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Fructosyltransferase mutants specify a function for the β-fructosidase motif of the sucrose-binding box in specifying the fructan type synthesized

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Abstract

The onion fructosyltransferase fructan:fructan 6G-fructosyltransferase (6G-FFT) synthesizes fructans of the inulin neo-series using 1-kestose as a substrate. 6G-FFT couples a fructosyl residue to either the terminal glucose via a β(2-6) linkage or a terminal fructose via a β(2-1) linkage. The sucrose-binding box is present at the N-terminus of invertases and fructosyltransferases. We tested its function by producing swaps of the first 36 amino acids of 6G-FFT with that of onion sucrose:sucrose 1-fructosyltransferase (1-SST) (SST-GFT) and vacuolar invertase (INV-GFT). In contrast to 6G-FFT, invertase and 1-SST are able to utilize sucrose as their only substrate. The chimerical enzymes were unable to use sucrose, but were active when incubated with 1-kestose. INV-GFT synthesized a similar array of fructans as 6G-FFT, in contrast, SST-GFT showed a dramatic shift in activity towards synthesis of β(2-1) linkages. Thus the region containing the sucrose-binding box is directing the fructan type synthesized. In invertases, the β-fructosidase motif, which is part of the sucrose-binding box, consists of NDPNG/A. This motif is variable in fructosyltransferases and consists of NDPSG in 6G-FFT and ADPNA in 1-SST of onion. We studied the importance of the 6G-FFT β-fructosidase motif using mutants S87N (NDPNG) and N84A;S87N (ADPNG). S87N has 6G-FFT activity, whereas N84A;S87N has a activity that was shifted towards synthesis of β(2-1) linkages. This is in agreement with the observed activities of the chimerical proteins and indicates that the β-fructosidase motif of the sucrose-binding box is specifying the fructan type synthesized.

Abbreviations: aa, amino acid; bp, base pair; DP, degree of polymerization; 1-FFT, fructan:fructan 1-fructosyltransferase; 6G-FFT, fructan:fructan 6G-fructosyltransferase; 1-SST, sucrose:sucrose 1-fructosyltransferase; INV, vacuolar invertase

Introduction

Fructans, or polyfructosylsucroses, are fructose polymers that are derived from sucrose. They are storage carbohydrates, similar to starch and sucrose. Fructans are synthesized and stored in the vacuole (Frehner *et al.*, 1984; Wiemken *et al.*, 1986; Darwen and John, 1989). The chain length of plant fructans ranges from three up to a few hundred fructose units. Different types of fructans can be distinguished based on the linkage types present.

Fructans are part of the human diet and are naturally present in many plants including onion, leek, and artichoke. Studies into the health aspects of fructans have resulted in the recognition of fructans as a beneficial food ingredient (Roberfroid, 2002). Fructans are non-digestible oligosaccharides

that have prebiotic qualities since they are preferably fermented by beneficial bowel bacteria. Because of their acknowledged health effects fructans are regarded a functional food and used as food additive in e.g. yoghurts, ice cream, and bread. The enzymes that synthesize fructans, fructosyltransferases, probably evolved from vacuolar invertases (β-fructofuranosidase, EC 3.2.1.26) (Hendry, 1993; Wei and Chatterton, 2001). These invertases are present in all plants where they hydrolyse sucrose into glucose and fructose.

A simple fructan type is linear inulin, which is present in plants belonging to the Asterales (e.g. chicory). Inulin consists of β(2-1) linked fructose residues. Two enzymes responsible for inulin synthesis were isolated from plants, sucrose:sucrose 1-fructosyltransferase (1-SST, EC 2.4.1.99) and fructan:fructan 1-fructosyltransferase (1-FFT, EC 2.4.1.100). 1-SST synthesizes the shortest inulin, the trisaccharide 1-kestose. 1-FFT uses the 1-kestose to synthesize higher DP fructan molecules (Edelman and Jefford, 1968; Koops and Jonker, 1996; Lüscher *et al*., 1996; Van den Ende *et al*., 1996; Van den Ende and Van Laere, 1996). In Liliaceae (e.g. onion) a different type of inulin is present, named inulin neo-series. In the inulin neo-series, two β(2-1) linked fructose chains are attached to the sucrose starter unit. One chain is linked to the C1 of the fructose residue (as is the case in inulin) and the other to the C6 of the glucose residue. As a result, several fructan types are distinguished in onion [1] an inulin series which is elongated at the fructose of the starter sucrose, designated Ix (x represents the degree of polymerisation); [2] an neokestose based series with elongation only at the glucose residue of the starter sucrose, designated *Nx*; [3] another neokestose based series with elongation on both the fructose and glucose residue of the starter sucrose, designated Nx (Figure 1) (Ernst *et al*., 1998). Synthesis is initiated with the conversion of sucrose to 1-kestose by 1-SST. Subsequently, fructan:fructan 6G-fructosyltransferase (6G-FFT) is responsible for the synthesis of neo-series inulin (Shiomi, 1989; Vijn *et al*., 1997). 6G-FFT uses short inulins, such as 1-kestose, as a fructose donor and sucrose or fructans as an acceptor. It was shown that all fructan types observed in onion can be synthesized by 6G-FFT (Ritsema *et al*., 2003). Dual activities can thus be assigned to 6G-FFT; these are [1] the coupling of a fructosyl residue to the terminal glucose C6, and [2] a 1-FFT-like activity, namely the coupling of a fructosyl residue to the C1 of terminal fructose residues.

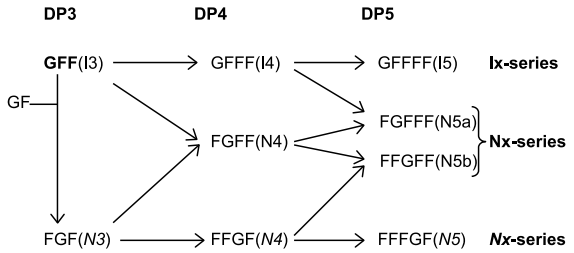

Figure 1. Scheme of the synthesis of different onion-type fructans by 6G-FFT. Every step uses 1-kestose or a higher DP fructan, as the fructosyl donor. I3, 1-kestose; *N3*, 6G-kestotriose; I4, 1,1-kestotetraose; *N4*, 1,6G-kestotetraose; N4, 1&6G-kestotetraose; I5, 1,1,1-kestopentaose; *N5*, 1,1,6G-kestopentaose; N5a, 1,1&6G-kestopentaose; N5b, 1&1,6G-kestopentaose.

Fructosyltransferases as well as vacuolar invertases belong to family 32 of glycosyl hydrolases. In invertases a so-called sucrose-binding box is highly conserved. It consists of the consensus: H-X-X-P-X-X-X-X-[LIVM]-N-D-P-N-[GA]. The NDPNG/A pattern of the sucrose-binding box is called the β-fructosidase motif, it contains the essential Asp residue that probably acts as a nucleophile. In fructosyltransferases the sucrose-binding box is also present although some variations occur, especially in the β-fructosidase motif, leading to a different consensus: H-X-X-[PTV]-X-X-X-X-[LIVMA]-[NSCAYG]-[DE]-P-[NDSC]-[GA] (Pons *et al*., 2000). In 6G-FFT the sucrose-binding box is formed by amino acids 75–88, located at the N-terminus of the mature protein. The first 62 amino acids of the protein are predicted to be involved in vacuolar targeting, and are therefore not present in the mature protein.

Sucrose binding boxes of enzymes originating from different plants, but encoding the same enzymatic specificity are often more homologous to each other than sucrose binding boxes of proteins originating from the same plant, but encoding different enzymatic activities (Figure 2) (Ritsema and Smeekens, 2003). Therefore, we decided to study the effect of swaps between sucrose binding box regions of proteins displaying different activities and originating from the same plant. We used the well-studied onion 6G-FFT (Ritsema *et al*., 2003) and exchanged its sucrose-binding box region with that of onion invertase

(A)

aa	motif	enzyme	plant species	accession number
163	NDPNG	invertase	*Allium cepa*	AJ006067
138	NDPNG	invertase	*Zea mays*	U16123
131	NDPNG	invertase	*Asparagus officinalus*	AF002656
137	NDPNG	invertase	*Cichorium intybus*	AJ419971
134	NDPNG	invertase	*Arabidopsis thaliana*	AY142666
96	ADPNA	1-SST	*Allium cepa*	AJ006066
96	GDPNA	1-SST	*Allium sativum*	AY098442
135	NDPNG	1-SST	*Triticum aestivum*	AB029888
135	NDPNG	1-SST	*Festuca arundinacea*	AJ297369
119	SDPDG	1-SST	*Cichorium intybus*	U81520
107	SDPDG	1-SST	*Heliantus tuberosus*	AJ009757
116	SDPDG	1-SST	*Cynara scolymnus*	Y09662
111	SDPDG	1-SST	*Taraxacum officinale*	AJ250634
95	SDPNG	6-SFT	*Hordeum vulgare*	83233
93	SDPNG	6-SFT	*Triticum aestivum*	AB029887
95	SDPNG	6-SFT	*Poa secunda*	AF192394
103	YDPNG	1-FFT	*Cichorium intybus*	U84398
101	YDPDG	1-FFT	*Heliantus tuberosus*	AJ009756
103	YDPNG	1-FFT	*Cynara scolymnus*	AJ000481
84	NDPSG	6G-FFT	*Allium cepa*	Y07838
			bacterium	
16	NDPNG	invertase	*Thermotoga maritima*	AJ001073

(B)

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63    TNDMLAWQRCGFHFRTVRNYMNDPSGPMYYKGWY 6G-FFT onion
75    TNEMLKWQRTGYHFQPPNHFMADPNAAMYYKGWY 1-SST onion
142   TNQMLSWQRTGFHFQPVKNWMNDPNGPLYYKGWY inv. onion

63    TNDMLAWQRCGFHFRTVRNYMNDPNGPMYYKGWY 6G-FFT S87N
63    TNDMLAWQRCGFHFRTVRNYMADPNGPMYYKGWY 6G-FFT S84A;S87N
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Figure 2. (A) Comparison of β-fructosidase motifs of vacuolar invertases and fructosyltransferases. (B) Sequence comparison of the swapped domains including sucrose-binding box consensus (in bold) and 6G-FFT point mutants. Predicted mature enzyme sequences without the vacuolar targeting signal are depicted. Inv., vacuolar invertase.

(INV-GFT) and 1-SST (SST-GFT). The substrate and product-specificities of the chimerical proteins were investigated. Both chimerical proteins used the same substrate as 6G-FFT, but the product profile of the SST-GFT chimera was shifted towards inulin-type fructans (Ix series, Figure 1). We conclude that the region containing the sucrose-binding box is important in determining product type, but not substrate specificity.

Subsequently, point mutations in the β-fructosidase motif of 6G-FFT were introduced in an attempt to pinpoint the amino acids responsible for the shift in activity observed for the SST-GFT chimera. Interestingly, the N84A;S87N double mutant, which resembles the 1-SST β-fructosidase motif showed a significant shift in activity towards inulin-type fructans. This indicates that the β-fructosidase motif of the sucrose-binding box is important in determining the fructan type synthesized.

Materials and methods

Construction of 6G-FFT chimera's

6G-FFT chimeras were constructed in pMON999, which carries the 35S promoter and NOS terminator sequences. The 3′ region (bp 288–1893) of the onion 6G-FFT gene (accession number Y07838) was cut from pBlue using *Kpn*I and *Xho*I and ligated into pMon999 digested with *Kpn*I and *Bam*HI. The 5′ regions from onion 1-SST (accession number AJ006066) and onion vacuolar invertase (accession number AJ006067) were cut from pBlue using *Sma*I–*Kpn*I and ligated to the 3′ 6G-FFT construct digested with *Stu*I–*Kpn*I, to create SST-GFT and INV-GFT, respectively. Chimerical constructs were confirmed by restriction analysis and PCR using specific primers.

pAPV1 is a pCAMBIA1380 in which the hygromycin resistance gene was exchanged for

kanamycin resistance derived from pCAMBIA2300, using the *Xho*I restriction sites. To enable *Agrobacterium*-mediated transformation the cassette containing the hybrid gene with 35S promoter and NOS terminator sequence was excised from pMON999 using *Not*I, and ligated to *Bsp*120I digested pAPV1.

Site-directed mutagenesis of 6G-FFT

Mutants were made according to the Altered sites II mutagenesis kit (Promega). The first 1337 bases of the 6G-FFT gene were cloned from pBlue into pALTER-1 using *Eco*RI. After mutagenesis the sequence was confirmed by sequencing. For expression in plants, the mutated gene was cloned into a pMON999 that already contained 3′ 6G-FFT (from the *Kpn*I site) using *Eco*RI, and the orientation was checked by restriction analysis. From pMON999 the cassette containing the gene with 35S-promotor and NOS-terminator was cloned into pAPV1 (see above).

Growth and transformation of BY2 cells

BY2 cells were grown as a suspension culture in modified Linsmaier and Skoog medium as described by Nagata *et al.* (1992). The cells were grown at 27 °C at 150 rpm and sub cultured once a week by a 70-fold dilution in fresh medium.

Agrobacterium-mediated transformation of BY2 cells was performed according to An (1985) and Genschik *et al.* (1998) as described before (Ritsema *et al.*, 2003). Transformed callus can be maintained as callus by transferring to a fresh plate with kanamycin (100 μg/ml) and vancomycin (750 μg/ml) once a month.

Assay for fructosyltransferase activity

Fructosyltransferases were isolated from growing BY2 callus by shaking Eppendorf tubes containing cells in a dismembrator (B. Braun Biotech International) two times for 1 min at 2800 rpm in the presence of glass beads (2 of ∅ 2 mm and 2 of ∅ 4 mm). Debris was spun down at 13 000 rpm for 15 min. Of the supernatant, 40 μl was combined with 5 μl of an 0.5 M MES buffer pH 5.7 and 5 μl of substrate to a total volume of 50 μl, and incubated at 28 °C. After incubation, products were analysed using a Dionex HPAEC-PAD (high performance anion exchange chromatography with pulsed amperometric detection) system with a PA100 column (Ritsema *et al.*, 2003). Solutions were: (A) water; (B) 0.5 M NaOH; and (C) 1M NaAc. Running profile applied: T = 0, 80% A 20% B; T = 5 min, 50% A 50% B; T = 15 min, 40% A 50% B 10% C; T = 20 min, 33% A 50% B 17% C; T = 35 min, 50% B 50% C. We used the peak identification as proposed by Shiomi (1993; 1997) and Ernst *et al.* (1998) and nomenclature as proposed by Waterhouse and Chatterton (1993).

All experiments were at least preformed three times; a representative sample is shown in the figures.

Results

The sucrose binding box in fructosyltransferases

Amino acid sequences of sucrose-binding boxes of different fructosyltransferases and invertases from fructan producing plants were compared. The so-called β-fructosidase motif (NDPNG/A), which is the core of the sucrose-binding box, was present in invertases and in some fructosyltransferases (Figure 2A). However, this central motif is usually altered in fructosyltransferases, as is the rest of the sucrose-binding box. Fructosyltransferases most likely evolved from vacuolar invertases. Compared to vacuolar invertase, onion 6G-FFT has Ser instead of the second Asn, whereas 1-SST has Ala instead of the first Asn. In onion, for 1-SST the last amino acid of the β-fructosidase motif is Ala, whereas this is Gly for vacuolar invertase and 6G-FFT. The presumed nucleophile Asp is invariably present in the β-fructosidase motif, as is the adjacent Pro.

To investigate the role of the sucrose-binding boxes in the activities of fructosyltransferases, we used the well-studied onion 6G-FFT, which enzymatic activities are depicted in Figure 1 (Vijn *et al.*, 1997; Ritsema *et al.*, 2003). We exchanged the region containing the sucrose-binding box for that of another onion enzyme involved in fructan synthesis, 1-SST, or for that of the closely related vacuolar invertase (Vijn *et al.*, 1998). A conserved *Kpn*I restriction site was used to make chimerical genes that resulted in proteins that are named SST-GFT and INV-GFT, respectively. The swaps

resulted in two constructs in which aa 1-96 of 6G-FFT were replaced with aa 1–175 of onion invertase or aa 1–108 of onion SST. Since the N-termini of these proteins differ, the different enzymes have a different vacuolar-targeting signal. Based on homologies these targeting signals are predicted to be aa 1–62 for 6G-FFT, aa 1–141 for INV-GFT, and aa 1–74 for SST-GFT. The amino acids that are swapped in the predicted mature proteins are shown in Figure 2B.

Fructosyltransferase chimeras produce different products from the substrate 1-kestose

BY2 cells can be used for high-level expression of fructosyltransferases as was described before for onion 6G-FFT (Ritsema *et al*., 2003). The chimeras INV-GFT and SST-GFT were expressed in this system. They show lower overall activities than that of the wild type 6G-FFT (Figures 3 and 4).

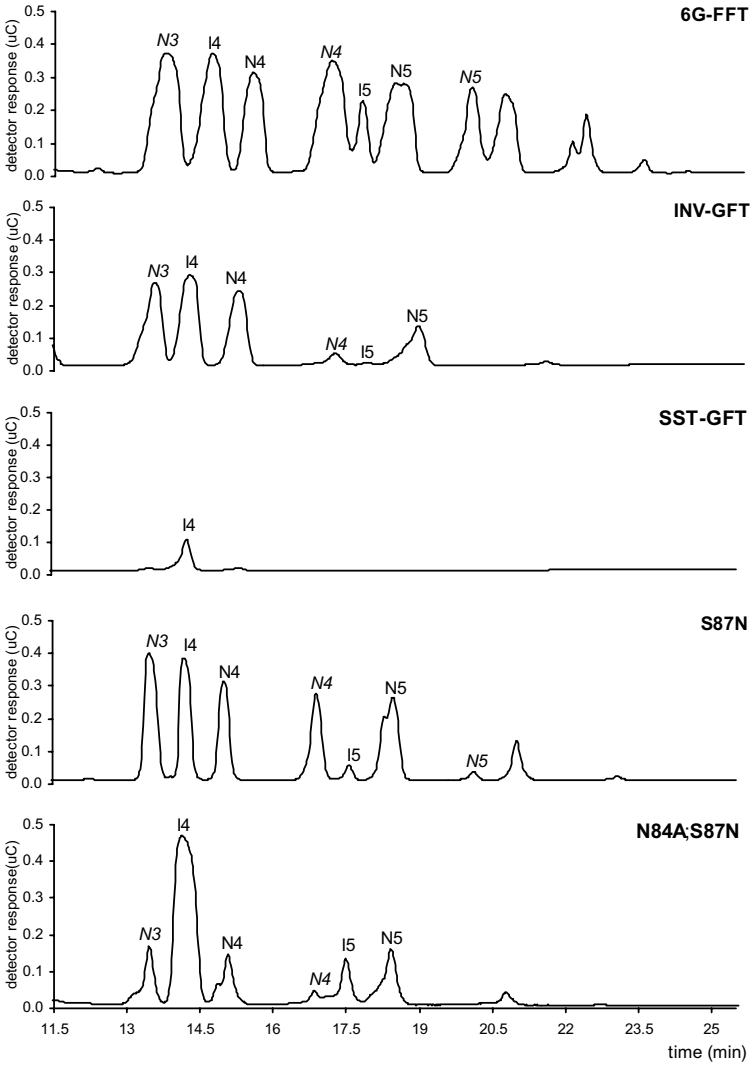

Figure 3. Comparison of the HPAEC-PAD profile of fructans synthesized by 6G-FFT wild type, chimerical fructosyltransferases and 6G-FFT mutants. Extracts from BY2 cells were incubated overnight at 28 °C with 200 mM 1-kestose. Peak annotation according to Ernst *et al*. (1998) *N3*, 6G-kestotriose; I4, 1,1-kestotetraose; *N4*, 1,6G-kestotetraose; N4, 1&6G-kestotetraose; I5, 1,1,1-kestopentaose; *N5*, 1,1,6G-kestopentaose; N5, mixture of 1,1&6G-kestopentaose and 1&1,6G-kestopentaose; I6, 1,1,1,1-kestohexose; DP6, mixture of DP6 fructans with chain elongation on both sites of the sucrose; rest, longer fructans.

Overnight incubation of the INV-GFT hybrid with 1-kestose gave an array of products also seen for 6G-FFT at shorter incubation times (Figure 4). These products consisted of 6G-kestotriose (neokestose, *N3*); 1,1-kestotetraose (nystose, I4); 1&6G-kestotetraose (N4); 1,6G-kestotetraose (*N4*); 1,1,1-kestopentaose (I5); 1&1,6G-kestopentaose and 1,1&6G-kestopentaose (N5). In this product profile the three fructan series defined in onion (Ix, *Nx*, and Nx) can be recognized (see also Figure 1), indicating that 6G-FFT and INV-GFT have similar product specificity.

The SST-GFT chimera produced only 1,1-kestotetraose (I4) after overnight incubation with 1-kestose, indicating a preference for the production of inulin-type (Ix) fructans. The preferred activity of this enzyme is the coupling of a fructosyl residue to another fructosyl residue via a β(2-1) bond instead of the coupling of a fructosyl residue to glucose via a β(2-6) bond. The latter activity is 6G-FFT-specific; the former can also be performed by 1-FFT.

After prolonged incubation – up to 8 days – the products made by INV-GFT were similar to those

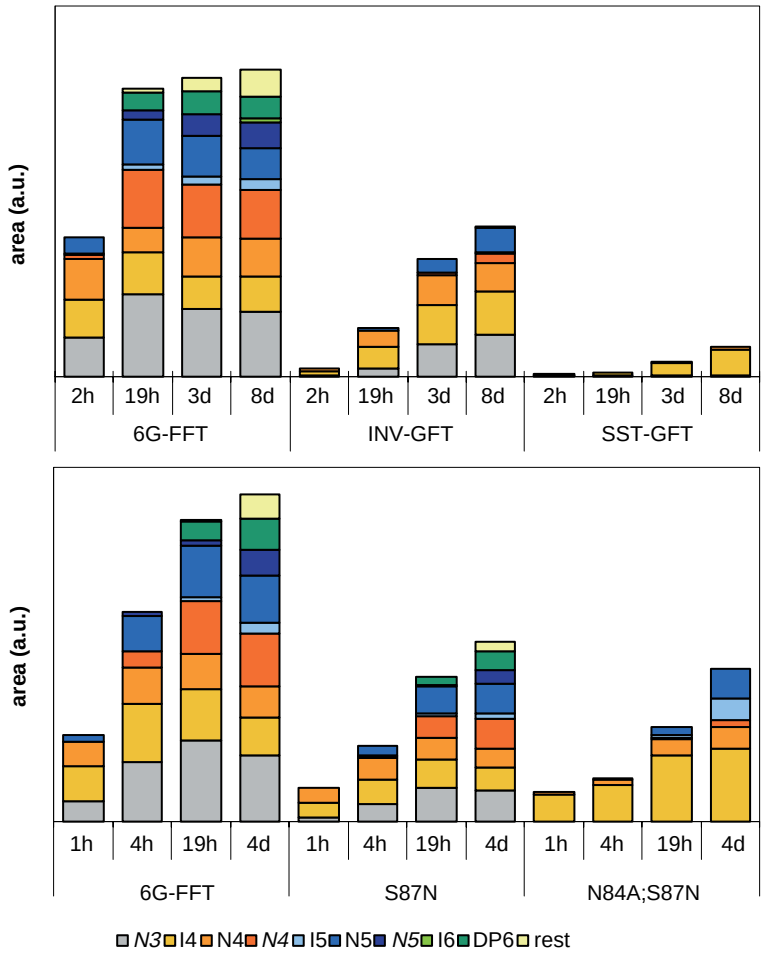

Figure 4. (a)Time series of fructan synthesis by 6G-FFT and the fructosyltransferase chimeras INV-GFT and SST-GFT. Incubation times were 2 h, 19 h, 3 days, or 8 days at 28 °C in the presence of 200 mM 1-kestose. (b) Time series of fructan synthesis by 6G-FFT wild type and mutants S87N and N84A;S87N. Incubation times were 1 h, 4 h, 19 h, or 4 days at 28 °C with 200 mM 1-kestose. Peak areas are measured in arbitrary units. Peaks: *N3*, 6G-kestotriose; I4, 1,1-kestotetraose; *N4*, 1,6G-kestotetraose; N4, 1&6G-kestotetraose; I5, 1,1,1-kestopentaose; *N5*, 1,1,6G-kestopentaose; N5a, 1,1&6G-kestopentaose; N5b, 1&1,6G-kestopentaose; I6, 1,1,1,1-kestohexose; DP6, mixture of DP6 fructans with chain elongation on both sites of the sucrose; rest, longer fructans.

made by 6G-FFT after a few hours (Figure 4). Especially relative amounts of the different onion series fructans were comparable to those made by 6G-FFT. In contrast, SST-GFT still gave predominantly 1,1-kestotetraose (Figure 4). No products other than those seen for wild type 6G-FFT were made by the chimeras.

To determine the ability of the SST-GFT protein to elongate 1,1-kestotetraose, we incubated SST-GFT, as well as 6G-FFT with 200 mM 1,1-kestotetraose as a substrate for up to 4 days. 6G-FFT produced the same array of products from 1,1-kestotetraose as it does from 1-kestose. In contrast, SST-GFT could not polymerise 1,1-kestotetraose (Figure 5).

Overall, the specificity of INV-GFT resembles that of the wild type 6G-FFT synthesizing all types of neo-series inulin, indicating that the sucrose-binding box region is not determining the polymerising ability. SST-GFT synthesizes predominantly inulin, which can be described as a shift towards 1-FFT activity, with only polymerisation up to DP4 observed.

Point mutations in the β-fructosidase motif influence product specificity

A very important region of the sucrose-binding box is the β-fructosidase motif in which the presumed nucleophile Asp is present. This motif differs between invertases and various fructosyltransferases (Figure 2A). To determine the importance of the dissimilar amino acids for 6G-FFT activity, site-directed mutagenesis was performed to mimic the vacuolar invertase or 1-SST β-fructosidase motif. A point mutant of 6G-FFT was created in which Ser-87 was changed to Asn, resulting in 6G-FFT with NDPNG as β-fructosidase motif, resembling that of vacuolar invertase. Subsequently another mutant of 6G-FFT was made from S87N. By introducing Ala for Asn-84 the double mutant N84A; S87N was created. This is a 6G-FFT with ADPNG as β-fructosidase motif, resembling that of 1-SST.

Overnight incubation of these 6G-FFT mutants with 1-kestose resulted in a range of products (Figure 3). The profile of the 6G-FFT S87N mutant resembles that of wild type 6G-FFT. A similar array of products is present, although amounts are lower. The profile of the double mutant 6G-FFT N84A;S87N is different. The same peaks can be seen as in wild-type 6G-FFT. However, the ratios between the peaks differ. Major peaks are those of the onion lx series. These resemble the inulin-type fructans as synthesized by 1-FFT. Also lower amounts of the other onion fructan series, *Nx* and Nx, can be observed.

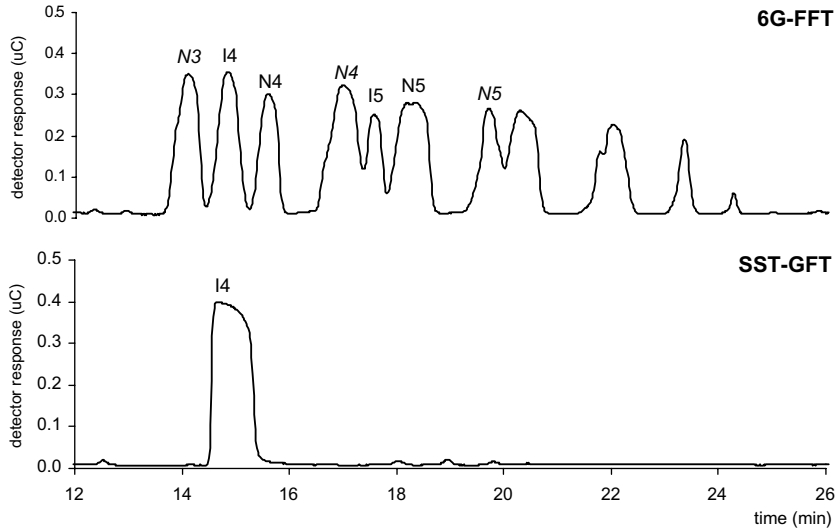

Figure 5. HPAEC-PAD profile of 6G-FFT and SST-GFT extracts incubated with 200 mM 1,1-kestotetraose overnight at 28 °C. Peaks: *N3*, 6G-kestotriose; I4, 1,1-kestotetraose; *N4*, 1,6G-kestotetraose; N4, 1&6G-kestotetraose; I5, 1,1,1-kestopentaose; *N5*, 1,1,6G-kestopentaose; N5a, 1,1&6G-kestopentaose; N5b, 1&1,6G-kestopentaose; I6, 1,1,1,1-kestohexose; DP6, mixture of DP6 fructans with chain elongation on both sites of the sucrose; rest, longer fructans.

After prolonged incubations, up to 4 days, the amounts of products increased (Figure 4). The S87N mutant showed the same relative peak areas as wild type 6G-FFT. The preference of the N84A;S87N double mutant for inulin type fructans remained.

The shift of activity seen in the N84A;S87N double mutant resembles that observed for the SST-GFT chimera, providing support for the conclusion that sucrose-binding box which harbours the β-fructosidase motif is important for specifying products made by fructosyltransferases.

The mutated enzymes are active at the same 1-kestose concentrations as 6G-FFT

A series of different 1-kestose concentrations indicated that the chimeras and the point mutants show activity in the same range as the wild type 6G-FFT (Figure 6). Lowering the concentration from 200 mM to 10 mM resulted in the synthesis of lower DP fructans, but the ratio between inulin-type (Ix) and neo-series-type (Nx and *Nx*) fructans stayed the same for all fructosyltransferases.

Sucrose is not a substrate for fructosyltransferases

6G-FFT cannot use sucrose as the sole substrate (Vijn *et al.*, 1997; Ritsema *et al.*, 2003). In contrast, the proteins that donated the sucrose-binding box region in the chimeras–invertase and 1-SST- are able to use sucrose as the sole substrate. Therefore, the activity of the fructosyltransferases with sucrose was tested. Endogenous invertases from tobacco BY2 cells hydrolyse sucrose, however, after 3 days incubation of BY2 cells in 0.2 M sucrose, 10%–40% of the sucrose remained. INV-GFT and SST-GFT showed no polymerising activity with sucrose as a substrate, not even at 0.5 M of sucrose and an incubation time of 8 days (data not shown). Also 6G-FFT mutants S87N and N84A;S87N did not polymerise sucrose. These results indicate that the sucrose-binding box in itself does not dictate the ability to use sucrose as the substrate for fructan production.

Discussion

The sucrose-binding box of invertase is essential for sucrose breakdown. Acting as the nucleophile is probably the Asp in the β-fructosidase region (NDPNG/A) of the sucrose-binding box (Reddy and Maley, 1990). In analogy to invertases, the sucrose-binding box of fructosyltransferases is proposed to be the binding site for the various fructans that are used as a substrate. The sucrose-binding box of fructosyltransferases differs substantially from the consensus sequence for invertases, and even the conserved β-fructosidase region is altered in fructosyltransferases (Figure 2A). This feature coincides with a different

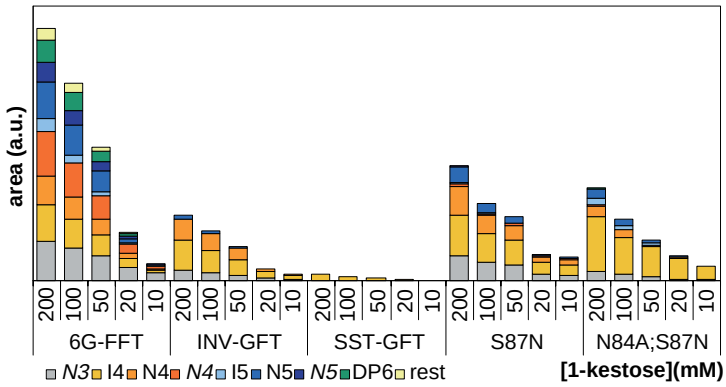

Figure 6. Fructan synthesis by 6G-FFT wild type, chimerical fructosyltransferases, and 6G-FFT mutants in response to different 1-kestose concentrations as indicated. Extracts were incubated overnight at 28 °C. Peak areas are measured in arbitrary units. Peaks: *N3*, 6G-kestotriose; I4, 1,1-kestotetraose; *N4*, 1,6G-kestotetraose; N4, 1&6G-kestotetraose; I5, 1,1,1-kestopentaose; *N5*, 1,1,6G-kestopentaose; N5a, 1,1&6G-kestopentaose; N5b, 1&1,6G-kestopentaose; I6, 1,1,1,1-kestohexose; DP6, mixture of DP6 fructans; rest, longer fructans.

substrate range of fructosyltransferases, since many fructosyltransferases would accept fructans of different length and structure as a substrate.

The importance of the sucrose-binding box region for fructosyltransferase activity and specificity was investigated using 6G-FFT, 1-SST and vacuolar invertase from onion. The chimeras INV-GFT and SST-GFT were active with 1-kestose. They did not retain the ability of their sucrose-box-donating parent to utilize sucrose. Also the 6G-FFT mutants S87N and N84A;S87N, which were mutated in the central β-fructosidase motif of the sucrose-binding box, did not show activity when sucrose was added as a substrate. Therefore, the sucrose-binding box does not appear to be a major determinant of the type of substrate used by fructosyltransferases.

Remarkably, the swapped N-terminal region, containing the sucrose-binding box, does affect the type of product(s) made from the supplied substrate 1-kestose. INV-GFT synthesized onion type neo-series inulin, whereas SST-GFT synthesized inulin DP4 (1,1-kestotetraose) only. Thus, INV-GFT shows the same activity as 6G-FFT, whereas SST-GFT shows a shift in activity towards that described for 1-FFT, namely the elongation of 1-kestose with β(2-1) linked fructose residues. SST-GFT also resembles 1-SST in that the substrate is elongated on a terminal fructose unit by a β(2-1) linked fructose. However, the substrates for 1-SST and SST-GFT differ; 1-SST uses sucrose, whereas SST-GFT requires 1-kestose.

The C-terminal part of the chimerical proteins is derived from 6G-FFT. This enzyme cannot use sucrose as the donor substrate for the fructosyl residue. Since the substrate-specificity of the chimeras resembles that of 6G-FFT, the 6G-FFT-derived part of the chimera is probably involved in shaping the substrate-binding pocket in such a way that only 6G-FFT specific donor substrates can enter.

The conclusion that the sucrose-binding box is of importance for product specification is strengthened and refined by the 6G-FFT point mutations in the β-fructosidase motif of the sucrose-binding box. Mutant S87N, which results in the β-fructosidase motif NDPNG, resembling invertase, has the dual activity of 6G-FFT. It is able to couple a fructosyl residue to a terminal fructose via a β(2-1) link as well as to a terminal glucose via a β(2-6) link. Since both INV-GFT and the 6G-FFT S87N mutant show synthesis of the same products as wild type 6G-FFT, we conclude that the changes in the sucrose-binding box are not essential for the presumed evolution of vacuolar invertase to 6G-FFT.

The N84A;S87N double mutant contains ADPNG, resembling the 1-SST β-fructosidase motif, and has its activity shifted towards synthesis of inulin-type fructans (Ix series). The synthesis of β(2-1) linked fructose residues is preferred over that of β(2-6) linked coupling of a fructose residue to the terminal glucose. Apparently, transfer of a fructosyl-residue to the terminal glucose is hampered in N84A;S87N as well as in the SST-GFT chimera. Probably, in both fructosyltransferase enzymes the acceptor substrate can only be bound in such a way that the terminal fructose is accessible for transfer, whereas the terminal glucose is not available.

The three-dimensional structure of a plant invertase or fructosyltransferase has not yet been determined. Recently, however, the three-dimensional structure of two related enzymes, a bacterial invertase and a bacterial fructosyltransferase became available. Levansucrase from the bacterium *Bacillus subtilis* is the first fructosyltransferase for which a structure is now available (Meng and Fütterer, 2003). The overall structure is a five-bladed β-propeller, in which every blade consists of four antiparallel β-strands. The active site consists of a negatively charged central pocket.

Although bacterial and plant fructosyltransferases share limited homology on the amino acid level, some similarities can be noted and a similar structure is predicted (Pons *et al.*, 2000). The putative sucrose-binding box in bacterial fructosyltransferases is not similar to those from plants. The VWD (aa 84–86) motif of *B. subtilus* levansucrase contains the presumed nucleophile and is therefore probably equivalent to (part of) the sucrose-binding box of plant fructosyltransferases. Both Trp85 and Asp86 of this motif are involved in binding the fructose residue of sucrose (Meng and Fütterer, 2003). In plant fructosyltransferases and invertases the aromatic residue closest to the nucleophile is present in the sucrose-binding box two amino acids N-terminal of the β-fructosidase motif.

The three-dimensional structure of the invertase of the bacterium *Termotoga maritima* (Alberto *et al.*, 2004) seems even more relevant for

fructosyltransferases, since it belongs to the same GH32 family (Alberto *et al.*, 2004). Furthermore, it has a sucrose-binding box that strongly resembles the sucrose-binding box of plant vacuolar invertases and fructosyltransferases. The sequence of the β-fructosidase motif is identical to that of vacuolar invertases, and in addition the aromatic amino acid tryptophan is present two amino acids in front of this motif.

The overall structure of the bacterial invertase is also a five bladed β-propeller with a central negatively charged active pocket, in addition a β-sandwich is attached to the C-terminus. The domain swapped in the INV-GFT and SST-GFT chimeras resembles the N-terminal surface loop and the first β-strand, which forms the outermost strand of the C-terminal blade, where it forms a short molecular velcro. In this first β-strand the β-fructosidase motif is present, and this is probably the part of the swapped domain that has most influence on enzymatic activity.

The bacterial invertase was crystallized without the substrate sucrose. However, based on the levansucrase structure the sucrose was modeled in the active site (Alberto *et al.*, 2004). The amino acids Asp17 (nucleophile) and Asn16 (see Figure 2A) of the β-fructosidase motif seem in close contact with the substrate and therefore appear to be involved in binding the sucrose residue. The other three residues of the β-fructosidase motif seem not to be involved in binding the substrate, however, these could be involved in shaping the binding pocket. Also the aromatic Trp14 was not predicted to be involved in substrate binding, although it is present at the rim of the binding pocket.

When we compare the β-fructosidase box of 6G-FFT to that of the bacterial invertase (Figure 2A) we could extrapolate that S87 is probably not in direct contact with the substrate, whereas N84 might be. In light of this it is conceivable that in 6G-FFT the mutation S87N has less effect on 6G-FFT activity than N84A. This tendency can indeed be observed in the data presented in this article. It is, however, hard to explain the change in product specificity observed in the 6G-FFT mutant N84A;S87N from the 3-dimensional structure of invertase. It is also not clear why 6G-FFT is only active when 1-kestose is present. We know it can use sucrose as a fructosyl acceptor (producing neokestose), but not as a fructosyl donor. This leads to the speculation that the substrate-binding pocket of 6G-FFT is differently shaped than that of invertase, leading to a positioning of the active residues more suitable for the cleavage of fructose from kestose than from sucrose. These questions can probably only be answered when a three-dimensional structure of 6G-FFT (in the presence of substrates) becomes available.

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