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Sucrose:Fructan 6-Fructosyltransferase, a Key Enzyme for Diverting Carbon from Sucrose to Fructan in Barley Leaves[1]

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Sucrose:sucrose 6-fructosyltransferase, an enzyme activity recently identified in fructan-accumulating barley (*Hordeum vulgare*) leaves, was further characterized. The purified enzyme catalyzed the transfer of a fructosyl group from sucrose to various acceptors. It displayed some β-fructosidase (invertase) activity, indicating that water could act as fructosyl acceptor. Moreover, it transferred the fructosyl residue of unlabeled sucrose to [U-^{14}C]Glc, producing [U-^{14}C]sucrose and unlabeled glucose. Most significantly for fructan synthesis, the enzyme used as acceptors but not as donors a variety of oligofructans containing β(2→1)- and β(2→6)-linked fructosyl moieties. Thus, it acted as a general sucrose:fructan fructosyltransferase. The products formed by the enzyme from sucrose and various purified, structurally characterized oligofructans were analyzed by liquid chromatography and identified by comparison with structurally characterized standards. The results showed that the enzyme formed exclusively β(2→6) fructosyl-fructose linkages, either initiating or elongating a fructan chain of the phlein type. We propose, therefore, to rename the purified enzyme sucrose:fructan 6-fructosyltransferase.

An estimated 15% of plant species accumulate fructans (oligo- and polyfructosyl-Sucs) instead of starch as their main carbohydrate reserves (Hendry, 1987). Most of them belong to such prominent and widespread orders as the Poales (grasses), Asterales, and Liliales. Hence, fructan-accumulating plants are believed to constitute the major components of about one-third of the global vegetation cover (Hendry, 1993). It is surprising that the enzymology of fructan metabolism is still poorly understood, particularly in the grasses, among which are representatives of paramount economic importance: wheat, barley (*Hordeum vulgare*), rye, oat, and most forage grasses of temperate and cool climate zones (for reviews, see Pontis and Del Campillo, 1985; Nelson and Spollen, 1987; and Pollock and Cairns, 1991).

It is generally agreed that plant fructans are derived from Suc. It is believed that the fructans are synthesized in essentially two steps according to the model of Edelman and Jefford (1968), which is based primarily on studies of tubers of the Jerusalem artichoke (*Helianthus tuberosus*), a member of the Asteraceae. In a first, essentially irreversible step catalyzed by 1-SST (EC 2.4.1.99), Suc acts as both donor and acceptor of a fructosyl residue, yielding Glc and the trisaccharide 1-kestose. In a second, readily reversible step, 1-kestose or another β(2→1)-linked fructan can function as fructosyl donor and either Suc or any fructan can function as acceptor. This reaction is catalyzed by 1-FFT (EC 2.4.1.100), which specifically cleaves and reforms β(2→1) fructosyl-Fru linkages. By repeated 1-FFT-mediated transfructosylations, linear fructans of the inulin type, containing exclusively β(2→1)-linked fructosyl residues attached to the initial Suc, are synthesized (reviewed by Pollock and Cairns, 1991).

Inulins are characteristic for the Asteraceae, whereas grasses contain fructans of more complex structure with β(2→6) in addition to β(2→1) fructosyl-Fru linkages. Fructans containing both β(2→1) and β(2→6) linkages in substantial amounts, such as those that occur in wheat and barley, are called graminans. Fructans containing primarily or exclusively β(2→6) linkages, such as those found in many forage grasses, as well as in bacteria, are called phleins or levans (Waterhouse and Chatterton, 1993). For convenience in discussion, the term "levan" will be used here to refer to bacterial fructan only.

Both 1-SST and 1-FFT activities have been measured, and the corresponding enzymes have been partially purified from cereals and other grasses (reviewed by Pollock and Cairns, 1991). Yet it remains unclear which enzymes are responsible for the β(2→6) fructosyl-Fru linkages prevailing in the fructan of grasses. It has been found, however, that barley leaves contain a 6-SST activity that produces 6-kestose (Wagner and Wiemken, 1987) in addition to 1-SST activity. Both 1-SST and 6-SST are strongly induced under conditions that lead to fructan accumulation in the leaves (Simmen et al., 1993). A partially purified preparation of 6-SST also exhibited a strong fructosyltransfer activity from Suc to 1-kestose (Simmen et al., 1993), producing the branched bifurcose (1- and 6-kestotetraose). Thus,

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Abbreviations: DP, degree of polymerization; 1-FFT, fructan: fructan 1-fructosyltransferase; 6-SFT, Suc:fructan 6-fructosyltransferase; 1-SST, Suc:Suc 1-fructosyltransferase; 6-SST, Suc:Suc 6-fructosyltransferase.

this preparation formed a β(2→6) fructosyl-Fru linkage not only with Suc, but also with the simplest inulin, 1-kestose, as a fructosyl acceptor. Here we show that the purified enzyme can transfer the fructosyl moiety of Suc to a wide variety of fructans of different structure and DP, whereby β(2→6) linkages are formed exclusively. Fructans function only as fructosyl acceptors and not as donors, differentiating the activity from that of FFT. The enzyme, initially called 6-SST, is therefore more appropriately designated a 6-SFT. Together with the previously described fructan-synthesizing activities present in barley and wheat, namely 1-SST (Wagner and Wiemken, 1987) and 1-FFT (Jeong and Housley, 1992), 6-SFT is capable of synthesizing all of the fructan species currently known to occur in these cereals (Bancal et al., 1991, 1992).

MATERIALS AND METHODS

Preparation of Enzyme

6-SST activity was prepared from primary leaves of 7-d-old barley (*Hordeum vulgare*) seedlings continuously illuminated for 24 h to induce fructan synthesis (Simmen et al., 1993). Purification generally followed the protocol of Simmen et al. (1993), using sequential HPLC on Mono Q HR 5/5 and on alkyl Superose (Pharmacia). However, this preparation was found to still contain some fructan hydrolase activity, releasing Fru from oligofructans. Therefore, for the experiments described in Figure 1 and Table I, the purification protocol was modified to obtain a preparation of similar specific 6-SST activity but essentially lacking fructan hydrolase activity (N. Sprenger, K. Bortlik, T. Boller, and A. Wiemken, unpublished data). For both purifications, fractions with peak enzyme activities from HPLC runs were pooled, concentrated on a Centricon-30 concentrator (Amicon, Beverly, CT), supplemented with glycerol to 50% (v/v) final concentration, and stored at −70°C. The purified enzyme had a molecular mass of 67 kD when analyzed by size-exclusion chromatography on Superdex 75 and yielded two polypeptides of approximately 50 and 20 kD on SDS-PAGE (N. Sprenger, K. Bortlik, T. Boller, and A. Wiemken, unpublished data).

Preparation of Fructan Substrates and Standards

Fructan oligomers are designated by their common names according to the proposition of Waterhouse and Chatterton (1993) (see Fig. 2). Neutral carbohydrates were extracted both from Jerusalem artichoke (*Helianthus tuberosus*) stored for 12 weeks at 4°C and from wheat (*Triticum aestivum*) stems 2 weeks after anthesis (Bancal et al., 1992). The Jerusalem artichoke extract comprised hexoses, Suc, and an oligoinulin series (DP3 to about DP30). The wheat extract was further purified to yield 6-kestose, 4,4-kestotetraose, and a mixture of bifurcose and 6,1-kestotetraose. 1-Kestose, nystose, and 1,1,1-kestopentaose were purified from Neosugar-P (Beghin Say, Toulouse, France), a commercial sweetener.

The fructan oligomers were purified by successive separations on normal and reverse-phase preparative HPLC. Normal-phase columns were packed with RSil NH_2 5 μm (Interchim, Montluçon, France) and eluted with water:acetonitrile (either 30:70 or 60:40, v/v). Reversed-phase columns were packed with Econosil C_{18} 10 μm (Altech, Deerfield, IL) and eluted with water:acetonitrile (either 99:1 or 100:0, v/v). The dimensions of the columns were 1 × 25 cm and they were eluted at a flow rate of 4 mL min^{-1}. After each step of separation, the collected fractions (up to 100 mL) were concentrated under vacuum before further chromatography. The final purities, as assessed by anion-exchange HPLC (as described below), were higher than 95%, except for 1,1,1-kestopentaose, which was 80% pure.

Labeled Suc Preparation

Commercial [U-^{14}C]Suc (Amersham) was found to contain radioactive contaminants that may interfere with the enzyme assay and also with the detection of labeled products. To purify the product, 80 μL were applied to a 10 × 20 cm F1500 silica gel plate and chromatographed with acetone:water (87:13, v/v) as described by Wagner et al. (1983). The area containing labeled Suc was identified as the main spot of the chromatogram after analyzing the plate on a GS-250 Molecular Imager System (Bio-Rad). It was scraped off the plate, suspended in 65 μL of water, and centrifuged. The supernatant containing pure [U-^{14}C]Suc was used as a substrate after appropriate dilution with unlabeled Suc.

Enzyme Assays

If not otherwise indicated, assays were done in the presence of 100 mM Suc either alone or with 100 mM Glc, Fru, or the purified fructan oligomers. Incubations were made in triplicate as follows: an aliquot of 5 μL of enzyme in 100 mM citric acid-Na_2HPO_4 buffer, pH 5.75, was incubated with the different substrates in a total volume of 15 μL at 27°C for 4 h. For testing Suc:fructosyltransferase activity with inulin acceptor, the substrates were 200 mM (final concentration) [U-^{14}C]Suc (18.5 kBq) and a 10% (w/v) extract of neutral sugars from Jerusalem artichoke tubers. For measuring frutosyltransfer from Suc to Glc, 100 mM [U-^{14}C]Glc (18.5 kBq, Amersham) and 200 mM unlabeled Suc served as substrates. The reactions were stopped by boiling for 3 min. Samples were then diluted by adding 30 μL of water containing 4 μg of trehalose as internal standard and stored at −20°C until analyzed by chromatography. For characterization of sugars released from defined oligofructans, no internal standard was added.

Analysis of Carbohydrates

The sugars obtained from the incubation with defined oligofructans were identified by co-chromatography with structurally characterized standards on both reverse-phase HPLC/refractometry and anion-exchange HPLC/amperometry (Bancal et al., 1992, 1993). Two anion-exchange separations were carried out for each sample, using two different gradients on a Carbopac PA-1 column in a DX300 chromatography system from Dionex (Sunnyvale, CA). The flow rate was 1 mL min^{-1} in both cases. With gradient

1 (Bancal and Triboï, 1993) the full range of fructans up to DP30 to DP50 was separated with lower resolution. With gradient 2, increased resolution was achieved for DP3 to DP6 oligofructans. For gradient 2, NaOH was kept at a constant 150 mM concentration. Sodium acetate was initially present at a 25 mM concentration and was raised to 50 mM within 0.1 min and then to 150 mM within 20 min; it was kept at this level for 5 min and then lowered again to 25 mM within 2 min.

Samples from experiments involving radiolabeling of inulin were similarly separated by anion-exchange chromatography using an initial gradient of NaOH from 100 to 300 mM within 5 min, followed by a sodium acetate gradient from 0 to 500 mM in 300 mM NaOH within 25 min at a flow rate of 1 mL min^{-1}. Radioactivity was monitored by a flow detector (Radiomatic FLO-ONE\BETA, model A-500 from Canberra Packard, Meriden, CT) following the pulsed amperometric detector. Samples from the assays with Suc and labeled Glc were similarly analyzed, eluting the anion-exchange column with the gradient described by Simmen et al. (1993).

RESULTS

In previous work (Simmen et al., 1993) it was found that barley leaf 6-SST activity used not only Suc but also 1-kestose as fructosyl acceptor. To test whether other fructans might function as acceptors as well, purified 6-SST activity was incubated with an extract of fructans from tubers of Jerusalem artichoke in the presence of [U-^{14}C]Suc. Samples taken from the assay at zero time of incubation and analyzed by anion-exchange HPLC showed the regular pattern of peaks characteristic for the inulin of low DP present in tubers during early spring before sprouting (Fig. 1A). After 4 h of incubation, a number of new peaks appeared that were clearly distinct from the inulin series (Fig. 1B). All of these new peaks contained labeled material, whereas the fructans of the inulin series did not (Fig. 1, C and D).

These results describe the enzyme activity as a general SFT. Enzyme likely does not form β(2→1) fructosyl-Fru linkages because the inulin series is not labeled (Fig. 1, B and D). The release of some labeled Fru in addition to Glc (Fig. 1, C and D) indicates the presence of β-fructosidase (invertase) activity.

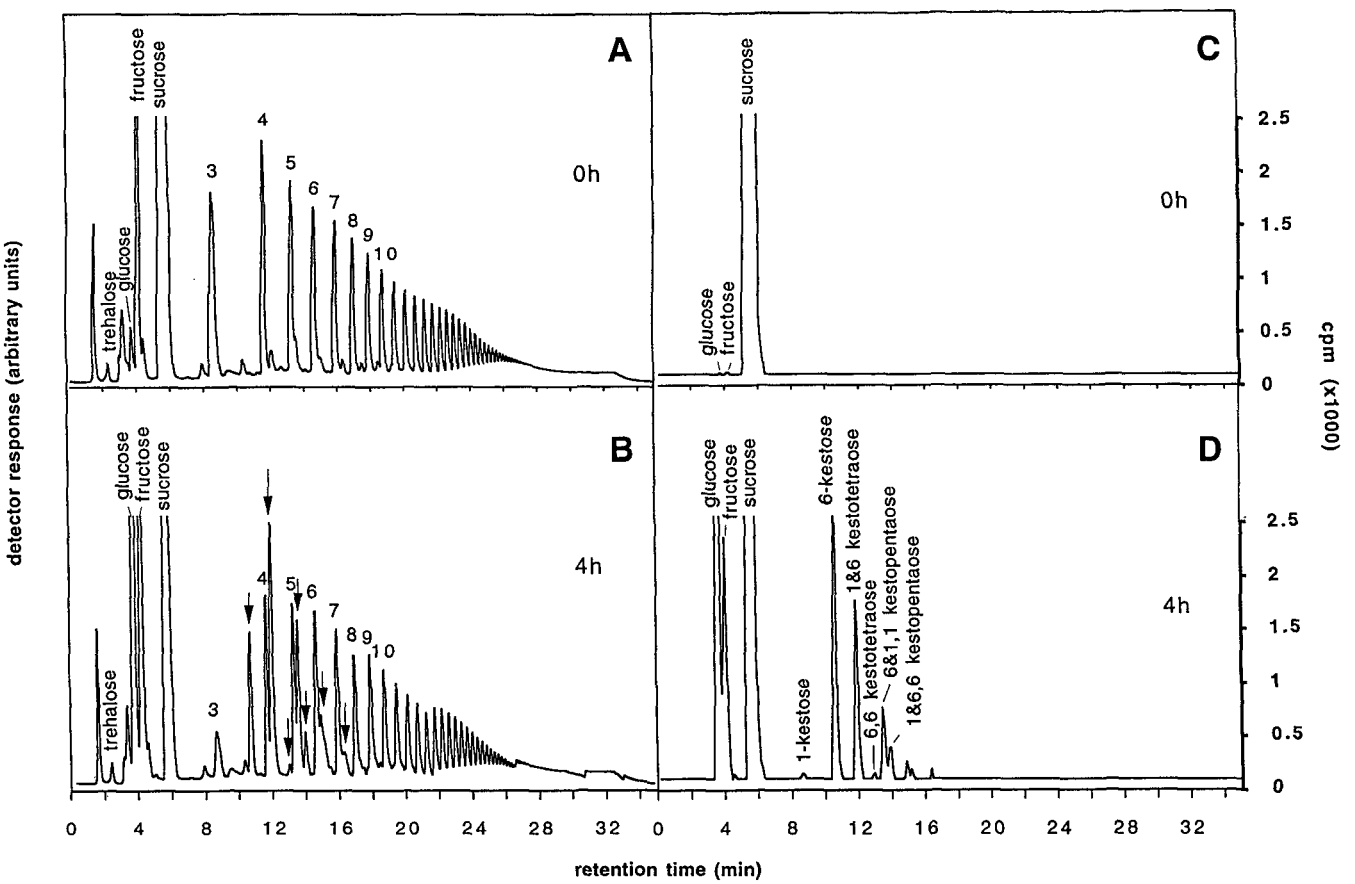

Figure 1. HPLC analysis of an assay mixture of purified barley 6-SST with [U-^{14}C]Suc and an inulin extract, incubated for 0 h (A and C) or 4 h (B and D). Analysis was carried out by anion-exchange HPLC followed by pulsed amperometric detection (A and B) or by radioactivity flow detection (C and D). Trehalose was added as an internal standard. The peaks representing the inulin series in A and B are numbered according to DP. Peaks appearing after 4 h of incubation are indicated by arrows (B). They correspond to the fructan oligomers as detected by radioactivity monitoring (D).

To obtain further insight into the activities exhibited by the enzyme, it was assayed in the presence or absence of Suc with a variety of oligofructans with defined structures (Fig. 2; Table I). In assay mixtures containing an oligofructan together with Suc, one primary, new oligomeric product was formed. This product, which appeared only in traces in assays without added Suc, was identified for each oligofructan substrate by co-chromatographies with structurally characterized standards in two HPLC systems (Fig. 2). In each case, the product contained a single, additional fructosyl residue, linked through a β(2→6) bond either to the fructosyl residue attached to the terminal Glc or to a β(2→6) fructosyl chain. Thus, the enzyme either initiated or extended a phlein chain. In addition to these fructosylated products and to Glc, peaks corresponding to Fru and 6-kestose were also identified in all the assays with oligofructans and Suc, indicating multiple activities of the enzyme.

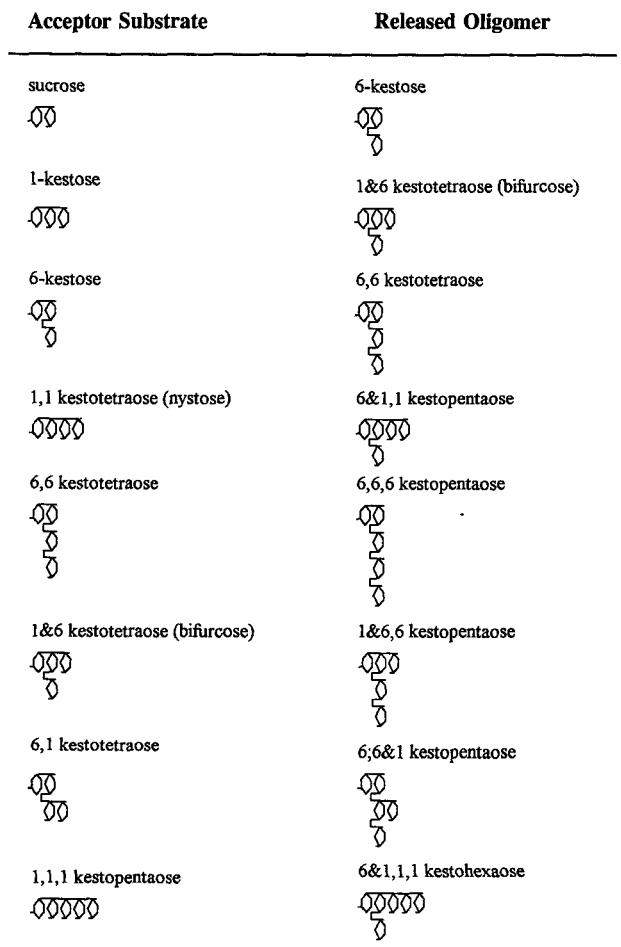

Figure 2. Substrates supplied as acceptors and products generated from them in the assays within 4 h by 6-SFT from barley. The nomenclature proposed by Waterhouse and Chatterton (1993) has been adopted. Common names are also given. Fructans were identified by co-chromatography with known standards on both anion-exchange and reverse-phase HPLC. Note that Fru and 6-kestose were also released in the assays, with the origin most likely coming from Suc and water acting as acceptor substrates (see Table I).

To evaluate these different activities quantitatively, Fru release was taken as a measure for invertase activity, 6-kestose production as a measure of 6-SST activity, and the production of fructans of DP > 3 as a measure of 6-SFT activity. The sum of these activities yielded the total fructosyltransfer activity. Representative results with different substrate combinations are presented in Table I. When Suc was offered as the only substrate, the enzyme acted predominantly as an invertase, with about 20% of the total fructosyltransferase activity represented by 6-SST. A small 6-SFT activity was likely due to further fructosylation of the 6-kestose formed by 6-SST. When the enzyme was incubated with only an oligofructan (1-kestose, 6-kestose, or 6,6-kestotetraose), or with a combination of oligofructans, there was virtually no fructosyltransfer activity: the small amount of 6-SFT activity observed with 1-kestose was in all likelihood due to a contamination of the 1-kestose substrate with Suc, as indicated both by the stoichiometric production of Glc and by the decrease of contaminating Suc during the assay. Remarkably, when Suc was supplied together with oligofructans, the total fructosyltransferase activity decreased only slightly compared to activities when incubation took place with Suc alone, but invertase and 6-SST activities were strongly reduced, and 6-SFT became the major activity. In the combination of Suc and 1-kestose, for example, 80% of the activity of the enzyme corresponded to 6-SFT, less than 20% to invertase, and less than 5% to 6-SST (Table I). Thus, in the presence of oligofructan acceptors, the enzyme behaved predominantly as SFT at the expense of invertase and SST activities.

An interesting result was observed when the enzyme preparation was incubated in the presence of Suc and Glc: each activity of the enzyme was inhibited by about 60% compared to inhibition when incubation took place with Suc alone (Table I). This observation prompted us to investigate whether Glc might act as a fructosyl acceptor substrate. The enzyme was incubated with unlabeled Suc in the presence of [U-^{14}C]Glc and the products formed were analyzed. As expected, Fru and 6-kestose were found to be produced by the invertase and 6-SST activities of the enzyme, respectively (Fig. 3, A and B). By monitoring radioactivity, it became evident that in addition, Suc had taken up label, indicating a Suc:Glc fructosyltransferase activity (Fig. 3, C and D). Labeled Suc was further identified by co-chromatography on TLC with Suc. When the radioactive spot was scraped off the TLC plate, resuspended in buffer, and incubated with invertase from *Candida utilis* (Sigma), all of the radioactivity was converted again to [U-^{14}C]Glc (data not shown).

DISCUSSION

Until now, the biosynthesis of grass fructans has been poorly understood. 1-SST is clearly present in grasses (Wagner and Wiemken, 1987; Obenland et al., 1991); however, the generation of grass fructans according to the SST/FFT model would require a 6-FFT, such as that observed in *Asparagus officinalis* (Shiomi, 1989). An FFT activity has been purified from wheat leaves, but it specifically forms β(2→1) linkages (Jeong and Housley, 1992). Despite

Table I. *β-Fructosidase (invertase), 6-SST, and 6-SFT activities of the purified enzyme, incubated with 0.1 M Suc alone or together with 0.1 M of an additional substrate*

Enzyme activities were determined by quantifying the products formed in the assays after anion-exchange HPLC. Release of Fru indicated the activity of invertase, production of 6-kestose was used to determine 6-SST activity, and the sum of other fructans generated represented 6-SFT activity. The sum of the three activities was taken as total fructosyltransfer activity. Data are given per mL of the enzyme preparation; 1 mL contained 10 μg of protein and corresponded to the amount of activity contained in 150 mg fresh weight of the original leaf material.

Substrate(s)	Activities			
	β-Fructosidase (invertase)	6-SST	6-SFT	Total fructosyl transfer
	nkat mL^{-1}			
Suc	2.65	0.67	0.06	3.38
1-Kestose	0.02[a]	—[b]	0.21[a]	0.24[a]
6-Kestose	0.02[a]	n.d.[c]	—	0.02[a]
6,6-Kestotetraose	0.01[a]	—	0.01[a]	0.02[a]
Suc + Glc	1.07	0.28	0.03	1.38
Suc + 1-kestose	0.52	0.11	2.37	3.01
Suc + 6-kestose	1.68	n.d.	0.71	2.39
Suc + 6,6-kestotetraose	1.19	0.25	1.29	2.73
1-kestose + 6-kestose	0.03[a]	n.d.	0.18[a]	0.21[a]
1-kestose + 6,6-kestotetraose	0.02[a]	—	0.11[a]	0.13[a]
6-kestose + 6,6-kestotetraose	0.01[a]	n.d.	0.01[a]	0.03[a]

[a] Most likely caused by the presence of a small amount of contaminating Suc in the oligofructan substrate preparations (see text). [b] —, Activities were below the detection limit. [c] n.d., Not determined because the samples already contained 0.1 M 6-kestose serving as substrate.

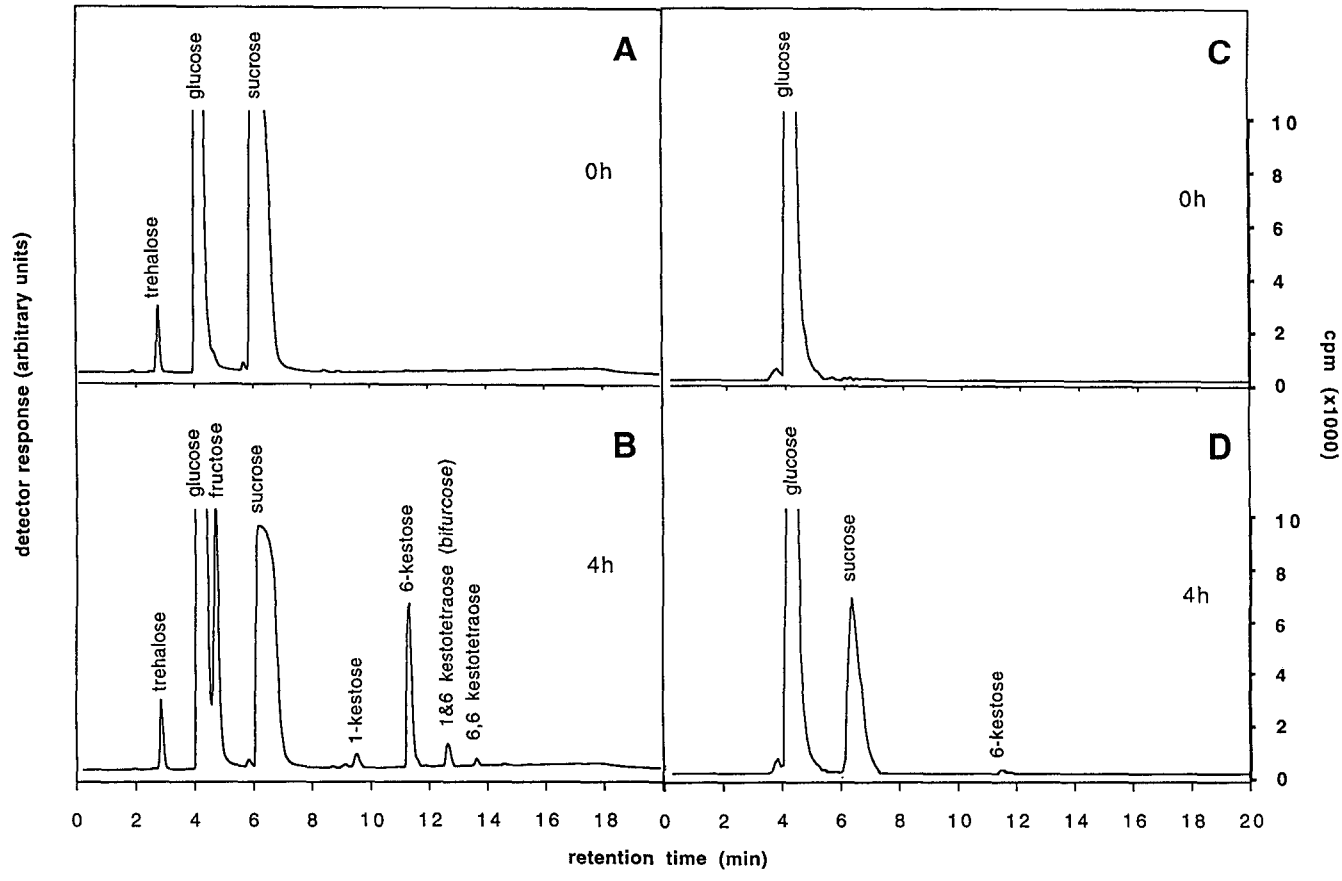

Figure 3. Label transfer from Glc to Suc by enzyme incubation with 200 mM Suc and 100 mM [U-^{14}C]Glc. Carbohydrates present in samples before (A and C) and after (B and D) incubation for 4 h were analyzed by anion-exchange HPLC coupled with pulsed amperometric detection (A and B) and radioactivity flow detection (C and D). Trehalose was added as internal standard. Sugars were identified by their retention times. All the radiolabel in Suc was determined to be in the Glc moiety.

intense searches, no 6-FFT activity has been isolated thus far in grasses (Pollock and Cairns, 1991; Housley and Pollock, 1993).

We now propose that 6-SFT, catalyzing the essentially irreversible transfer of a fructosyl residue to a fructan in a $\beta(2\rightarrow6)$ linkage, is the key enzyme for the formation of the graminans and phleins. The $\beta(2\rightarrow6)$ fructosyl-Fru linkages by far prevail in grasses (Carpita et al., 1991). Thus, most of the flux of carbon from Suc to fructan is probably mediated by 6-SFT.

In grasses, 1-SST plays a role in synthesizing 1-kestose, which is the fructosyl acceptor favored by 6-SFT. Then 6-SFT transforms 1-kestose into the branched bifurcose, which appears to hold a central position in fructan reserves of wheat and barley (Bancal et al., 1992) as well as in other grasses (Chatterton et al., 1993a, 1993b). According to our new model, all the fructans known at present in these grasses are synthesized by a concerted action of 6-SFT and the fructan-metabolizing enzymes previously identified. Therefore, 1-SST and 6-SFT act as key enzymes for net fructan synthesis, whereas 1-FFT and possibly fructan hydrolases are responsible for fructan processing. The existence of a 6-SFT makes it superfluous to speculate about any additional enzymes that might explain the synthesis of the barley and wheat fructan. For instance, there is no longer a need to speculate about either a 6-FFT (Housley and Pollock, 1993) or a specific branching enzyme (Bancal et al., 1992).

Clearly, the relative activities of the different enzymes for fructan metabolism have a profound influence on the specific fructan mixture accumulated in a given grass species. Changes in the level of the respective enzyme activities are most probably responsible for the differences observed in the fructan oligomer content within a single plant species during plant development (Bancal et al., 1992) or under the influence of climatic factors (Bancal and Triboï, 1993). Thus, 1-SST has been found to be induced rapidly upon excision and with continuous illumination of barley and wheat leaves, leading to initial accumulation of 1-kestose. Later, 6-SFT is also induced (Simmen et al., 1993), and consequently the 1-kestose pool declines, whereas bifurcose as well as fructans with a high proportion of $\beta(2\rightarrow6)$ linkages accumulate (Fig. 4).

It has not escaped our attention that in many respects the 6-SFT resembles the bacterial transfructosylases generally designated levan sucrases (reviewed by Cote and Ahlgren, 1993). Actually, in an earlier report an SFT activity resembling bacterial levan sucrases was found in the particulate fraction from wheat and other grasses and named "phlein sucrase" (Suzuki and Nass, 1988). This activity was exceedingly low (about 1 pkat/g fresh weight in wheat, at least 5000 times lower than the activity reported in the present work), necessitating incubation times of up to 16 h. Thus, it was difficult to exclude other explanations for the net accumulation of $\beta(2\rightarrow6)$ linkages in assays, such as a bacterial origin. Like bacterial levan sucrases, the 6-SFT here described can use Glc, fructan, and even water as fructosyl acceptors, leading to fructosyl exchanges at Glc, fructan polymerization, or hydrolytic release of Fru, respectively.

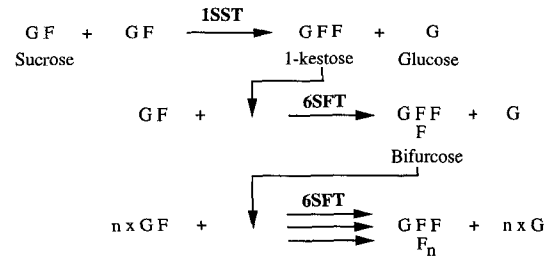

Figure 4. Scheme of the proposed activities of 1-SST and 6-SFT during fructan biosynthesis in excised barley leaves exposed to continuous light.

Kinetic studies on the levan sucrase of *Bacillus subtilis* (Chambert et al., 1974) and the isolation of a fructosyl-enzyme intermediate (Chambert and Goncy-Tréboul, 1976) suggested that the bacterial enzyme operates by a double-displacement mechanism (Lehninger, 1981). In a first step Suc is cleaved and Glc is released, and in a second step the fructosyl residue still covalently bound to the enzyme is transferred to an acceptor substrate.

The activity of 6-SFT also shows clear differences with bacterial levan sucrases. The barley enzyme appears to work best with oligofructans, whereas the bacterial enzymes produce primarily long-chain fructans. The polymerase activity of the latter is stimulated relative to its hydrolytic activity by binding the enzyme to hydroxy apatite (Chambert and Petit-Glatron, 1993) or by progressively substituting water with acetonitrile in the enzyme assays (Chambert and Petit-Glatron, 1989). These effects are not observed with the barley enzyme (K. Bortlik, unpublished results). Specific binding to Con A Sepharose (U. Simmen, unpublished results) demonstrates that 6-SFT is a glycoprotein characteristic for eukaryotes. The enzyme sequencing is actually underway: partial data on tryptic fragments of 6-SFT showed similarities not to bacterial levan sucrases but rather to plant invertases (P. Jenö and N. Sprenger, unpublished results). However, our results stress the basic similarity in the enzymatic mechanism and suggest that fructan synthesis of levan in bacteria and of phlein in grasses proceed along a similar pathway.

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