer reaction (Fig. 1B). In this reaction 1-FFT transfers fructosyl units from inulin to sucrose and, by doing so, directly incorporates glucose and fructose residues into the existing inulin pool, bypassing the 1-SST mediated route. The two routes of inulin synthesis can theoretically be distinguished by the rates of glucose and fructose incorporation into the inulin pool. If during a certain period of time (T1-T2) only the back transfer reaction would occur, equal amounts of glucose and fructose would be incorporated into the inulin pool, or:

$$(G2-G1)/(T2-T1) = (F2-F1)/(T2-T1) \quad (1)$$

In which G and F represent the amounts (mol) of glucose and fructose bound in the inulin pool. To get an indication of the relative contribution of the two routes leading to inulin biosynthesis (1-SST + 1-FFT versus 1-FFT back transfer), we analysed the dynamics of the amounts of inulin-bound fructose and glucose (Fig. 7). Curve fitting was used to determine the equations describing the observed kinetics, and the slopes of the curves were calculated using the corresponding derivatives, dF/dT and dG/dT. Figure 7B shows that dF/dG reached its maximum just before the end of phase 1, implying a maximum rate of inulin biosynthesis via the 1-SST plus 1-FFT route at this stage. During phase 2, dF/dG decreased continuously to reach the value of 1 in November, implying that towards the end of phase 2 the biosynthesis of inulin proceeds entirely via the 1-FFT back transfer reaction. However, the contribution of 1-FFT back transfer to inulin production may be overestimated in this period, since 1-FEH may also affect the ratio of inulin-bound fructose and glucose by releasing fructose from inulin. This interference may have been particularly relevant in November, when 1-FEH activity increased (Fig. 3). Involvement of 1-FEH would imply that polymerization by the combined reactions of 1-SST and 1-FFT still played a role in inulin polymerization during phase 2. The occurrence of the back transfer reaction in field

grown plants was suggested earlier to explain a shift from high DP inulin to lower DP inulin at the end of September [39].

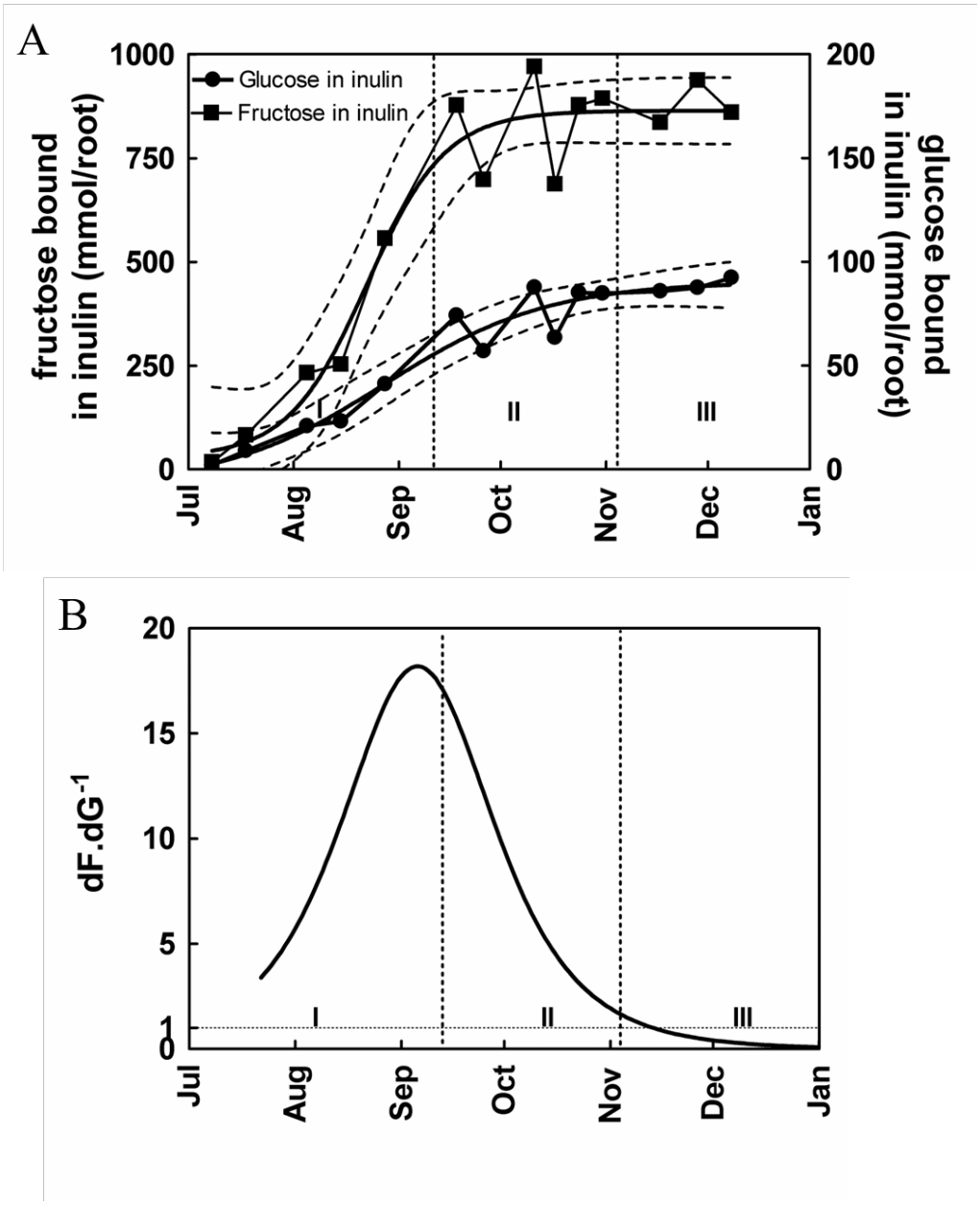

Figure 7. Fructose and glucose units present in inulin in taproots during the growing season.
Panel A: The amount (mmol/taproot) of fructose units (squares) plotted on the left y-axis, and glucose units (circles), plotted on the right y-axis, bound in inulin was analysed during the growing season of 2003. The lines represent Boltzmann sigmoid curves for glucose and fructose in inulin. The dotted lines indicate the 95% confidence intervals of the equations.
Panel B: Representation of dF/dG during the growing season. The dotted vertical lines indicate the transition to the next phase, the horizontal line indicates dF/dG=1.

Interestingly, the occurrence of 1-FEH activity in November may be linked to a decrease in temperature during that month, as was already suggested [23, 40]. Van den Ende showed that the induction of 1-FEH activity was probably not mediated through leaf damage or lower influx of sucrose into the root, but was the result of a direct effect of low temperatures [33]. It is however difficult to assess the effect of 1-FEH on inulin in the field because the temperature fluctuates substantially during the 24 h daily cycle: inulin degradation by 1-FEH during cold night may be compensated by inulin biosynthesis during the day. Therefore, the relationship between temperature and 1-FEH activity and gene expression was also investigated under controlled conditions. Chicory plants were grown for three to four months under standard growing conditions and then were exposed to night temperature regimes of 3.5 and 6^0C. The 1-FEH activity, 1-*FEH I* and 1-*FEH II* expression, mDP of inulin and the concentrations of monosaccharides were monitored during the three weeks following the transfer to the cold regime. 1-*FEH I* expression was clearly induced after exposure to the cold nights (Fig. 8). Other effects of the low temperatures were an increase of sucrose, 1-kestose and 1-nystose, and a decrease in mDP. Free fructose, however, did not increase. The expression of 1-*FEH II* was not induced, even at 3.5^0C, the lowest temperature used in these growth cabinets experiments (Fig. 8).

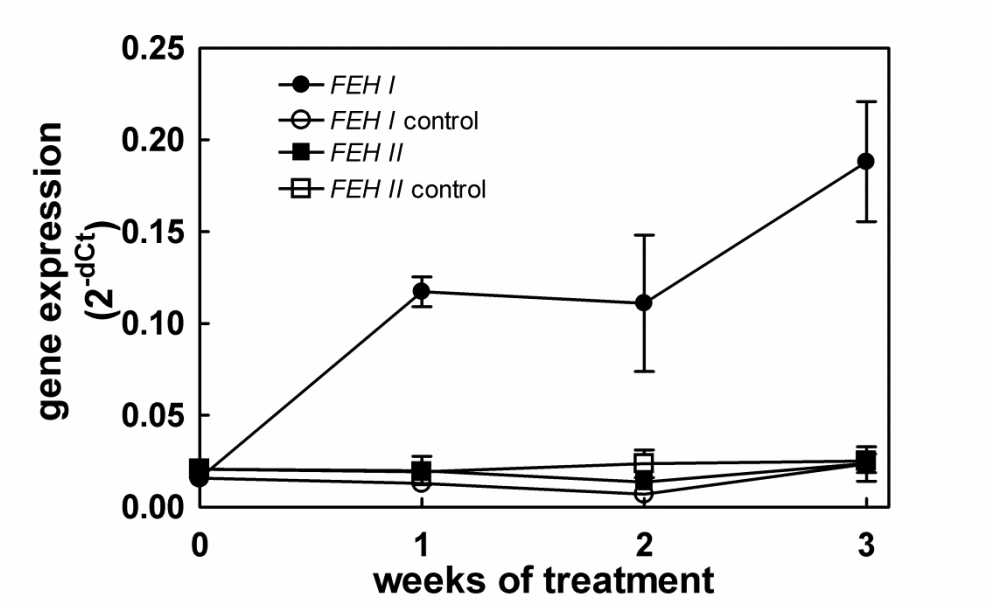

Figure 8. Relative gene expression of *1-FEH I* and *1-FEH II* measured in taproots grown under controlled climatic conditions. The relative gene expression of *1-FEH I* (circles) and *1-FEH II* (squares) was measured in chicory taproots grown in growth chambers under control conditions (open symbols) and at low temperatures (13°C/3.5 - 6°C during 12h/12h) (closed symbols). The error bars show the SEM of the tree replicate experiments.

Similarly, in the field, *1-FEH I* expression was induced when the night temperature decreased below 10^0C, and from September onwards the minimum day temperature

negatively correlated with *1-FEH I* expression (Fig. 9A). The results are in accordance with earlier experiments where mature chicory plants raised under field conditions were moved to a warm greenhouse, thereby preventing the induction of 1-FEH in autumn [33]

In conclusion, the mDP decreases in this period because of 1-FFT back transfer and 1-FEH activities. Despite 1-FEH activity, inulin yield increases, probably due to a combination of 1-FFT back transfer activity and residual 1-SST and 1-FFT polymerisation activity.

Phase 3, inulin breakdown

This period is characterised by degradation of inulin. Indicators are: stable inulin yield, slow taproot growth, increased 1-FEH activity, decreased mDP, increased free fructose and formation of inulo-*n*-oses. In 2003, phase 3 started at the beginning of November after a period of cold nights (no frost) after which the inulin yield did not increase anymore (Fig. 2B). The inulin concentration remained constant (Fig. 2A) even though taproot weight still increased, showing that inulin synthesis still must have occurred in phase 3. *1-FEH II* expression was only induced by frost (Fig. 9A), resulting in an increase of free fructose (Fig. 6). In 1999, phase 3 started mid-November after a period of frost (see Appendix E in Supplementary Data) also resulting in the formation of free fructose. In contrast, in 2004 no frost occurred, *1- FEH II* was not induced and only small amounts of free fructose were formed (Appendix E in Supplementary Data). In the field experiments of 2003 and, more clearly, in the field experiment of 2004 it was observed that the expression of *1-FEH I* was induced by low temperatures (Fig. 9A and B). From October onwards a negative correlation was seen between the temperature and the expression of *1-FEH I*: in periods of frost the expression of *1-FEH I* was higher. Part of the fructose released by 1-FEH was trapped in inulo-*n*-oses. Inulo-*n*-oses are formed by 1-FFT mediated transfer of inulin-bound fructose to free fructose [23]. The accumulation of inulo-*n*-oses could obscure the decrease in mDP since these molecules are also degraded during the acid hydrolysis used to determine the mDP and their degradation only results in the liberation of fructose. The formation of inulo-*n*-ose is not only described for chicory [4, 41] but also for *Helianthus tuberosus* [42, 43], Burdock (*Arctium lappa*) [44] and Allium species [45]

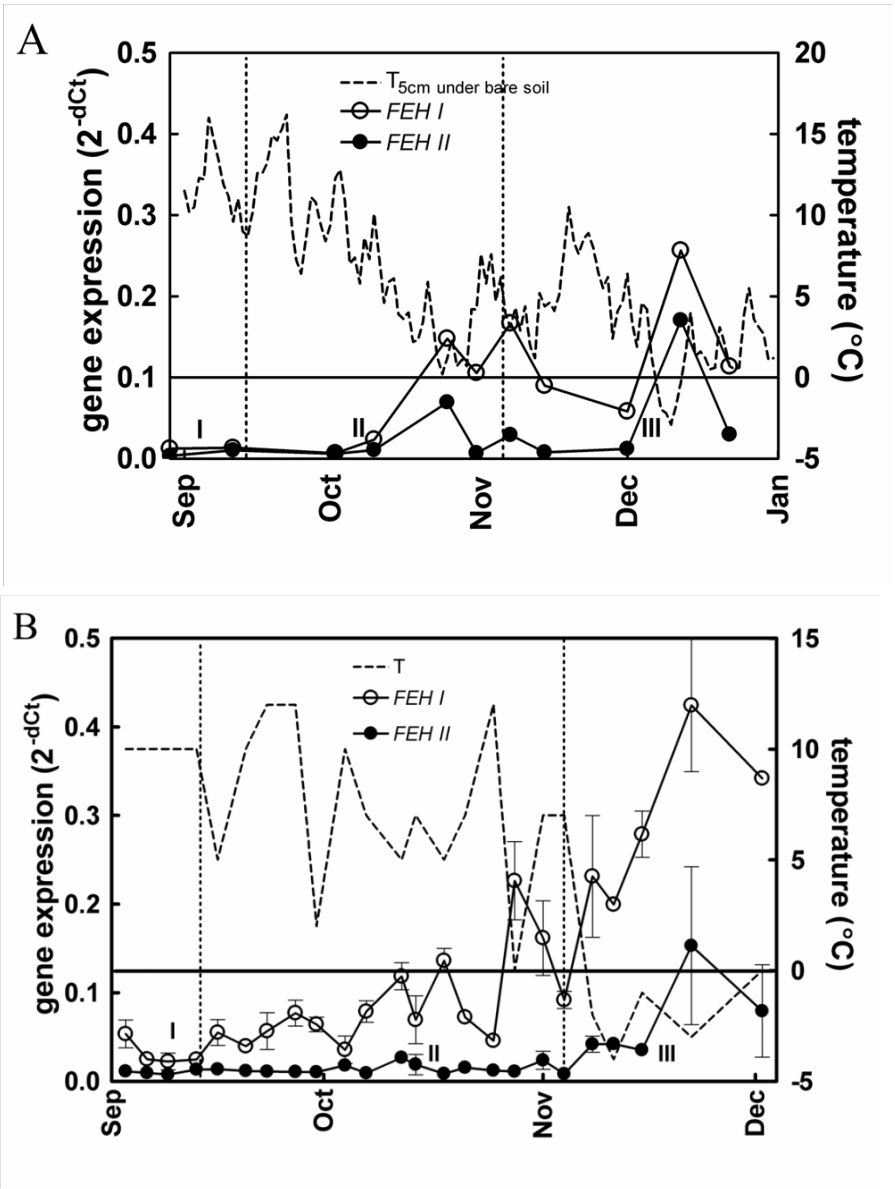

Figure 9. Relative gene expression of *1-FEH I* and *1-FEH II* in field-grown taproots and the minimum day temperature, during the growing season.
Panel A: The relative expression of *1-FEH I* and *1-FEH II* was measured in taproots grown during the growing season of 2003 (open and closed circles resp.) and plotted on the left axis. The minimum day temperature 5 cm under bare soil (striped line) is plotted on the right y-axis. The dotted vertical lines bound the different phases of the growing season 2003 described in this paper. Panel B: The relative expression of *1-FEH I* and *1-FEH II* was measured in taproots grown during the growing season of 2004 (open and closed circles resp.) and plotted on the left axis. The error bars show the SEM of the three replicate experiments. The error bars may be smaller than the symbol and not visible. The minimum day temperature on ground level (dotted line) of the day before harvest is plotted on the right axis.

In these plant species inulo-*n*-ose was formed during cooler temperatures or cold storage. Some of these studies also showed that the accumulation of inulo-*n*-ose was linked to 1-FFT activity and high fructose concentrations. It could be speculated that the physiological role of this reaction is to prevent the accumulation of high concentrations of fructose in the cells and helps regulate the osmotic adjustment during cold period. Although all these observations point towards a process of inulin degradation, the inulin concentration remained constant. The fact that the concentrations of 1-kestose and 1-nystose increased during this period (Fig. 6) might indicate that 1-kestose and 1-nystose were formed by 1-FEH at the expense of longer polymers. However, Van den Ende showed that 1-FEH has equal affinity for long and short inulin [40]. Purified 1-FEH II incubated with chicory inulin only formed fructose and sucrose and not 1-kestose or 1-nystose. Taking this property of 1-FEH into consideration, the increase of 1-kestose and 1-nystose observed during phase 3 in the field may not result from the degradation of long polymers by 1-FEH, but may, more likely, be the result of *de novo* synthesis mediated by 1-FFT activity, that remained high in this period. Such remnant 1-FFT activity in the absence of inulin polymerisation was also found in *Vernonia herbacea* after a cold treatment [46]. 1-FFT back transfer reaction, although reducing the mDP, increases the total amount of inulin molecules which may play a role in osmotic adjustment in plants under cold stress as suggested before [47].

In conclusion: 1-FEH and 1-FFT activities seemed to be in balance in phase 3, combined with a minor role for 1-SST, resulting in a constant inulin concentration in the taproot but with a decreasing mDP.

Modification of inulin biosynthesis in fructan accumulating crops

We studied the relationships between carbohydrate content, mDP, yield, gene expression and enzyme activities related to inulin metabolism in order to get insight in the regulation of the DP by internal and external factors and to provide tool for steering the inulin biosynthesis.

One way of increasing the mDP of chicory inulin at harvest is to prevent the reduction of 1-SST activity during phase II and III. We 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. Two different *1-SST* genes (*SST-I* and *SST-II*), isolated from *Helianthus tuberosus* [17, 48], were expressed under the 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 introduced genes. The mDP of inulin and the activity of 1-SST, 1-FFT and 1-FEH were monitored from September until November, when the reduction of 1-SST activity normally occurs in wild-type chicory, and the fructosyltransferase activity of 1-FFT onto sucrose also takes place. No impairment on growth was observed. Comparison of the control plants with plants harbouring the *Helianthus 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 in the first

8 weeks of the experiment. Nevertheless, plants harbouring the *SST-I* gene did not show elevated levels of 1-SST activity; neither did they show a significant alteration in mDP. Unfortunately, low temperatures had a comparable effect on the decrease of mDP in both types of transgenics (containing *SST-I* or *SST-II)* and on the wild-type plants. At the end of the growing season the extra *1-SST* gene did not result in longer chain inulin, most probably because of the (early) induction of FEH by low temperatures by the end of the experiment. Another approach to enhance the mDP of chicory inulin at harvest was to decrease the 1-FEH activity. A *FEH I* antisense fragment driven by the constitutive CaMV 35S promoter was introduced into chicory. In three independent transgenic lines cold induction of *FEH I* expression could be reduced by the antisense *FEH I* expression. However, this decreased 1-FEH induction had only minor effects on the mDP when the transgenics were subjected to a cold treatment, normally 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. Other studies that attempted to change the inulin composition of chicory reported also that the biosynthesis was difficult to influence. In a study performed by Sprenger *et al.* [49] a sucrose: fructan 6-fructosyltransferase (6-SFT) from barley was introduced into chicory. The aim was to produce the mixed type fructan. Unfortunately, the composition of inulin in the taproot of the transgenic plants had not been changed. However, excised leaves that were placed in sucrose solutions and illuminated continuously, accumulated ß(2-1) inulin and ß(2-6) fructan (1-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 modifying the fructan linkage-type in chicory, the gene encoding onion 6G-FFT was introduced [50]. 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. Similar studies in other crops showed that the native fructan composition can significantly be altered and that the effect on fructan yield and composition was more pronounced than in the transgenic chicory. A study in *Lolium* showed that the expression *6G-FFT* or *1-SST* from barley in *Lolium perenne* yielded up to 15% more fructan than in wild-type plants; this increased fructan content did not impair the growth of the plant. Differences of the linkage type or changes in the DP profile were not reported. Another example of fructan modification in *Lolium* showed that native high DP fructan was depleted and the profile of lower DP fructan was altered upon introduction of *sacB*, that was targeted to the vacuole [51]. The modification of the linkage-type however negatively affected the fructan yield. The modification also slowed down plant growth, the flowering plants were stunted, had narrower leaves and poorly developed roots. In transgenic lettuce, overexpressing an

Asparagine Synthase from *Escherichia coli*, rather unexpectedly accumulated 30 times more fructan than wild-type plants lettuce [52]. 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.

In conclusion, although the introduced genes were functional and the knockdown could be detected in the transgenic chicory plants, only slight changes of the expected effect on the mDP or inulin composition were observed. The reasons for this discrepancy 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 mechanism of fructan degradation, which involves three exohydrolase enzymes and 1-FFT, is highly complex. More effective alternatives for the antisense of 1-*FEH* would be the RNAi strategy or a site-directed mutagenesis strategy that would be applied to three *FEH*-genes (*FEH Ia, b* and *FEH II*). A second strategy to increase mDP would be the over expression of an extra 1-FFT with a higher affinity for high molecular weight inulin as acceptor. This would result in the accumulation of higher DP inulin. As a third option, the large influence of cold (temperature below 10°C) on the degradation of inulin could be partially prevented by growing chicory in more moderate climates, for example in Chile or India. Selecting for the desired properties, so low 1-FEH, continues 1-*SST* and high affinity of 1-FFT for HDP inulin in breeding would also be a good strategy.

Conclusions

To gain insight into the regulation of fructan biosynthesis and mobilization in chicory, especially in relation to fructan chain length, different aspects of fructan metabolism were studied. The relationships between carbohydrate content, gene expression and the activity of enzymes involved in inulin metabolism were analysed in plants grown under field conditions and hypotheses were validated with experiments performed under controlled conditions in growth chambers. To facilitate the description and discussion of the processes involved in inulin polymerisation during the growing season, we defined three different phases based on the changes observed in the pattern of inulin accumulation. Phase 1 of inulin metabolism is characterized by inulin anabolism. The maximum mDP was reached at the beginning of September, at the end of phase I. The coinciding onset of root thickening, high photosynthesis rate and the initiation of inulin metabolism resulted in high amounts of storage carbohydrates. This process is most probably induced by an excess of sucrose, produced by photosynthesis and exported to the root. Although the enzymes 1-SST and 1-FFT appeared to be activated at the same time, the activity profiles of both enzymes strongly differed. The reduction in activity of 1-SST seems to be regulated at the post-transcriptional level as suggested by the discrepancy between mRNA level and enzyme activity. Furthermore, the regulation of 1-SST is not

directly controlled by the changes in day length and temperature as shown by growth cabinet experiments. During phase 2, 1-FFT back transfer activity mediates a decrease in mDP, although the contribution of 1-FEH to this decrease in mDP cannot be ruled out since 1-FEH I was induced when temperatures dropped below 10°C during that period. The continuous increase of the inulin yield during that phase is most probably the result of the contribution of both the back transfer by 1-FFT and the residual polymerisation by 1-SST and 1-FFT. The ability of 1-FFT to use both inulin and sucrose as acceptor allows the plant to accumulate inulin under different circumstances during the growing season (compare phases one and two). It could well be that these reactions are regulated by the concentrations of the substrates and the kinetic property of the enzyme. During phase 3, low ambient temperatures play an important role in inducing 1-FEH which releases fructose from the inulin, resulting in a decreasing mDP. While the mDP decreases mainly due to the activity of 1-FEH, 1-FFT activities (anabolic and back-transfer) and the remaining low 1-SST activity contribute to keep the inulin concentration in the taproot constant. The constant inulin concentration in combination with the fructose release may play a role in osmotic adjustment to protect the plant from the cold. The enzymes involved in inulin metabolism were shown to have specific regulation. 1-SST activity, during the growing season, increased sharply with the onset of the thickening of the root and reached its maximum in the middle of phase 1. Afterward, 1-SST activity only decreased and at the end of phase 2 the activity was only 10% of the level in phase 1. Constant *1-SST* mRNA levels in phase 1 showed that 1-SST activity down-regulation is not transcriptionally mediated. In contrast to 1-SST, the regulation of both 1-FEH and 1-FFT activity seems to take place at the transcriptional level. The 1-FFT protein, however, appeared to be very stable since 1-FFT activity remained constant during phase 3 whereas the expression of *1-FFT* in field grown plants decreased in this phase. The cold night experiments in the growth cabinet and the field experiments showed that *1-FEH I* expression is regulated by temperature. The observed threshold temperature for the induction of *1-FEH I* was 10°C. In contrast, *1-FEH II* requires lower temperatures and is only induced by sub 0°C temperatures (this study and [53]), although *1-FEH II* is also induced by other factors such as wounding and defoliation [27, 54]. Transgenic chicory plants were generated aiming at improved inulin biosynthesis, both gene constructs, aiming at resp. heterologous expression of *1 SST* and antisense expression of *FEH I*, were successfully introduced and the expression of the target genes was influenced; The 1-SST activity was increased and the expression of *FEH I* was decreased. Unfortunately the final goal to increase the inulin mDP at harvest was not reached.

Concluding: this study shows that the changes in mDP during the growing season are the result of the interplay of the three major enzymes 1-SST, 1-FFT and 1-FEH. The biosynthetic pathway appeared to be very robust as modification appeared to be difficult. Overall, this provides key information on how to improve inulin yield and quality in chicory and sheds light on the physiological role of inulin metabolism.

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Appendix A. Supplementary data

Supplementary data associated with this chapter can be found in the online version of the original article, at http://dx.doi.org/10.1016/j.jplph.2012.06.005.

References

[1] D. Wittouck, K. Boone, S. Bulcke, S. Debosschere, A. Vandaele, M. Demeulemeester, A. Calus, Industriële cichorei, in, Onderzoeks- en voorlichtingscentrum voor land- en tuinbouw, Rumbeke, 2002, pp. 79-84.

[2] R.E. Sévenier, J. van Arkel, J.C. Hakkert, A.J. Koops, Fructan: nutritional significance, application, biosynthesis, molecular biology and genetic engineering, in: P.K. Jaiwal (Ed.) Plant Genetic Engineering, Metabolic engineering and molecular farming-I, Studium Press, Houston, 2006, pp. 31 - 55.

[3] J. Edelman, T.G. Jefford, The Mechanism of Fructosan Metabolism in Higher Plants as Exemplified in *Helianthus tuberosus*, New Phytologist, 67 (1968) 517-531.

[4] W. Van den Ende, J. De Roover, A. Van Laere, In vitro synthesis of fructofuranosyl-only oligosaccharides from inulin and fructose by purified chicory root fructan:fructan fructosyl transferase, Physiologia Plantarum, 97 (1996) 346-352.

[5] R. Vergauwen, A. Van Laere, W. Van den Ende, Properties of Fructan:Fructan 1-Fructosyltransferases from Chicory and Globe Thistle, Two Asteracean Plants Storing Greatly Different Types of Inulin, Plant Physiology, 133 (2003) 391-401.

[6] W. Van den Ende, A. Michiels, D. Van Wonterghem, R. Vergauwen, A. Van Laere, Cloning, Developmental, and Tissue-Specific Expression of Sucrose:Sucrose 1-Fructosyl Transferase from *Taraxacum officinale*. Fructan Localization in Roots, Plant Physiology, 123 (2000) 71-80.

[7] C.J. Pollock, A.J. Cairns, Fructan Metabolism in Grasses and Cereals, Annual Review of Plant Physiology and Plant Molecular Biology, 42 (1991) 77-101.

[8] P.P. Rutherford, E.W. Weston, Carbohydrate changes during cold storage of some inulin-containing roots and tubers, Phytochemistry, 7 (1968) 175- 180.

[9] W. Praznik, R.H.F. Beck, Inulin composition during growth of tubers of *Heliantus tuberosus*, Agricultural and Biological Chemistry, 51 (1987) 1593- 1599.

[10] A.J. Koops, H.H. Jonker, Purification and characterization of the enzymes of fructan biosynthesis in tubers of *Helianthus tuberosus* 'Colombia': I. Fructan: fructan fructosyl transferase, Journal of Experimental Botany, 45 (1994) 1623-1631.

[11] A.J. Koops, H.H. Jonker, Purification and Characterization of the Enzymes of Fructan Biosynthesis in Tubers of *Helianthus tuberosus* Colombia (II. Purification of Sucrose:Sucrose 1-Fructosyltransferase and Reconstitution of Fructan Synthesis in Vitro with Purified Sucrose:Sucrose 1-Fructosyltransferase and Fructan:Fructan 1-Fructosyltransferase), Plant Physiology, 110 (1996) 1167-1175.

[12] W. Van den Ende, A. van Laere, De-novo synthesis of fructans from sucrose in vitro by a combination of two purified enzymes (sucrose: sucrose 1-fructosyl transferase and fructan: fructan 1-fructosyl transferase) from chicory roots (*Cichorium intybus L.*), Planta, 200 (1996) 335-342.

[13] R.E. Sévenier, R.D. Hall, I.M. van der Meer, J.C. Hakkert, A.J. van Tunen, A.J. Koops, High level fructan accumulation in a transgenic sugar beet, Nature Biotech, 16 (1998) 843-846.

[14] W. Van den Ende, A. Michiels, D. Van Wonterghem, S.P. Clerens, J. De Roover, A.J. Van Laere, Defoliation Induces Fructan 1-Exohydrolase II in Witloof Chicory Roots. Cloning and Purification of Two Isoforms, Fructan 1-Exohydrolase IIa and Fructan 1-Exohydrolase IIb. Mass Fingerprint of the Fructan 1-Exohydrolase II Enzymes, Plant Physiology, 126 (2001) 1186-1195.

[15] E.M. Hellwege, S. Czapla, A. Jahnke, L. Willmitzer, A.G. Heyer, Transgenic potato (*Solanum tuberosum*) tubers synthesize the full spectrum of inulin molecules naturally occurring in globe artichoke (*Cynara scolymus*) roots, Proceedings of the National Academy of Sciences of the United States of America, 97 (2000) 8699-8704.

[16] J.M. Stoop, J. van Arkel, J.C. Hakkert, C. Tyree, P.G. Caimi, A.J. Koops, Developmental modulation of inulin accumulation in storage organs of transgenic maize and transgenic potato, Plant Science, 173 (2007) 172-181.

[17] I.M. Van der Meer, A.J. Koops, J.C. Hakkert, A.J. van Tunen, Cloning of the fructan biosynthesis pathway of Jerusalem artichoke, The Plant Journal, 15 (1998) 489-500.

[18] G. Weyens, T. Ritsema, K. Van Dun, D. Meyer, M. Lommel, J. Lathouwers, I. Rosquin, P. Denys, A. Tossens, M. Nijs, S. Turk, N. Gerrits, S. Bink, B. Walraven, M. Lefèbvre, S. Smeekens, Production of tailor-made fructans in sugar beet by expression of onion fructosyltransferase genes, Plant Biotechnology Journal, 2 (2004) 321-327.

[19] T. Ritsema, L. Hernández, A. Verhaar, D. Altenbach, T. Boller, A. Wiemken, S. Smeekens, Developing fructan-synthesizing capability in a plant invertase via mutations in the sucrose-binding box, The Plant Journal, 48 (2006) 228-237.

[20] B. Lasseur, L. Schroeven, W. Lammens, K. Le Roy, G. Spangenberg, H. Manduzio, R. Vergauwen, J. Lothier, M.-P. Prud'homme, W. Van den Ende, Transforming a Fructan:Fructan 6G-Fructosyltransferase from Perennial Ryegrass into a Sucrose:Sucrose 1-Fructosyltransferase, Plant Physiology, 149 (2009) 327-339.

[21] N.M. Itaya, A.F. Asega, M.A.M. Carvalho, R.d.C.L. Figueiredo-Ribeiro, Hydrolase and fructosyltransferase activities implicated in the accumulation of different chain size fructans in three Asteraceae species, Plant Physiology and Biochemistry, 45 (2007) 647-656.

[22] K. Koch, R. Andersson, I. Rydberg, P. Åman, Influence of harvest date on inulin chain length distribution and sugar profile for six chicory (*Cichorium intybus L.*) cultivars, Journal of the Science of Food and Agriculture, 79 (1999) 1503-1506.

[23] W. Van den Ende, A. Van Laere, Fructan Synthesizing and Degrading Activities in Chicory Roots (*Cichorium Intybus L.*) during Field-growth, Storage and Forcing, Journal of Plant Physiology, 149 (1996) 43-50.

[24] N.J. Chatterton, P.A. Harrison, W.R. Thornley, J.H. Bennett, Separation and quantification of fructan (inulin) oligomers by anion exchange chromatography, in: A. Fuchs (Ed.) Inulin and Inulin Containing Crops, Elsevier Science Publishers B.V, Amsterdam, 1993, pp. 93-99.

[25] J.W. Timmermans, M.B. van Leeuwen, H. Tournois, D. de Wit, J.F.G. Vliegenthart, Quantitative Analysis of the Molecular Weight Distribution of Inulin by Means of Anion Exchange HPLC with Pulsed Amperometric Detection, Journal of Carbohydrate Chemistry, 13 (1994) 881 - 888.

[26] K.J. Livak, T.D. Schmittgen, Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-DDCT Method, Methods, 25 (2001) 402-408.

[27] N. Druart, J. De Roover, W. Van den Ende, P. Goupil, A. Van Laere, S. Rambour, Sucrose assimilation during early developmental stages of chicory (*Cichorium intybus L.*) plants, Planta, 212 (2001) 436-443.

[28] A. Van Laere, W. Van den Ende, Inulin metabolism in dicots: chicory as a model system, Plant, Cell & Environment, 25 (2002) 803-813.

[29] T. Rausch, S. Greiner, Plant protein inhibitors of invertases, Biochimica et Biophysica Acta (BBA) - Proteins & Proteomics, 1696 (2004) 253-261.

[30] U. Kusch, K. Harms, T. Rausch, S. Greiner, Inhibitors of plant invertases do not affect the structurally related enzymes of fructan metabolism, New Phytologist, 181 (2009) 601-612.

[31] P. Brodersen, L. Sakvarelidze-Achard, M. Bruun-Rasmussen, P. Dunoyer, Y.Y. Yamamoto, L. Sieburth, O. Voinnet, Widespread Translational Inhibition by Plant miRNAs and siRNAs, Science, 320 (2008) 1185-1190.

[32] J. De Roover, K. Vandenbranden, A. Van Laere, W. Van den Ende, Drought induces fructan synthesis and 1-SST (sucrose: sucrose fructosyltransferase) in roots and leaves of chicory seedlings (*Cichorium intybus* L.), Planta, 210 (2000) 808-814.

[33] W. Van den Ende, A. Van Laere, Induction of 1-FEH in Mature Chicory Roots Appears to be Related to Low Temperatures Rather than to Leaf Damage, TheScientificWorldJournal, 2 (2002) 1750-1761.

[34] R. Améziane, C. Richard-Molard, E. Deléens, J.-F. Morot-Gaudry, A.M. Limami, Nitrate ($^{15}NO_3$) limitation affects nitrogen partitioning between metabolic and storage sinks and nitrogen reserve accumulation in chicory (*Cichorium intybus* L.), Planta, 202 (1997) 303-312.

[35] A.P. Sobolev, A.L. Segre, D. Giannino, D. Mariotti, C. Nicolodi, E. Brosio, M.E. Amato, Strong Increase of Foliar Inulin Occurs in Transgenic Lettuce Plants (*Lactuca sativa L.*) Overexpressing the Asparagine Synthetase A Gene from Escherichia coli, Journal Agriculture and Food Chemistry, 55 (2007) 10827-10831.

[36] W. Van den Ende, J. De Roover, A. Van Laere, Effect of nitrogen concentration on fructan and fructan metabolizing enzymes in young chicory plants (*Cichorium intybus*), Physiologia Plantarum, 105 (1999) 2-8.

[37] U. Kusch, S. Greiner, H. Steininger, A.D. Meyer, H. Corbière-Divialle, K. Harms, T. Rausch, Dissecting the regulation of fructan metabolism in chicory (*Cichorium intybus L.*) hairy roots, New Phytologist, 184 (2009) 127-140.

[38] I.S. Bhatia, S.K. Nann, R. Singh, Biochemical changes in the water-soluble carbohydrates during the development of chicory (*Cichorium intybus* L.) roots., journal of Science of Food and Agriculture, 25 (1974) 535-539.

[39] W. Van den Ende, A. Mintiens, H. Speleers, A.A. Onuoha, A. Van Laere, The metabolism of fructans in roots of *Cichorium intybus* during growth, storage and forcing, New Phytologist, 132 (1996) 555-563.

[40] W. Van den Ende, A. Michiels, J. De Roover, P. Verhaert, A. Van Laere, Cloning and functional analysis of chicory root fructan1-exohydrolase I (1-FEH I): a vacuolar enzyme derived from a cell-wall invertase ancestor? Mass fingerprint of the 1-FEH I enzyme, The Plant Journal, 24 (2000) 447-456.

[41] M.K. Ernst, N.J. Chatterton, P.A. Harrison, Carbohydrate changes in chicory (*Cichorium intybus* L. var. foliosum) during growth and storage, Scientia Horticulturae, 63 (1995) 251-261.

[42] W. Saengthongpinit, T. Sajjaanantakul, Influence of harvest time and storage temperature on characteristics of inulin from Jerusalem artichoke (*Helianthus tuberosus* L.) tubers, Postharvest Biology and Technology, 37 (2005) 93-100.

[43] A.K. Gupta, N. Kaur, G. Anil Kumar, K. Narinder, Fructan metabolism in jerusalem artichoke and chicory, in: Developments in Crop Science, Elsevier, 2000, pp. 223-248.

[44] Y. Ishiguro, K. Ueno, S. Onodera, N. Benkeblia, N. Shiomi, Effect of temperatures on inulobiose and inulooligosaccharides in burdock roots during storage, Journal of Food Composition and Analysis, 24 (2011) 398-401.

[45] M.K. Ernst, N.J. Chatterton, P.A. Harrison, G. Matitschka, Characterization of fructan oligomers from species of the genus Allium L, Journal of Plant Physiology, 153 (1998) 53-60.

[46] M.T. Portes, R.d.C.L. Figueiredo-Ribeiro, M.A.M. de Carvalho, Low temperature and defoliation affect fructan-metabolizing enzymes in different regions of the rhizophores of *Vernonia herbacea*, Journal of Plant Physiology, 165 (2008) 1572-1581.

[47] D. Hincha, K, E. Hellwege, M, A. Heyer, G, J. Crowe, H, Plant fructans stabilize phosphatidylcholine liposomes during freeze-drying, European Journal of Biochemistry, 267 (2000) 535-540.

[48] A.J. Koops, R.E. Sévenier, A.J. Van Tunen, L. De Leenheer, Transgenic plants presenting a modified inulin producing profile, in, United states of America, 2003.

[49] N. Sprenger, L. Schellenbaum, K. van Dun, T. Boller, A. Wiemken, Fructan synthesis in transgenic tobacco and chicory plants expressing barley sucrose:fructan 6-fructosyltransferase, FEBS Letters, 400 (1997) 355-358.

[50] I. Vijn, A. Van Dijken, N. Sprenger, K. Van Dun, P. Weisbeek, A. Wiemken, S. Smeekens, Fructan of the inulin neoseries is synthesized in transgenic chicory plants (*Cichorium intybus L.*) harbouring onion (*Allium cepa L.*) fructan:fructan 6G-fructosyltransferase, The Plant Journal, 11 (1997) 387-398.

[51] X.Y. Ye, X.W. Wu, H.Z. Zhao, M.F. Frehner, J.N. Nösberger, I.P. Potrykus, G.S. Spangenberg, Altered fructan accumulation in transgenic *Lolium multiflorum* plants expressing a *Bacillus subtilis sacB* gene, Plant Cell Reports, 20 (2001) 205-212.

[52] A.P. Sobolev, A.L. Segre, D. Giannino, D. Mariotti, C. Nicolodi, E. Brosio, M.E. Amato, Strong Increase of Foliar Inulin Occurs in Transgenic Lettuce Plants *(Lactuca sativa L.*) Overexpressing the Asparagine Synthetase A Gene from *Escherichia coli*, Journal of Agricultural and Food Chemistry, 55 (2007) 10827-10831.

[53] A. Michiels, A. Van Laere, W. Van den Ende, M. Tucker, Expression analysis of a chicory fructan 1-exohydrolase gene reveals complex regulation by cold, Journal of Experimental Botany, 55 (2004) 1325-1333.

[54] J. De Roover, A. Van Laere, W. Van den Ende, Effect of defoliation on fructan pattern and fructan metabolizing enzymes in young chicory plants (*Cichorium intybus L.*), Physiologia Plantarum, 106 (1999) 158-163.

Chapter 3

Carbohydrate-mediated regulation of fructan biosynthesis in chicory (Cichorium intybus L.*)*

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Submitted

Abstract

In this study the regulation of fructan biosynthesis in chicory was analysed. Fructan gene expression and carbohydrate concentrations were studied in axial sections of mature chicory roots, revealing that the highest expression levels and carbohydrate levels were found in the phloem. Correlations were found between the gene expression patterns of *sucrose: sucrose 1-fructosyl transferase (1-SST), fructan: fructan 1-fructosyl transferase (1-FFT)* and the carbohydrate levels, suggesting a possible involvement of sugars in the regulation of *1-SST* and *1-FFT* gene expression. The expression of *1-SST* and *1-FFT* in excised chicory leaves was induced by sucrose, which suggests that both genes are at least partly regulated in the same way. Glucose also induced fructan gene expression, while induction by fructose was less pronounced compared to sucrose. Expression of *1-FFT* was for example not induced by fructose in contrast to *1-SST* expression. To further unravel the mechanism of induction, the promoters of *1-SST* and *1-FFT* from chicory were isolated and characterized through *in silico* and *in planta* (only *1-FFT*) analysis. Computational analysis of several fructosyltransferase (FT) promoters from different species revealed elements that were common in fructan biosynthesis-promoters among different species and also occur in Arabidopsis promoter sequences. One of the elements was predominantly present in genes involved in sugar metabolism and transport. This element did also contain a core sequence involved in MYB transcription factor binding important for fructosyltransferase activation in wheat, as was published recently. An 1100bp *1-FFT* upstream promoter fragment was shown to be functional in transgenic chicory and in the non-fructan plants, Arabidopsis and potato. Induction by exogenous supplied carbohydrates resulted in expression of the reporter gene *GUS* comparable to *1-FFT* expression upon sucrose feeding in chicory. The main conclusion of this study is that fructan biosynthesis in chicory is induced by sucrose and/or glucose, and that the induction mechanism for fructan biosynthesis seems to represent a universal plant signalling pathway present in chicory, potato and Arabidopsis.

Introduction

Most temperate plants deposit carbohydrates in sink tissues to overcome conditions unfavourable for growing, like grazing, low temperatures in winter or high temperatures in summer. The most prominent carbohydrate storage is starch; whereas mainly in Gramineae and Asteraceae fructan is used as carbohydrate storage molecule. Fructan occurs in many economical important crops, for example onion, rye, oat, yacon and chicory. Chicory (*Cichorium intybus* L.) is a biennial taproot-bearing crop plant that is grown for the production of inulin on an industrial scale. Inulin, a ß(2,1) linked linear fructan with a terminal glucose residue, serves as a reserve carbohydrate in taproots. Inulin is isolated and used for a wide range of food and non-food applications (Chapter 1).

The biosynthesis of inulin is catalysed by the activity of three different classes of enzymes: sucrose: sucrose 1-fructosyl transferase (1-SST), fructan: fructan 1-fructosyl

transferase (1-FFT) and fructan exohydrolase (1-FEH) [1]. 1-SST primarily catalyses the synthesis of the smallest inulin, 1-kestose from two molecules of sucrose. In this reaction also glucose is formed, in equimolar amounts to 1-kestose. 1-FFT catalyses the transfer of fructosyl units from 1-kestose and any other inulin molecule onto 1-kestose and longer inulin molecules. The third class of enzymes, 1-FEH, catalyses the degradation of inulin by the hydrolyses of terminal fructosyl units, which results in the formation of fructose and lower DP inulin [2]. See also Chapter 2, figure 1 for a schematic overview of the biosynthetic pathway. The biosynthesis occurs in the taproot and is induced when radial root growth starts. The induction of fructan biosynthesis in chicory has been the subject of several studies. Kusch *et al*.[3] studied the regulation of 1-SST and 1-FFT in relation to the nitrogen availability in hairy root cultures of chicory. When the hairy roots were transferred from the standard culture medium to a medium with high-carbon and low-nitrogen, expression of *1-SST* and *1-FFT* was strongly induced and inulin accumulated. Excised leaves showed to be another powerful model to study fructan regulation, as shown by several studies in grasses [4-6]. Excised leaves of chicory do not contain fructan initially, but they start to accumulate fructan upon illumination or supply of sugars. Under these conditions, they display strongly enhanced activities of fructan biosynthetic enzymes. The use of the excised leaf system to study fructan biosynthesis was first applied in chicory by Améziane [7] to show the inducibility of *1-SST* by light and the correlation of *1-SST* enzyme activity with the concentration of sucrose.

Studies on the biosynthesis of fructan during the growing season have been performed for several crops, as described in Chapter 2 for chicory, showing patterns of gene expression and enzyme activity in relation to factors like endogenous carbohydrate concentration and ambient temperature. However, the question which molecular mechanisms are triggering the expression of *1-SST* and *1*-FFT at the onset of inulin biosynthesis is still unanswered. In this study we combine different laboratory experiments, such as analysis of endogenous enzymes and product levels, the excised leaf system and the use of transgenic plants, with computational experiments *in silico*. This resulted in a detailed study with the objective to better understand the regulation of *1-SST* and *1-FFT* in chicory and to unravel the role of sugars as regulators of fructan biosynthesis.

Results

Localization of inulin biosynthesis in root tissue

Inulin is stored in the taproot of chicory, but it is unclear in which part of the root fructan biosynthesis takes place and what the relation is between the imported sugars, fructan gene expression level and the regulation and induction of fructan biosynthesis. To answer these questions, the gene expression levels of 1-SST and 1-FFT and the levels of soluble carbohydrates were determined in different layers of tissue composing the chicory taproot. Roots from plants grown under field conditions were harvested in September. A

transversal section was made from just below the root shoulder, and six concentric rings were dissected (Fig. 1). The outer rings 1 and 2 contained mostly storage parenchyma, rings 3, 4 and 5 phloem and ring 6 phloem and xylem. The dissected tissues were used for the analysis of soluble sugars and fructan gene expression.

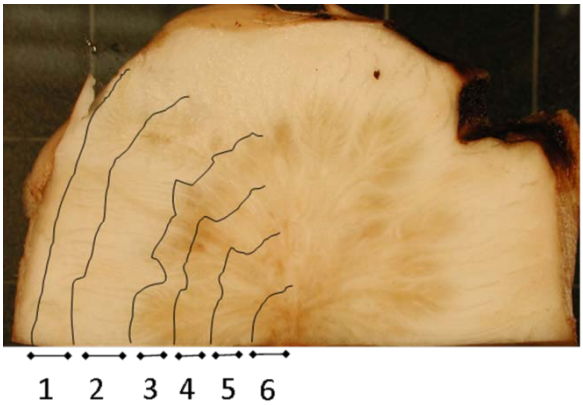


Figure 1. Example of sampling along the horizontal axis through the chicory taproot. Lines indicate the borders of the different rings, numbered from the outside to the inside of the root.

Soluble sugar analysis revealed that the concentration of sucrose was not significantly different across the rings (Fig. 2), although the two inner rings (rings 5 and 6) tended to have a slightly higher sucrose concentration. The concentration of glucose was low in rings 1 and 2 and increased towards the inside of the root, where it was up to 7-fold higher (Fig. 2). The concentration of fructose was low compared to sucrose and glucose, but the distribution pattern of fructose was comparable to the one of glucose. The two inner rings (5 and 6) showed about 4-fold higher fructose concentrations than the rings more to the periphery of the root (Fig. 2).

Gene expression was analysed by qRT-PCR analysis and showed that *1-SST* and *1-FFT* were expressed in all parts of the root (Fig. 3), but expression was highest in the inner part: rings 5 and 6 (Fig. 3). Comparison of the sugar content (Fig. 2) and the gene expression (Fig. 3) revealed a positive correlation between the expression of both genes and the concentration of glucose and fructose, but not sucrose. To determine the effect of the developmental stage of the plant on the localization of inulin biosynthesis, similar analyses were also performed in older roots. These analyses showed that the expression patterns of *1-SST* and *1-FFT* and the co-localization with the carbohydrates over the rings did not change in time (data not shown).

In conclusion: the positive correlations between the gene expression patterns of *1-SST* and *1-FFT* with glucose and fructose levels suggest an involvement of sugars in the regulation of *1-SST* and *1-FFT* gene expression.

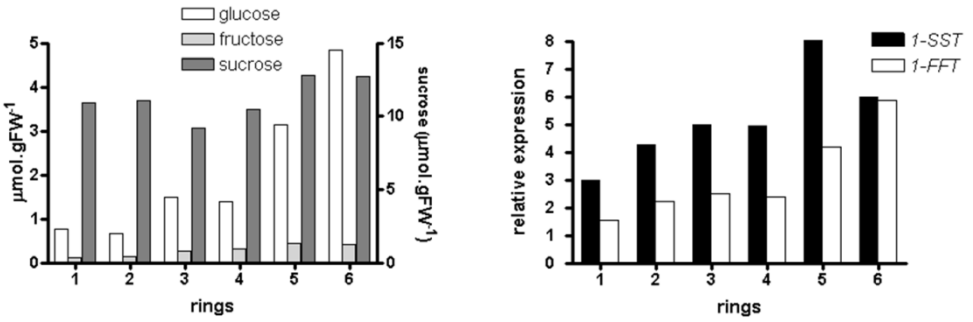


Figure 2. (left figure) The sugar composition in the rings along the horizontal axis through the chicory taproot. The concentration (µmol.gFW^{-1}) of glucose (white bars) and fructose (light gray bars) are plotted on the left Y-axis, the concentration of sucrose (gray bars) is plotted on the right y-axis. The concentrations represent the means of three different taproots

Figure 3. (right figure) Relative gene expression of *1-SST* and *1-FFT* in the rings along the horizontal axis through the chicory taproot. The relative gene expression of *1-SST* (black bars) and *1-FFT* (white bars) is measured in different rings taken along the horizontal axis of chicory taproot and is relative to the expression of the ribosomal protein *L19* homologue. The amounts represent the means of measurements on three different taproots.

Ectopic expression of fructan biosynthesis in leaves of chicory

To study the effects of carbohydrates on the induction of *1-SST* and *1-FFT* expression, the excised leaf system was used. Under normal vegetative growing conditions, glucose, fructose and sucrose concentrations are low in leaves of chicory and fructan biosynthesis is absent. However, fructan biosynthesis in chicory was shown to be induced in excised leaves upon sucrose induction [7]. This system allowed us to study the induction of fructan biosynthesis in chicory in a tissue where fructan synthesis normally does not occur. Time course experiments with excised leaves using sucrose in the (feeding) solution showed that the expression of *1-SST* was induced 13h after the start of the experiment (Fig. 4A). At t=24h, expression of *1-FFT* was induced and *1-SST* expression was increased. The expression of both genes further increased with time and the highest expression was found at 48h after the onset of the experiment.

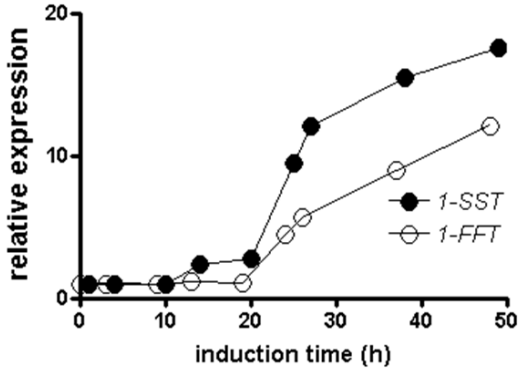


Figure 4a. Expression of *1-SST* and *1-FFT* in excised leaves of chicory, measured in time. The relative expression of *1-SST* (closed circle) and *1-FFT* (open circle) upon feeding on 25% (730mM) sucrose was analysed at t=0, 3, 10, 18, 25, 27, 36 and 48h after the start of the experiment. The expression is relative to the expression at t=0.

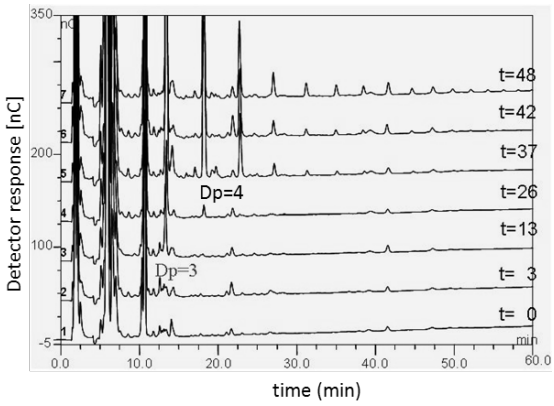


Figure 4b. Carbohydrate HPAEC-PAD analysis of carbohydrate extracts of excised chicory leaves incubated in a 25% (730mM) sucrose solution for different induction periods (indicated in the figure). 1-kestose and 1-nystose are indicated with "Dp=3", "Dp=4"resp. above the specific peak Chromatograms are nudged along the y-axis to prevent overlap.

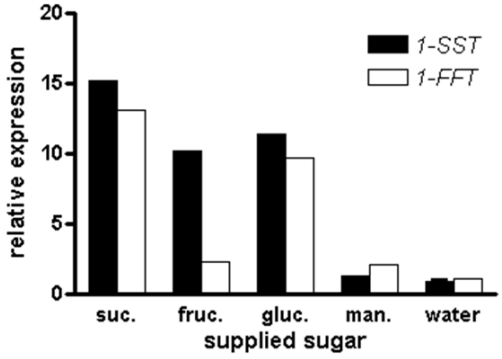


Figure 4c. Relative gene expression of *1-SST* and *1-FFT* measured in excised chicory leaves that were fed with sugar solutions (1 Os.kg-1). The relative expression of *1-SST* (black bars) and *1-FFT* (white bars) are relative to the expression of the ribosomal protein *L19* homologue of chicory.

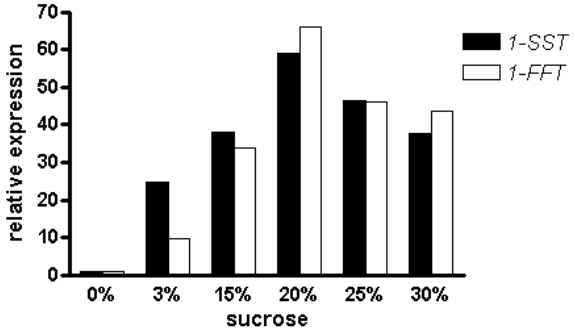


Figure 4d. Relative gene expression of *1-SST* and *1-FFT* measured in excised chicory leaves fed with different concentrations of sucrose. The relative expression of *1-SST* (black bars) and *1-FFT* (white bars) are relative to the expression at zero percent sucrose

Carbohydrate analysis showed that the initial accumulation of 1-kestose coincided with the initial expression of *1-SST*, at t=13h. Low levels of 1-nystose were detected at t=26h (Fig. 4B), higher DP and higher concentrations of fructan were found after 48 hours of induction. Also glucose induced the expression of both fructan genes, but to a slightly lower level than sucrose (Fig. 4C). Induction by fructose resulted in an increase of *1-SST*

expression, while the expression of *1-FFT* remained comparable to the water control (Fig. 4C). The expression of *1-SST* and *1-FFT* was not increased by mannitol, showing that the gene expression in the experiments was not the result of osmotic stress.

Experiments with a range of sucrose concentrations showed an increase of *1-SST* and *1-FFT* gene expression upon increasing sucrose concentrations up to 20% (580 mM). At higher concentrations, the expression was reduced somewhat but still about 40-fold higher than at 0% sucrose (Fig. 4D). The concentrations of sucrose, glucose, fructose and inulin in the leaves increased significantly upon addition of sucrose, glucose and fructose, whereas no significant effect was seen upon mannitol feeding (Fig. 5). The effects of sucrose and glucose feeding were comparable, both resulting in increased amounts of glucose, fructose and sucrose. When fructose was added, lower amounts of sucrose accumulated while 6-fold more fructose accumulated in contrast to the experiments with glucose or sucrose supply (Fig. 5).

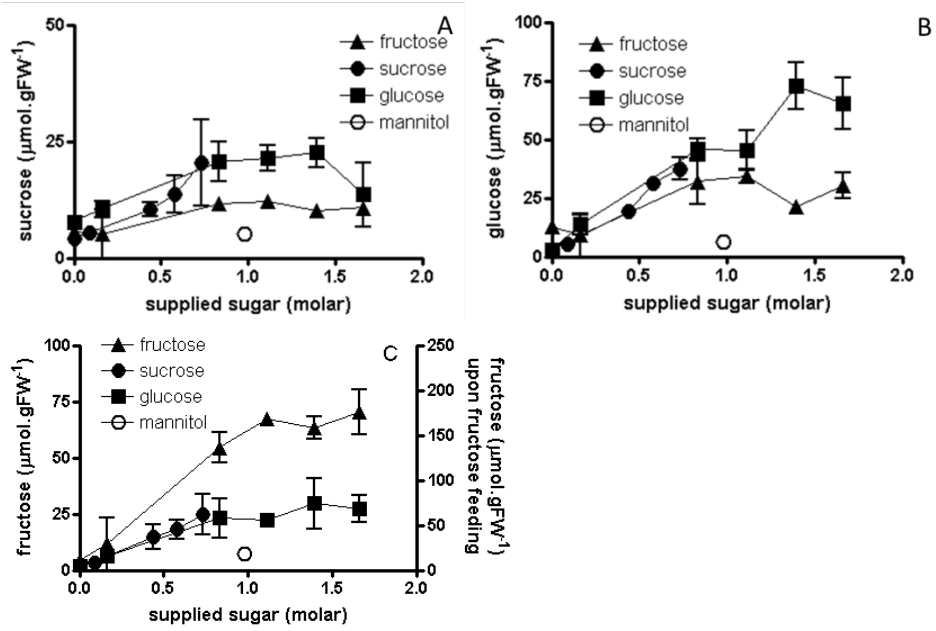


Figure 5. Sugar contents of excised leaves after feeding on various concentrations of different sugars. The concentration of sucrose (a), glucose (b) and fructose (c) upon incubation in fructose (triangle), sucrose (closed circle), glucose (square) or mannitol (open circle). In 5c the fructose accumulated upon fructose feeding is plotted at the right axis. The error bars represent the SEM of 2 replicate experiments.

The concentration of fructan was determined in the same samples and comparison with the gene expression upon sucrose feeding showed that the levels of 1-kestose (DP3) and 1-nystose (DP4) plus 1F-β-fructofuranosylnystose (DP5) correlated with *1-SST* and *1-FFT* gene expression, respectively (compare Fig. 4C with 6a resp. 6b). Fructan

biosynthesis was induced to a low level by three percent of sucrose, glucose and fructose (87, 166 and 166 mM, respectively), resulting in the accumulation of small amounts of 1-kestose (Fig. 6a). Increasing the concentrations of glucose and sucrose in the solution resulted in an almost linear increase of the level of DP3 (product of 1-SST) and in accumulation of DP4 and 5 (products of 1-FFT) (Fig. 6b). In contrast, incubation with fructose resulted in only a minor increase of DP3 content and did not induce the production of DP4 and DP5 (Fig. 6b) (*P*-value- fructose feedings vs. control- for DP3 and DP4+5 was *P*=0.001 and *P*=0.108, respectively). In conclusion: Sucrose or/and glucose concentrations in the tissue seem to be correlated with *1-SST* and *1-FFT* expression and fructan accumulation.

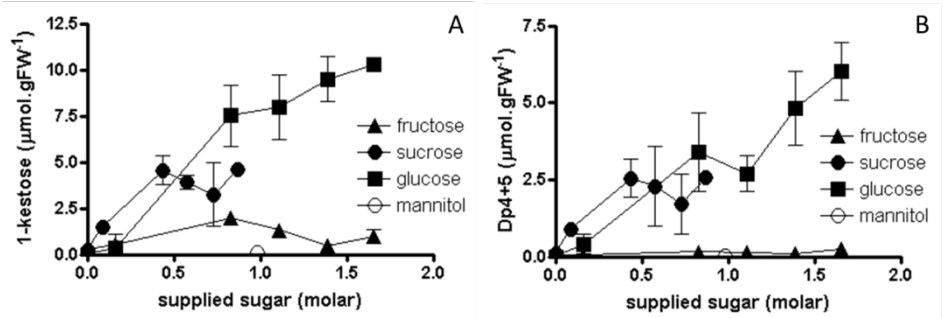


Figure 6. Fructan contents of excised leaves after feeding on different concentrations of different sugars. The concentration of 1-Kestose (a) and the sum of concentrations of 1-nystose (DP4) and 1F-β-fructofuranosylnystose (DP5) (b) upon incubation in fructose (triangle), sucrose (closed circle), glucose (square) or mannitol (open circle). The error bars represent the SEM of 2 replicate experiments.

Isolation and bioinformatics analysis of the 1-SST and 1-FFT promoters

To allow analysis of promoter elements of *1-SST* and *1-FFT*, promoter sequences of *1-SST* and *1-FFT* were cloned from genomic libraries. For *1-SST* a 1350 bp upstream fragment was cloned. For *1-FFT* two fragments were cloned: a 450 bp and an 1100 bp fragment. The TATA box and CAAT box, commonly found in the 5′ non-coding region of eukaryotic genes (Hanley and Schuler, 1988), were found in the distal region of the promoter sequence using the PLACE signal scan search [8]. For *1-SST* a putative TATA-box is located 85 bp upstream of the ATG. A putative CAAT box is present at 229 bp upstream of the ATG. The putative TATA and CAAT box of *1-FFT* are located more close to the ATG, respectively 72 and 94 bp upstream of the translation start site (Fig. 7). The elements SURE, DOF, GT-1 and I-Box that have previously been described as putatively involved in carbohydrate mediated regulation of gene expression were also found in the promoter sequences of both genes (Fig. 7). In order to identify other putative, new *cis*-motifs on the isolated promoter sequences, we applied a bioinformatics strategy consisting of a *de novo* motif search; MEME [9] was applied to the sequences listed in table S1 supplementary data, with the criteria that the element should be present in promoter sequences of chicory *1-SST* and *1-FFT*, but not in *1-FEH* and *Invertase*. In total six motifs were selected for further

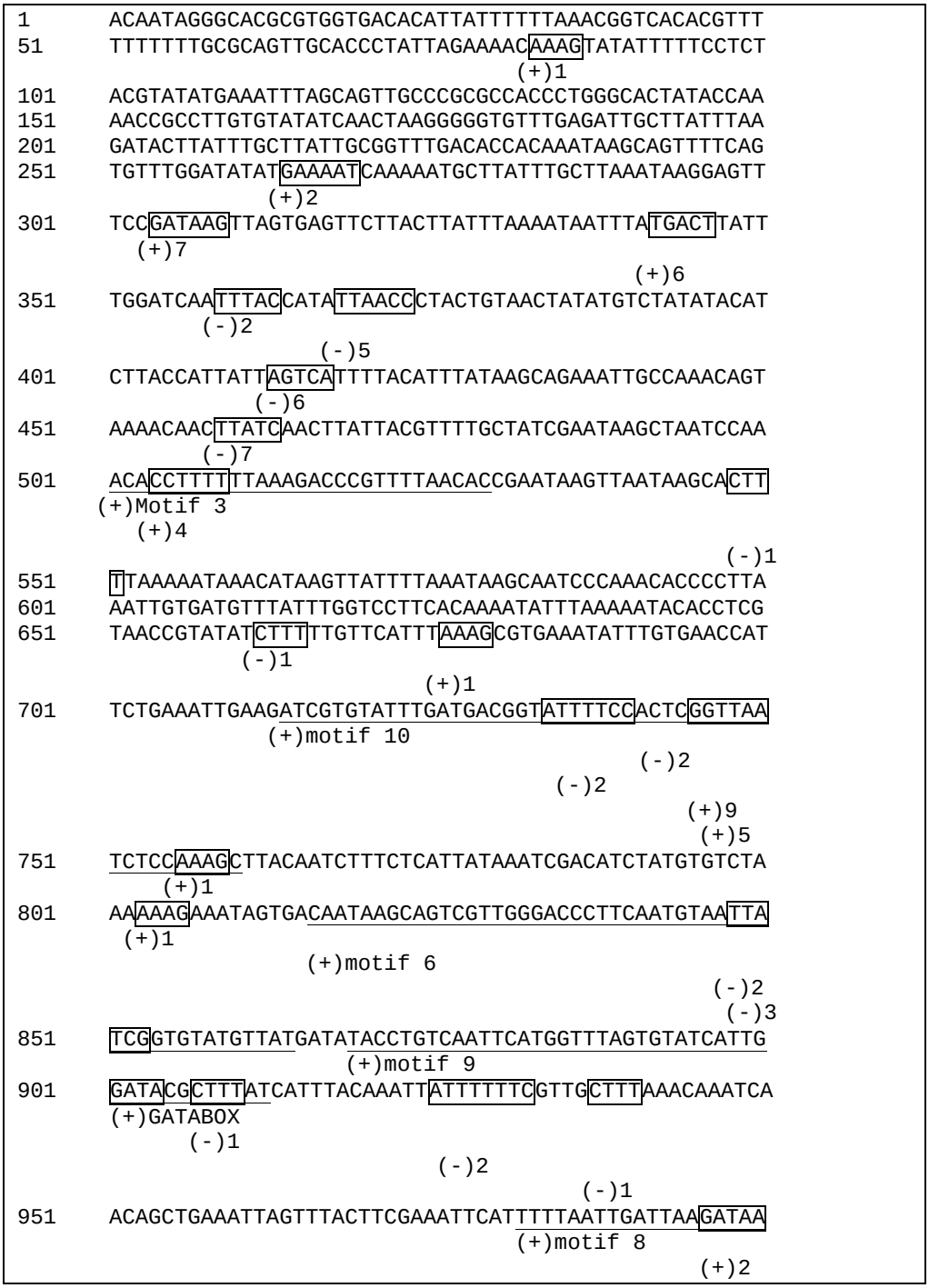
analysis, nr. 3, 5, 6, 7, 8, 9, and 10 (Fig. S1, supplementary data). Comparison of the identified motifs with existing motifs in JASPAR [10] showed no similarity to motifs in that database. However, from literature a recently experimentally validated binding motif in *Ta1-SST* and *Ta6-SFT* genes for a MYB transcription factor was retrieved [11]. The core of this binding motif is TTxGGT, which is an integral part of one of the binding motifs we predicted; motif 9. The sequence was found in the motif 9 region of *1-SST*, but not in *1-FFT* (Fig. 7). To obtain further supporting evidence for putative functionality of these motifs in the common carbohydrate mediated gene regulation, the occurrence in promoter regions in Arabidopsis, detected with FIMO [12], was analysed. About 500 Arabidopsis genes contained one or more of the motifs in the promoter sequences.

1-SST

```
1       ACAAGTTGTCATATTTGGTTAGATCAAAAGTTCTAATCCATTTTTATCAA
                                  (+)1
                                                    (-)2
                                                     (-)3
51      ATCTTCTAACATTTAAACTCAATATTCTTTTAGGATGTTACAATTCGAAT
                                  (-)1
101     TTATGAAGTATTCCCTAAGCCATAAATAGAAATGAAGAAGAGGAGTGAAG
151     AATTGCTGCTTTATAGAAATGAAGAAAAGGAGTGAATATTTAAGTTCCAA
              (-)1
                                (-)4
                                 (+)1
201     ACCTTCAATTAAACTTCAATCTAGCTTCAACTCCAATTAAATATCTCAAA
251     CCCACTTCTCAATCCTTTTTTAGATCCTAAATCATTTAAATGAAAAGAAT
                    (+)4
                     (-)1
                                                      (+)1
301     GGGAAGGATTTGGGTTTAAGTATAAGTGCTTAGGTATTTAACCATAATAG
                                               (-)5
351     TAATTTATTCCCCAACTAACTCTCAATTCTCATAGAATTGTGGAAATTCT
                                                (+)2
401     TAGTTAAACCCAGATAAGTTAGTATATTAACAATTAACATCCAAATTCTA
                   (+)7
451     GTTTGACCAAGTTAGTTTCAAGTCCAAACCTAAGTTTTCTTACTTGGCAC
501     ATTTGGTCCCTTAAATATTCAAATACTAGAAATATACCTTTTGAGGTGGA
                                           (+)4
                                            (-)1
                                                        (+)2
                                                         (+)2
551     AAAACAAAAGTGGGTCGGAGATGAACCATGCACTGCATCATTTTTTATTCA
            (+)1
601     AAAAATTGAGTAAAAAATGATGGTGAGTTGGAGATGATCTGACCATTGTA
                                (+)motif 5
651     GTTGTGGGACAATCCACAATCCCTTGGGCTATTCAAATATATATGAAGAT
701     CTTGGAATCTCATATATTGATTTGGATGTTCCAAATTCAGAATACGTATA
751     TTGGATTTTAATTTACATTATTGTATTTTACACAACCTATTCGTATATAG
            (+) motif 10
```


801 TTATATATCACTTAACATTATTTGATTATACCTACTCATCACATAAGAAA
851 TCTAAACACAAGTGACTTTTACGCTACAAAATTTTCATGATAATATTTTT
(+)6
(+)motif 7
(-)1
(-)2
(+)2
(+)3
901 TGAAAATATGGACGTTGATTTAATATTGCTTGGTGTTAAAAGATGTAGAA
(+)2
(+)motif 8
(+)1
951 CAGAATTACTCTATATACGATACAAACCCAATACATACACATCCAAAGAC
(+)1
1001 ATACACACAAGACAAACCAATACATAAACATACACTGATACACGGCTACG
1051 ACACAAAATACACGCACATAGTATACATGCAACATGTTAAACCATATATT
1101 TGTGGTAGAAAGCAATAGGCACGTTTATGCTCTTTTCCAATTAATATATA
(+)1
(+) motif6
(-)1
(-)2
(+)CCAATBOX1
1151 TTCATTTTTTTATTTTTATTTTAATTTTCGGTTCATGGTTTAGTACTTTA
(+)motif 9
(-)2
(-)1
1201 ATTAGTCCGGTTTAGTTTACGATGTCTGAAGAGAAAAAACAAAGCTGTCA
(+)2
(+)8
(+)1
1251 CTTTCATGGTATGGAAGTATCCTGTATTAAAAATTATATAAAAACTCCAT
(-)1
(+) motif 3
(-)TATABOX4
(+)TATABOX4
1301 TAATCACCCCTTACCACGACTCACCTTTAGACAGTAAAGTGTGCGTAGTA
(-)1
(+)1
1351 GAGAGACCACCCCACATG
M

1-FFT


1 ACAATAGGGCACGCGTGGTGACACATTATTTTTTAAACGGTCACACGTTT
51 TTTTTTTGCGCAGTTGCACCCTATTAGAAAACAAAGTATATTTTTCCTCT
(+)1
101 ACGTATATGAAATTTAGCAGTTGCCCGCGCCACCCTGGGCACTATACCAA
151 AACCGCCTTGTGTATATCAACTAAGGGGGTGTTTGAGATTGCTTATTTAA
201 GATACTTATTTGCTTATTGCGGTTTGACACCACAAATAAGCAGTTTTCAG
251 TGTTTGGATATATGAAAATCAAAAATGCTTATTTGCTTAAATAAGGAGTT
(+)2
301 TCCGATAAGTTAGTGAGTTCTTACTTATTTAAAATAATTTATGACTTATT
(+)7
(+)6
351 TGGATCAATTTACCATATTAACCCTACTGTAACTATATGTCTATATACAT
(-)2
(-)5
401 CTTACCATTATTAGTCATTTTACATTTATAAGCAGAAATTGCCAAACAGT
(-)6
451 AAAACAACTTATCAACTTATTACGTTTTGCTATCGAATAAGCTAATCCAA
(-)7
501 ACACCTTTTTTAAAGACCCGTTTTAACACCGAATAAGTTAATAAGCACTT
(+)Motif 3
(+)4
(-)1
551 TTAAAAATAAACATAAGTTATTTTAAATAAGCAATCCCAAACACCCCTTA
601 AATTGTGATGTTTATTTGGTCCTTCACAAAATATTTAAAAATACACCTCG
651 TAACCGTATATCTTTTTGTTCATTTAAAGCGTGAAATATTTGTGAACCAT
(-)1
(+)1
701 TCTGAAATTGAAGATCGTGTATTTGATGACGGTATTTTCCACTCGGTTAA
(+)motif 10
(-)2
(-)2
(+)9
(+)5
751 TCTCCAAAGCTTACAATCTTTCTCATTATAAATCGACATCTATGTGTCTA
(+)1
801 AAAAAGAAATAGTGACAATAAGCAGTCGTTGGGACCCTTCAATGTAATTA
(+)1
(+)motif 6
(-)2
(-)3
851 TCGGTGTATGTTATGATATACCTGTCAATTCATGGTTTAGTGTATCATTG
(+)motif 9
901 GATACGCTTTATCATTTACAAATTATTTTTTCGTTGCTTTAAACAAATCA
(+)GATABOX
(-)1
(-)2
(-)1
951 ACAGCTGAAATTAGTTTACTTCGAAATTCATTTTTAATTGATTAAGATAA
(+)motif 8
(+)2

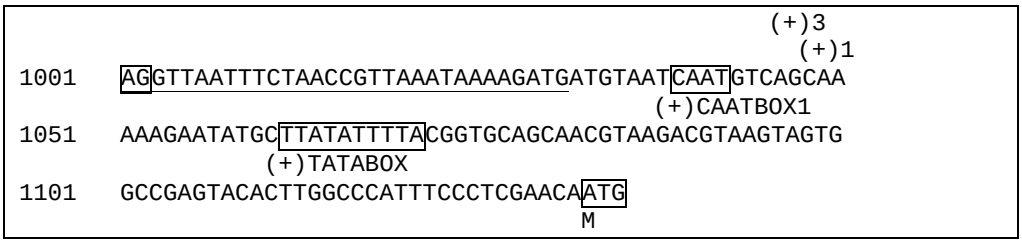


Figure 7. Nucleotide sequences of the *1-SST* and the *1-FFT* promoter.
The nucleotides are numbered from the end of the promoter fragment. Putative cis-acting elements are boxed and numbered, 1= DOFCOREZM, 2= GT1CONSENSUS, 3= IBOXCORE, 4= PYRIMIDINEBOXOSRAMY1A, 5= GT1CORE, 6= WBOXHVISO1, 7= IBOX , 8= GT1GMSCAM4, 9=MYBCORE. The start of transcription is boxed and indicated with an "M". The motifs that were found in the de novo motif discovery are underlined. The MYB core binding motif on motif 9 of *1-SST* is marked in grey. The (+) or (-) symbol indicates the current resp. the opposite strand.

In order to identify putative relations between the motifs identified and Arabidopsis gene expression, co-expression analysis was performed with the genes having the same motif using the co-expression analysis tool of CORNET 2.0 ARABIDOPSIS on seven sets of pre-defined microarray expression data [13]. Genes that were selected for the presence of motifs 3 and 10 in their promoter did not seem to be co-regulated. None of the motifs seemed to be involved in the regulation by abiotic stress and no clear common expression pattern was found with this set of data. However, many genes selected for the presence of motifs 5,6,7,8 and 9 were differentially regulated by hormones (Table S2, supplementary data). Several genes were shown to be differentially expressed upon day length and light intensity, mostly in embryo, seed or seedlings, sometimes in roots but not in leaves. As light tends to directly affect the concentration of sucrose in plant tissue [14], further transcriptional profiling experiments were performed on individual microarray datasets from different sugar feeding experiments. This showed that motif 9, containing the MYB-core, was overrepresented in genes that are (≥2x) up-regulated upon sugar feeding in the experiments E-GEOD-3704 and E-GEOD-5723 (P = 0,05 and 0,07, respectively) (table 1a). The Arabidopsis genes that possess motif 9 in their promoter are given in table 1b. Among them are genes involved in sugar transport and carbohydrate regulation, like sucrose-phosphatase, 60S and 40S ribosomal proteins and receptor kinases (table 1b). In conclusion, the *in silico* promoter study provides evidence for the hypothesis that the fructan biosynthesis are regulated by sugars.

Experiment	Description
E-GEOD-3704	Transcription profiling of Arabidopsis dark grown seedlings response to 6h-long treatment with 90 mM sucrose
E-GEOD-4022	Transcription profiling of Arabidopsis response to sucrose feeding to detached leaves
E-GEOD-5723	Transcription profiling by array of Arabidopsis after treatment with 3-O-methylglucose and 6-deoxyglucose

Table 1a: Microarray experiments with the Affimetrix GeneChip Arabidopsis Genome array that were used to study the carbohydrate regulated expression of the selected Arabidopsis genes that share motives in their promoter with fructan biosynthesis-genes. Data were made available through the ArrayExpress Archive from EBI.

name	description
AT1G02960	AT1G02960 >AT1G02960.1 | Symbols: | unknown protein | chr1:667002-669773 REVERSE >AT1G02960.2 | Symbols: | unknown protein | chr1:667002-669773 REVERSE >AT1G02960.3 | Symbols: | unknown protein | chr1:667325-669773 REVERSE
AT1G09157	AT1G09157 >AT1G09157.1 | Symbols: | unknown protein | chr1:2951665-2952396 REVERSE
AT1G15670	AT1G15670 >AT1G15670.1 | Symbols: | kelch repeat-containing F-box family protein | chr1:5390119-5391198 FORWARD
AT1G21250	AT1G21250 >AT1G21250.1 | Symbols: WAK1, PRO25 | WAK1 (CELL WALL-ASSOCIATED KINASE); kinase | chr1:7439512-7441892 FORWARD
AT1G26640	AT1G26640 >AT1G26640.1 | Symbols: | aspartate/glutamate/uridylate kinase family protein | chr1:9207620-9209766 REVERSE
AT1G26650	AT1G26650 >AT1G26650.1 | Symbols: | unknown protein | chr1:9210335-9211342 FORWARD
AT1G64260	AT1G64260 >AT1G64260.1 | Symbols: | zinc finger protein-related | chr1:23847756-23849915 FORWARD
AT1G74500	AT1G74500 >AT1G74500.1 | Symbols: | bHLH family protein | chr1:27998298-27998668 REVERSE
AT1G76630	AT1G76630 >AT1G76630.1 | Symbols: | tetratricopeptide repeat (TPR)-containing protein | chr1:28759699-28764786 FORWARD
AT2G11778	AT2G11778 >AT2G11778.1 | Symbols: | unknown protein | chr2:4728072-4728239 FORWARD
AT2G18938	AT2G18938 >AT2G18938.1 | Symbols: | unknown protein | chr2:8202216-8202581 FORWARD
AT2G23400	AT2G23400 >AT2G23400.1 | Symbols: | dehydrodolichyl diphosphate synthase, putative / DEDOL-PP synthase, putative | chr2:9963978-9965229 REVERSE
AT2G37600	AT2G37600 >AT2G37600.1 | Symbols: | 60S ribosomal protein L36 (RPL36A) | chr2:15774410-15775203 REVERSE >AT2G37600.2 | Symbols: | 60S ribosomal protein L36 (RPL36A) | chr2:15774410-15775203 REVERSE
AT2G46910	AT2G46910 >AT2G46910.1 | Symbols: | plastid-lipid associated protein PAP / fibrillin family protein | chr2:19272427-19273856 FORWARD
AT3G27630	AT3G27630 >AT3G27630.1 | Symbols: | unknown protein | chr3:10231119-10231418 REVERSE
AT3G27640	AT3G27640 >AT3G27640.1 | Symbols: | transducin family protein / WD-40 repeat family protein | chr3:10232307-10235467 FORWARD
AT3G47370	AT3G47370 >AT3G47370.2 | Symbols: | 40S ribosomal protein S20 (RPS20B) | chr3:17453671-17454437 REVERSE >AT3G47370.1 | Symbols: | 40S ribosomal protein S20 (RPS20B) | chr3:17453671-17454437 REVERSE >AT3G47370.3 | Symbols: | 40S ribosomal protein S20 (RPS20B) | chr3:17453671-17454437 REVERSE
AT3G47720	AT3G47720 >AT3G47720.1 | Symbols: SRO4 | SRO4 (SIMILAR TO RCD ONE 4); NAD+ ADP-ribosyltransferase | chr3:17592771-17593966 FORWARD
AT3G48880	AT3G48880 >AT3G48880.1 | Symbols: | F-box family protein | chr3:18127873-18129008 FORWARD >AT3G48880.2 | Symbols: | F-box family protein | chr3:18127873-18129008 FORWARD
AT3G54804	AT3G54804 >*AT3G54804.1* | Symbols: | unknown protein | chr3:20293147-20293251 REVERSE
AT3G61550	AT3G61550 >AT3G61550.1 | Symbols: | zinc finger (C3HC4-type RING finger) family protein | chr3:22776444-22777082 FORWARD
AT4G02320	AT4G02320 >AT4G02320.1 | Symbols: | pectinesterase family protein | chr4:1022725-1026118 REVERSE
AT4G13615	AT4G13615 >AT3G24100.1 | Symbols: | FUNCTIONS IN: molecular_function unknown; INVOLVED IN: biological_process unknown; LOCATED IN: cellular_component unknown; EXPRESSED IN: male gametophyte, pollen tube; EXPRESSED DURING: L mature pollen stage, M germinated pollen stage; CONTAINS InterPro DOMAIN/s: Four F5 protein (InterPro:IPR007513); BEST Arabidopsis thaliana protein match is: four F5 protein-related / 4F5 protein-related (TAIR:AT4G13615.1); Has 195 Blast hits to 195 proteins in 53 species: Archae - 0; Bacteria - 0; Metazoa - 136; Fungi - 12; Plants - 43; Viruses - 0; Other Eukaryotes - 4 (source: NCBI BLink). | chr3:8703373-8704045 FORWARD >AT4G13615.1 | Symbols: | four F5 protein-related / 4F5 protein-related | chr4:7924353-7925017 FORWARD
AT4G22380	AT4G22380 >AT4G22380.1 | Symbols: | ribosomal protein L7Ae/L30e/S12e/Gadd45 family protein | chr4:11812041-11812922 REVERSE
AT4G26130	AT4G26130 >AT4G26130.1 | Symbols: | unknown protein | chr4:13240403-13241263 REVERSE
AT4G31890	AT4G31890 >AT4G31890.1 | Symbols: | armadillo/beta-catenin repeat family protein | chr4:15427290-15429049 REVERSE
AT4G39170	AT4G39170 >AT4G39170.1 | Symbols: | SEC14 cytosolic factor, putative / phosphoglyceride transfer protein, putative | chr4:18240887-18243621 FORWARD
AT5G09920	AT5G09920 >AT5G09920.1 | Symbols: RPB15.9, ATRPB15.9, RPB15.9.9, NRPB4 | NRPB4; DNA-directed RNA polymerase | chr5:3096276-3097370 FORWARD
AT5G15060	AT5G15060 >AT5G15060.1 | Symbols: | FUNCTIONS IN: molecular_function unknown; INVOLVED IN: biological_process unknown; LOCATED IN: cellular_component unknown; CONTAINS InterPro DOMAIN/s: Lateral organ boundaries, LOB (InterPro:IPR004883); BEST Arabidopsis thaliana protein match is: LBD4 (LOB DOMAIN-CONTAINING PROTEIN 4) (TAIR:AT1G31320.1); Has 430 Blast hits to 427 proteins in 15 species: Archae - 0; Bacteria - 0; Metazoa - 0; Fungi - 0; Plants - 430; Viruses - 0; Other Eukaryotes - 0 (source: NCBI BLink). | chr5:4875381-4875806 REVERSE
AT5G15070	AT5G15070 >AT5G15070.1 | Symbols: | acid phosphatase/ oxidoreductase/ transition metal ion binding | chr5:4876898-4885615 FORWARD
AT5G26730	AT5G26730 >AT5G26730.1 | Symbols: | FUNCTIONS IN: molecular_function unknown; INVOLVED IN: biological_process unknown; LOCATED IN: mitochondrion; CONTAINS InterPro DOMAIN/s: FAS1 domain (InterPro:IPR000782); BEST Arabidopsis thaliana protein match is: unknown protein (TAIR:AT5G16920.1); Has 15 Blast hits to 15 proteins in 4 species: Archae - 0; Bacteria - 0; Metazoa - 0; Fungi - 0; Plants - 15; Viruses - 0; Other Eukaryotes - 0 (source: NCBI BLink). | chr5:9300422-9301990 FORWARD >AT5G16920.1 | Symbols: | FUNCTIONS IN: molecular_function unknown; LOCATED IN: endomembrane system; EXPRESSED IN: leaf whorl, sepal, flower, seed; EXPRESSED DURING: petal differentiation and expansion stage, E expanded cotyledon stage; CONTAINS InterPro DOMAIN/s: FAS1 domain (InterPro:IPR000782); BEST Arabidopsis thaliana protein match is: unknown protein (TAIR:AT5G26730.1); Has 24 Blast hits to 24 proteins in 6 species: Archae - 0; Bacteria - 0; Metazoa - 0; Fungi - 0; Plants - 18; Viruses - 0; Other Eukaryotes - 6 (source: NCBI BLink). | chr5:5567084-5567854 FORWARD
AT5G36140	AT5G36140 >AT5G36140.1 | Symbols: CYP716A2 | CYP716A2; electron carrier/ heme binding / iron ion binding / monooxygenase | chr5:14212212-14213259 REVERSE
AT5G38390	AT5G38390 >AT5G38390.1 | Symbols: | F-box family protein | chr5:15366432-15368053 FORWARD
AT5G44550	AT5G44550 >AT5G44550.1 | Symbols: | integral membrane family protein | chr5:17942100-17943174 REVERSE
AT5G45930	AT5G45930 >AT5G45930.1 | Symbols: CHLI2, CHL I2, CHLI-2 | CHLI2 (MAGNESIUM CHELATASE I2); ATPase/ magnesium chelatase | chr5:18628095-18629565 FORWARD
AT5G48680	AT5G48680 >AT5G48680.1 | Symbols: | sterile alpha motif (SAM) domain-containing protein | chr5:19744725-19746053 REVERSE

Table 1b: Arabidopsis genes that contain the motif 9

pFFT driven GUS expression in transgenic chicory

Since at the onset of inulin biosynthesis both *1-SST* and *1-FFT* seemed to be regulated in a more or less comparable way, and since the expression of *1-FFT* stayed constant throughout the growing season in chicory [15] we chose to analyse the action of the *1-FFT* promoter in transgenic plants. Two *1-FFT* 5' upstream fragments, of respectively 450bp and 1100bp, were cloned in a binary vector upstream of the *GUS* gene and were introduced into chicory. Young transgenic, rooted plantlets did not express *GUS* in roots and leaves. Three-months-old taproot bearing plants harbouring the 450 bp promoter fragment construct still did not show any GUS activity in taproot and leaf tissue. Interestingly, taproots of 3-months old plants harbouring the 1100 bp *1-FFT* promoter fragment construct did show GUS activity (Fig. 8a). No activity was detected in leaves. Comparison of the relative gene expression of *1-FFT* and *GUS* in concentric rings taken from the taproot showed that the expression pattern of *GUS* driven by the 1100 bp *1-FFT* promoter fragment resembled the expression pattern of *1-FFT* (Fig. 8b). The measured levels of *GUS* transcripts were, however, lower than the levels of *1-FFT* transcripts.

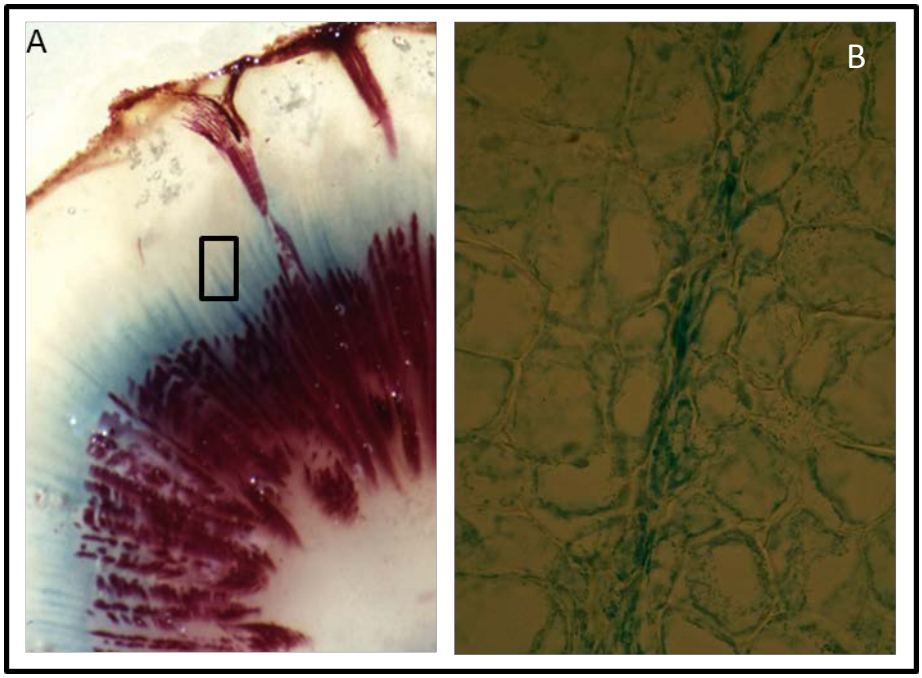


Figure 8a. GUS expression patterns of *1-FFT* promoter *GUS* fusion (pFickbin3) in different tissues of Chicory, (A) root transversal section. The box indicates the phloem tissue shown in (B) with 100 x magnification.

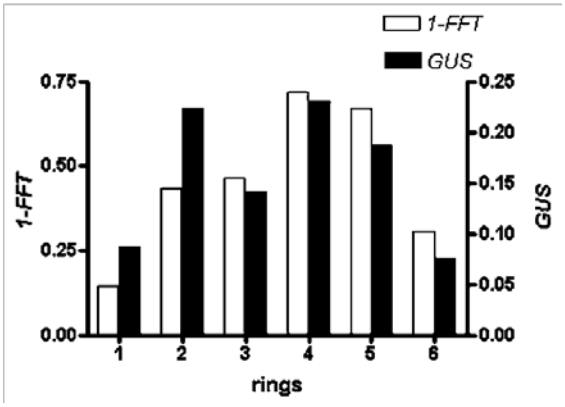


Figure 8b. Relative gene expression of *1-SST* and *1-FFT* in the taproot of a transgenic chicory expressing GUS under control of the *1-FFT* promoter. Relative gene expression of *GUS* (black bars, plotted on the right axis) and *1-FFT* (white bars, plotted on the left axis) is measured in different rings taken along the horizontal axis of chicory taproot and is relative to the expression of the ribosomal protein *L19* homologue of chicory. The figure shows the expression in a representative single transgenic individual

Following the same strategy for the induction of fructan accumulation as used with wild type chicory, excised leaves of plants containing the 1100 bp *1-FFT* promoter *GUS*-construct were incubated with sucrose solutions. Forty-eight hours after the start of the incubation, GUS activity showed that the promoter was induced in the leaves upon sucrose addition, although the expression pattern was sometimes patchy. No GUS activity was detected in leaves on 0% sucrose, or in non-transgenic leaves to which sucrose was applied (data not shown).

In conclusion, the results described above show that the 1100 bp *1-FFT* promoter fragment is able to drive gene expression in a pattern similar to the gene expression of endogenous *1-FFT*, both in the taproot as well as in the excised leaf system. This suggests that this promoter fragment contains all the important regulatory elements required for sugar induction and spatial expression.

pFFT driven GUS expression in non fructan-accumulating species

To test the hypothesis that the regulation of fructan biosynthesis is based on a common mechanism also present in other plant species, the 1100 bp *1-FFT*-promoter GUS construct was expressed in two non-fructan accumulating model species, potato and Arabidopsis. GUS assay analysis on transgenic potato tubers and 8-10 week old Arabidopsis plants showed no promoter activity (data not shown). However, when potato tuber slices were incubated in sucrose they showed GUS activity. Almost 90% of the transgenic lines showed GUS-activity when potato slices were incubated in 25% (730 mM) sucrose (Fig. 9a). Lower concentrations of sucrose, 5% (146 mM) and 10% (292 mM), could induce *GUS* expression driven by the *1-FFT* promoter in 53 and 60% of the lines, respectively. GUS activity was not induced in potato slices incubated in water (Fig. 9a). The capacity of different carbohydrates to induce *1-FFT* promoter activity was assessed in Arabidopsis. Seeds of six independent transgenic Arabidopsis lines harbouring the 1100 bp *1-FFT*-promoter-GUS construct were germinated on medium supplemented with different sugars. On average 40% of the seedlings per line showed GUS activity at 11 days after sowing (DAS) when germinated on sucrose or glucose (Fig. 9b). The GUS was localised in the root,

hypocotyls and/or cotyledons of the seedlings (Fig. 10). The inhibition of early seedling development by glucose described by Dekkers *et al*. [16] did not influence our results as we scored only at 11 DAS, whereas the delaying effect was seen only until 3 DAS. GUS activity upon fructose feeding was only seen in one or two seedlings of four of the six lines tested. Mannitol, which was used as a control for osmotic stress, induced GUS activity only in two lines, one seedling per line. No GUS activity was detected in seedlings germinated in the absence of sugar.

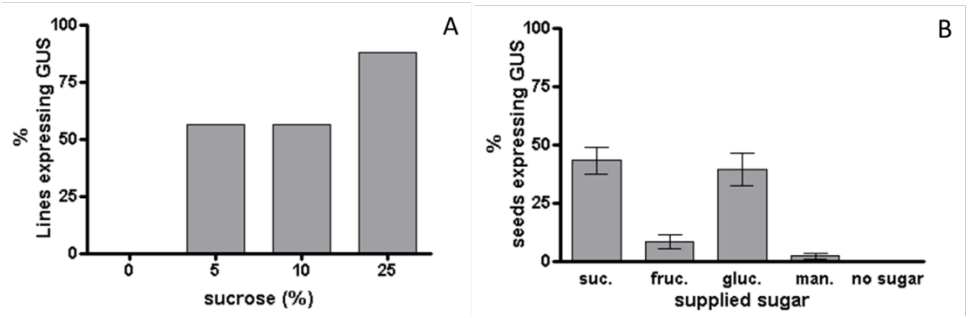


Figure 9a. Percentage of potato lines expressing GUS upon sucrose feeding. The percentage of transgenic potato lines (n=10), harboring the *1-FFT*-GUS construct, expressing GUS in potato slides that were incubated in solutions with 0%, 5% (146mM) , 10% (292mM) or 25% (730mM) of sucrose.

Figure 9b. Percentage of Arabidopsis seedlings expressing GUS upon germination on sugar. The percentage of transgenic Arabidopsis seedlings per line that is expressing GUS after germination on sucrose, fructose, glucose or mannitol containing media or a medium without sugar. The error bars represent the SEM over six independent Arabidopsis lines, per treatment 14 seeds were used per line.

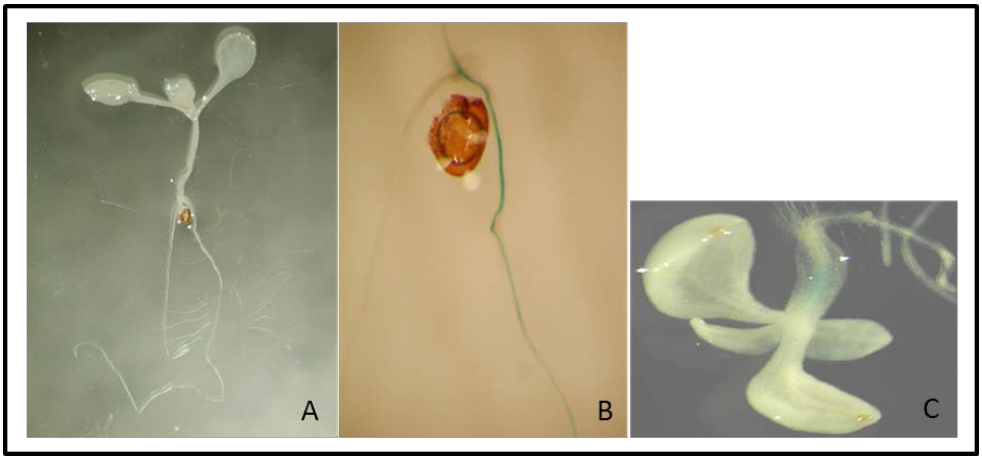


Figure 10. GUS expression patterns of *1-FFT* promoter *GUS* fusion (pFickbin3) in different tissues of Arabidopsis, Arabidopsis WT seedling (A). Panel B and C represent examples of GUS staining in pFickbin3 overexpressing Arabidopsis plants, B: seedling root, C: hypocotyl.

Discussion

Localization of inulin biosynthesis in roots

To determine the localization of inulin biosynthesis in the taproot of chicory, sugar and gene expression analysis was performed in different concentric rings of field grown taproots. The gene expression analysis of *1-SST* and *1-FFT* in chicory taproot showed the highest expression in the inner part of the root, the phloem and xylem. In the other rings *1-SST* and *1-FFT* were also expressed, although to a lower level. The pattern of expression in the different root-tissues was in accordance with the finding that 1-SST and 1-FFT activity increased towards the inside of the roots as was shown by our own enzyme activity analyses (data not shown) and by Kusch [17]. Van Wonterghem *et al*. showed that in 10 week-old seedlings the highest concentrations of 1-FFT protein was located in parenchyma cells surrounding the phloem and more to the periphery of the root around the laticifers concluding that 1-FFT is not restricted to a certain region in the root [18]. A histological study on chicory taproots, harvested in September, showed inulin crystals in a radial axis throughout the root [19], and in a study on the localization of fructan biosynthesis in *Taraxacum officinale* higher fructan accumulation was found around the phloem but it was not restricted to that region [2]. Inulin was also observed in xylem vessels, similar as was seen in *Helianthus tuberosus* [20] and *Gomphrena macrocephala* [21]. Our results confirm these findings by detecting mRNA of *1-SST* and *1-FFT* throughout the whole root. The concentration of glucose and fructose correlated with the level of expression of the fructan genes in the root. The correlation of the fructan biosynthesis gene expression and fructose cannot be explained by the mode of action of the fructan biosynthesis genes. The good correlation of *1-SST* expression and glucose in the different root tissues, however, may be explained by the fact that glucose is one of the products of 1-SST.

Ectopic expression of fructan biosynthesis

The excised leaf system has been widely used to study fructan biosynthesis in grasses [4, 6, 22-24]. In our experiments, the excised leaf system was used to further study the induction of fructan metabolism in chicory. Untreated leaves did not express *1-SST* and *1-FFT* and did not accumulate inulin. Accumulation of 1-kestose and expression of *1-SST* was observed in detached leaves 13h after sucrose induction. This shows that 1-SST can be induced and that fructan biosynthesis can take place in the leaves upon supply of an extra carbon source. This conclusion is in accordance with the findings of Améziane [7] who showed 1-kestose production and 1-SST activity in the excised leaf system after 8h of illumination. A longer induction period resulted in increased *1-SST* expression and increased 1-kestose concentrations most probably due to the increased level of sucrose in the leaves taken up from the medium as was shown in figures 4A, 5, 6 and as described by Améziane [7]. The expression of *1-FFT* and accumulation of 1-nystose and DP5 occurred later than *1-SST* expression, as also described in hairy root cultures, where 1-FFT activity could only be detected after 168h of feeding with sucrose [3]. A role for 1-kestose in the

induction of *1-FFT* expression is not described in literature and would be in conflict with our observation that the *1-FFT* promoter is induced in transgenic plants in the absence of 1-kestose. We did not observe the lag phase for enzyme induction, described by Kusch to explain the time between *1-FFT* induction and the activity. In our excised leaf system the high *1-FFT* expression at 26h perfectly coincided with the initiation of 1-nystose accumulation. However, it cannot be entirely excluded that this 1-nystose was formed by 1-SST as described earlier in *in vitro* assays [25]. The time difference between the induction of *1-SST* and *1-FFT* expression could be explained by (1) a faster regulation mechanism for 1-SST compared with 1-FFT, since it has been shown for barley that 1-SST reacts quickly on changing conditions, whereas the second enzyme, in the case of barley, 6-SFT, reacts much later [26]. It could also be explained by (2) a lower threshold for the induction of *1-SST*, meaning that *1-SST* would be induced at lower levels of sucrose or glucose in the tissue than *1-FFT*, which could be deduced from our data (Figs 5 and 6). A threshold concentration for the carbohydrate induction of fructan biosynthesis was suggested earlier [4]. The concentrations of sucrose and glucose used for feeding affected gene expression, fructan accumulation and the concentration of sucrose and glucose in the detached leaves. A tight regulation of the concentration of glucose and sucrose in leaves during the diurnal cycle at the start of photosynthesis, as suggested by Nägele *et al*. [27], could be an explanation for the increase of the concentrations of both carbohydrates, upon feeding with one or the other. Edelman and Jefford [28] showed that [^{14}C]-hexoses, injected into tubers of *Helianthus tuberosus,* were converted into glucose-6-phosphate, fructose-6-phosphate and uridine diphosphate glucose, precursors for sucrose, revealing a rapid turnover of the carbohydrate pools. This turnover was also shown in Lolium by Lattanzi *et al*. [29]. The low expression of *1-FFT* upon fructose feeding compared to sucrose and glucose feeding (Fig. 4C) could be the result of the lower concentration of sucrose or glucose in the leaves upon fructose feeding (Fig. 5A,B). As a consequence, the threshold level for induction of *1-FFT* expression was possibly not reached, as suggested earlier [26]. However, the threshold level of sucrose for 1-SST enzyme activity (20 µmol/gFW), for example, mentioned by Van den Ende [30], is in the same range as the sucrose concentrations (10-30 µmol/gFW) we found in the excised leaves after feeding on carbohydrates (Fig. 5A). The same range of concentration was found in fructan accumulating taproots (fig. 6, chapter 2). The effect of fructose on 1-SST is different since *1-SST* is expressed, but 1-kestose is not formed even though sucrose is available, and it seems that the 1-SST activity is inhibited by fructose. Although inhibition of 1-SST and 1-FFT by fructose has not been described before, inhibition by fructose was reported for FEH and invertase, two enzymes evolutionary related to 1-SST and 1-FFT [31, 32]. Inhibition of 1-SST is described only *in vitro*, by high concentrations of sucrose or high ionic strength [33, 34]. It seems that the regulation differs over plant species and tissues. In barley, fructan synthesis was induced in excised leaves by incubation in 30 mM fructose under illumination, showing that *1-SST* can be induced by fructose [35]. The difference in effect between fructose and glucose on 1-SST induction was not seen in experiments on orchard grass, where

comparable amounts of fructan accumulated upon induction with both of these carbohydrates [36]. In chicory hairy root cultures, fructose could induce both *1-SST* and *1-FFT* expression after 4 to 8 days of induction, whereas glucose could only induce a low expression. Differences in root tissue morphology or invertase activity may explain the different results compared with the excised leaves. Fructose did not stimulate the growth of the hairy roots [3] which could mean that like in the excised leaves, fructose accumulated in hairy roots but was not metabolised.

The results from our excised leaf experiments are confirmed by the experiments with transgenic chicory plants harboring the *1-FFT* promoter GUS fusions that showed a similar expression pattern of GUS compared with the endogenous *1-FFT* gene upon carbohydrate induction in the leaves. The expression study in the two non-fructan accumulating plants, Arabidopsis and potato, showed activity of the chicory 1-*FFT* promoter fragment upon carbohydrate induction comparable to the activity in chicory, from which we can conclude that the promoter fragment contains the proper regulatory elements for carbohydrate regulation and that the actors in the regulation pathway are present in Arabidopsis and potato. The expression study in the non-fructan accumulating model plants also showed that the *1-FFT* promoter was not induced under normal growing conditions in Arabidopsis and potato, probably because the endogenous sugar concentration is too low. Sucrose, the most abundant sugar in both species, is maximally 5.8 and 10 µmol/gFW in resp. potato tuber [37] and 6-8 week old Arabidopsis plants [38]. This is low compared with the levels of sucrose in chicory roots at the onset of inulin biosynthesis, 30-40 µmol/gFW [15], and in leaves after feeding carbohydrates (20 µmol/gFW). The correlation between the endogenous concentration of carbohydrates and inulin biosynthesis induction was also shown in our experiments with excised chicory leaves. It could be hypothesized that the resulting sucrose or/and glucose levels in the leaves are the regulating factors for fructan biosynthesis. Although we could measure glucose and sucrose levels in the leaves, we could not study their effect on fructan metabolism separately because of the sucrose to glucose inter-conversion. In barley similar experiments have been performed with sucrose in the presence of an invertase inhibitor, preventing the formation of glucose. The result was an increased gene expression level of *6-SFT*, indicating that the formation of glucose had a negative effect, most probably due to reduced sucrose content [39]. Another study in barley showed a positive correlation between the accumulation of fructan upon sucrose feeding, but no correlation between fructan and glucose feeding when complete plants were illuminated [40]. Müller suggested also that the induction of fructan biosynthesis in barley occurs independently from hexoses and most likely occurs via interaction of sucrose with transporters [22]. This provides strong evidence for a regulating role for sucrose rather than glucose, although this was only shown in monocots. In the dicot plant dahlia it was shown that when light conditions changed from long to short day, the concentration of sucrose increased by 20%, which coincided with an increase of the accumulation of fructan. The concentration of fructose and glucose remained unaffected by the day length change. This is, as Legnani and Miller stated, also evidence for a regulating role of sucrose [41].

Apart from the effect of sucrose on fructan accumulation, we showed a positive correlation between glucose and fructose and mRNA levels of *1-SST* and *1-FFT* in chicory taproots. In addition, Kusch [3] showed that fructose has a strong potential to induce expression of *1-SST* and *1-FFT*. So it cannot be excluded that also these hexoses have an effect on the expression of *1-SST* and *1-FFT* in the roots.

Further analysis; promoter analysis

Comparison of the *1-SST* and *1-FFT* promoter sequences from chicory with earlier published sequences showed that several well described regulatory elements involved in sugar regulation are present in the *1-SST* and *1-FFT* promoters and are also found in the barley *6-SFT* promoter [42]. Some elements that have been described previously, and are putatively involved in carbohydrate mediated regulation, were found in the promoters as well. The SURE element (WBOXHVISO1) is a recognition site for the WRKY transcription factor involved in sugar regulation in barley [43]. The DOF transcription factor binding site [44] was found in *1-FFT,* in combination with the pyrimidine box. The GT-1 and I-Box were also found in the barley 6-SFT promoter [42] and were shown to be involved in light responsive gene expression [45, 46]. The *de novo* motif search resulted in several putative *cis* acting elements that were present in *1-SST* and *1-FFT*. One of the motifs we identified, motif 9, showed homology to a MYB binding site recently discovered in the *Ta6-SFT* promoter [11]. The MYB transcription factor TaMYB13 was shown to be a transcriptional activator of the fructan biosynthesis genes in wheat. Xue *et al*. suggested AtMYB48 and AtMYB59 to be the Arabidopsis homologues of TAMYB13 [11]. Both are expressed upon sucrose feeding and continuous light as shown in the eFP browser of the Bio-array resource for plant Biology (BAR) [47]. It could be hypothesized that these TFs are regulating the expression of the chicory *1-FFT* promoter in the transgenic Arabidopsis. Transcriptional profiling using Arabidopsis microarray experiments revealed a correlation between motif 9 and sugar regulation. A significant part of the genes having motif 9 were differentially expressed upon sugar feeding. Examples are 60S and 40S ribosomal proteins (RPS20B, RPL36A). The accumulation of more ribosomal protein may be needed for faster translation of genes expressed upon increased levels of carbohydrate, resulting from sugar feeding or from photosynthesis [48]. Motif 9 was also found in the receptor kinase, WAK1 that was found to be co-related with the concentration of trehalose-6-phosphate upon sugar feeding [49] and involved in plant energy signalling. Kinases are also shown to be involved in, or co-regulated with, fructan biosynthesis genes, as shown by Ruuska *et al*. who studied the effects of nitrogen nutrition on accumulation of carbohydrates and fructan in wheat [50].

In conclusion; we showed that transcriptional profiling on co-expression of genes is a valuable tool for studies on gene expression regulation mechanisms and the discovery of regulatory proteins. The results, however, should be validated by experiments such as ChIP-seq and Y1H screens. The identified regulatory sequences may be valuable tools to isolate regulatory proteins and subsequently discover the transcriptional regulators of

fructan biosynthesis. The failure of the 450 bp fragment of the *1-FFT* promoter in expressing GUS showed that apparently this part of the promoter lacks key regulatory elements. A more extensive deletion study of the promoter could give more evidence for the involvement of the identified motives in the regulation of fructan biosynthesis genes.

Material en methods

Cloning of the 1-SST and 1-FFT-promoters

To clone the promoters of *1-SST* and *1-FFT* from chicory, DNA was isolated from leaves of the chicory variety "Cassel", and using GenomeWalking™ kit (Clontech laboratories) twelve different genomic libraries were constructed. The libraries served as template for PCR using adapter primers supplied by Clontech in combination with gene specific primers that were designed on the 5'end of the *1-SST* and *1-FFT* gene (accession no. resp. U81520 and U84398). PCR products were cloned into the PCR-script vector (Stratagene; Agilent Technologies, CA) and the nucleotide sequence was determined.

Construction of 1-FFT promoter-GUS reporter constructs

The initial step in the preparation of the 1-*FFT* promoter-GUS reporter constructs was the introduction of restriction sites into the 1-FFT promoter fragment. Recognition sites for *Pst*I and *Nco*I were introduced by PCR resp. at the 5'end of the genomic fragments and at start of transcription and used to ligate the fragments in front of a GUS-intron gene in pCambia1301 binary vector (Cambia, Australia) as *Pst*I x *Nco*I fragments. The promoter-GUS fragments were subsequently transferred, as *Pst*I x *Afl*II fragments, to pCAMBIA 2301 (Cambia, Australia) harboring the *NPTII* gene for kanamycin resistance, resulting in the chimeric gene constructs pFickbin3 and pFickbin4, harboring resp. the 1100bp and 450bp *1-FFT* promoter fragment. Binary vectors pTofgus and pCambia2301 (Cambia, Australia), both having the Gus-intron gene under control of the 35S-promoter, were taken along as controls in plant transformation of chicory (pTofgus), potato (pCambia2301) and Arabidopsis (pCambia2301). The binary gene constructs were transferred to *Agrobacterium tumefaciens*, strain AGL0, using a heat shock transformation method [51].

Plant transformation

Chicory (*Cichorium intybus* L.) line 95/9 was transformed with the chimeric gene constructs pFickbin3, pFickbin4 and pTofgus according to the protocols of Vermeulen and Desprez [52, 53].Transformed plants were subsequently micro-propagated and kept *in vitro*. Rooted plants were tested for the presence of the transgenes by PCR and further grown in Desch C 17cm H pots filled with potting soil (nr.103967, Lentse potgrond) in the greenhouse. Temperature and day length was set at 18 °C daytime and 15 °C night-time temperature with a photoperiod of 14 h provided by Philips lamps HPT-T 400 W (Philips, Eindhoven, The Netherlands). otato (*Solanum tuberosum L*. cv. Kardal) plants were

transformed according to the method described by Dietze [54], using the chimeric gene constructs pFickbin3, pFickbin4, and pCambia2301. Plants were grown in 18 cm pots in the greenhouse at 18 °C daytime and 15 °C night-time temperature with a photoperiod of 14 h provided by Philips lamps HPT-T 400 W (Philips, Eindhoven, The Netherlands). Transgenic Arabidopsis (Col-0) plants harboring chimeric gene construct pFickbin3 and pCambia2301 were generated by floral dip [55].Seeds were selected on MS-agar plates supplemented with kanamycin (25mg/L).After two weeks, rooting seedlings were transferred to soil and grown until seed set. The following T2 generation was checked for the presence of the construct by PCR.

Localization study in taproot

Cichorium intybus L. (cv. Orchies) was grown on experimental fields in Wageningen (The Netherlands) in 2003. Plants were sown in the beginning of May and crop husbandry was performed comparable to commercial fields. The soil was classified as Fluvisol (FAO soil group). Roots were harvested in September. Harvested roots were peeled and transversal slices of about 1 cm thick were taken from just below the shoulder of the root and divided into six different rings. Ring one and two contained mostly storage parenchyma. Ring 3, 4, 5 contained mainly phloem tissue, surrounded by parenchyma, ring 6 contained phloem and xylem. Transgenic plants used in this study were grown in the greenhouse for four months and subsequently harvested and divided in six rings. Ring 1 contained the rhizoderm, ring 2 and 3 storage parenchyma and phloem tissue. Ring 4 and 5 contained mainly phloem tissue, surrounded by parenchyma, ring 6 contained phloem and xylem. After preparation, the material was immediately frozen in liquid nitrogen and used for RNA extraction and carbohydrate analyses.

The excised leaf system

In the excised leaf system young fully-developed leaves were cut from greenhouse cultured plants and immediately used in induction experiments. The leaves were placed with the cut ends in 50 ml disposable centrifuge tubes, filled with 40 ml of the appropriate solution. The leaves were illuminated (80µmol photons m^{-2} s^{-1}) with TLD 36W/840 TL-bulbs (Philips, Eindhoven, The Netherlands) in a growth chamber at 20°C under long day conditions for 48 hours. For each sample five leaves from three different plants were used. All experiments were repeated at least two times. The sugar solutions, using pure chemicals (Sigma-Aldrich), were prepared with tap water. When comparing the effect of different sugars the osmolality of the solution was kept the same.

Carbohydrate induction in potato and Arabidopsis

Freshly grown potato tubers were peeled and tissue was collected throughout the tuber using a 5 mm cork bore. The plugs were cut into 3 mm slices and subsequently incubated in 0%, 5% (146mM), 10% (292mM) or 25% (730mM) sucrose. GUS activity in the slices was assayed after 6 and 24 hours of incubation. In total, five transgenic lines harboring the pFickbin3 construct and two lines harboring the pCambia2301 control plasmid were tested in duplicate. Transgenic T3 Arabidopsis seeds harboring the pFickbin3 or pCambia2301 constructs were plated on MS medium containing kanamycin (25 mg/l) supplemented with 0, or 3% sucrose (88mM), glucose (167mM) or fructose (167mM). Per line 14 seeds were used per treatment. In total six lines harboring the pFickbin3 lines were tested along with several control lines. The plants were grown at 25°C under short day conditions in a climate room. After 11 days the seedlings were assays on GUS-activity.

Carbohydrate and RNA isolation from plant material

Fresh plant material was quickly frozen in liquid nitrogen and ground using an IKA A11 analytical mill (IKA®-Werke GmbH & Co. KG, Germany). Carbohydrates were extracted and analysed by anion exchange chromatography-pulsed-amperometric detection [15]. Total RNA was isolated using TRI reagent (Sigma-Aldrich) according to the protocol supplied. The RNeasy Mini Kit (Qiagen) was used to purify the isolated RNA. The RNA quality and quantity was determined spectrophotometrically.

Histochemical GUS-assay

Histochemical determination of GUS activity in plant tissue was performed by submerging the tissue in a solution containing 0,5 mg/ml 5-Bromo-4-chloro-3-indolyl-ß-D-glucuronic acid (X-Glc), 1% Triton X-100, 1mM K^+ ferricyanide/ferrocyanide mixture as an oxidation catalyst [56] , 10 mM NaEDTA in 100 mM Phosphate buffer (pH 7.5), vacuum-infiltrating for 5 min, and incubating at 37°C overnight. Plant tissue was cleared in 70% ethanol after staining.

Analysis of steady-state mRNA: quantitative real-time PCR

The method for quantification of mRNA using quantitative real-time PCR for *GUS*, *1-SST* and *1-FFT* has been described by van Arkel *et al*. [15]. The mRNA levels of the studied genes were normalized using the comparative C_T method [57]. A chicory homologue of ribosomal protein L19 served as a reference gene. The oligonucleotide primers were designed with Primer Express 1.5 software (Applied Biosystems). The following primers were used:

Ribosomal protein L19 sense, CTGCCAGCGTCCTCAAGTG
Ribosomal protein L19 antisense, CATTGGGATCAAGCCAAACCT
1-SST sense, CCAACAACCATCAGGGAGAAG
1-SST antisense, AGCAACGGAGCTGTGAACGT
1-FFT sense, GAACCAGCAAGATTCCACGAA

1-FFT antisense, TTCTCCGGCAGTGCTAAATTG
GUS sense, CGGAAGCAACGCGTAAACTC
GUS antisense, TGAGCGTCGCAGAACATTACAT

Northern blot analysis

Total RNA (10μg) was size-fractionated on 1% agarose phosphate gel and transferred to Hybond-N^+ (GE healthcare, USA). Specific DNA probes were made from cDNA fragments, generated by PCR, labeled with isotope ^{32}P following the RadPrimer DNA labeling System (Life Technologies) protocol. The cDNA information was derived from Genbank libraries (*1-SST* accession no. U81520, *1-FFT* Accession no. U84398, *Ribosomal protein L19* homologue from chicory). The probes were hybridized to the RNA blots in hybridization buffer [58] for 16 hours at 60 °C and washed three times 30 minutes in 0,1XSSC, 0,1% SDS afterwards. The hybridized blots were exposed to Xomat^{tm} Kodak film. To visualize *1-SST* and *1-FFT*, exposure was performed for 16 hours. Primers that were used to make the probe for *1-SST* accession no. U81520 were TGTCATCTGTGTTCTGGTTGC and ACCACGATTCCTAATGGTCC. Primers used for *1-FFT* accession no. U84398 were GTGACCTTGAGGATGCATCC and TCGGTTGCACCCGCGCTCG. With primers T3 and T7 located around the cDNA clone in the pBluescript SK+ vector the probe was synthesized for the *ribosomal protein L19* from chicory.

Bioinformatics analyses

To obtain sequence motifs overrepresented in the promoters of *1-SST* and *1-FFT* like genes, several fructan biosynthesis-promoter sequences (Table S1, supplementary data) were used in a MEME-search with default settings. The FIMO software tool [9] was used and subsequently the occurrence of MEME motifs was analysed in the Arabidopsis genome. Arabidopsis promoter regions were defined based on the TAIR10 description (TAIR10_upstream_1000_20101104) and motif occurrences in those promoters were extracted from the FIMO output. The gene expression patterns of the Arabidopsis genes containing the MEME motifs in the promoter were determined using microarray data available in the CORNET database [13] and at the Array Express Archive from EMBL-EBI. Using CORNET a series of experiments was screened in order to identity putative co-expression of the selected genes. To identify genes that were regulated upon sugar feeding, transcriptional profiling was performed using experiments E-GEOD-3704, E-GEOD-4022 and E-GEOD-5723 from the Array Express Archive. Genes that were up-regulated upon sugar feeding were further studied in a literature search.

Data presentation and curve fitting

Northern blots were quantified using Quantity One 1-D analyses software (Biorad laboratories). Data plotting and curve fitting was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA).

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References

[1] J. Edelman, T.G. Jefford, The Mechanism of Fructosan Metabolism in Higher Plants as Exemplified in *Helianthus tuberosus*, New Phytologist, 67 (1968) 517-531.

[2] W. Van den Ende, A. Michiels, D. Van Wonterghem, R. Vergauwen, A. Van Laere, Cloning, Developmental, and Tissue-Specific Expression of Sucrose:Sucrose 1-Fructosyl Transferase from *Taraxacum officinale*. Fructan Localization in Roots, Plant Physiology, 123 (2000) 71-80.

[3] U. Kusch, S. Greiner, H. Steininger, A.D. Meyer, H. Corbière-Divialle, K. Harms, T. Rausch, Dissecting the regulation of fructan metabolism in chicory (*Cichorium intybus L.*) hairy roots, New Phytologist, 184 (2009) 127-140.

[4] K. Maleux, W. Van den Ende, Levans in Excised Leaves of *Dactylis glomerata* : Effects of Light, Sugars, Temperature and Senescence, Journal of Plant Biology, 50 (2007) 671-680.

[5] D.M. Obenland, U. Simmen, T. Boller, A. Wiemken, Regulation of Sucrose-Sucrose-Fructosyltransferase in Barley Leaves, Plant Physiology, 97 (1991) 811-813.

[6] A.L. Winters, J.H.H. Williams, D.S. Thomas, C.J. Pollock, Changes in gene-expression in response to sucrose accumulation in leaf tissue of *Lolium temulentum* L, New Phytologist, 128 (1994) 591-600.

[7] R. Améziane, A. Limami, J.-F. Morot gaudry, Fructan biosynthesis and SST activity in excised leaves of *Cichorium intybus* L., in: U. Rennes (Ed.) Plant Sciences 1994, Saint malo (France), 1994, pp. 123.

[8] K. Higo, Y. Ugawa, M. Iwamoto, T. Korenaga, Plant cis-acting regulatory DNA elements (PLACE) database: 1999, Nucleic Acids Research, 27 (1999) 297-300.

[9] T.L. Bailey, E. C., "Fitting a mixture model by expectation maximization to discover motifs in biopolymers", in: Second International Conference on Intelligent Systems for Molecular Biology, AAAI Press, Menlo Park, California, 1994, pp. 28-36.

[10] J.C. Bryne, E. Valen, M.-H.E. Tang, T. Marstrand, O. Winther, I. da Piedade, A. Krogh, B. Lenhard, A. Sandelin, JASPAR, the open access database of transcription factor-binding profiles: new content and tools in the 2008 update, Nucleic Acids Research, 36 (2008) D102-D106.

[11] G.-P. Xue, M. Kooiker, J. Drenth, C.L. McIntyre, TaMYB13 is a transcriptional activator of fructosyltransferase genes involved in β-2,6-linked fructan synthesis in wheat, The Plant Journal, 68 (2011) 857-870.

[12] C.E. Grant, T.L. Bailey, W.S. Noble, FIMO: scanning for occurrences of a given motif, Bioinformatics, 27 (2011) 1017-1018.

[13] S. De Bodt, D. Carvajal, J. Hollunder, J. Van den Cruyce, S. Movahedi, D. Inzé, CORNET: A User-Friendly Tool for Data Mining and Integration, Plant Physiology, 152 (2010) 1167-1179.
[14] J.A. Tognetti, H.G. Pontis, G.M.A. Martínez-Noël, Sucrose signaling in plants: A world yet to be explored, Plant Signaling & Behavior, 8 (2013) e23316.
[15] J. van Arkel, R. Vergauwen, R. Sévenier, J.C. Hakkert, A. van Laere, H.J. Bouwmeester, A.J. Koops, I.M. van der Meer, Sink filling, inulin metabolizing enzymes and carbohydrate status in field grown chicory (*Cichorium intybus* L.), Journal of Plant Physiology, 169 (2012) 1520-1529.
[16] B.W. Dekkers, J.M.J. Schuurmans, S.M. Smeekens, Glucose delays seed germination in *Arabidopsis thaliana*, 218 (2004) 579-588.
[17] U. Kusch, Dissecting the regulation of fructan active enzymes in *Cichorium intybus*, in: Combined faculties for natural Science and for mathematics of the Ruperto-Carola University, University of heidelberg, heidelberg, 2009.
[18] D. Van Wonterghem, W. Van den Ende, A. Van Laere, Proceedings of the Eight Seminar on Inulin, in: A. Fuchs (Ed.) The Eight Seminar on Inulin, European fructan Association, Lille, France, 1999, pp. 59-62.
[19] D. Desmet, Histologische studie van de witloofwortelstructuur en van *in vitro* geïnduceerde meristemen, in: Toegepaste plantenwetenschappen, Katholieke Universiteit Leuven, Leuven, 1997, pp. 133.
[20] M. Ernst, Histochemische Untersuchungen auf Inulin, Stärke und Kallose bei *Helianthus tuberosus* L. (Topinambur), Angewandte Botanik, 65 (1991) 319-330.
[21] C. Vieira, R. Figueiredo-Ribeiro, Fructose-containing carbohydrates in the tuberous root of *Gomphrena macrocephala* St.-Hil.(Amaranthaceae) at different phenological phases, Plant, Cell & Environment, 16 (1993) 919-928.
[22] J. Müller, R.A. Aeschbacher, N. Sprenger, T. Boller, A. Wiemken, Disaccharide-mediated regulation of sucrose: fructan-6-fructosyltransferase, a key enzyme of fructan synthesis in barley leaves, Plant Physiology, 123 (2000) 265-274.
[23] A.J. Cairns, G.D. Bonnett, J.A. Gallagher, R.J. Simpson, C.J. Pollock, Fructan biosynthesis in excised leaves of *Lolium temulentum* VII. Sucrose and fructan hydrolysis by a fructan-polymerizing enzyme preparation, New Phytologist, 136 (1997) 61-72.
[24] J.-Z. Wei, N. Jerry Chatterton, S.R. Larson, Expression of sucrose: fructan 6-fructosyltransferase (6-SFT) and myo -inositol 1-phosphate synthase (MIPS) genes in barley (*Hordeum vulgare*) leaves, Journal of Plant Physiology, 158 (2001) 635-643.
[25] A.J. Koops, H.H. Jonker, Purification and Characterization of the Enzymes of Fructan Biosynthesis in Tubers of *Helianthus tuberosus Colombia* (II. Purification of Sucrose:Sucrose 1-Fructosyltransferase and Reconstitution of Fructan Synthesis in Vitro with Purified Sucrose:Sucrose 1-Fructosyltransferase and Fructan:Fructan 1-Fructosyltransferase), Plant Physiology, 110 (1996) 1167-1175.
[26] V.J. Nagaraj, D. Altenbach, V. Galati, M. Lüscher, A.D. Meyer, T. Boller, A. Wiemken, Distinct regulation of sucrose: sucrose-1-fructosyltransferase (1-SST) and sucrose: fructan-6-fructosyltransferase (6-SFT), the key enzymes of fructan synthesis in barley leaves: 1-SST as the pacemaker, New Phytologist, 161 (2004) 735-748.
[27] T. Nägele, S. Henkel, I. Hörmiller, T. Sauter, O. Sawodny, M. Ederer, A.G. Heyer, Mathematical Modeling of the Central Carbohydrate Metabolism in Arabidopsis

Reveals a Substantial Regulatory Influence of Vacuolar Invertase on Whole Plant Carbon Metabolism, Plant Physiology, 153 (2010) 260-272.
[28] J. Edelman, T.G. Jefford, The mechanism of fructosan metabolism in higher plants as exemplified in *Helianthus tuberosus*, New Phytologist, 67 (1968) 517-531.
[29] F.A. Lattanzi, U. Ostler, M. Wild, A. Morvan-Bertrand, M.-L. Decau, C.A. Lehmeier, F. Meuriot, M.-P. Prud'homme, R. Schäufele, H. Schnyder, Fluxes in central carbohydrate metabolism of source leaves in a fructan-storing C3 grass: rapid turnover and futile cycling of sucrose in continuous light under contrasted nitrogen nutrition status, Journal of Experimental Botany, 63 (2012) 2363-2375.
[30] W. Van den Ende, D. VanWonterghem, E. Dewil, P. Verhaert, A. De Loof, A. Van Laere, Purification and characterization of 1-SST, the key enzyme initiating fructan biosynthesis in young chicory roots (*Cichorium intybus*). Physiologia Plantarum, 98(1996), 455-466.
[31] W. Van den Ende, A. Michiels, J. De Roover, P. Verhaert, A. Van Laere, Cloning and functional analysis of chicory root fructan1-exohydrolase I (1-FEH I): a vacuolar enzyme derived from a cell-wall invertase ancestor? Mass fingerprint of the 1-FEH I enzyme, The Plant Journal, 24 (2000) 447-456.
[32] W. Van den Ende, A. Van Laere, Purification and properties of a neutral invertase from the roots of *Cichorium intybus*, Physiologia plantarum, 93 (1995) 241-248.
[33] P.M. Chevalier, R.A. Rupp, Inhibition of sucrose: sucrose fructosyl transferase by cations and ionic strength, Plant Physiology, 101 (1993) 589-594.
[34] W. Van den Ende, A. Van Laere Purification and properties of an invertase with sucrose: sucrose fructosyltransferase (SST) activity from the roots of *Cichorium intybus* L, New Phytologist, 123 (1993) 31-37.
[35] W. Wagner, A. Wiemken, Enzymology of Fructan Synthesis in Grasses: Properties of Sucrose-Sucrose-Fructosyltransferase in Barley Leaves (*Hordeum vulgare* L. cv Gerbel), Plant Physiology, 85 (1987) 706-710.
[36] K. Maleux, W. Van den Ende, Levans in Excised Leaves of *Dactylis glomerata*; Effects of Light, Sugars, Temperature and Senescence, Journal of Plant Biology, 50 (2007) 671-680.
[37] C. Matsuura-Endo, A. Kobayashi, T. Noda, S. Takigawa, H. Yamauchi, M. Mori, Changes in sugar content and activity of vacuolar acid invertase during low-temperature storage of potato tubers from six Japanese cultivars, Journal of Plant Research, 117 (2004) 131-137.
[38] K. Uematsu, N. Suzuki, T. Iwamae, M. Inui, H. Yukawa, Expression of Arabidopsis plastidial phosphoglucomutase in tobacco stimulates photosynthetic carbon flow into starch synthesis, Journal of Plant Physiology, 169 (2012) 1454-1462.
[39] J. Müller, R.A. Aeschbacher, N. Sprenger, T. Boller, A. Wiemken, Disaccharide-Mediated Regulation of Sucrose:Fructan-6-Fructosyltransferase, a Key Enzyme of Fructan Synthesis in Barley Leaves, Plant Physiology, 123 (2000) 265-274.
[40] O.A. Koroleva, A.D. Tomos, J.F. Farrar, J. Gallagher, C.J. Pollock, Carbon allocation and sugar status in individual cells of barley leaves affects expression of Sucrose: Fructan 6-Fructosyltransferase gene, Annals of Applied Biology, 138 (2001) 27-32.
[41] G. Legnani, W.B. Miller, Short photoperiods induce fructan accumulation and tuberous root development in Dahlia seedlings, New Phytologist, 149 (2002) 449-454.

[42] V.J. Nagaraj, R. Riedl, T. Boller, A. Wiemken, A.D. Meyer, Light and sugar regulation of the barley sucrose: fructan 6-fructosyltransferase promoter, Journal of Plant Physiology, 158 (2001) 1601-1607.
[43] C. Sun, S. Palmqvist, H. Olsson, M. Borén, S. Ahlandsberg, C. Jansson, A Novel WRKY Transcription Factor, SUSIBA2, Participates in Sugar Signaling in Barley by Binding to the Sugar-Responsive Elements of the iso1 Promoter, The Plant Cell Online, 15 (2003) 2076-2092.
[44] L. Shaw, C. McIntyre, P. Gresshoff, G.-P. Xue, Members of the Dof transcription factor family in Triticum aestivum are associated with light-mediated gene regulation, Functional & Integrative Genomics, 9 (2009) 485-498.
[45] E. Lam, N.H. Chua, GT-1 binding site confers light responsive expression in transgenic tobacco, Science (New York, N.Y.), 248 (1990) 471-474.
[46] G. Giuliano, E. Pichersky, V.S. Malik, M.P. Timko, P.A. Scolnik, A.R. Cashmore, An evolutionarily conserved protein binding sequence upstream of a plant light-regulated gene, Proceedings of the National Academy of Sciences, 85 (1988) 7089-7093.
[47] D. Winter, B. Vinegar, H. Nahal, R. Ammar, G.V. Wilson, N.J. Provart, An "Electronic Fluorescent Pictograph" Browser for Exploring and Analyzing Large-Scale Biological Data Sets, PLoS ONE, 2 (2007) e718.
[48] M. Hummel, J.H.G. Cordewener, J. de Groot, S. Smeekens, A.H.P. America, J. Hanson, Dynamic protein composition of Arabidopsis thaliana cytosolic ribosomes in response to sucrose feeding as revealed by label free MSE proteomics, Proteomics, 12 (2012) 1024-1038.
[49] H. Schluepmann, A. Van Dijken, M. Aghdasi, B. Wobbes, M. Paul, S. Smeekens, Trehalose mediated growth inhibition of Arabidopsis seedlings is due to trehalose-6-phosphate accumulation, Plant Physiology, 135 (2004) 879-890.
[50] S.A. Ruuska, D.C. Lewis, G. Kennedy, R.T. Furbank, C.D. Jenkins, L.M. Tabe, Large scale transcriptome analysis of the effects of nitrogen nutrition on accumulation of stem carbohydrate reserves in reproductive stage wheat, 66 (2008) 15-32.
[51] R. Hofgen, L. Willmitzer, Storage of competent cells for Agrobacterium transformation, Nucleic acids research, 16 (1988) 9877-9877.
[52] A. Vermeulen, H. Vaucheret, V. Pautot, Y. Chupeau,; *Agrobacterium* mediated transfer of a mutant Arabidopsis acetolactate synthase gene confers resistance to chlorsulfuron in chicory (*Cichorium intybus* L.), Plant Cell Reports, 11 (1992) 243-247.
[53] B. Desprez, Recherche de methods de d'obtention de plantes haploïdes chez la chicories (*Cichorium intybus* L.), in: Sus-Orsay, Paris, 1994, pp. 174.
[54] J. Dietze, A. Blau, L. Willmitzer, Agrobacterium-mediated transformation of potato (*Solanum tuberosum*), in: I. Potrykus, Spangenberg, G (Ed.) Gene Transfer to Plants XXII, Springer-Verlag, Berlin, 1995, pp. 24-29.
[55] S.J. Clough, A.F. Bent, Floral dip: a simplified method forAgrobacterium-mediated transformation ofArabidopsis thaliana, The Plant Journal, 16 (1998) 735-743.
[56] R.A. Jefferson, T.A. Kavanagh, M.W. Bevan, GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants, The EMBO journal, 6 (1987) 3901.
[57] K.J. Livak, T.D. Schmittgen, Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-DDCT Method, Methods, 25 (2001) 402-408.

[58] G.M. Church, W. Gilbert, Genomic Sequencing, Proceedings of the National Academy of Sciences of the United States of America, 81 (1984) 1991-1995.

Supplementary data

Table S1: The names, origin and description of the promoters that has been used in the *de novo* motif search performed with the MEME software

Name	plant species	Description
AB072442.1	*Triticum aestivum*	*wft2* gene for sucrose: sucrose 1-fructosyltransferase.
AJ306962.1	*Hordeum vulgare*	*sucrose: fructan 6-fructosyltransferase* gene promoter region
AJ585414.1	*Festuca arundinacea*	*sucrose: sucrose 1-fructosyltransferase* promoter region
AM407401.1	*Lolium perenne*	*fructan: fructan 6G-fructosyltransferase* promoter region
AM407403.1	*Lolium perenne*	putative *(sucrose/fructan) 6-fructosyltransferase* promoter region
NC_003076.8	*Arabidopsis thaliana*	chromosome 5, inv promoter region Chr5:3838631..3839630
AY323935.1	*Cichorium intybus*	*fructan 1-exohydrolase IIa* promoter
EF442112.2	*Cichorium intybus*	*sucrose: sucrose fructosyl transferase (1-SST),* promoter region
EU545647.1	*Cichorium intybus*	*fructan: fructan-1 fructosyltransferase (1-FFT),* promoter region
EU545648.1	*Cichorium intybus*	*sucrose: sucrose-1 fructosyltransferase (1-SST)* promoter region
FJ361762.1	*Triticum aestivum*	*fructan: fructan 1-fructosyltransferase (1-FFT)* gene, promoter region
-	*Cichorium intybus*	*fructan 1-exohydrolase I* promoter
-	*Cichorium intybus*	*fructan: fructan-1 fructosyltransferase (1-FFT),* promoter region
-	*Cichorium intybus*	*vacuolar invertase (C40)*, promoter region
-	*Cichorium intybus*	*sucrose: sucrose fructosyl transferase (1-SST)* promoter region
-	*Helianthus tuberosus*	*sucrose: sucrose fructosyl transferase* (*1-SST I*) promoter region
-	*Helianthus tuberosus*	*sucrose: sucrose fructosyl transferase* (*1-SST II*) promoter region, fragment 1
-	*Helianthus tuberosus*	*sucrose: sucrose fructosyl transferase* (*1-SST II*) promoter region, fragment II

Figure S1: The motifs resulted from the *de novo* motif search performed with the MEME software. Motifs 3,5,6,7,8 and 9 were selected for further analysis. The putative MYB binding site is underlined in motif 9.

Motif 3

Motif 5

Motif 6

Motif 7

Motif 8

Motif 9

motif	*Remark*	Abiotic stress TAIR10 (256 exp)	AtGenExpress All TAIR10 (425 exp)
3	*28 out of 117 are missing on the arrays*	No correlations	Little correlations
6	*27 out of 90 are missing on the arrays*	Little correlations	Low expression
7	*30 out of 78 are missing on the arrays*	Two groups with different expression pattern	No correlations
8	*32 out of 133 are missing on the arrays*	Two groups with different expression pattern	No correlations
9	*9 out of 36 are missing on the arrays*	No correlations	Low expression
10	*15 out of 56 are missing on the arrays*	Little correlations	Low expression

motif	Development TAIR10 (135 exp)	Hormone treatment TAIR10 (140 exp)	Leaf TAIR10 (212 exp)
3		Positive correlation with ABA, methyl Jasmonate, and GA	No correlations
6	Little correlations	Positive correlation with IAA, NAA, and brassinosterols	Correlation with light
7	pollen, embryo and cotelydon	Positive correlation with ABA, GA, and brassinosterols	Little correlations, overall high expression
8	Embryo, LD and light in cotelydon	Little correlation with NAA, ABA, light and brassinosterols	No correlations
9	No correlations	Little correlation with, GA, JA, ethylene and brassinosterols	Little correlations, overall high expression
10	Little correlations	No correlations	Little correlations

motif	Root TAIR10 (258 exp)	Seed TAIR10 (83 exp)
3	Positive correlation with LD	Correlation with LD in cells and cotelydon
6	Correlation with daylenght	Correlation with Light, LD, and type of light
7	Good correlations, gene clusters	Correlation with Light, LD, and continious light
8	Clusters of genes	Expresson differences in embryo
9	LD	Positive corelation with LD and continious light
10	Little correlations	Positive corelation with LD and continious light

Table S2: Remarks and findings of the expression analysis study performed using the co-expression analysis tool of CORNET 2.0 ARABIDOPSIS using the *de novo* identified motifs on the different sets of microarray experiments, as shown in the headings of the columns.

Chapter 4

Developmental modulation of inulin accumulation in storage organs of transgenic maize and transgenic potato

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Abstract

Many important health promoting and functional characteristics are attributed to the non-digestible polysaccharide, inulin. Its use as a prebiotic in functional food and feed has further increased inulin demand worldwide. Inulin production in crops used for food and feed application, such as maize and potato, may provide a more direct and cost-effective route to provide functional food or feed when compared to native inulin producers. Towards this end we have expressed the inulin synthesizing enzymes, 1-SST and 1-FFT from Jerusalem artichoke in maize and potato. Transgenic maize plants produced inulin type fructan (at 3.2 mg/g kernel) when expressing an endosperm targeted gene cassette. Kernel development and germination were not affected. Potato tubers expressing *1-SST* accumulated 1.8 mg inulin/g tuber while tubers with a combined expression of *1-SST* and *1-FFT* accumulated 2.6 mg inulin/g tuber. Inulin accumulation in maize kernels was modulated by kernel development. Inulin levels peaked and then underwent moderate degradation by late kernel development. In potato, inulin production was relatively stable throughout tuber development and little evidence of degradation was observed. The accumulation of 1-kestose in transgenic maize was positively correlated with kernel sucrose concentration. Introduction of the fructan synthetic pathway in a high-sucrose maize background increased inulin accumulation to 41 mg/g kernel. Evidence is presented indicating that sucrose availability is limiting fructan production in transgenic maize.

Introduction

Fructans are linear or branched polymers of repeating fructose residues connected by ß(2-1) and/or ß(2-6) fructosyl-fructose linkages, optionally including one terminal glucosyl unit. Fructans are widely distributed in nature occurring in bacteria, fungi and over 40,000 higher plant species [1-2]. Plants generally contain fructans with a degree of polymerization (DP) of 3 to 200 [2], while bacteria can produce very large fructan polymers with a DP greater than 5000 [3]. Inulin, the best characterized fructan, contains predominantly linear molecules with ß(2-1) linkages and is found in chicory (*Cichorium intybus* L.), sunflower (*Helianthus annuus* L.), and Jerusalem artichoke (*Helianthus tuberosus* L.), among others. It is generally believed that inulin biosynthesis in plants occurs through the concerted action of two vacuolar enzymes, 1-SST (sucrose: sucrose 1-fructosyltransferase, EC 2.4.1.99) and 1-FFT (fructan: fructan 1-fructosyltransferase, EC 2.4.1.100 [4-5]. 1-SST catalyzes the conversion of sucrose to the trisaccharide 1-kestose (DP3). The elongation of 1-kestose to inulin (of DP up to 200) is catalyzed by 1-FFT. 1-SST has also been reported to produce small inulin of DP3 to DP5 [6] or DP7 [7] and thus may have some 1-FFT activity.

Many important nutritional and functional characteristics are attributed to inulin-type fructan. Inulin can increase calcium absorbance, act as a low calorie sweetener or be used as a water-soluble dietary fiber in food ingredients. Functional characteristics include among others taste improvement, added mouth feel, high water holding capacity and fat replacement [8-9]. More importantly, inulin is not metabolised by humans and animals and can act as a prebiotic [10]. Prebiotics enhance human and animal health performance by being selectively fermented by beneficial organisms such as Bifidobacteria in the large intestine of animals, at the expense of pathogenic or non-beneficial organisms such as Salmonella, *E. coli*, Clostridium species [8,9,11]. Because of this diverse range of nutritional and functional characteristics inulin is increasingly being used for improving food quality and added health benefits (functional foods) [11]. Interest in the use of prebiotics has increased among feed manufacturers, livestock and poultry producers as well [12]. Inulin type prebiotics have shown a positive effect on the health status and growth performance of piglets [13], pets, livestock and poultry has been reported [12,14,15]. Addition of low levels (1 mg/g) of low molecular weight inulin to the diet of chicks resulted in a reduction of Salmonella colonization [16].

The major industrial sources of inulin are the chicory root and Jerusalem artichoke tuber. The commercial potential for inulin is high, however, its use is limited by the high costs of production. Transgenic crops accumulating fructan would have a significant advantage over native fructan-storing plants by making use of established breeding programs, pest resistance and adaptation to a variety of growing regions throughout the world. Transgenic approaches to produce inulin in commercial crops have been successful to a limited extent. Transgenic potato expressing the globe artichoke *1-SST* produced the short chain inulin, 1-kestose [17]. Transgenic potato plants expressing both *1-SST* and *1-FFT*

produced inulin molecules of DP>60, similar to the inulin profile found in the globe artichoke [18]. Transgenic sugar beet accumulated high levels of short chain inulin (DP3, DP4 and DP5) by expressing the *1-SST* of Jerusalem artichoke [7]. The prebiotic effect of inulin, even at low doses in feed, makes its potential use in feed crops, such as corn, very attractive.

Here we report on the production of inulin and its developmental modulation in the commercially important crops maize and potato. Our data indicates that the dicot genes *1-SST* and *1-FFT*, from Jerusalem artichoke, are fully functional in a monocot. Furthermore, the developmental modulation of inulin production in maize and potato are reported.

Methods

Chimeric constructs for endosperm specific expression of the Jerusalem artichoke 1-SST and/or 1-FFT in transgenic maize

A construct designed for tissue specific expression of the Jerusalem artichoke *1-SST* gene in maize endosperm was assembled by replacing the Cauliflower Mosaic Virus (CaMV) 35S promoter in the plasmid pSST403 [19] with a maize endosperm-specific 10 kD zein, seed storage gene promoter [20]. The complete *1-SST* coding sequence contained in pSST403 [19] was isolated by digesting with the restriction endonuclease enzymes NcoI and HindIII. The isolated sequence was added to the plasmid pCyt-SacB [21] containing a 10 kD zein promoter and 3' termination region. The maize endosperm expression cassette, containing the 10 Kd promoter, *1-SST* coding sequence (including the native secretory and vacuole targeting signals) and the 10 kD 3' end, designated 10 kD-SST, was isolated by digesting with SmaI and SalI, then ligated into the plasmid KS17. The KS17 vector contains a hygromycin resistance gene (*HPT*) used as the selectable marker. The final vector was designated 10 kD-SST-17. The second construct designed for tissue specific expression of the Jerusalem artichoke *1-FFT* gene in maize endosperm was assembled by replacing the Cauliflower Mosaic Virus (CaMV) 35S promoter in the plasmid pSST405 [19] with a maize endosperm-specific 10 kD zein, seed storage gene promoter [20]. The complete *1-FFT* coding sequence contained in pSST405 was isolated by digesting with NcoI and BamHI. The isolated sequence was added to the plasmid pCyt-SacB containing a 10 kD zein promoter and 3' termination region. PCyt-SacB was digested was NcoI and BamHI to remove the SacB region. The maize endosperm expression cassette, containing the 10 kD promoter, *1-FFT* coding sequence (including the native secretory and vacuole targeting signals) and the 10 kD 3' end, designated 10 kD-FFT, was isolated by digesting with SmaI and SalI, then ligated into the plasmid KS17. The final vector was designated 10 kD-FFT-17.

Chimeric constructs for expression of the Jerusalem artichoke 1-SST and/or 1-FFT in Transgenic potato

For the transgenic expression of *1-SST* and *1-FFT* from Jerusalem artichoke two binary constructs were made. The construct harboring the *1-SST* was pSST331, the construct harboring the *1-FFT* was pFFT301 [19]. In both constructs the expression was driven by the (CAMV) 35S promoter. To generate transgenic control plants a third construct was made, in which the *1-SST* from pSST331 was removed, resulting in pFB2.

Plant material and plant transformation

To generate transgenic maize plants the plant expression vector 10 kD-SST-17 or the combined expression vectors 10kD-SST-17 + 10kD-FFT-17, and a plasmid vector encoding a selectable marker (pDetric) were co-transformed into embryogenic maize callus derived from crosses of the inbred lines A188 and B73 by micro projectile bombardment [22]. Transformed embryogenic cells were recovered on medium containing either glufosinate-ammonium or chlorsulfuron. The selectable marker pDetric contains the BAR gene, coding for phosphinothricin acetyltransferase, under the control of the (CAMV) 35S promoter. A mutant acetolactate synthase gene contained in pALSLUC confers resistance to chlorsulfon. Transgenic shoots were transferred to 30 cm pots containing Metromix (Scotts-Sierra Company) media and grown to maturity in the greenhouse. Mature kernels from original transformants (1^{st} generation transformants, T1) and subsequent maize generations (T2 to T4, generated through self-pollination) were grown in the greenhouse or planted directly in the field (Newark, DE, USA). Potato (*Solanum tuberosum* L. cv. Kardal) plants were transformed via *Agrobacterium tumefaciens* AGL0 as described by Dietze *et al*. [23] using the chimeric gene constructs pSST331, pFFT101 or pFB2. Plants were grown in 18 cm. pots in the greenhouse at 18°C daytime and 15°C night-time temperature with a photoperiod of 14h provided by Philips lamps HPT-T 400W (Philips, Eindhoven, The Netherlands).

Analysis of transgenic plants expressing the 1-SST or 1-FFT genes

Detection of the 1-SST gene and the 1-FFT gene in transgenic maize plants was accomplished by PCR analysis on leaf tissue, using oligonucleotide primers specific for the 1-SST or 1-FFT gene (data not shown). Three-months-old potato plants were tested for the presence and expression of the transgenes by Northern and Southern blotting. Potato plants expressing only one copy of the transgenes were used as parent plants to generate progeny harboring both 1-SST and 1-FFT or were self-pollinated to produce 1-SST or 1-FFT progeny. From the progeny, the inulin producing plants were propagated by cuttings. After 100 days the nutrition was stopped to force the plants to produce tubers. At day 175, the plants were withheld from water to ripen the tubers. At day 190, the dried plants were

harvested and tubers stored at 4°C. At day 105, 125, 140, 153 and 189 two plants were harvested from each line and tubers analysed. A post-harvest sample was taken after a storage time of 3 months. Fresh weight was determined from each harvested tuber and a representative sample was taken using a cork borer of 4 mm diameter for carbohydrate analyses.

Carbohydrate extraction and analysis

In an initial screen, individual kernels from transgenic maize lines were harvested at 25-35 days after pollination (DAP) for detection of fructose polymers. Kernels were crushed with a mortar and pestle. Water (200 to 400 µL) was added and the mixture heated to 80°C for 10 minutes. The homogenized tissue was centrifuged at 10,000 x g for 10 minutes and 2 µL of aqueous solution spotted on to HP-K silica TLC plates (Whatman Scientific, Clifton, NJ). TLC plates were developed twice in butanol: propanol: water (3:14:4). Fructan was detected by urea-phosphoric acid stain [24]. Analysis demonstrated that control kernels (untransformed) did not contain fructan. In subsequent studies, individual kernels (4 to 10 kernels per event) were harvested at different time points during development or at maturity, frozen in liquid nitrogen and stored at –80°C until further analysis. The carbohydrate profile of sugars and fructan up to DP30 was determined by HPAE/PAD using a Carbopac^{TM} PA-1 guard column (4x50 mm) and a Carbopac^{TM} PA-1 (4x250 mm) column and an ED40 Electrochemical detector (Dionex, Sunnyvale, CA, USA). To quantify glucose, fructose, sucrose, 1-kestose (DP3), DP4 and DP5 a mixture of pure compounds was used. Oligofructans of > DP5 were quantified using acid hydrolysis as described by Gruters *et al*. [25]. Fresh potato tuber material (125 mg) was homogenized in 750 µl of 20 mM sodium phosphate buffer, pH 7.0 with a pestle in an eppendorf tube. Soluble carbohydrates were extracted at 85°C during 30 minutes with intermittent agitation. After cooling to room temperature the extract was centrifuged at 10.000 x g. From the supernatant a 50-fold dilution was made and 100 µl thereof was analysed by HPAE. For analysis of sugars to DP5 the sugars were eluted from the column using 50mM NaOH supplemented with increasing concentrations of NaAc as described by Sévenier *et al*. [6]. For analyses of fructan longer than DP5 the extract was analysed using a 20 minutes linear gradient of 500mM NaOH, 50mM sodium acetate and deionized water running from 20:0:80 (vol:vol:vol) to 20:30:50 (vol:vol:vol) followed by a 5 minutes linear gradient of the same solutions running from 20:30:50 (vol:vol:vol) to 20:80:0 (vol:vol:vol). Potato inulin polymers of DP> 5 were quantified using DP3 as a reference. Since longer polymers have a decreased detector it is necessary to correct the amounts based on the DP3 reference for this decreased detector response [26]. These correction factors were extracted from the work of Chatterton *et al*. [26] and consist of multiplying the response by 1.36, 1.40 and 1.44 for DP6, DP7, DP8 and DP> 8 respectively. To measure the mean DP (mDP) of inulin, the amount of fructose and glucose residues per inulin-peak was calculated by multiplying the

amount (in moles) of each inulin peak by the amount of fructose and/or glucose residues present in the polymer represented by this specific peak. This calculation was performed for every peak in the inulin chromatogram. The ratio between the total glucose and glucose + fructose represents the mDP.

Results

Endosperm-targeted inulin production in transgenic maize

In order to introduce a fructan biosynthetic pathway into the main storage organs of the monocot maize, plant vectors expressing either the Jerusalem artichoke *1-SST* or *1-FFT* genes in the vacuole of maize endosperm were constructed. Inulin synthesis was expected to take place in endosperm vacuoles, due to the vacuole targeting signals present in each construct. A total of 44 transgenic events containing the 10kD-SST construct and 77 events containing the 10kD-SST and/or the 10kD-FFT constructs were produced. Transgenic plants expressing either *1-SST* alone or *1-SST+1-FFT* showed normal growth phenotype and fertility. Mature kernels of these transgenic events showed normal (wild-type) dent phenotype. Furthermore, kernel size and number per ear were not affected when compared to wild type (data not shown).

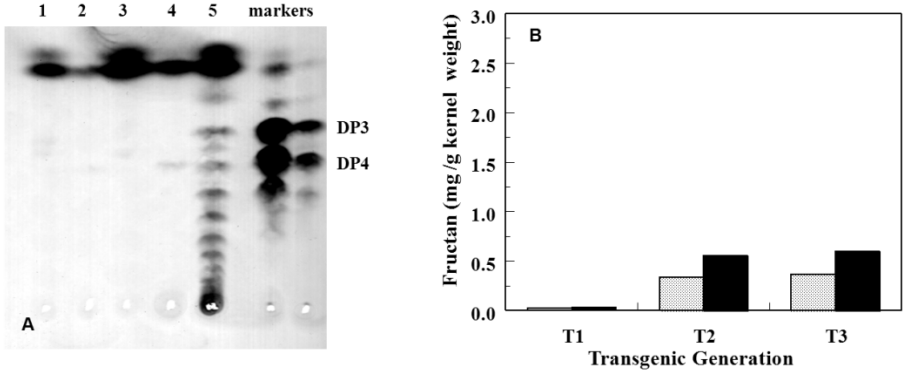

Figure 1. a: TLC analysis of individual maize kernels from transgenic lines containing intact copies of both the 10kD-SST and 10kD-FFT expression cassettes. Kernel 1 to 4 did not accumulate fructan while kernel 5 contained fructan of DP3 to DP20. Fructan polymers containing 1 and 2 additional fructose residues (DP3 and DP4, respectively) are indicated. A marker lane (M) containing fructose, sucrose, DP3 and DP4 fructan is indicated.
b: Mean fructan concentration (gray) and highest fructan concentration (black) in mature kernels of three generations (T1, T2, T3) of transgenic plants expressing both 1-SST + 1-FFT.

Maize kernels from transgenic lines expressing the *1-SST* accumulated inulin averaging from 0.13 to 3.2 mg/g fresh weight (FW) (Table 1) and having a DP ranging from DP3 to DP7 (data not shown). This data unequivocally shows that a fructosyltransferase

derived from a dicot can be expressed successfully in monocot kernels when driven by a seed specific maize promoter. Fructan in kernels expressing both the *1-SST* and *1-FFT* genes ranged greatly in DP with most events accumulating inulin of DP3 to DP8. Some events showed accumulation of inulin with a DP much larger than in lines containing the *1-SST* gene alone (Fig. 1A). The amount of inulin accumulation in T1 kernels (T1 is the first transgenic generation that produces kernels) varied between events as is shown in Table 1.

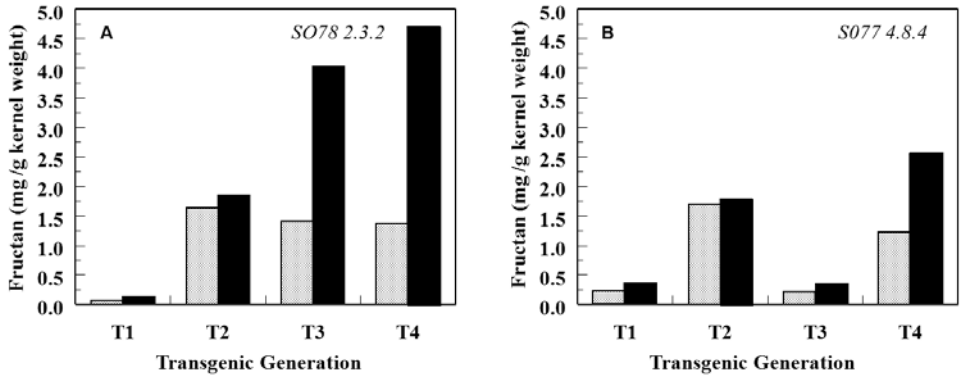

Figure 2. Fructan concentration in mature kernels from two transgenic maize lines, S078 2.3.2 (A) and S077 4.8.4 (B), expressing the Jerusalem artichoke *1-SST*. Mean fructan concentration (shown in gray) and highest fructan concentration (shown in black) of four generations of transgenic plants (T1 to T4).

To determine if this novel trait was stably inherited in maize and to produce homozygous plants, multiple events were grown and self-pollinated for up to 4 generations. Low-molecular weight inulin accumulated in kernels from all 4 generations indicating that the trait was inherited in a stable manner. Inulin accumulation in maize kernels varied between generations of the same transgenic event as well as between different transgenic events (Figs.1 and 2). Transgenic maize kernels expressing *1-SST* accumulated up to 5 mg inulin/gFW (event S078 2.3.2, black bars show highest accumulation, Fig. 2A) and showed a relatively stable average accumulation around 1.5 mg/gFW (gray bars) over several generations. Other SST-only events such as S077.4.8.4 showed a more variable phenotype (Fig. 2B).

Table 1. Soluble carbohydrates (mg/g kernel wt) of mature kernels from transgenic maize plants expressing *1-SST* or *1-SST* + *1-FFT*.

event	gene target	Glucose	Fructose	Sucrose	inulin
wild type	-	2.12 ± 1.27	1.20 ± 0.95	15.73 ± 4.93	0.00 ± 0.00
S078 2.3.2	*1-SST*	1.33 ± 0.51	0.90 ± 0.17	19.82 ± 6.65	0.13 ± 0.09
S077.4.8.4	*1-SST*	0.97 ± 0.11	0.42 ± 0.01	11.08 ± 0.10	0.23 ± 0.16
S078.3.2.2	*1-SST*	2.19 ± 1.96	1.91 ± 1.33	13.41 ± 2.53	1.54 ± 0.89
F014 4.38.21	*1-SST*	3.27 ± 0.15	3.23 ± 0.30	26.74 ± 3.53	3.20 ± 0.68
F014.4.38.7	*1-SST*	2.71 ± 0.23	2.64 ± 0.08	15.45 ± 0.71	1.85 ± 0.71
F021 3.4.4	*SST+FFT*	2.25 ± 0.88	1.43 ± 0.65	10.14 ± 1.12	0.62 ± 0.44
F022 2.2.3	*SST+FFT*	1.45 ± 0.39	0.70 ± 0.23	7.28 ± 1.05	0.36 ± 0.25
F022 2.4.2	*SST+FFT*	0.47 ± 0.01	0.20 ± 0.01	9.64 ± 0.78	0.02 ± 0.02

Inulin production in transgenic potato

The inulin biosynthetic pathway was introduced in potato to study the accumulation, stability and degradation of fructan in potato, a plant that normally does not accumulate fructan. The carbohydrate composition of transgenic tubers was determined in duplicate samples taken from 6 different transgenic lines at 6 different developmental stages. Potato tubers harboring 1-SST accumulated inulin of DP3 to DP8 at 1.8 mg/g tuber (Table 2). The mDP was between DP3 and DP4. These results confirm that the *1-SST* gene from Jerusalem artichoke is fully functional in other dicot species, as was shown in Petunia by Van der Meer *et al*. [19]. The combined expression of *1-SST* and *1-FFT* in potato resulted in an inulin accumulation at 2.6 mg/g tuber (Table 2). These lines also produce inulin polymers up to DP29 (Line F96a X S312 nr.6 (data not shown). The mDP of the SST+FFT plants ranged between 5 and 7.

Sucrose availability limits fructan accumulation in maize endosperm

A strong correlation (r^2 = 0.80) between the substrate for 1-SST, sucrose, and its product, 1-kestose was observed in transgenic maize kernels (Fig. 3A). Total fructan concentration was also positively correlated to sucrose concentration of the kernel, however to a weaker extent (r^2 = 0.59, Fig. 3B) when compared to 1-kestose. To further investigate this relationship, inulin producing maize lines were crossed with a high sucrose maize mutant *sh2* [27]. The *sh2* mutant is characterized by a mutation in ADP-glucose pyrophosphorylase, resulting in an abolishment of starch production and accumulation of sucrose. Mature kernels of this super-sweet corn have a shriveled phenotype. T1 kernels from the cross between fructan producing transgenic maize lines and *sh2* were grown in a

greenhouse, selected for the presence of the transgene, and kernels from these crosses were field planted to produce 2nd generation (T2) kernels. T2 kernels, homozygous for *sh2* genotype, were selected based on their shriveled phenotype. The carbohydrate profiles of 1-SST only or 1-SST+1-FFT transgenic kernels in a high-sucrose background are shown in Table 3. Inulin accumulation in kernels expressing both *1-SST* and *1-FFT* ranged from 5 to 41 mg/g (Table 3) with DP ranging from DP3 to DP10 (data not shown) and an average inulin accumulation of 20.05 mg/g. (Table 3). Kernels expressing only 1-SST showed a lower inulin accumulation (6.3 mg/g, Table 3) with a DP of 3 to 6 (data not shown). Interestingly, the progeny of the transgenic event F022.2.2.3 in a high sucrose genetic background (F022.2.2.3 x sh2, Table 3) accumulated substantially higher levels of inulin (20.05 mg/g) as compared to its parent in a wild-type genetic background (F022 2.2.3, Table 1) which accumulated 0.36 mg/g. Furthermore, these transgenic events showed a positive correlation between sucrose and 1-kestose (r^2=0.63) similar to what was observed in dent transgenics. Taken together, these data suggest that sucrose availability is limiting inulin production in transgenic maize kernels.

Table 2. Soluble carbohydrates (mg/gFW) of transgenic potato tubers expressing *1-SST* or *1-SST* + *1-FFT* harvested 125 days after planting.

Event	gene target	Glc	Fru	Suc	DP3	DP4	DP5	inulin
wild type	-	4.68	0.52	3.07	0.00	0.00	0.00	0.00
S312a nr.1	*1-SST*	0.83	0.37	2.69	0.89	0.10	0.04	1.04
S312a nr.4	*1-SST*	0.08	0.01	2.86	1.42	0.08	0.29	1.79
S312a nr.7	*1-SST*	0.56	0.21	1.98	0.15	0.19	0.33	0.66
F96a X S312 nr.6	*SST+FFT*	2.00	0.75	3.11	0.77	0.47	1.31	2.58
F6b X S312a nr.25	*SST+FFT*	0.05	0.14	3.40	0.45	0.74	0.67	1.86
S312a X F6b nr.31	*SST+FFT*	2.37	0.87	2.61	0.40	0.31	0.59	1.30

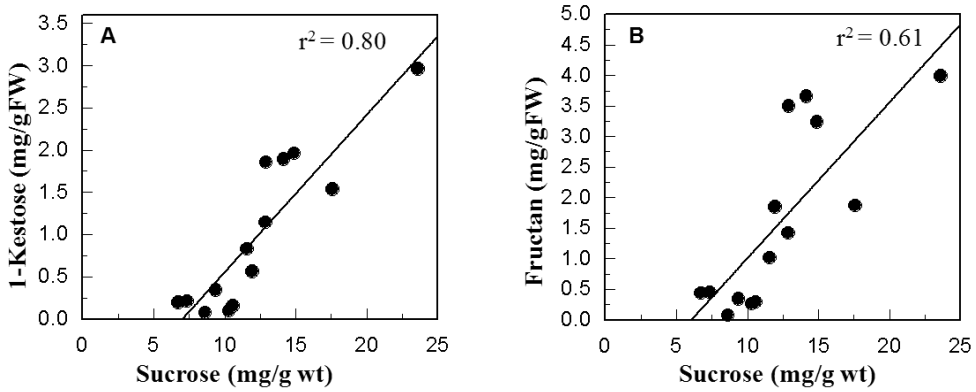

Figure 3. Correlation between sucrose and 1-kestose (A) or sucrose and fructan (B) in transgenic maize kernels expressing the Jerusalem artichoke *1-SST*.

Sucrose availability in fructan accumulating potato

The carbohydrate content of tubers, harvested at different time points, of three potato lines expressing *1-SST* was determined (Fig 4A). Sucrose concentrations ranged from 2.1 to 19.6 mg/g, possibly caused by the difference in genetic background of the lines due to the crosses that were made to produce the lines. This wide range of sucrose is not unusual and can be observed in commercial potato cultivars as shown by Amrein *et al*. [28]. Figure 4 shows that the correlation between the concentration of sucrose and 1-kestose (r^2 =0.17) or total fructan (r^2=0.17) was low. Sucrose and fructose concentrations were similar in fructan and non-fructan producing lines whereas glucose concentration was more variable and reduced in transgenic lines when compared to wild type lines (data not shown). This suggests that sucrose pool observed in developing tubers may not be the limiting factor for fructan accumulation in transgenic potato.

Developmental modulation of inulin accumulation in transgenic maize

To better understand the cause of the variable inulin levels at kernel maturity, we characterized the soluble carbohydrate profile during kernel development in transgenic maize expressing 1-SST (Fig. 5). Sucrose accumulated early in kernel development (from 7 to 14 DAP) and decreased during the mid to late phases (Fig. 5A). Glucose and fructose concentrations were highest in the early stages of kernel development and decreased rapidly as the kernel matured. Interestingly, inulin accumulation in maize kernels expressing the 1-SST was modulated by kernel development (Fig. 5B). The immediate product of 1-SST,

1-kestose, accumulated early in kernel development and decreased during the later phases. 1-kestotetraose (DP4) and 1-kestopentaose (DP5) also increased during the early stages and decreased in late development. Surprisingly, all types of fructan molecules (DP3, DP4 and DP5) decreased substantially during the late stages of development. A similar profile of sucrose and fructan accumulation and degradation is observed when the data is expressed on a kernel basis (data not shown), indicating that the decrease is due to degradation of previously accumulated fructan. This developmental study was repeated with three independent events and all showed a similar profile indicating that this developmental regulation of fructan accumulation and degradation was common to all tree events. Interestingly, at 20 DAP, developing kernels of transgenic maize expressing 1-SST and 1-FFT in a high sucrose background (F022.2.2.3 x sh2) showed a similar high inulin accumulation (17 mg/g) when compared to their mature kernels (20 mg/g, Table 3). This indicates that in a high sucrose background, degradation may be negligible. Hence sucrose may play a critical role in regulating fructan accumulation and turnover.

Table 3. Inulin and carbohydrate content (mg/gFW) of mature transgenic maize kernels expressing *1-SST* and *1-FFT* in a dent (wild type) or high sucrose (*sh2*) background.

event	gene	Glc	Fru	Suc	DP3	DP4	DP5	Inulin	TotCHO
wild type (dent)		2.11	0.99	15.72	-	-	-	-	20.98
sh2 (super sweet)		3.99	2.5	35.96	-	-	-	-	44.93
sh2 x S078 2.3.2	*1-SST*	4.04	5.17	33.35	0.83	2.19	3.29	6.32	51.26
F022.2.2.3 x sh2	*SST,FFT*	4.03	3.83	23.70	1.51	7.79	8.48	20.05	51.97

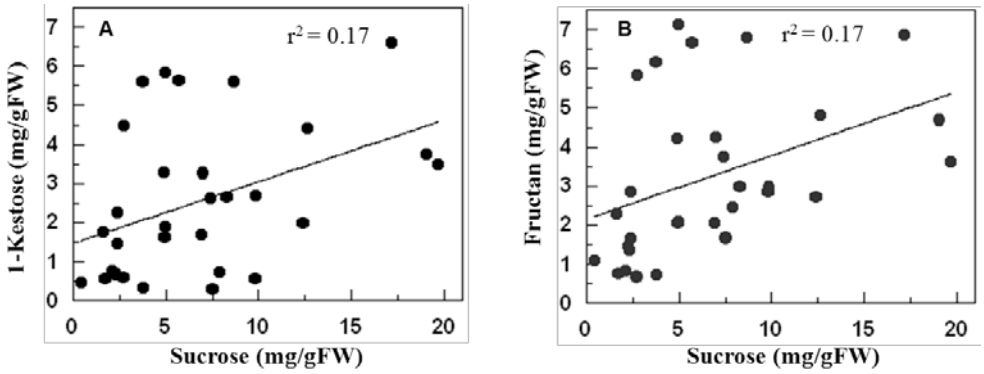

Figure 4. Correlation between sucrose and 1-kestose (A) or sucrose and fructan (B) in transgenic potato tubers expressing the Jerusalem artichoke *1-SST*.

Developmental modulation of inulin accumulation in transgenic potato

In order to study the stability of fructan produced in potato tubers, 1-SST and 1-SST + 1-FFT fructan producing tubers were sampled during a period of 100 days. 1-kestose accumulated early in tuber development but, in contrast to maize, decreased only slightly during tuber maturation (Fig. 6). Tubers expressing 1-SST accumulated increasing amounts of 1-kestotetraose and 1-kestopentaose during tuber development (Fig. 6A). Plants expressing both 1-SST and 1-FFT accumulated showed a slight decrease in DP3 and DP4 fructan during tuber development. The DP5 decrease was even less (Fig. 6B). The mDP of 1-SST tubers (mDP=3.4) and 1-SST + 1-FFT tubers (mDP=6.0) remained constant during tuber development (Fig. 7). This data indicates that inulin production in potato tubers is rather stable with little inulin degradation occurring.

Post –harvest modulation of inulin accumulation in transgenic potato

When potato tubers were stored at 4°C for 3 months a near total degradation of fructan was observed (Fig. 6, cold storage). A similar degradation pattern was observed in both 1-SST and 1-SST+1-FFT expressing tubers. Only trace levels of DP3, 4 and 5 and no higher DP fructan was left after storage. Together with the decrease in fructan an increase of reducing sugar concentration was found (data not shown).

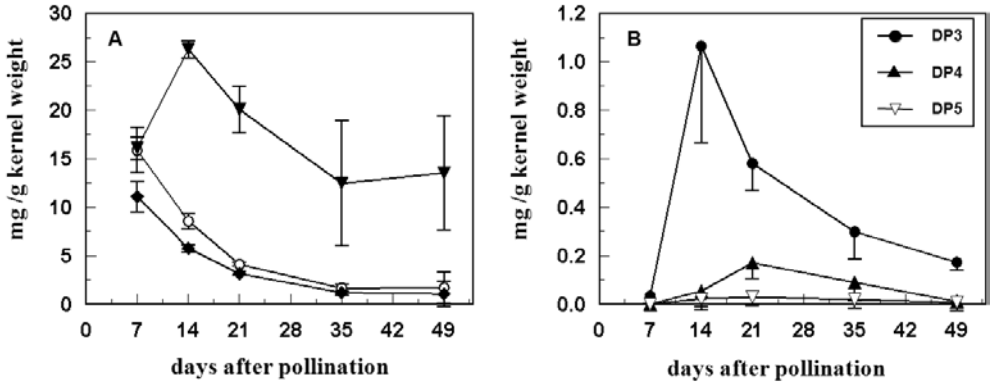

Figure 5. Carbohydrate profile of fructan accumulating maize kernels. *A:* Glucose (open circle), fructose (diamond) and sucrose (triangle turned upside down) accumulation during kernel development of transgenic maize expressing the Jerusalem artichoke 1-SST. *B:* Accumulation of DP3 (closed circle), DP4 (triangle) and DP5 (triangle turned upside down) during kernel development of transgenic maize expressing the Jerusalem artichoke *1-SST*.

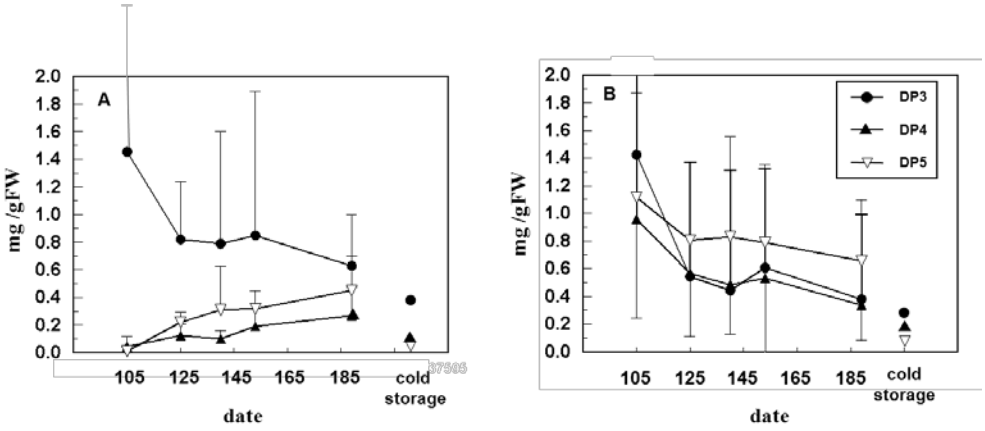

Figure 6. Carbohydrate profile of fructan accumulating potato tubers. *A:* Inulin accumulation (DP3 (closed circle), DP4 (triangle) and DP5 (triangle turned upside down) during tuber development of transgenic potato expressing the Jerusalem artichoke *1- SST*. *B:* Accumulation of DP3 (closed circle), DP4 (triangle) and DP5 (triangle turned upside down), during tuber development of transgenic potato expressing the Jerusalem artichoke *1- SST* and *1-FFT*.

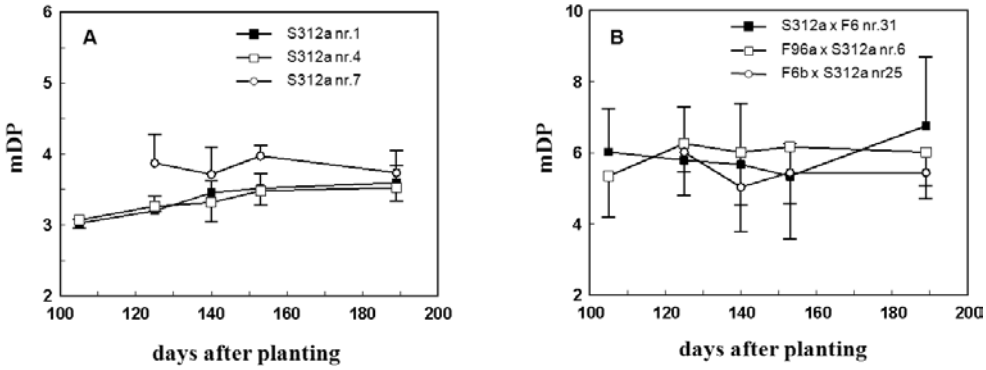

Figure 7. Mean DP of fructan in tubers of three independent potato lines (S312 nr.1, S312a nr.4, S312a nr.7) expressing *1-SST* (A). Mean DP of fructan in tubers of three independent potato lines (F96a x S312 nr.6, F6b x S312a nr.25, S312a x F6 nr 31) expressing *1-SST* and *1-FFT* (B).

Discussion

The inulin biosynthetic pathway was successfully introduced into the monocot maize by expressing two dicot genes (*1-SST* and *1-FFT*) isolated from Jerusalem artichoke. Transgenic maize plants were able to convert sucrose into inulin type fructan. Inulin synthesis is expected to take place in endosperm vacuoles, due to the vacuole targeting signals present in the (endosperm targeted) gene cassettes. The presence of vacuole-like organelles in maize endosperm was first demonstrated by Woo *et al*. [29] and later confirmed by Kim and Krishman [30]. Since the endosperm represents the largest seed storage tissue we chose to target the inulin pathway to this tissue. Successful targeting of a bacterial fructosyltransferase in maize endosperm vacuoles has been reported before [21] and resulted in a stable accumulation of the levan polymer. In contrast, maize plants expressing a cytosolic targeted version of the same fructosyltransferase were severely affected in their development [21]. Vacuolar targeting of the inulin pathway in maize endosperm did not affect kernel development or germination indicating that tissue and organelle specificity are critical for stable fructan accumulation in crops that normally do not accumulate fructan. This study also provides further evidence that sucrose accumulates in maize endosperm and can act as a substrate to produce novel compounds. Transgenic maize expressing the *1-SST* accumulated low molecular weight inulin (DP3 to DP7) at 3.2 mg/g kernel in a dent background and at 6.32 mg/g in a high sucrose background. Although this inulin concentration is relatively low with regard to inulin processing for food uses, it falls well in the range for use as a prebiotic in animal feed. Addition of low doses of inulin (1 mg/g) to the diet of chicks resulted in reduced Salmonella colonization [16]. Animal feed supplements with fructan concentrations of less than 1% have improved gut microbial ecology and enhanced stool quality resulting in increased animal health [31-32]. Transgenic maize expressing both *1-SST* and *1-FFT* accumulated higher DP inulin indicating that 1-FFT is fully functional in maize endosperm and acts as a chain elongation factor. Multiple events of transgenic maize expressing *1-SST* or *1-SST* + *1-FFT* were grown for up to 4 generations and analysis showed that the inulin trait was stably inherited. When these lines were crossed with a high sucrose maize mutant *sh2* inulin accumulated in mature kernels at up to 4 % of the kernel weight. Since maize is a major component of feed in livestock and poultry, the inclusion of inulin and its prebiotic effects may provide a cost effective alternative to feed supplements used for improved animal health

The inulin pathway was also introduced in potato. Potato tubers expressing *1-SST* accumulate inulin of DP3 to DP8 at 1.8 mg/g tuber with a mean DP between 3 and 4. These data confirm that the *1-SST* from Jerusalem artichoke is fully functional in other dicot species, as was shown in Petunia [19] and sugar beet [7]. The combined expression of *1-SST* and *1-FFT* in potato resulted in inulin with a higher DP (up to 28) and a mean DP between 5 and 7. This data further supports the role of 1-FFT in the production of high molecular weight inulin as was seen for potato tubers expressing *1-SST* and *1-FFT* from globe artichoke [18].

Sucrose concentration of transgenic maize kernels expressing *1-SST* was highly correlated to the concentration of its direct product, 1-kestose (r^2 = 0.80), and the total inulin content (r^2 = 0.6). The correlation between sucrose and 1-kestose or inulin was weaker in transgenic potato than it was in maize. The different response in maize and potato may be explained by different carbohydrate metabolic processes in a monocot versus dicot background. The high K_m for sucrose of 1-SST may prevent significant fructan accumulation in crops that have low sucrose pool as postulated by Ritsema T. and S. Smeekens [33]. Furthermore, the maize vacuolar-organelles accumulate 18-kD alpha-globulin and forms protein accretions [29] whereas potato has a typical dicot storage vacuole. Differences between these vacuoles may be responsible for the differential response seen in maize and potato but further studies are necessary to better understand the metabolic processes that occur in vacuoles of maize endosperm. To further study the sucrose vs. inulin relationship, the inulin synthetic pathway was introduced into a super sweet corn line [27] using standard breeding practices. Shrunken-2 (*sh2*) is characterized by a 10 fold increase of soluble sucrose content compared to dent maize genotypes [34]. Inulin accumulation in transgenic high sucrose maize resulted in a similar correlation between sucrose and inulin. Furthermore a substantial increase of inulin accumulation up to 49 mg/g kernel wt was observed. Increased sucrose availability for fructan biosynthesis may be related to a decreased competition with starch synthesis in a *sh2* background. High inulin accumulation has also been reported in transgenic sugar beet [7] although no data was shown regarding the correlation between sucrose and inulin levels in sugar beet. Taken together, our data indicates that sucrose availability is limiting overall fructan accumulation.

Inulin accumulation in maize kernels was modulated by kernel development. Accumulation of 1-kestose peaked early in kernel development and decreased during the later phases. Sucrose accumulation showed a very similar profile when compared to 1-kestose, further supporting a strong correlation between sucrose content and 1-kestose production. The peak of 1-kestose accumulation preceded that of 1-kestotetraose and 1-kestopentaose suggesting a sequential elongation of inulin polymers with DP>3. The accumulation of the fructan molecules of DP>3 also decreased during late stages of kernel development indicating that the inulin polymers were partly degraded. The origin of this inulin degradation is unknown but may be related to activity of invertase. Vacuolar invertases are ß -fructofuranosidases and can hydrolyse ß-Fru-containing oligosaccharides [35]. Although little is known about the physiological processes in maize endosperm vacuoles, inulin degradation may be due to the catabolic activity of soluble invertase. In maize, soluble acid invertase genes (*Ivr-1* and *Ivr-2*) are sugar-modulated with *Ivr-2* being up regulated by increasing carbohydrate supply and *Ivr-1* being up regulated by depleting sucrose supply [36]. Furthermore, maize *Ivr*-1 is predominantly expressed during reproductive development and has a high expression in the kernel crown whereas sugar-enhanced genes (Ivr-2) are expressed in many importing organs [36]. Inulin production and sucrose depletion during mid to late development may provide the circumstance to allow

for Ivr-2 up regulation and inulin degradation. Increased starch production may further limit or destroy the maize vacuolar compartment possibly further increasing fructan turnover. The latter is supported by the observation that inulin production in high sucrose-low starch maize lines results in limited or no inulin degradation.

Compared to maize, inulin producing potato tubers showed only modest fructan degradation during tuber development. Potato lines expressing *1-SST* showed highest DP3 accumulation in young tubers. During tuber development the concentration of DP3 declined while the DP4 and DP5 polymer concentration increased. This indicated that 1-SST is using DP3 as a substrate to produce DP4 and DP5 inulin in a similar fashion as was shown for the maize transgenics. Potato lines expressing both *1-SST* and *1-FFT* show the highest inulin accumulation early in tuber development followed by a decrease in DP3, DP4 and to a lesser extent DP5 inulin during tuber development. The decrease of small inulin and the stability of the mDP (Fig. 7) suggest that the small inulin is mostly used by 1-FFT as a substrate for the production of higher DP inulin. Cold-storage of potato tubers resulted in an almost complete breakdown of the inulin in both the 1-SST and 1-SST + 1-FFT potato lines (Fig. 6). Storage of potato tubers at low temperatures is known to cause accumulation of reducing sugars, sucrose and increased invertase activity [37-39]. QTL analysis for hexose and sucrose content in cold induced potato revealed QTLs linked to invertase [40]. Ectopic expression of an invertase inhibitor in potato tubers reduced cold-induced hexose accumulation by up to 75 [41] further supporting a role of invertase in cold sweetening. Since invertase also possesses ß-fructofuranosidous activity, the induction of invertase activity during cold storage may also lead to the total degradation of accumulated fructan.

This data together with our maize data suggest that enzymes possessing beta-fructofuranosidases activity, such as invertase, may play a critical role in determining the stability and level of novel inulin polymers produced in both transgenic monocot and dicot crops. Sucrose itself may also play a key role in the production and stability of inulin production as is evident from its tight correlation with inulin accumulation and reduced inulin degradation observed in high sucrose maize lines. More studies are needed to better understand the physiological basis of the interaction between sucrose, hexose and inulin metabolism.

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References

[1] G.A.F. Hendry, R.K. Wallace, The origin, distribution and evolutionary significance of fructans. In: M. Suzuki, N. Chatterton, (Eds.), Science and Technology of Fructans, Boca Raton: CRC Press Inc., 1993, pp.119-139.

[2] C.J. Pollock, A.J. Cairns, Fructan metabolism in grasses and cereals, Ann. Rev. Plant Physiol. Plant Mol. Biol. 42 (1991) 77-101.

[3] R Lieberman, M. Potter, W.Jr. Humphrey, C.C. Chien, Idiotypes of inuline-binding antibodies and myeloma proteins controlled by genes linked to the allotype locus of the mouse. J. Immunol. 117 (1976) 2105-2111.

[4] J. Edelman, T.G. Jefford, The mechanism of fructosan metabolism in higher plants as exemplified in Helianthus tuberosus, New Phytol. 67 (1968) 517-531.

[5] I. Vijn, S. Smeekens, Fructan more than a reserve carbohydrate? Plant Physiol. 120 (1999) 351-360.

[6] R. Sévenier, R.D. Hall, I.M. van der Meer, H.J.C. Hakkert, A.J. van Tunen, A. Koops, High level fructan accumulation in a transgenic sugar beet, Nat. Biotech. 16 (1998) 843-846.

[7] G. Weyens, T. Ritsema, K. Van Dun, D. Meyer, M. Lommel, J. Lathouwers, I. Rosquin, P. Denys, A. Tossens, M. Nijs, S. Turk, N. Gerrits, S. Bink, B. Walraven, M. Lefevre, S. Smeekens, Production of tailor-made fructans in sugar beet by expression of onion fructosyltransferase genes, Plant Biotechnology, 2 (2004) 321-327.

[8] P.D. Meyer, Nondigestible oligosaccharides as dietary fiber, Journal of AOAC International, 87 (2004) 718-726.

[9] B. C. Tungland, Fructooligosaccharides and other fructan structures and occurrence, production, regulatory aspects, food application, and nutritional health significance, American Chemical Society Symposium series, 849 (2003) 135-152.

[10] G. R. Gibson, M. B. Roberfroid, Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. Journal of Nutrition 125 (1995) 1401-1412.

[11] J. Van Loo. J. Cummings, N. Delzenne, H. Englyst, A. Franck, M. Hopkins, N. Kok, G. Macfarlane, D. Newton, M. Quigley, M. Roberfroid, T. van Vliet, E. van den Heuvel. Functional food properties of nondigestible oligosaccharides: a consensus report from the ENDO project (DGXII AIRII-CT94-1095). British Journal of nutrition 81 (1999) 121-132

[12] E.A. Flickinger, G.C. Fahey Jr., Pet food and feed applications of inulin, oligofructose and other oligosaccharides. British Journal of Nutrition (2002) 87, S297-S300.

[13] C. L. Kien, J.C. Chang, J.R. Cooper, W.L. Frankel, Effects of prefeeding a prebiotic on diarrhea and colonic cell proliferation in piglets fed lactulose. Journal of Parental and Enteral Nutrition. 28 (2004) 22-26.

[14] A.J. Mul, F.G. Perry, The role of fructo-oligosaccharides in animal nutrition. Recent advances in animal nutrition. (1994) 57-79.

[15] Z. Zdunczyk, Physiological effects of low digestible oligosaccharides in diets for animal and humans. Pol. J. Food Nutr. Sci 13 (2004) 115-130

[16] T. Fukata, K. Sasai, T. Miyamoto, E. Baba, Inhibitory effect of competitive exclusion and fructooligisaccharide, singly and in combination on Salmonella colonization of chicks. J. Food Protect., 1999, 62 229-233

[17] E.M. Hellwege, D. Gritscher, L. Willmitzer, A.G. Heyer, Transgenic potato tubers accumulate high levels of 1-kestose and nystose: functional identification of a sucrose: sucrose 1-fructosyltransferase of artichoke (*Cynara scolymus*) blossom discs, Plant J. 12 (1997) 1057-1065.

[18] E.M. Hellwege, S. Czapla, A. Jahnke, L. Willmitzer, A. Heyer, Transgenic potato (Solanum tuberosum) tubers synthesize the full spectrum of inulin molecules naturally occurring in globe artichoke (*Cynara scolymus*) roots, PNAS 97 (2000) 8699-8704.

[19] I.M. van der Meer, A. Koops, J.C. Hakkert, A.J. van Tunen, Cloning of the fructan biosynthetic pathway of Jerusalem artichoke, Plant J. 15 (1998) 489-500.

[20] J.A. Kirihara, J.B. Petri, J.W. Messing, Isolation and sequence of a gene encoding a methionine-rich 10-kDa zein protein from maize, Gene 71 (1988) 359-370.

[21] P.G. Caimi, L.M. McCole, T.M. Klein, P.S. Kerr, Fructan accumulation and sucrose metabolism in transgenic maize endosperm expressing a *Bacillus amyloliquefaciens* SacB gene, Plant Physiol. 110 (1996) 355-363.

[22] T.M. Klein, E.D. Wolf, R. Wu, J.C. Sanford, High-velocity microprojectiles for delivering nucleic acids into living cells, Nature 327 (1987) 70-73.

[23] J. Dietze, A. Blau, L. Willmitzer, Agrobacterium-mediated transformation of potato (Solanum tuberosum). In: I. Potrykus, G. Spangenberg, (Eds.), Gene Transfer to Plants XXII. Springer-Verlag, Berlin, 1995, pp 24–29.

[24] C. Wise, R. Dimler, H. Davis, C. Rist, Determination of easily hydolyzable fructose units in dextran preparations, Anal. Chem. 27 (1955) 33-36.

[25] U. Gruters, On the role of wheat stem reserves when source-sink balance is disturbed by elevated CO2. Journal of applied Botany 73 (1999) 55-62.

[26] N.J. Chatterton, P.A. Harrison, W.R. Thornley, J.H. Benett, Separation and quantification of fructan (inulin) oligomers by anion exchange chromatography. In: A. Fuchs (Ed.) Inulin and Inulin-Containing Crops. Elsevier, Amsterdam (1993) pp 93–99.

[27] J.R. Laughnan, The effect of the sh2 factor on carbohydrate reserves in the mature endosperm of maize. Genetics 38 (1953) 485-499.

[28] T.M. Amrein, S. Bachmann, A. Noti, M. Biedermann, M.F. Barbosa, S. Biedermann-Brem, K. Grob, A. Keiser, P. Realini, F. Escher, R. Amado, Potential of Acrylamide Formation, Sugars, and Free Asparagine in Potatoes: A Comparison of Cultivars and Farming Systems. J. Agric. Food. Chem., 51 (2003) pp5556-5560.

[29] Y. Woo, D.W. Hu, B.A. Larkins, R. Jung, Genomics analysis of genes expressed in maize endosperm identifies novel seed proteins and clarifies pattern of zein gene expression, The plant cell 13 (2001) 2297-2317.

[30] W.S. Kim, H.B. Krishnan, Allelic variation and differential expression of methionine-rich delta-zeins in maize inbred lines B73 and W23a1, Planta 217 (2003) 66-74.

[31] J.S. Bailey, L.C. Blankenship, N.A. Cox, Effect of fructooligosaccharide on Salmonella colonization of the chicken intestine, Poultry Sci. 70 (1991) 2433-2438.

[32] M.D. Willard, R.B. Simpson, E.K. Delles, N.D. Cohen, T.W. Fossum, D. Kolp, G. Reinhardt, Effects of dietary supplementation of fructo-oligosaccharides on small intestinal bacterial overgrowth in dogs, Am. J. Vet. Res. 55 (1994) 654-658.

[33] T. Ritsema, S. Smeekens, Fructans: beneficial for plants and humans. Curr. Opinion in Plant Biol. 6 (2003) 223-230.

[34] R.G. Creech, Genetic control of carbohydrate synthesis in maize endosperm, Genetics 52 (1965) 1175-1186.
[35] A. Sturm, Invertases. Primary structures, functions, and roles in plant development and sucrose partitioning, Plant Physiol. 21 (1999) 1-7.
[36] J. Xu, W.T. Avigne, D.R. McCarthy, K.E. Koch, A similar dichotomy of sugar modulation and developmental expression affects both paths of sucrose metabolism: evidence from a maize invertase gene family, Plant cell 8 (1996) 1209-1220.
[37] N.J.G. Appeldoorn, S.M. de Bruijn, E.A.M. Koot-Gronsveld, R.G.F Visser, D. Vreugdenhil, L.H.W. van der Plas, Developmental changes of enzymes involved in conversion of sucrose to hexose-phosphate during early tuberization of potato, Planta, 202 (1997) 220-226.
[38] W.G. Burton, The sugar balance in some British potato varieties during storage. II. The effects of tuber age, previous storage temperature, and intermittent refrigeration upon low-temperature sweetening, Eur. Potato. J. 12 (1969) 81-95.
[39] R. Pressey, R. Shaw, Effect of temperature on invertase, invertase inhibitor and sugars in potato tubers, Plant Physiol. 41 (1966) 1657-1661.
[40] C.M. Menendez, E. Ritter, R. Schafer-Pregl, B. Walkemeier, A. Kalde, F. Salamini, C. Gebhardt, Cold sweetening in diploid potato: mapping quantitative trait loci and candidate genes. Genetics 162, (2002)1423-1434.
[41] S. Greiner, Ectopic expression of a tobacco invertase inhibitor homolog prevents cold-induced sweetening of potato tubers. Nature Biotech. 17 (1999) 708-711.

Chapter 5

General discussion

Towards tailor-made fructan

Fructan

Fructan is a polymer of fructose. It occurs in a variety of organisms; bacteria, fungi and in approximately 15% of flowering plants. Fructans can be divided into three groups based on their linkage type: 1) levan, with ß(2-6) linked fructosyl units; this mainly occurs in bacteria [1], monocotyledonous plants (where it is also called phlein) [2] and also in a member of the Buxales order as was recently reported [3]; 2) inulin, a ß(2-1) linear polymer present in dicotyledonous plants [4]; and 3) fructan neo-series, a mixed type of fructan found in Liliaceae [5] in which ß(2-1) chain elongation occurs at the C1 and C6 positions of the glucose residue.

Bacteria use fructan as an energy storage molecule [6] and as a protective layer outside the cell. This fructan layer is used by plant pathogenic bacteria to prevent host-pathogen recognition and protect against bacteriostatic compounds released by collapsed plant cells [7]. S*treptococci*, present in the oral cavity, use the fructan layer as an adhesive leading to the formation of dental plaques, which consist largely of levan-type fructan [8]. The biosynthesis of levan in bacteria is performed by a single enzyme, levansucrase (E.C. 2.4.1.10). Some classes of bacteria produce inulin-type fructan by an enzyme called inulosucrase (EC 2.4.1.9).

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 stress such as drought and cold [5, 9]. The length of plant fructan varies from 10 to approximately 200 fructosyl units. This variation highly depends on the taxonomic background of the fructan producing plant species.

Plant fructan is used for a range of food and non-food applications [10] depending on the DP. Short chain inulin can be used to produce fructose syrup for the sweetening of cold drinks. Long chain inulin (mDP ≥ 25) is used as a fat replacer, prebiotic and foam stabilizer in food products. Long chain inulin also provides the starting material for the production of carboxymethylinulin, a scavenger of divalent cations in household detergents.

Tailor-made fructan

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, which is sown in spring and the taproots are harvested in autumn of the same year, for inulin extraction. At harvest, the mean inulin polymer length is 9-10 and the average carbohydrate yield is about 11,000 kg per hectare [11]. One of the most important quality parameters of inulin is the polymer length. For some applications - such as fat replacer and for the production of derivatised inulin which require a minimum DP>25 - the raw inulin extracted from chicory is unsuitable and needs to be enriched for long molecules, which is a costly process.

Interestingly, it has been observed that the polymer length in chicory is much higher early in the growing season than in autumn when it is harvested. The decrease in polymer length at harvest is caused by catalytic reactions of 1-FFT and 1-FEH, the activity of both of these enzymes varies with plant development and environmental factors. A second important parameter is the concentration of low molecular weight carbohydrates in the taproot that negatively influence the quality of the extracted inulin. At the end of the growing season and during storage of the taproots high concentrations of low molecular weight carbohydrates accumulate. Another factor that negatively influences the quality of the inulin is inulo-*n*-ose, a fructan polymer consisting of only fructosyl-residues that is produced by 1-FFT at the end of the growing season when a high concentration of fructose is present in the root. The inulo-*n*-ose cannot be separated from inulin and hence negatively affect the purity of inulin.

What regulates the fructan yield and the degree of polymerisation, and how can we modify this?

Since fructan chain length is crucial for its industrial application the ability to steer fructan biosynthesis for the production of tailor-made fructan would be of great interest. This might be accomplished by six different strategies:

- First, the production of inulin with higher mDP could be obtained by preventing the degradation of inulin, which could be achieved by down-regulation of the enzymes involved through RNAi strategies or site-directed mutagenesis.
- A second strategy to influence the degree of polymerization and yield would be by enhancing the fructan biosynthesis during the growing season. This could be accomplished by introducing a heterologous fructosyl transferase gene that is not regulated by the endogenous regulatory mechanism or by overexpression of the transcription factor that induces the fructosyltransferase (FT) gene expression.
- Another strategy to increase the mDP would be the over expression of a fructan biosynthesis gene with a higher affinity for high molecular weight inulin as acceptor. This would result in the accumulation of higher DP inulin.
- As a fourth strategy the degradation of inulin by cold could be partially prevented by growing chicory in more moderate climates.
- A strategy to steer the expression pattern of the introduced genes and thereby the degree of polymerisation is to drive the expression of heterologous genes by promoters with a pre-defined spatial and temporal expression.
- The final strategy is to introduce the pathway in crops that do not metabolise or catabolise fructan and that therefore do not exhibit the problems we are facing in chicory. Moreover, the introduction of fructan biosynthesis in non-fructan species might render new types and different sizes of fructan not yet present in normal fructan-producing plants.

Fructan biosynthesis in chicory

In order to create the knowledge basis for the modification of inulin DP and yield in chicory we studied the regulation of fructan biosynthesis. We analysed the relationships between carbohydrate content, mDP, yield, gene expression and the activities of the enzymes involved in inulin biosynthesis. 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) [12]. 1-SST primarily catalyses the biosynthesis 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 fructan molecules with a higher DP. 1-FFT increases the mDP when using 1-kestose (the shortest fructan) as a fructosyl donor. This reaction converts 1-kestose into sucrose, which is not a fructan, and a fructosyl unit that is used to elongate a pre-existing fructan molecule. Under 1-kestose limiting conditions such as low 1-SST activity, 1-FFT can instead catalyse the transfer of fructosyl units from a fructan molecule onto sucrose [13, 14]. 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 resulting in the formation of fructose and a lower DP inulin [15]

In this thesis I defined three successive phases in the growing season of chicory, based on changes observed in the pattern of inulin accumulation. Phase one starts about four weeks after sowing with the onset of taproot thickening and inulin biosynthesis. This phase is characterised by an increase of inulin mDP and yield. In the second phase, the mDP decreases, while the inulin yield still increases. The third phase starts while the mDP continues to decrease, the yield becomes constant and the taproot growth slows down. Concomitant with the onset of taproot thickening in phase I the activity of the fructan synthesis enzymes, 1-SST and 1-FFT is induced. The activity of 1-SST increases rapidly and reaches a maximum three weeks later [16]. Ten weeks after sowing the activity decreases until the end of phase II (in November) when only 10% of the activity remains. We showed that this decrease is not regulated by temperature or day length (Chapter 2). The activity of 1-FFT follows a different pattern, the activity increases slowly and stabilises after four weeks. The activity of 1-FFT remains constant during the rest of the growing season. The activity of 1-FEH is induced by low ambient temperatures and plays an important role in phase III, at the end of the growing season. In the first few months mainly short inulin chains are formed as a direct result of the fructosyltransferase activities. In the second part of phase I, inulin molecules with a DP of up to 25 are accumulated. At the beginning of September, at the end of phase I, the mDP reaches a maximum of about 16, and then decreases. The extent of the decrease is dependent on the cultivar [17] and growing conditions (Chapter 2). 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 [18]. The result is

a decrease in mDP but an increase of yield per taproot. We show that activity of 1-FEH and the remaining 1-SST may also have an influence on inulin biosynthesis in phase II. Later in the season, the mDP further decreases because of the fructan exohydrolase activity of FEH I that is induced at low temperatures as shown in our climate room experiments where different induction temperatures for FEH I were tested (Chapter 2). The degradation of inulin is further enhanced by FEH II later in the season, when temperatures of 4°C and below induce this second exohydrolase. Although all these observations point towards a process of inulin degradation, the inulin concentration remains constant, mainly because the concentrations of 1-kestose and 1-nystose increase. This is most probably not a result of FEH activity but a result of *de novo* synthesis by 1-FFT activity.

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 of 1-FEH activity negatively influences the mDP. Based on this knowledge we tried to bring the first two strategies, to increase yield and polymer length, into practice. The first strategy was to prevent the degradation of inulin in autumn, which might be achieved by down-regulation of FEH I. It was envisioned that the absence of FEH I activity might prevent depolymerisation, although a reduction of mDP by 1-FFT and FEH II might still occur. An *FEH I* antisense fragment driven by the constitutive CaMV 35S promoter was introduced into chicory and *FEH I* expression was indeed reduced by the antisense *FEH I* expression. However, this decreased *1-FEH* expression level had only minor effects on the mDP, when the transgenic plants were subjected to a cold treatment, normally inducing specifically *FEH I.* The second approach to increase inulin yield and polymer length was to enhance inulin biosynthesis. We tried to accomplish this by the overexpression of a heterologous *1-SST* during the growing season. The additional polymerisation activity by the extra 1-SST enzyme would maintain a higher concentration of 1-kestose and a lower concentration of sucrose in phase II and III; two factors that might contribute to limiting 1-FFT-mediated depolymerisation. Plants harbouring an extra *1-SST* gene were grown under field resembling conditions to study the effect of the introduced gene. Comparison of the control plants with transgenic plants harbouring the *Helianthus tuberosus SST-II* showed that the transgene significantly contributed to the total activity of 1-SST. However, this seemed not to affect the mDP at the end of the growing season.

In conclusion, our attempts to modify the inulin metabolism in chicory in order to increase the mDP comprised the introduction of extra genes and the knockdown of an exohydrolase gene. Although the introduced genes were functional and the knockdown did result in a reduction in mRNA expression in the transgenic plants, only slight changes in the mDP or inulin composition were observed. The reasons for this 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 mechanism of fructan degradation, which involves three exohydrolase enzymes and 1-FFT, is highly complex.

Regulation of fructan biosynthesis in chicory

To develop improved strategies for tailor-made fructan production we studied the localization and regulation of fructan biosynthesis in chicory. A result of this study might be to come up 1) promoters that can be used to fine-tune the spatial and temporal expression of transgenes and 2) transcription factors that are putatively involved in fructan biosynthesis regulation that could be used to enhance complete fructan biosynthesis in transgenic plants.

Localization of inulin biosynthesis in the root

The study on the localization of inulin biosynthesis in the roots of chicory showed that 1-SST and 1-FFT enzyme activity increased towards the inside of the roots and that the highest expression occurred around the phloem tissue (Chapter 3) confirming the results of Kusch *et al*.[19]. In the outer tissues, however, 1-SST and 1-FFT enzyme activity was not absent. I showed that *1-SST* and *1-FFT* expression were maximally four times lower at the outside of the root. The presence of inulin biosynthesis throughout the root agrees with the results of Van Wonterghem *et al*, who showed that the 1-FFT protein was located in parenchyma cells surrounding the phloem and more to the outside of the root [20]. Inulin crystals were also found throughout the root; in radial axis [21]. In *Taraxacum officinale* higher enzyme activity and higher inulin accumulation was found around the phloem tissue, but inulin was also found in xylem vessels [15]. *S*imilar results were found in *Helianthus tuberosus* [22] and *Gomphrena macrocephala* [23]. I showed that the concentration of glucose and fructose correlates with the level of fructosyl transferase expression in the root (Chapter 3). The correlation of the biosynthesis-gene expression and fructose cannot be explained by the mode of action of the biosynthesis proteins. However, the good correlation of *1-SST* expression and glucose may be explained by the fact that glucose is one of the products of the 1-SST reaction as shown earlier in chicory [24] (Chapter 2) and in *Taraxacum officinale* [15]. Transport of inulin throughout the plant has only been demonstrated for 1-kestose [25] but not for longer inulin [26], suggesting that biosynthesis is present throughout the root. In conclusion: most inulin accumulation occurs near the phloem, where sucrose is imported into the root, but is not restricted to this area.

Carbohydrate-mediated regulation of fructan biosynthesis

The regulation of fructan biosynthesis has been subject of several studies [19, 27-29] and (Chapter 2). The degradation of fructan was shown to be regulated by temperature, drought and sucrose. Several studies showed that an increase of *FEH* expression and corresponding enzyme activity correlate with drought and temperature [19, 29-34]. Other studies have shown that the expression of fructan biosynthesis genes in excised leaves was enhanced upon feeding with sucrose [31, 35-38] or/ and glucose [19, 39]. The feeding experiments on excised leaves of chicory that I describe in Chapter 3 revealed a positive regulation of fructan biosynthesis genes upon sucrose and glucose

feeding. Induction of inulin biosynthesis by fructose feeding was much lower, especially for 1-FFT. Secondly, using the promoter of *1-FFT* driving GUS-expression showed that the promoter is induced upon carbohydrate feeding in transgenic chicory, potato and Arabidopsis and thereby showed that the regulation is most probably a common mechanism in the plant kingdom. Comparison of the promoters to other fructan biosynthesis promoters showed that several well defined regulatory elements involved in sugar regulation are present in the *1-SST* and *1-FFT* promoter. The *de novo* sequence motif search that we applied on the promoter sequences from chicory and other plants resulted in several putative TF-binding sites. In Arabidopsis, a significant overrepresentation of genes, possessing motif 9, were up regulated upon light and sugar feeding, indicating that the motif could play a role in carbohydrate regulation, both in chicory and Arabidopsis. Recent results of an expression correlation study in wheat revealed a positive correlation of *1-SST* and *6-SFT* with a *MYB* gene, TaMYB13 [40]. The core DNA-binding sequence was present in *1-SST* and *6-SFT* from wheat and shared homology with the motif 9 that I found. The Arabidopsis homologues of TaMYB13 are AtMYB48 and AtMYB59 [40] and they are expressed in the cotyledons and the root of the seedling, as shown in the eFP browser of the Bio-array resource for plant Biology (BAR) [41]. In conclusion: my work resulted in a promoter possibly suitable for heterologous gene expression in chicory and showed that among others, the MYB transcription factors could be involved in fructan biosynthesis regulation in chicory just as in Arabidopsis and wheat. Isolation of the MYB homologue of chicory and its overexpression in chicory would be an interesting experiment and would show the relevance of strategy five.

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

There has been an array of alternative industrial crops genetically modified with fructosyltransferase genes, from either bacterial or plant origin, for the production of fructan. Table I summarises these studies and shows the fructan yield and the characteristics of the transgenic plants. 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 plant phenotype and fitness was in most cases negatively influenced. The yield remained low in most transgenics, probably because the levansucrase was not targeted to the vacuole.

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

The expression of fructosyltransferase genes of plant origin in non-fructan accumulating plants allowed the accumulation of substantial amounts of fructan in the transgenics and did not lead to altered performance of the host plant (see Table I for an overview).

In order to explore the 6th strategy: the introduction of the fructan biosynthetic pathway in non-fructan metabolizing and catabolizing plant species we introduced the *SST-I* from Jerusalem artichoke into potato and maize. Carbohydrate analysis of the transgenic plants showed that in both species the gene is functional and inulin accumulates in the respective storage organs, the maize kernel and potato tuber. The maximal inulin yield for potato, maize, and super sweet maize was approximately 3, 5, and 9 µmol/gFW resp., and consisted mostly of 1-kestose, 1-nystose and DP5 fructan. The production of the latter two molecules is normally attributed to 1-FFT activity. However, it has been shown before that *in vitro* 1-SST alone can catalyse the formation of fructan larger than 1-kestose [4]. In another study, *SST-I* from Jerusalem artichoke was introduced into 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/g FW fructan [42]. The 1-SST from globe artichoke was successfully introduced into sugarcane [43]. The yield in sugarcane, however, was only 8 -112 nmol/g FW. This small amount of fructan did not negatively influence the sucrose concentration. Moreover, the sucrose levels in the 1-SST plants were even higher than in wild-type plants. The author assumed that carbon partitioning was changed by the accumulation of 1-kestose. He also showed that invertase was not the cause of this low level of 1-kestose, neither the expression level of the introduced *1-SST*. In rice three different *1-SST* genes were introduced and shown to be functional, resulting in detectable amounts of fructan in the leaves [44, 45]. Kawakami *et al*. showed that leaves from transgenic rice expressing *1-SST* from *Triticum spp*. accumulated fructan up to 16 mg/g FW. 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 [44]. The general conclusion from these studies is that 1-kestose and longer inulin can be produced by 1-SST in non-fructan accumulating plants.

The production of other types of fructan was also shown to be possible. The heterologous expression of *sucrose: fructan 6-fructosyltransferase* (*6-SFT*) in tobacco and rice resulted in the accumulation of kestose and a series of unbranched fructan of the phlein type [44, 46]. 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/g FW) [44]. This low yield could relate to the findings of Duchateau who showed that the enzyme exhibits much higher invertase activity than fructosyltransferase activity when incubated with sucrose as the sole substrate *in vitro* [47]. Since wild-type tobacco and rice plants do not accumulate fructan but do contain sucrose, the introduced 6-SFTs were probably not able to exert sufficient fructosyltransferase activity to accumulate large amounts of fructan in these crops.

In order to further explore the 6th strategy we introduced both 1-SST and 1-FFT from Jerusalem artichoke into potato and maize and demonstrated that the fructan pathway is fully transferable. The transgenic plants containing both genes did not only accumulate fructan with a higher DP than the *1-SST*-expressing plants but showed inulin

chain length distributions comparable to that of the species from which 1-FFT originated. Our results on potato and maize transgenic plants and the results on transgenic sugar beet also enriched with the fructan biosynthesis pathway from Jerusalem artichoke by Koops *et al*. [48, 49] showed the same inulin chain length distribution in the transgenic plants as in Jerusalem artichoke. These results are supported by the findings of Hellwege *et al*. [50] 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 into sugar beet resulted in the accumulation of a branched fructan with a profile closely resembling that of onion [51], 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 into non-fructan-accumulating crop plants. The genes were fully functional and their expression and accumulation of fructan did not affect the plant phenotype. The chain length distribution of the fructan produced was dependent on the origin of the FFT.

Table I: Fructan production in genetically engineered crops that originally do not accumulate fructan.
N.R.: not reported; eq. hex.: equivalent hexoses.
This table was based on a previously published table [10].

Crop transformed	Gene introduced	Gene's origin	Enzyme targeting	Tissue analyzed	Soluble sugar content of the transformed tissue			Phenotype changes	Literature cited
					Sucrose	Fructan	Glucose		
Maize	*sacB*	*Bacillus amyloliquefaciens*	Vacuole	Seeds	N.R.	10 – 80 mg/g DW	N.R.	no effect on the phenotype	Caimi et al., 1996
Maize	*sacB*	*Bacillus amyloliquefaciens*	Cytoplasm	Seeds	N.R.	16 – 18 mg/g DW	N.R.	severe reduction of seed DW	Caimi et al., 1997
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 + 1-FFT*	*Helianthus tuberosus*	Vacuole	Seeds	24 mg/gFW	20 mg/gFW	4 mg/gFW	N.R.	Stoop et al.,2007
Petunia	*1-SST*	*Helianthus tuberosus*	Vacuole	Leaf	0.41 mg/gFW	0.47 mg/FW	N.R.	no effect on the phenotype	Van der meer et al., 1998
Petunia	*1-FFT*	*Helianthus tuberosus*	Vacuole	Leaf	N.R.	0 mg/FW	N.R.	no effect on the phenotype	Van der meer et al., 1998
Potato	*sacB*	*Bacillus subtilis*	Vacuole	Leaves (old)	N.R.	350 mg/g DW	N.R.	reduced starch	Van der Meer et al., 1994
Potato	*sacB*	*Bacillus subtilis*	Vacuole	Microtubers	N.R.	50 mg/g DW	N.R.	reduced starch	Van der Meer et al., 1994
Potato	*sacB*	*Bacillus subtilis*	Vacuole	Leaves	N.R.	5 mg/g FW	N.R.	stunted growth	Pilon-Smits et al. 1996
Potato	*sacB*	*Bacillus subtilis*	Vacuole	Tubers	3-11 mg/gFW	11 mg/gFW	0.5-5 mg/gFW	reduced starch content and browning	Pilon-Smits et al. 1997
Potato	*sacB*	*Bacillus amyloliquefaciens*	Cytoplasm	Tubers	N.R.	5-50mg/g DW	N.R.	reduced starch and tuber DW	Caimi et al., 1997
Potato	*1-SST*	*Cynara scolymus*	Vacuole	Tubers	14 µmol/g FW	19 µmol/gFW	N.R.	N.R.	Hellwege et al., 1997
Potato	*1-SST + 1-FFT*	*Cynara scolymus*	Vacuole	Tubers	15 µmol/g FW	50 mg/g DW	3,9 µmol/g FW	no effect on the phenotype	Hellwege et al., 2000
Potato	*sacB*	*Bacillus subtilis*	plastid	Leaf	90 µmol/g FW	66 mg/gFW	25 µmol/gFW	N.R.	Gerritis et al. 2001
Potato	*1-SST*	*Helianthus tuberosus*	Vacuole	Tubers	0.08 mg/gFW	1.4 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
- (starch-deficient)	*LSC*	*Erwinia amylovora*	Vacuole	Tubers	N.R.	70-120 mg/gDW	82-201 mg/gDW	no effect on the phenotype	Röber et al., 1996
- (starch-deficient)	*LSC*	*Erwinia amylovora*	Apoplasm	Tubers	N.R.	190 mg/gDW	50 mg/gDW	reduced tuber FW	Röber et al., 1996
- (starch-deficient)	*LSC*	*Erwinia amylovora*	Cytoplasm	Tubers	N.R.	0 mg/gDW	N.R.	N.R.	Röber et al., 1996
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., 2009
Rice	*1-SST*	*Smallanthus sonchifilus*	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., 2010

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*	*Bacillus subtilis*	Vacuole	roots/shoot	N.R.	0.5 mg/g DW	N.R.	enhanced drought resistence	Pilon-Smits et al. 1999
Sugar beet	*1-SST + 6-FFT*	*Allium cepa*	Vacuole	Roots	29 mg/g FW	66.4 mg/g FW	34 mg/g FW	no effect on the phenotype	Weyens et al., 2004
Sugarcane	*lsdA*	*Acetabacter diazotrophicus*	Vacuole	N.R.	N.R.	N.R.	N.R.	no effect on the phenotype	Truijllo 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 et al. 2007
Sugarcane	*ftfA*	*Lactobacilus sanfranciscensis*	cytosol/cel wall	Internodes	32-40mg/gW	0.01 mg/g f	N.R.	reduced total carbohydrate	Bauer et al. 2012
Sweet potato	*lsdA*	*Acetabacter diazotrophicus*	Vacuole	N.R.	N.R.	N.R.	N.R.	N.R.	Truijllo et al.,2000
Tobacco	*sacB*	*Bacillus subtilis*	Vacuole	Leaves	0.14mg/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 resistence	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	50mg/gDW	0.5-3 mg/gDW	N.R.	no effect on the phenotype	Sprenger et al., 1997
Tobacco	*sacB*	*Bacillus 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*	*Bacillus amyloliquefaciens*	Cytoplasm (inducible promotor)	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*	*Zymomonas mobilis*	Cytoplasm	Leaves	N.R.	visible on TLC	N.R.	enhanced osmotic stress resistence	Park et al. 1999
Tobacco	*sacB*	*Bacillus subtilis*	plastid	Leaf	N.R.	20 mg/gFW	N.R.	N.R.	Gerrits et al. 2001
Tobacco	*1-SST*	*Lactuca sativa*	Vacuole	Leaves	0,1-3.8 mg/gFW	40-110 ug/gFW	N.R.	enhanced freezing tolerance	Hui-Juan et al 2007
Tobacco	*LsdA*	*Gluconacetobacter diazotrophicus*	Vacuole	Leaves	N.R.	70 mg/gFW	N.R.	older leaves bleached prematurely and became rigid	Banguela et al, 2011
Tobacco	*1-SST, 1-FFT or 6-FFT*	*Triticum spp.*	Vacuole	shoots	N.R.	6.1–10.2 mg/gFW	N.R.	tolerance to abiotic stresses	Bie et al. 2012
Tobacco	*1-SST+ 1-FFT*	*Triticum spp.*	Vacuole	shoots	N.R.	36,3 mg/gFW	N.R.	tolerance to abiotic stresses	Bie et al. 2012
Tobacco	*1-SST+ 6-SFT*	*Triticum spp.*	Vacuole	shoots	N.R.	610,6 mg/gFW	N.R.	tolerance to abiotic stresses	Bie et al. 2012
Tobacco	*1-FFT+ 6-SFT*	*Triticum spp.*	Vacuole	shoots	N.R.	29,9 mg/gFW	N.R.	tolerance to abiotic stresses	Bie et al. 2012
Tobacco	*1-SST+ 1-FFT + 6-FFT*	*Triticum spp.*	Vacuole	shoots	N.R.	75,7 mg/gFW	N.R.	tolerance to abiotic stresses	Bie et al. 2012
White clover	*ftf*	*Streptococcus salivarius*	Vacuole	Leaves	1.3 mg/g FW	3.1 mg/g FW	0.6 mg/g FW	reduced growth	Jenkins et al., 2002

Physiological factors influencing tailor-made fructan yield in heterologous crops

Tailor-made fructan, the synthesis of fructan with the desired chain length and linkage type, largely relies on the choice of the appropriate genes as we showed in the previous paragraphs. The proof of principal for tailor-made fructan was delivered with transgenic sugar beet as shown by Koops *et al*. [48]. Different combinations of *1-SST* genes and *1-FFT* genes resulted in transgenic sugar beet lines 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 [51]. Beside the origin of the genes used for biosynthesis, the availability of substrate and competition with other carbohydrate biosynthesis pathways are important for the accumulation of fructan.

Sucrose availability determines fructan yield

The studies described above show that the host crop is crucial in determining yield. When comparing the transgenic potato described in Chapter 3 with the transgenic sugar beet described by Sévenier *et al*. [42], both harboring the same *1-SST* from Jerusalem artichoke, it became clear that there was a big difference in yield between these two production platforms. Sugar beet accumulated 40 times more inulin than potato. Similarly, a 55 times higher inulin yield was found in a starch deficient maize line expressing *1-SST* and *1-FFT* from Jerusalem artichoke than in the starch accumulating parent expressing the same genes (Chapter 3). Both sugar beet and the starch deficient maize accumulated high levels of sucrose. This positive correlation between the amount of fructan accumulated and the availability of sucrose is also found by Xue *et al*. in barley-outcrossing populations [52]. In contrast to maize, where we found that sucrose is the limiting factor, our results on potato do not show that sucrose is the limiting factor for inulin biosynthesis. This could also mean, as stated by Morandini *et al*., that the enzymes require a sufficiently high concentration of sucrose to attain a significant catalytic rate [53]. In general, it can be concluded that sucrose availability appears to be a determining factor for the fructan yield in these transgenic plants.

Competition of fructan accumulation with starch accumulation

Many plant species used as a platform crop for the production of fructan also accumulated starch next to sucrose, which might be a competing carbohydrate synthesis pathway using the same substrate as the fructan biosynthesis pathway. Our results in maize clearly demonstrated this competition between endogenous starch and fructan biosynthesis when we compared the amounts of fructan accumulated in transgenic starch-deficient maize (storing sucrose) with the amount accumulated in transgenic dent maize (starch accumulating) expressing the same fructosyltransferase gene. While the transgenic starch deficient maize accumulated on average 20 mg/g FW fructan in the kernel, the starch accumulating transgenics accumulated 55-fold less fructan. We did not determine the starch content in the transgenic potato plants, but in other studies describing potato as host for the production of fructan, reduced amounts of starch were reported [54-57].

Interestingly, Pilon-Smits and co-workers showed that expression of *sacB* in potato caused an inverse correlation of starch with fructan content [56]. It can be concluded from these studies that production of fructan in starch accumulating crops is not favorable because the competition for sucrose between the starch production and the fructan biosynthesis will affect the synthesis of both or one of the storage carbohydrates leading to a reduction in the potential fructan yield.

Fructan stability in the new fructan storage organs

In combination with the availability of substrate and the presence of competing pathways, the absence of degrading enzymes is important for the production of tailor-made inulin in new platform crops. Enzymes capable of degrading fructan are fructan exohydrolases (FEH), present in fructan accumulating plants, where they are important for the remobilization of the stored carbohydrates [58]. In non-fructan plants FEH is not present, although in sugar beet (leaf) and Arabidopsis FEH homologues have been found [58] that could play a role in degradation of the produced fructan. However, invertases may play a more important role in degrading fructan in putative platform crops. Invertases are ß-fructofuranosidases that can hydrolyse ß-Fru-containing oligosaccharides [59] like inulin. Our study on the stability of fructan in transgenic potato expressing *1-SST* and *1-FFT* showed no degradation of inulin during tuber development and maturation. Only cold-storage of potato tubers resulted in an almost complete breakdown of the accumulated fructan. Storage of potato tubers at low temperatures is known to increase invertase activity and hence cause accumulation of reducing sugars and sucrose [60]. In transgenic maize expressing fructan biosynthesis genes inulin is broken down during the late stages of kernel development. One explanation is that maize fructan and starch biosynthesis deplete the sucrose pool, which up-regulates soluble acid invertase *Ivr-1*. Another explanation is that the degradation of fructan is the result of mechanical damage of the storage cell-compartment, as starch granules may destroy the maize vacuolar compartment where the fructan is synthesized and stored, resulting in the degradation of inulin by invertase in the cytoplasm. This latter explanation was supported by the observation that inulin production in 'high sucrose-low starch' maize lines results in limited or no inulin degradation. The accumulation of fructan in sugar beet expressing *1-SST* was not hindered by invertase in the taproot due to the complete absence of the enzyme. In leaves however, the fructan yield was only 8% of that in the root, most probably due to fructan degradation by vacuolar invertase [42]. In rice, invertase activity was found in the panicle and the flag leaf. In rice it is known that several vacuolar invertases have the ability to degrade fructan polymers. However, in peduncles grown under normal conditions only one invertase gene is expressed [61]. The possibility to accumulate large amounts of fructan in the peduncle has to be investigated.

In conclusion, these data suggest that invertase activity and morphological changes of the storage organ may play a critical role in determining the stability and level of tailor-made inulin polymers produced in both transgenic monocot and dicot crops.

Platform crops for tailor-made fructan

A suitable platform crop for fructan production preferably shows a high productivity, possesses a large storage organ, accumulates sucrose and produces little or no starch. Furthermore, a processing chain for extraction of raw material should be available. The crops that have been evaluated in this chapter are having a suitable processing chain. In the production process of starch from potato the protamylasse is concentrated and added to feed, used as fertilizer or further processed for the extraction of protein. Isolation of inulin from the protamylasse could be feasible. Potato, however, produces large quantities of starch that compete for sucrose in the tuber. Maize is a well know industrial crop with an established processing chain. However, substantial amounts of inulin only accumulated in super sweet corn that is harvest immaturely, cannot be stored and misses a processing chain as it is eaten fresh. Tobacco was used in several studies for fructan production, but is more a model organism than a platform crop for tailor-made fructan production. The disadvantages of the crops described above are missing in other crops like sugar beet. Sugar beet is able to accumulate high levels of sucrose (200 mg/g FW), resulting in a sugar yield of 10-14 tons/hectare, which almost certainly explains why sugar beet was also shown to be a successful platform for the production of fructan [42, 48, 51]. 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 [51]. Another potentially powerful platform crop for fructan production is sugarcane (*Saccharum spp L.*) because it has a high sucrose content, 500 mg/g DW in mature internodes [62], and a well-established agronomic and processing chain. The sugar production by sugarcane (6-14 tons/hectare) is comparable to sugar beet. Nicholson *et al*. successfully introduced *1-SST* from globe artichoke into sugarcane and achieved 1-kestose production under field resembling conditions [43]. The yield, however, was at most 112 nmol/g FW, which is 1000 times lower than in transgenic sugar beet expressing *1-SST.* Additional studies will be necessary to address the full potential of sugar cane as a 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 continents than the crops mentioned previously. It has been shown that *FT* genes could be expressed in rice. Transgenic rice expressing *1-SST* accumulated up to 16 mg/g FW of fructan in leaves [44]. Although the fructan concentration was 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, adding extra value. In that case, a new processing chain should be developed 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.

General conclusions and outlook

In this chapter I discussed the research on the regulation and modification of fructan synthesis in *fructan accumulating* plants as well as on the introduction of fructan synthesis in *non-fructan accumulating* plants. I evaluated the reported effects of fructan accumulation on endogenous storage carbohydrates and on phenotypes and discussed the different genes and crops that have been used with a focus on the fructan yield.

To date, mostly chicory is used for the commercial production of fructan. We studied the relationships between carbohydrate content, mDP, yield, gene expression and enzyme activities related to inulin metabolism in order to get insight in the regulation of the DP by internal and external factors. Our transgenic strategies to increase the mDP at harvest were overexpression of *1-SST* and down-regulation of FEH I by an antisense approach. Even though the overexpression of *1-SST* and down-regulation of *FEH I* was successful, the effect on the mDP at harvest was minimal. More effective alternatives for the silencing of 1-*FEH* would be the RNAi strategy or a site-directed mutagenesis strategy that should be applied to all three *FEH*-genes (*FEH Ia, b* and *FEH II*). The absence of 1-FEH I activity would reduce the inulin depolymerisation process even though reduction of mDP by 1-FFT might still occur. The absence of 1-FEH II activity would reduce this process even further, especially later in the season. Moreover, the absence of any 1-FEH activity would limit the production of free fructose, which in turn would result in the reduction of inulo-*n*-ose amounts.

The study on the regulation of inulin biosynthesis in chicory showed that it is induced by sucrose and/or glucose, and that the induction mechanism for fructan biosynthesis is part of a universal plant signaling pathway also present in chicory, potato and Arabidopsis. A strategy to enhance fructan biosynthesis in chicory based on this knowledge would be the expression of heterologous genes under control of the *1-FFT* promoter. In addition to the sugar inducibility, the transcript levels of *1-FFT* are high (unpublished results), which makes this promoter very well suitable to enhance 1-SST activity in the later part of the growing season of chicory when the sucrose concentration is high and the 1-SST activity in wild-type plants is low. Also the introduction of a heterologous 1-FFT with higher affinity for high DP inulin would be ideally performed under control of the *1-FFT* promoter as the expression would coincide with the availability of high amounts of substrate. Another strategy to enhance the mDP in chicory would be the overexpression of transcription factors involved in inducing fructan biosynthesis. Tools for this strategy resulted from the *in silico* analysis of the promoters, pinpointing several transcription factors putatively involved in regulation of fructan synthesis gene expression. A candidate would be the TaMYB133 homologue of which we found the recognition site in our *de novo* sequence motif search. TaMYB13 was shown to be involved in wheat fructan biosynthesis [40]. The overexpression of such a transcription factor could result in an enhanced activation of 1-SST and 1-FFT and an increased fructan accumulation or extended period of fructan accumulation. As a next strategy, the large influence of cold (temperature

below 10°C) on the degradation of inulin could be partially prevented by growing chicory in more moderate climates, for example in Chile or India. Although other stresses (for example drought and nutritional stress) may still be important factors in degradation of fructan polymers. Selecting for the desired properties, so low 1-FEH, continuous 1-SST and high affinity of 1-FFT for HDP inulin in breeding would also be a good strategy. The disadvantages of chicory during the growing season concerning break-down of fructan DP, and the so far encountered difficulty to modify the native inulin biosynthesis pathway via genetic modification, make 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 /hectare). On the other hand, the new crops have the advantage of lacking a breakdown mechanism and providing a clean starting point for the tailor-made fructan production. We showed that potato and maize are putative production platforms, although sugar beet, sugarcane and rice seem to be the most promising alternative production platforms. The production of fructan in these crops could be increased by optimizing the sugar availability in the crops by selecting cultivars with a natural high sugar content as shown by Xue *et al*. [52] or by using starch deficient mutants, as was shown in starch deficient maize (chapter 3). To date, a wide spectrum of genes has been isolated from different species allowing the fine-tuning of 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 activity with two different 1-FFTs, one having high affinity for 1-kestose for the synthesis of short polymers, and the second having a relatively 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 *Helianthus tuberosus* [4, 63] combined with the high DP *1-FFT* from *Echinops ritro* [14]. 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 [46]. 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 could possibly lower the affinity for sucrose and fructose as acceptor substrate and prevent the back-transfer of fructosyl units onto these acceptor molecules, thereby preventing 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*. when changing a 6G-FFT/1-FFT into an 1-SST [64]. Fructosyltransferase enzyme engineering might allow designing enzyme tools for tailor-made fructan synthesis with desired properties such as a specific linkage type and polymer length. We showed that many possibilities for tailor-made fructan synthesis in crops exist and that some already have been proven.

References

[1] R. Dedonder, E.F. Neufeld, V. Ginsburg, Levansucrase from *Bacillus subtilis*, in: Methods in Enzymology, Academic Press, 1966, pp. 500-505.

[2] G.D. Bonnett, I.M. Sims, R.J. Simpson, A.J. Cairns, Structural diversity of fructan in relation to the taxonomy of the Poaceae, New Phytologist, 136 (1997) 11-17.

[3] W. Van den Ende, M. Coopman, S. Clerens, R. Vergauwen, K. Le Roy, W. Lammens, A. Van Laere, Unexpected Presence of Graminan- and Levan-Type Fructans in the Evergreen Frost-Hardy Eudicot *Pachysandra terminalis* (Buxaceae): Purification, Cloning, and Functional Analysis of a 6-SST/6-SFT Enzyme, Plant Physiology, 155 (2011) 603-614.

[4] A.J. Koops, H.H. Jonker, Purification and Characterization of the Enzymes of Fructan Biosynthesis in Tubers of *Helianthus tuberosus Colombia* (II. Purification of Sucrose: Sucrose 1-Fructosyltransferase and Reconstitution of Fructan Synthesis in Vitro with Purified Sucrose: Sucrose 1-Fructosyltransferase and Fructan: Fructan 1-Fructosyltransferase), Plant Physiology, 110 (1996) 1167-1175.

[5] C.J. Pollock, Tansley Review No. 5. Fructans and the Metabolism of Sucrose in Vascular Plants, New Phytologist, 104 (1986) 1-24.

[6] R.A. Burne, Y.-Y.M. Chen, D.L. Wexler, H. Kuramitsu, W.H. Bowen, Cariogenicity of *Streptococcus mutans* Strains with Defects in Fructan Metabolism Assessed in a Program-fed Specific-pathogen-free Rat Model, Journal of Dental Research, 75 (1996) 1572-1577.

[7] S. Kasapis, E.R. Morris, M. Gross, K. Rudolph, Solution properties of levan polysaccharide from *Pseudomonas syringae pv. phaseolicola*, and its possible primary role as a blocker of recognition during pathogenesis, Carbohydrate Polymers, 23 (1994) 55-64.

[8] G.L. Cote, J. Ahlgren, Metabolism in microorganisms, Part I. Levan and Levansucrase, in: M. Suzuki, N. Chatterton (Eds.) Science and Technology of Fructans, CRC Press, Boca Raton, FL, 1993, pp. 141-169.

[9] E. Pilon-Smits, M. Ebskamp, M.J. Paul, M. Jeuken, P.J. Weisbeek, S. Smeekens, Improved Performance of Transgenic Fructan-Accumulating Tobacco under Drought Stress, Plant Physiology, 107 (1995) 125-130.

[10] R.E. Sévenier, J. van Arkel, J.C. Hakkert, A.J. Koops, Fructan: nutritional significance, application, biosynthesis, molecular biology and genetic engineering, in: Plant Genetic Engineering, Metabolic engineering and molecular farming, Studium Press, Houston, 2006, pp. p. 31 - 55.

[11] D. Wittouck, K. Boone, S. Bulcke, S. Debosschere, A. Vandaele, M. Demeulemeester, A. Calus, Industriële cichorei, in, Onderzoeks- en voorlichtingscentrum voor land- en tuinbouw, Rumbeke, 2002, pp. 79-84.

[12] J. Edelman, T. Jefford, The mechanism of fructosan metabolism in higher plants as exemplified in *Helianthus tuberosus*, New Phytologist, 67 (1968) 517-531.

[13] W. Van den Ende, J.D. Roover, A. Van Laere, In vitro synthesis of fructofuranosyl-only oligosaccharides from inulin and fructose by purified chicory root fructan: fructan fructosyl transferase, Physiologia Plantarum, 97 (1996) 346-352.

[14] R. Vergauwen, A. Van Laere, W. Van den Ende, Properties of Fructan: Fructan 1-Fructosyltransferases from Chicory and Globe Thistle, Two Asteracean Plants Storing Greatly Different Types of Inulin, Plant Physiology, 133 (2003) 391-401.
[15] W. Van den Ende, A. Michiels, D. Van Wonterghem, R. Vergauwen, A. Van Laere, Cloning, Developmental, and Tissue-Specific Expression of Sucrose: Sucrose 1-Fructosyl Transferase from *Taraxacum officinale*. Fructan Localization in Roots, Plant Physiology, 123 (2000) 71-80.
[16] N. Druart, J. De Roover, W. Van den Ende, P. Goupil, A. Van Laere, S. Rambour, Sucrose assimilation during early developmental stages of chicory (*Cichorium intybus L.*) plants, Planta, 212 (2001) 436-443.
[17] K. Koch, R. Andersson, I. Rydberg, P. Åman, Influence of harvest date on inulin chain length distribution and sugar profile for six chicory (*Cichorium intybus L.*) cultivars, J. Sci. Food Agric., 79 (1999) 1503-1506.
[18] W. Van den Ende, A. Michiels, J. De Roover, A. Van Laere, Fructan biosynthetic and breakdown enzymes in dicots evolved from different invertases. Expression of fructan genes throughout chicory development, TheScientificWorldJournal-[electronic-resource], 2 (2002) 1281-1295.
[19] U. Kusch, Dissecting the regulation of fructan active enzymes in *Cichorium intybus*, in: Combined faculties for natural Science and for mathematics of the Ruperto-Carola University, University of Heidelberg, Heidelberg, 2009.
[20] D. Van Wonterghem, W. Van den Ende, A. Van Laere, Proceedings of the Eight Seminar on Inulin, in: A. Fuchs (Ed.) The Eight Seminar on Inulin, European fructan Association, Lille, France, 1999, pp. 59-62.
[21] D. Desmet, Histologische studie van de witloofwortelstructuur en van *in vitro* geïnduceerde meristemen, in: Toegepaste plantenwetenschappen, Katholieke Universiteit Leuven, Leuven, 1997, pp. 133.
[22] M. Ernst, Histochemische Untersuchungen auf Inulin, Stärke und Kallose bei *Helianthus tuberosus* L. (Topinambur), Angewandte Botanik, 65 (1991) 319-330.
[23] C. Vieira, R. Figueiredo-Ribeiro, Fructose-containing carbohydrates in the tuberous root of *Gomphrena macrocephala* St.-Hil.(Amaranthaceae) at different phenological phases, Plant, Cell & Environment, 16 (1993) 919-928.
[24] W. Van den Ende, A. Van Laere, De-novo synthesis of fructans from sucrose in vitro by a combination of two purified enzymes (sucrose: sucrose 1-fructosyl transferase and fructan: fructan 1-fructosyl transferase) from chicory roots (*Cichorium intybus* L.), Planta, 200 (1996) 335-342.
[25] H.G. Pontis, P. González, E. Etxeberria, Transport of 1-kestose across the tonoplast of Jerusalem artichoke tubers, Phytochemistry, 59 (2002) 241-247.
[26] S.C.H.J. Turk, K. De Roos, P.A. Scotti, K. Van Dun, P. Weisbeek, S.C.M. Smeekens, The vacuolar sorting domain of sporamin transports GUS, but not levansucrase, to the plant vacuole, New Phytologist, 136 (1997) 29-38.
[27] R. Morcuende, S. Kostadinova, P. Pérez, D. Molino, I. Martín, R. Martínez-Carrasco, Nitrate is a negative signal for fructan synthesis, and the fructosyltransferase-inducing trehalose inhibits nitrogen and carbon assimilation in excised barley leaves, New Phytologist, 161 (2004) 749-759.

[28] W. Van den Ende, J. De Roover, A. Van Laere, Effect of nitrogen concentration on fructan and fructan metabolizing enzymes in young chicory plants (*Cichorium intybus*), Physiologia Plantarum, 105 (1999) 2-8.

[29] J. van Arkel, R. Vergauwen, R. Sévenier, J.C. Hakkert, A. Van Laere, H.J. Bouwmeester, A.J. Koops, I.M. van der Meer, Sink filling, inulin metabolizing enzymes and carbohydrate status in field grown chicory (*Cichorium intybus* L.), Journal of Plant Physiology, (2012).

[30] J. De Roover, K. Vandenbranden, A. Van Laere, W. Van den Ende, Drought induces fructan synthesis and 1-SST (sucrose: sucrose fructosyltransferase) in roots and leaves of chicory seedlings (*Cichorium intybus* L.), Planta, 210 (2000) 808-814.

[31] J.-Z. Wei, J.N. Chatterton, S.R. Larson, Expression of sucrose: fructan 6-fructosyltransferase (6-SFT) and myo -inositol 1-phosphate synthase (MIPS) genes in barley (*Hordeum vulgare*) leaves, Journal of Plant Physiology, 158 (2001) 635-643.

[32] H. Hisano, A. Kanazawa, A. Kawakami, M. Yoshida, Y. Shimamoto, T. Yamada, Transgenic perennial ryegrass plants expressing wheat fructosyltransferase genes accumulate increased amounts of fructan and acquire increased tolerance on a cellular level to freezing, Plant Science, 167 (2004) 861-868.

[33] R.S.P. Rao, J.R. Andersen, G. Dionisio, B. Boelt, Fructan accumulation and transcription of candidate genes during cold acclimation in three varieties of *Poa pratensis*, Journal of Plant Physiology, 168 (2011) 344-351.

[34] F. del Viso, A.F. Puebla, C.M. Fusari, A.C. Casabuono, A.S. Couto, H.G. Pontis, H.E. Hopp, R.A. Heinz, Molecular Characterization of a Putative Sucrose:Fructan 6-Fructosyltransferase (6-SFT) of the Cold-Resistant Patagonian Grass *Bromus pictus* Associated With Fructan Accumulation Under Low Temperatures, Plant and Cell Physiology, 50 (2009) 489-503.

[35] A.J. Cairns, G.D. Bonnett, J.A. Gallagher, R.J. Simpson, C.J. Pollock, Fructan biosynthesis in excised leaves of *Lolium temulentum* VII. Sucrose and fructan hydrolysis by a fructan-polymerizing enzyme preparation, New Phytologist, 136 (1997) 61-72.

[36] K. Maleux, W. Van den Ende, Levans in Excised Leaves of *Dactylis glomerata* : Effects of Light, Sugars, Temperature and Senescence, Journal of Plant Biology, 50 (2007) 671-680.

[37] A.L. Winters, J.H.H. Williams, D.S. Thomas, C.J. Pollock, Changes in gene-expression in response to sucrose accumulation in leaf tissue of *Lolium temulentum* L, New Phytologist, 128 (1994) 591-600.

[38] J. Müller, R.A. Aeschbacher, N. Sprenger, T. Boller, A. Wiemken, Disaccharide-mediated regulation of sucrose: fructan-6-fructosyltransferase, a key enzyme of fructan synthesis in barley leaves, Plant Physiology, 123 (2000) 265-274.

[39] T. Suzuki, T. Maeda, S. Grant, G. Grant, P. Sporns, Confirmation of fructans biosynthesized in vitro from [1-13C]glucose in asparagus tissues using MALDI–TOF MS and ESI–MS, Journal of Plant Physiology, (2012).

[40] G.-P. Xue, M. Kooiker, J. Drenth, C.L. McIntyre, TaMYB13 is a transcriptional activator of fructosyltransferase genes involved in β-2,6-linked fructan synthesis in wheat, The Plant Journal, 68 (2011) 857-870.

[41] D. Winter, B. Vinegar, H. Nahal, R. Ammar, G.V. Wilson, N.J. Provart, An "Electronic Fluorescent Pictograph" Browser for Exploring and Analyzing Large-Scale Biological Data Sets, PLoS ONE, 2 (2007) e718.
[42] R.E. Sévenier, R.D. Hall, I.M. van der Meer, H.J.C. Hakkert, A.J. van Tunen, A.J. Koops, High level fructan accumulation in a transgenic sugar beet, Nature Biotech, 16 (1998) 843-846.
[43] T.L. Nicholson, Carbon turnover and sucrose metabolism in the culm of transgenic sugarcane producing 1-kestose, in: Genetics, University of Stellenbosch, Stellenbosch, 2007, pp. 116.
[44] A. Kawakami, Y. Sato, M. Yoshida, Genetic engineering of rice capable of synthesizing fructans and enhancing chilling tolerance, Journal of Experimental Botany, 59 (2008) 793-802.
[45] W. Pan, Y. Sunayama, Y. Nagata, M. Taniguchi, M. Takano, E. Inoue, H. Tamagake, H. Anzai, Cloning of a CDNA Encoding the Sucrose: Sucrose 1-Fructosyltransferase (1-SST) from Yacon and its Expression in Transgenic Rice, Biotechnology & Biotechnological Equipment, 23 (2009) 1479-1484.
[46] N. Sprenger, L. Schellenbaum, K. van Dun, T. Boller, A. Wiemken, Fructan synthesis in transgenic tobacco and chicory plants expressing barley sucrose: fructan 6-fructosyltransferase, FEBS Letters, 400 (1997) 355-358.
[47] N. Duchateau, K. Bortlik, U. Simmen, A. Wiemken, P. Bancal, Sucrose:Fructan 6-Fructosyltransferase, a Key Enzyme for Diverting Carbon from Sucrose to Fructan in Barley Leaves, Plant Physiology, 107 (1995) 1249-1255.
[48] A.J. Koops, R.E. Sévenier, A.J. Van Tunen, L. De Leenheer, Transgenic plants presenting a modified inulin producing profile, in, United states of America, 2003.
[49] J.M. Stoop, J. van Arkel, J.C. Hakkert, C. Tyree, P.G. Caimi, A.J. Koops, Developmental modulation of inulin accumulation in storage organs of transgenic maize and transgenic potato, Plant Science, 173 (2007) 172-181.
[50] E.M. Hellwege, S. Czapla, A. Jahnke, L. Willmitzer, A.G. Heyer, Transgenic potato (*Solanum tuberosum*) tubers synthesize the full spectrum of inulin molecules naturally occurring in globe artichoke (*Cynara scolymus*) roots, Proceedings of the National Academy of Sciences of the United States of America, 97 (2000) 8699-8704.
[51] G. Weyens, T. Ritsema, K. Van Dun, D. Meyer, M. Lommel, J. Lathouwers, I. Rosquin, P. Denys, A. Tossens, M. Nijs, S. Turk, N. Gerrits, S. Bink, B. Walraven, M. Lefèbvre, S. Smeekens, Production of tailor-made fructans in sugar beet by expression of onion fructosyltransferase genes, Plant Biotechnology Journal, 2 (2004) 321-327.
[52] G.P. Xue, J. Drenth, D. Glassop, M. Kooiker, C.L. McIntyre, Dissecting the molecular basis of the contribution of source strength to high fructan accumulation in wheat, Plant Molecular Biology, 81, (2013) 71-92.
[53] P. Morandini, Control limits for accumulation of plant metabolites: brute force is no substitute for understanding, Plant Biotechnology Journal, 11 (2013).253-267
[54] I.M. van der Meer, M. Ebskamp, R. Visser, P.J. Weisbeek, S. Smeekens, Fructan as a New Carbohydrate Sink in Transgenic Potato Plants, The Plant Cell Online, 6 (1994) 561-570.

[55] P.G. Caimi, L.M. McCole, T.M. Klein, H.P. Hershey, Cytosolic expression of the *Bacillus amyloliquefaciens* SacB protein inhibits tissue development in transgenic tobacco and potato, New Phytologist, 136 (1997) 19-28.
[56] E.A.H. Pilon-Smits, M.J.M. Ebskamp, M.J.W. Jeuken, I.M. van der Meer, R.G.F. Visser, P.J. Weisbeek, S.C.M. Smeekens, Microbial fructan production in transgenic potato plants and tubers, Industrial Crops and Products, 5 (1996) 35-46.
[57] P.G. Caimi, L.M. McCole, T.M. Klein, P.S. Kerr, Fructan Accumulation and Sucrose Metabolism in Transgenic Maize Endosperm Expressing a *Bacillus amyloliquefaciens SacB* Gene, Plant Physiology, 110 (1996) 355-363.
[58] W. Van den Ende, B. De Coninck, A. Van Laere, Plant fructan exohydrolases: a role in signaling and defense?, Trends in Plant Science, 9 (2004) 523-528.
[59] A. Sturm, Invertases. Primary Structures, Functions, and Roles in Plant Development and Sucrose Partitioning, Plant Physiology, 121 (1999) 1-8.
[60] R. Zrenner, K. Schüler, U. Sonnewald, Soluble acid invertase determines the hexose-to-sucrose ratio in cold-stored potato tubers, 198 (1996) 246-252.
[61] X. Ji, W. Van den Ende, L. Schroeven, S. Clerens, K. Geuten, S. Cheng, J. Bennett, The rice genome encodes two vacuolar invertases with fructan exohydrolase activity but lacks the related fructan biosynthesis genes of the Pooideae, New Phytologist, 173 (2007) 50-62.
[62] D. Glassop, L. Ryan, G. Bonnett, A. Rae, The Complement of Soluble Sugars in the *Saccharum* Complex, Tropical Plant Biology, 3 (2010) 110-122.
[63] A.J. Koops, H.H. Jonker, Purification and characterization of the enzymes of fructan biosynthesis in tubers of *Helianthus tuberosus* 'Colombia': I. Fructan: fructan fructosyl transferase, Journal of Experimental Botany, 45 (1994) 1623-1631.
[64] B. Lasseur, L. Schroeven, W. Lammens, K. Le Roy, G. Spangenberg, H. Manduzio, R. Vergauwen, J. Lothier, M.-P. Prud'homme, W. Van den Ende, Transforming a Fructan: Fructan 6G-Fructosyltransferase from Perennial Ryegrass into a Sucrose: Sucrose 1-Fructosyltransferase, Plant Physiology, 149 (2009) 327-339.

Summary

Fructan is a polymer of fructose produced by plants and microorganisms. Within the plant kingdom about 45.000 species accumulate fructan as storage carbohydrate in addition to, or instead of, starch. Fructan accumulating species are mainly found in temperate and sub-tropical regions with seasonal or sporadic rainfall. During the last decades, the use of fructan in the (food) industry has rapidly evolved, because of its health promoting characteristics and interesting functional properties. Chicory (*Cichorium intybus* L.) is a biennial taproot-bearing crop plant that is grown for the production of inulin on an industrial scale. Inulin, a ß(2,1) linked linear fructan with a terminal glucose residue, is stored in the chicory taproots. The degree of polymerisation (DP) determines the application of the inulin and hence the value of the crop. This leads us to the central question of this thesis:

What regulates the fructan yield and the degree of polymerisation, and how can we modify this?

The DP is highly dependent on the field conditions and harvest time, and therefore the first step in answering this question was to study the regulation of fructan (inulin) metabolism throughout the growing season. This is described in *Chapter 2*. Metabolic aspects of inulin production and degradation in chicory were monitored in the field and under controlled conditions. We determined the concentrations of soluble carbohydrates, the inulin mean degree of polymerisation (mDP), inulin yield, gene expression and activity of enzymes involved in inulin metabolism in the taproots. Inulin biosynthesis - catalysed by sucrose: sucrose 1-fructosyltransferase (EC 2.4.1.99) (1-SST) and fructan: fructan 1-fructosyltransferase (EC 2.4.1.100) (1-FFT) - started at the onset of taproot development. Inulin yield increased with time following a sigmoid curve reaching a maximum in November. The maximum inulin mDP of 15 was reached in September and then gradually decreased. Based on the changes observed in the pattern of inulin accumulation, we defined three phases in the growing season and analysed product formation, enzyme activity and gene expression in these defined periods. The results were validated by performing experiments under controlled conditions in climate rooms. Our results show that the decrease in 1-SST is not regulated by day length and temperature. From mid-September onwards the mDP decreased gradually although inulin yield still increased. This is most probably the result from back-transfer activity of 1-FFT and fructan exohydrolase activity (EC 3.2.1.153) (1-FEH). In plants 1-FEH catalyses the breakdown of fructan in order to release the stored carbohydrates necessary in periods of stress, like cold or drought periods or flowering. This information was used to design two strategies to obtain the desired, increased inulin DP and yield. Overexpression of *1-SST* was performed to increase

the mDP and to keep the sucrose concentration low, to prevent 1-FFT from depolymerizing inulin. The result was a higher mDP during the growing season. Unfortunately, no effect on the mDP was seen at the end of the growing season, most probably due to activity of FEH. Secondly, an *FEH I* antisense fragment was introduced into chicory in order to block depolymerisation at the end of the growing season. This resulted in a reduction in *FEH I* expression upon cold induction, but had only minor effects on the mDP. The degradation of inulin was most probably caused by the remaining 1-FEH activity. Overall this study showed that inulin metabolism in chicory is tightly regulated, but also revealed options to further steer inulin metabolism in chicory.

The next step in answering the central question was to study the regulation of the genes involved in fructan biosynthesis. In ***Chapter 3*** this was studied at three different levels. Firstly, fructan gene expression and carbohydrate concentrations were studied in axial sections of mature chicory root, revealing the highest expression levels and carbohydrate levels in the phloem. Correlations were found between the gene expression patterns of *1-SST, 1-FFT* and the carbohydrate levels, suggesting a possible involvement of sugars in the regulation of *1-SST* and *1-FFT* gene expression. Secondly, the induction of *1-SST* and *1-FFT* expression was studied in excised chicory leaves. Expression of both *1-SST* and *1-FFT* was induced upon sucrose and glucose feeding, suggesting that both genes are at least partly regulated in the same way. Upon fructose feeding, the induction of fructan biosynthesis was less pronounced than with sucrose. The expression of *1-SST* was induced by fructose but this resulted in only low amounts of 1-kestose. The expression of *1-FFT* was not induced upon fructose feeding. Thirdly, to further unravel the mechanism of induction, the promoters of *1-SST* and *1-FFT* from chicory were isolated and characterized through *in silico* and *in planta* (only *1-FFT*) analysis. Computational analysis of fructosyltransferase (FT) promoters revealed elements that are common in fructan biosynthesis-promoters among different species and also occur in Arabidopsis promoter sequences. One of these elements is predominantly present in genes involved in sugar metabolism and transport. This element did also contain a core sequence involved in MYB transcription factor binding important for fructan biosynthesis activation in wheat, as was published recently. An 1100bp *1-FFT* promoter fragment was shown to be functional in transgenic chicory and in the non-fructan accumulating plants species, Arabidopsis and potato. Application of carbohydrates resulted in the expression of the reporter gene *GUS* comparable to *1-FFT* expression upon carbohydrate feeding in chicory. This study provides information on the regulation of inulin biosynthesis, suggestions for studies on transcription factors, and provides a promoter for steering the expression of fructan biosynthetic genes in transgenic plants. An alternative way for the production of inulin with the desired DP and yield, circumventing the problems in chicory rather than trying to solve them, is the introduction of the fructan biosynthetic pathway in non-fructan metabolizing and catabolizing plant species.

Towards this end we have expressed the inulin synthesizing enzymes, 1-SST and 1-FFT from Jerusalem artichoke, in maize and potato, as described in ***Chapter 4***. Transgenic

maize plants produced inulin type fructan (at 3.2 milligram per gram kernel) and kernel development was not affected. Potato tubers expressing *1-SST* accumulated 1.8 milligram inulin per gram tuber while tubers with a combined expression of *1-SST* and *1-FFT* accumulated 2.6 milligram inulin per gram tuber. Inulin accumulation in maize kernels was modulated by kernel development, first peaking in young seeds and then decreasing again through degradation during late kernel development. In potato, inulin mDP was relatively stable throughout tuber development and little evidence of degradation was observed. The accumulation of 1-kestose in transgenic maize correlated positively with kernel sucrose concentration and introduction of the fructan biosynthetic pathway in a high-sucrose maize background increased inulin accumulation to 41 milligram per gram kernel kernel. This study shows the importance of sugar availability and the absence of degradation mechanisms in platform crops for tailor-made fructan production.

Further evaluation of the production of tailor-made inulin and putative platform crops is discussed in ***Chapter 5***. Here we come to the conclusion that the mDP, the distribution and yield depend on the origin of the fructan biosynthesis genes and the availability of sucrose in the host. The combination of genes from different origins could result in new types and different lengths of fructan molecules resulting in (new) specific properties of fructan. Limitations for the production of tailor-made fructan in chicory are not seen in putative new platform crops, such as sugar beet, sugarcane and rice.

The work described in this thesis on fructan biosynthesis in chicory and in new platform crops has resulted in new insights that will lead new applied and fundamental research in this field.

Samenvatting

Fructaan is een polymeer van fructose die kan worden geproduceerd door zowel planten als micro-organismen. Ongeveer 45.000 plantensoorten gebruiken fructaan als opslagmolecuul ter aanvulling of vervanging van zetmeel. Deze soorten komen vooral voor in gebieden met een gematigd of een subtropisch klimaat met sporadische of seizoensgebonden regenval. Door het toegenomen inzicht in de gezondheidsbevorderende- en functionele eigenschappen van fructaan, heeft het gebruik ervan in de (voedingsmiddelen) industrie zich snel ontwikkeld. Zo wordt de penwortel dragende plant cichorei (*Cichorium intybus* L.) commercieel geteeld voor de productie van inuline. Inuline is een lineair fructaan met ß (2,1)-gebonden fructose eenheden en een eindstandig glucose molecuul. Gedurende de groei van de plant wordt inuline opgeslagen in de penwortel. De polymerisatiegraad (DP) van inuline bepaalt in hoge mate de industriële toepassing en daarmee de waarde van het gewas.
Dit leidt ons tot de centrale vraag van dit proefschrift:

Wat bepaalt de polymerisatiegraad en de opbrengst van fructaan, en hoe zouden we dit kunnen veranderen?

Om deze vraag te kunnen beantwoorden zijn we als volgt te werk gegaan. Als eerste werd het fructaan (inuline) metabolisme gedurende het groeiseizoen bestudeerd, daar in cichorei de DP van inuline sterk afhankelijk is van de teeltomstandigheden en de oogsttijd. Dit onderzoek wordt beschreven in hoofdstuk 2. Voor dit onderzoek zijn, zowel gedurende het groeiseizoen op het veld als onder gecontroleerde omstandigheden in klimaatcellen, metingen gedaan aan de penwortel van cichorei. Naast het meten van de suiker concentraties en de concentratie van inuline zijn bovendien de gemiddelde polymerisatiegraad (mDP) en de opbrengst van inuline bepaald. Daarnaast zijn het genexpressie niveau en de activiteit van enzymen, die betrokken zijn bij inuline metabolisme, in de penwortels bepaald. De inuline biosynthese start gelijk met de uitgroei van de penwortel en wordt gekatalyseerd door de enzymen sucrose: sucrose 1-fructosyltransferase (EC 2.4.1.99) (1-SST) en fructaan: fructaan-fructosyltransferase 1 (EC 2.4.1.100) (1-FFT). De maximale inuline opbrengst wordt in november bereikt, terwijl de hoogste mDP al in september wordt bereikt, die vervolgens geleidelijk afneemt. Op basis van de waargenomen veranderingen in het patroon van de inuline accumulatie werden drie fasen in het groeiseizoen gedefinieerd waarin successievelijk productvorming, enzymactiviteit en genexpressie werden bestudeerd. De resultaten werden gestaafd met experimenten met cichorei planten die onder gecontroleerde omstandigheden in klimaatkamers werden geteeld. Met deze resultaten is aangetoond dat de geobserveerde daling van 1-SST gedurende het groeiseizoen niet door daglengte en temperatuur wordt

veroorzaakt. De mDP daalde in fase twee van het groeiseizoen terwijl de inuline opbrengst nog verder steeg. Waarschijnlijk wordt dit veroorzaakt door een combinatie van de depolymerisatie activiteit van 1-FFT en de fructaan exohydrolase activiteit van het enzym fructan exohydrolase (EC 3.2.1.153) (1-FEH). In planten katalyseert 1-FEH de afbraak van fructaan als de plant de energie, die opgeslagen zit in fructaan, weer nodig heeft. Dit kan zijn in periodes van stress zoals bijvoorbeeld bij droogte of koude, of als de plant gaat bloeien. Op basis van de bovengenoemde observaties werden twee strategieën ontwikkeld om een verhoogde inuline mDP en opbrengst te krijgen. De eerste was het tot overexpressie brengen van *1-SST*, met als doel een hogere mDP en een lagere sucrose concentratie. De lagere sucrose concentratie zou moeten voorkomen dat 1-FFT inuline gaat depolymeriseren. Het resultaat was een hogere mDP tijdens het groeiseizoen. Aan het eind van het groeiseizoen was de verhoging van de mDP echter niet meer te meten, waarschijnlijk als gevolg van exohydrolase activiteit van FEH I. De tweede strategie was het blokkeren van de depolymerisatie van 1-FEH door de introductie van een *FEH-I* antisense fragment in cichorei. Het resultaat was een lagere *FEH-I* expressie na koude behandeling vergeleken met niet getransformeerde koude behandelde planten. De invloed op de mDP was echter gering. De afbraak van inuline werd blijkbaar veroorzaakt door de resterende 1-FEH activiteit. Kortom uit deze studie bleek dat inuline metabolisme in cichorei strak gereguleerd is. Desondanks gaf het toch ideeën om het inuline metabolisme in cichorei te veranderen. Een voorbeeld is het gebruik van betere promotoren voor de heterologe 1-SST, zoals bijvoorbeeld de cichorei *1-FFT* promotor, of het blokkeren van meerdere *FEH*-genen tegelijk, om zo residuelle exohydrolase activiteit te voorkomen. De volgende stap in het beantwoorden van de centrale vraag was het bestuderen van de regulatie van de genen die betrokken zijn bij de fructaan biosynthese.

In hoofdstuk 3 staat beschreven hoe de regulatie werd onderzocht op drie verschillende niveaus. Ten eerste werd in dwarsdoorsnedes van volwassen cichorei penwortels de koolhydraten concentraties gemeten en het genexpressie niveau van *1-SST* en *1-FFT* bepaald. Hieruit bleek dat in het floëem de hoogste genexpressie niveaus en de hoogste koolhydraat concentraties aanwezig zijn. Deze co-lokalisatie duidt op een mogelijke betrokkenheid van de koolhydraten in de regulatie van *1-SST* en *1-FFT* genexpressie. Deze hypothese werd vervolgens getoetst door het induceren van de fructaan biosynthese in blad, het tweede niveau. De fructaan biosynthese werd geïnduceerd door afgesneden cichorei bladeren in oplossingen van de te bestuderen suikers te zetten. Zowel sucrose als glucose bleek de expressie van zowel *1-SST* als *1-FFT* te kunnen induceren. Dit suggereert dat beide genen tenminste gedeeltelijk op dezelfde manier worden gereguleerd. De inductie van de fructaan biosynthese door fructose was minder uitgesproken dan door die sucrose en glucose. De expressie van *1-SST* werd geïnduceerd door fructose, maar dit resulteerde in slechts lage hoeveelheden van 1-kestose. De expressie van *1-FFT* werd daarentegen niet geïnduceerd door fructose. Ten derde werden de promotors van *1-SST* en *1-FFT* uit cichorei geïsoleerd en *in silico* en *in planta* (alleen *1-FFT*) geanalyseerd en gekarakteriseerd. De computeranalyse van de

fructosyltransferase (FT) promotors leverde een aantal elementen op die ook in FT-promotors van andere plantensoorten voorkomen. Daarnaast werden deze elementen ook gevonden in niet-FT-promotor sequenties uit Arabidopsis. Eén van deze elementen is in Arabidopsis voornamelijk aanwezig in genen die betrokken zijn bij het suiker metabolisme en transport. Dit element bevat bovendien ook een kernsequentie die betrokken is bij de MYB transcriptiefactor binding. De betrokkenheid van de MYB bij de fructaan biosynthese blijkt uit een studie aan tarwe die onlangs werd gepubliceerd. Een 1100bp *1-FFT* promotor fragment bleek functioneel te zijn in transgene cichorei en in de niet-fructaan accumulerende plantensoorten, Arabidopsis en aardappel. De incubatie van de transgene planten of plantendelen, in oplossingen van de te bestuderen suikers, resulteerde in de expressie van het *GUS* reporter gen. Het expressiepatroon van *GUS* was vergelijkbaar met het *1-FFT* expressiepatroon na incubatie van cichorei blad met dezelfde suikers. Dit onderzoek geeft inzicht in regulatie van de inuline biosynthese en levert suggesties op voor studies naar transcriptiefactoren. Daarnaast leverde het ook een suiker gereguleerde promotor op die gebruikt kan worden voor het sturen van de expressie van fructaan biosynthese genen in transgene planten. Uit hoofdstuk 2 blijkt dat inuline metabolisme in cichorei strak gereguleerd en daardoor moeilijk is te beïnvloeden is. Alternatieven voor cichorei als productiegewas voor inuline zijn gewassen die *van nature* geen inuline maken en/of afbreken maar waarin de synthese route is geïntroduceerd.

Om dit te bereiken werden de inuline biosynthese genen *1-SST* en *1-FFT* uit aardpeer in maïs en aardappel geïntroduceerd, zoals beschreven staat in hoofdstuk 4. De transgene maïs produceerde 3,2 milligram inuline per gram maiskorrel waarbij het fenotype niet veranderde. De aardappel knollen van de planten met 1-SST produceerden 1,8 milligram inuline per gram knol, terwijl knollen met een gecombineerde expressie van *1-SST* en *1-FFT* 2,6 milligram inuline per gram knol produceerden. De inuline accumulatie in maïskorrels werd beïnvloed door de korrel ontwikkeling. De concentratie van inuline in de jonge zaden is hoog en neemt vervolgens af door afbraak tijdens de latere ontwikkeling van de maiskorrel. In aardappel was de inuline mDP relatief stabiel gedurende de knol ontwikkeling en er is geen reden om aan te nemen dat er afbraak plaats vindt. De concentratie van 1-kestose en sucrose waren positief gecorreleerd in de korrel. De introductie van de fructaan biosynthese route in suikermais, een variëteit met een hoge sucrose concentratie, leverde een hogere inuline concentratie op, tot wel 41 milligram per gram maiskorrel. Deze studie toont aan dat de beschikbaarheid van suiker en het ontbreken van een afbraak mechanisme belangrijk is voor “tailor-made” fructaan productie gewassen.

Een verdere evaluatie van de “tailor-made” fructaan en de productie gewassen komt ter sprake bij de algemene discussie in hoofdstuk 5. Daarin wordt geconcludeerd dat de mDP, de verhoudingen tussen de polymeerlengtes en de opbrengst afhankelijk zijn van de plantensoort waaruit de gebruikte FT-genen zijn geïsoleerd. Het combineren van genen uit verschillende plantensoorten zou nieuwe fructanen met mogelijk (nieuwe) specifieke eigenschappen en andere polymeerlengtes kunnen opleveren. Verder worden in hoofdstuk

5 mogelijke productie gewassen voor “tailor-made” fructaan besproken. Zoals onder andere uit dit proefschrift is gebleken is een voorwaarde voor “tailor-made” fructaan productie gewassen, dat deze gewassen niet al fructaan produceren en/of afbreken, maar wel grote hoeveelheden suiker accumuleren. Potentieel interessante gewassen zijn suikerbiet, suikerriet en rijst.

Het onderzoek dat beschreven is in dit proefschrift, over de fructaan biosynthese in cichorei en in nieuwe productie gewassen, resulteerde in nieuwe inzichten die een aanzet geven tot nieuw toegepast en fundamenteel onderzoek.

Dankwoord

Andries, jou wil ik als eerste bedanken voor je positieve invloed op mijn loopbaan. Als “Melkert-jongen“ had ik een jaarcontract en werkte ik op kleine projectjes, totdat jij me nodig had om FEH-genen uit cichorei te isoleren. Dat cichorei een hard(nekkig) gewas is om mee te werken illustreerde je met een enorme knal toen je de grote porseleinen mortier kapot stampte waarin je diepgevroren stukjes penwortel wilde vermalen voor mijn eerste experimenten. (Andries, ik heb nu ook zelf ondervonden dat het een hardnekkig gewas is.) Na 4 jaar werken aan cichorei was het EU-project afgelopen en vroeg je mij of ik de resultaten zou willen opschrijven in een proefschrift. Toen veranderde je rol van projectleider in co-promotor. In deze rol heb je mij de kern van het schrijven van een artikel geleerd door elke keer de vraag te stellen “wat is de boodschap van je verhaal?”. Die boodschap leek onvindbaar in die enorme berg data die de metingen aan cichorei, gedurende 4 groeiseizoenen, had opgeleverd. En dan noemde jij het artikel ook nog steevast “het kul-paper”. Later veranderde je functie naar BU-manager en droeg je het co-promotor schap over aan Ingrid.

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Robert, je nauwkeurige manier van kijken naar resultaten heeft mij veel geholpen. De discussies over de suikeranalyses op de Dionex, de fysiologische proeven aan cichorei die je met mij (frost-experiments) en met Hanny hebt gedaan (CK 1,2,3,4,5,6,7…n), hebben veel bijgedragen aan dit proefschrift. Bedankt voor je (geduldige) correctiewerk, je hebt het gedaan tot het einde maar eerder kwamen de fructanen je de keel al uit. En je bent er nog niet vanaf, want nu moet je ook nog voor paranimf spelen ;-). Wie weet werk ik binnenkort wel niet meer aan fructanen en kunnen we in het café over andere dingen praten.

Hanny, als jij niet op wereldreis was gegaan was ik waarschijnlijk nooit bij de fructaangroep terecht gekomen. Dit proefschrift was er dan ook zeker niet gekomen. Bedankt dat je zoveel proeven gedaan hebt die volgens jou “toch niet gepubliceerd zouden worden”. Al je aardappel kruisingen waren een mooie basis voor mijn werk aan de fructaan accumulatie in aardappel. Het cichoreiwerk dat je met Robert hebt gedaan was een mooie aanvulling op mijn veldproeven. Ik vind het leuk dat je paranimf wilt zijn.

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De studenten en voluntairs die meegewerkt hebben aan mijn promotieproject wil ik ook graag bedanken: Ad, Alex, Dean, Dianne, Dionne, Emmanuel, Jeroen, Marieke, Rob, Roy, Vicky en Zewen. Bedankt allemaal.

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Mijn oudste raadgever Sinterklaas, wil ik bedanken voor zijn blijvende interesse in mijn proefschrift. Sorry, dat ik me niet aan de afgesproken promotie data heb gehouden. De mooie data: 6-6-'06, 8-8-'08 en 10-10-'10 heb ik allemaal niet gehaald. Het afgelopen jaar was ik "te druk" om bij uw bezoek op het werk aanwezig te zijn. Dit jaar durf ik weer te komen.

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Verder wil ik iedereen bedanken, familie en vrienden, die altijd geïnteresseerd bleven in, de verhalen over en de vorderingen van, het proefschrift.

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Curriculum vitae

Jeroen van Arkel was born on the 13th of May 1972 in Leiden, the Netherlands. In 1990 he obtained his secondary school diploma (HAVO, Visser 't Hoofd Lyceum, Leiden). In 1992 he finished the study "Flower and vegetable breeding" at the MTuS Huis te Lande, Rijswijk. In 1996 he finished a bachelor study in plant biotechnology at the Hogeschool van Rotterdam & Omstreken, Delft. After his graduation he started working as research assistant at CPRO-DLO (currently Plant Research International) at the department of Cell biology (currently Bioscience) in Wageningen. In 1998 he got involved in the fructan research and in 2001 he was invited to write a thesis on the fructan project. During the following years, next to his PhD work, he was involved in a wide range of projects; aiming at the production of polymers and chemicals in plants; the analysis of carbohydrates and other metabolites in fungi, plants and other organic material and research on pathway regulation mechanisms and plant development in crops. The results from the fructan project are described in this thesis.

Publications

Related to this thesis

R.E. Sévenier, **J. van Arkel**, J.C. Hakkert, A.J. Koops, Fructan: nutritional significance, application, biosynthesis, molecular biology and genetic engineering, in: Plant Genetic Engineering, Metabolic engineering and molecular farming, Studium Press, Houston, 2006, pp. p. 31 - 55.

J.M. Stoop*, **J. van Arkel***, J.C. Hakkert, C. Tyree, P.G. Caimi, A.J. Koops, Developmental modulation of inulin accumulation in storage organs of transgenic maize and transgenic potato, Plant Science, 173 (2007) 172-181.

(*) Both authors contributed equally to this paper

J. van Arkel, R. Vergauwen, R. Sévenier, J.C. Hakkert, A. van Laere, H.J. Bouwmeester, A.J. Koops, I.M. van der Meer, Sink filling, inulin metabolizing enzymes and carbohydrate status in field grown chicory (*Cichorium intybus* L.), Journal of Plant Physiology, 169 (2012) 1520-1529.

J. van Arkel, R. Sévenier, J.C. Hakkert, H.J. Bouwmeester, A.J. Koops, I.M. van der Meer, Tailor-made fructan synthesis in plants: A review, Carbohydrate Polymers, 93 (2013) 48-56.

J. van Arkel, R. Sévenier, J.C. Hakkert, H.J. Bouwmeester, A. Koops, I.M. van der Meer, Fructan biosynthesis regulation and the production of tailor-made fructan in plants, in: N. Benkeblia (Ed.) Polysaccharides: Natural Fibers in Food and Nutrition, CRC Press, *Accepted manuscript,* 2013.

J. van Arkel, R. Sévenier, P. Devaux, A.D.J. van Dijk, J.C. Hakkert, H.J. Bouwmeester, A.J. Koops, I.M. van der Meer, Carbohydrate-mediated regulation of fructan biosynthesis in chicory (*Cichorium intybus* L.), *submitted*, (2013)

.

Other publications

J. Beekwilder, I.M. van der Meer, A. Simic, J. Uitdewilligen, J. van Arkel, R.C. de Vos, H. Jonker, F.W. Verstappen, H.J. Bouwmeester, O. Sibbesen, Metabolism of carotenoids and apocarotenoids during ripening of raspberry fruit, BioFactors, 34 (2008) 57-66.

P. Passarinho, T. Ketelaar, M. Xing, J. van Arkel, C. Maliepaard, M. Hendriks, R. Joosen, M. Lammers, L. Herdies, B. den Boer, L. van der Geest, K. Boutilier, BABY BOOM target genes provide diverse entry points into cell proliferation and cell growth pathways, Plant Molecular Biology, 68 (2008) 225-237.

F.R. Smiderle, G.L. Sassaki, J. van Arkel, M. Iacomini, H.J. Wichers, L.J.L.D.V. Griensven, High Molecular Weight Glucan of the Culinary Medicinal Mushroom *Agaricus bisporus* is an α-Glucan that Forms Complexes with Low Molecular Weight Galactan, Molecules, 15 (2010) 5818-5830.

F. Smiderle, A. Ruthes, J. van Arkel, W. Chanput, M. Iacomini, H. Wichers, L. Van Griensven, Polysaccharides from *Agaricus bisporus* and *Agaricus brasiliensis* show similarities in their structures and their immunomodulatory effects on human monocytic THP-1 cells, BMC Complementary and Alternative Medicine, 11 (2011).

D. Haenen, J. Zhang, C.S. da Silva, G. Bosch, I.M. van der Meer, J. van Arkel, J.J. van den Borne, O.P. Gutiérrez, H. Smidt, B. Kemp, A Diet High in Resistant Starch Modulates Microbiota Composition, SCFA Concentrations, and Gene Expression in Pig Intestine, The Journal of nutrition, 143 (2013) 274-283.

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: **Jeroen van Arkel**
Date: **11 October 2013**
Group: **PRI - Bioscience and Plant Physiology, Wageningen University & Research Centre**

1) Start-up phase	*date*
► **First presentation of your project**	
Presentation for the business unit Bioscience on the PhD topic	Dec 18, 2012
► **Writing or rewriting a project proposal**	
"Fructan accumulation in chicory and transgenic plants"	2010
► **Writing a review or book chapter**	
Fructan: Nutritional significance, application, biosynthesis, molecular biology and genetic engineering, In: Plant Genetic Engineering, volume 7, Metabolic engineering and molecular farming-I /, P.K. Jaiwal, .p. 31 - 55.	2006
Tailor-made fructan synthesis in plants: A review, Carbohydrate Polymers 93 (2013), pp 48-56	2012
► **MSc courses**	
► **Laboratory use of isotopes**	
Veilig werken met radioactieve stoffen en stralingsbronnen, niveau 5B, Internationale Agrarische Hogeschool Larenstein	Jan 08-11 & 22, 1996
Subtotal Start-up Phase	*8.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD Student day, University of Amsterdam	Nov 30, 2012
EPS Career day, Wageningen University	Feb 01, 2013
► **EPS theme symposia**	
Theme 4 'Genome Biology', Radboud University Nijmegen	Dec 07, 2012
Theme 1 'Developmental Biology of Plants', Leiden University	Jan 17, 2013
► **NWO Lunteren days and other National Platforms**	
NWO-ALW 'Experimental Plant Sciences', Lunteren (NL) (4x attendance)	Apr 2008-2011

►	**Seminars (series), workshops and symposia**	
	EU meeting FAIR5-CT97-Transformation of chicory into.. Leuven, Belgium	1999
	EU meeting FAIR5-CT97-Transformation of chicory into.. Lille, France	2000
	EU meeting FAIR5-CT97-Transformation of chicory into.. Wageningen, The Netherlands	2001
	Genemaths user training, Applied Maths (one day)	2003
	ABI 310 sequence analyser user training, Applied Biosystems (one day)	2004
	EU meeting FAIR7-Transcontainer Vienna, Austria	Apr 15-17, 2007
	EU meeting FAIR7-Transcontainer Plovdiv, Bulgaria	May 05-07, 2008
	EU meeting FAIR7-Transcontainer Brussels, Belgium	Oct 12-14, 2009
	Tools in polysaccharide Engineering, VLAG graduate school	Aug 28, 2011
	Agilent GCMS maintenance training, JSB Nederland	Jun 14, 2012
	Plant Sciences Seminars	2009-2012
►	**Seminar plus**	
►	**International symposia and congresses**	
	Ninth seminar about inulin, Budapest, Hungary	Apr 18-19, 2002
	Fifth International fructan Symposium, Havana, Cuba	Dec 05-09, 2004
	2nd International polysaccharide conference EPNOE 2011, Wageningen	Aug 29-Sep 02, 2011
	7th Intern. fructan Symposium, Saint-Jean-le-Thomas, France	Jul 02-06, 2012
	9th Intern. Conference on Renewable Resources and Biorefineries (RRB-9), Antwerp, Belgium	Jun 05-07, 2013
►	**Presentations**	
	Oral presentation on EU meeting FAIR5-CT97-Transformation of chicory into.. Leuven, Belgium	1999
	Oral presentation on EU meeting FAIR5-CT97-Transformation of chicory into.. Lille, France	2000
	Oral presentation on EU meeting FAIR5-CT97-Transformation of chicory into.. Wageningen, NL	2001
	Poster presentation Ninth seminar on inulin, Budapest, Hungary	Apr 18-19, 2002
	Poster presentation NWO Lunteren dagen, Wageningen, NL	2005
	Oral presentation on 5th International fructan Symposium, Havana, Cuba	Dec 05-09, 2004
	Oral presentation on EU meeting FAIR7-Transcontainer Vienna, Austria	Apr 15-17, 2007
	Oral presentation on EU meeting FAIR7-Transcontainer Plovdiv, Bulgaria	May 05-07, 2008
	Oral presentation on EU meeting FAIR7-Transcontainer Brussels, Belgium	Oct 12-14, 2009

	Oral presentation on 2nd International polysaccharide conference EPNOE 2011, Wageningen, NL	Aug 29-Sep 02, 2011
	Oral presentation on 7th Intern. fructan Symposium, Saint-Jean-le-Thomas, France	Jul 02-06, 2012
	Poster presentation on RRB-9 conference, Antwerp, Belgium	Jun 05-07, 2013
►	**IAB interview**	Nov 15, 2012
►	**Excursions**	
	Subtotal Scientific Exposure	*19.0 credits**

3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	Advanced Applications of DNA Amplification Techniques	Mar 15-19, 1999
	Bioinformatics	Nov 19-23, 2001
	Summer Course Glycosciences	Jun 28 Jun-Jul 01, 2004
	Basiscursus Gaschromatografie	May 24-26, 2011
	Massaspectrometrie voor chromatografisten	Jun 21-23, 2011
	PhD Course 'Systems Biology: Statistical Analysis of ~Omics Data'	Dec 10-14, 2012
►	**Journal club**	
►	**Individual research training**	
	Subtotal In-Depth Studies	*3.3 credits**

4) Personal development		*date*
►	**Skill training courses**	
	Techniques for Writing and Presenting Scientific Papers	Jan 2004
	Toegepaste statistiek, Wageningen Business School (8 Days)	Feb -Apr 2006
►	**Organisation of PhD students day, course or conference**	
►	**Membership of Board, Committee or PhD council**	
	Subtotal Personal Development	*3.6 credits**

TOTAL NUMBER OF CREDIT POINTS*	**34,4**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

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