Transgenic potato (*Solanum tuberosum*) tubers synthesize the full spectrum of inulin molecules naturally occurring in globe artichoke (*Cynara scolymus*) roots

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The ability to synthesize high molecular weight inulin was transferred to potato plants via constitutive expression of the *1-SST* (sucrose:sucrose 1-fructosyltransferase) and the *1-FFT* (fructan: fructan 1-fructosyltransferase) genes of globe artichoke (*Cynara scolymus*). The fructan pattern of tubers from transgenic potato plants represents the full spectrum of inulin molecules present in artichoke roots as shown by high-performance anion exchange chromatography, as well as size exclusion chromatography. These results demonstrate *in planta* that the enzymes sucrose:sucrose 1-fructosyltransferase and fructan:fructan 1-fructosyltransferase are sufficient to synthesize inulin molecules of all chain lengths naturally occurring in a given plant species. Inulin made up 5% of the dry weight of transgenic tubers, and a low level of fructan production also was observed in fully expanded leaves. Although inulin accumulation did not influence the sucrose concentration in leaves or tubers, a reduction in starch content occurred in transgenic tubers, indicating that inulin synthesis did not increase the storage capacity of the tubers.

Inulin is a highly water-soluble carbohydrate consisting of linear $\beta(2\rightarrow1)$-linked fructose chains attached to a sucrose molecule. It belongs to the fructan group of polysaccharides, which serves as alternative storage carbohydrate in the vacuole of approximately 15% of all flowering plant species (1–7). Recent gain of interest comes from observations that inulin positively influences the composition of the gut microflora (reviewed in ref. 8), and there is indication for beneficial effects on mineral absorption, blood lipid composition, and prevention of colon cancer (9). In addition, inulin is a low-calorie fiber that could be used in the production of fat-reduced foods (10). Currently, the main source for inulin production are chicory (*Cichorium intybus*) and Jerusalem artichoke (*Helianthus tuberosus*), both of which produce a fructan with a relatively low average molecular weight (11).

In 1968, Edelman and Jefford (12) proposed a model for inulin synthesis in *H. tuberosus* involving two enzymes: sucrose:sucrose 1-fructosyltransferase (1-SST) and fructan:fructan 1-fructosyltransferase (1-FFT). The model postulates that 1-SST catalyzes the synthesis of the trisaccharide 1-kestose from two molecules of sucrose, thereby releasing glucose. In subsequent steps, 1-FFT reversibly transfers fructosyl residues from one fructan to another, producing a mixture of fructans with different chain lengths. In recent years, evidence in support of the model was obtained from *in vitro* inulin synthesis experiments with purified 1-SST and 1-FFT enzymes (13–15). However, only polymers of chain lengths of up to 25 fructose units were synthesized, whereas the full range of molecule sizes in inulin-producing plants like *H. tuberosus* or *C. intybus* comprises a degree of polymerization (DP) of more than 60 (11). Transgenic petunia plants expressing the *1-SST* and *1-FFT* genes from *Helianthus tuberosus* also produce only inulin molecules of up to DP 25 (16). Therefore, unequivocal proof that 1-SST and 1-FFT are sufficient to catalyze the synthesis of fructans with a DP higher than that is still lacking.

Globe artichoke (*Cynara scolymus*) synthesizes inulin molecules with a chain length of up to 200 (11), which is the highest DP of inulin molecules known for plants. In the present contribution we show that expression of 1-SST and 1-FFT genes from globe artichoke in transgenic potato plants leads to substantial accumulation of inulin in tubers with a maximum chain length that is indistinguishable from inulin isolated from artichoke roots.

Materials and Methods

Transformation of Plants. The *1-SST* (17) and *1-FFT* cDNAs (18) were subcloned separately into the binary vector pBinAR, a derivative of the pBin19 vector (19). The cDNAs contain endogenous signal sequences for a predicted vacuolar localization of the encoded enzymes. The cDNAs were inserted between the constitutive cauliflower mosaic virus 35S RNA promoter (20) and the octopine synthase terminator sequence (21). Transformation of potato was performed as described by Rocha-Sosa *et al.* (22). Wild-type potato plants (*Solanum tuberosum* var. Désirée) were first transformed with the chimeric construct containing the *1-SST* gene. Positive transformants displaying 1-SST activity were selected and supertransformed with the *1-FFT* gene.

Plant Material. Plants grown in tissue culture were transferred into pots (30 cm in diameter) with soil and cultivated in the greenhouse under natural daylight and temperature regime. Leaf samples were taken from 6- to 7-week-old plants. The tubers were harvested after 12 weeks of growth. In the case of plants transformed with the *1-SST* gene, tuber discs were prepared (1 cm diameter, approximately 2 mm thick), immediately frozen in liquid nitrogen, and homogenized. Of five different plants per *1-SST*/*1-FFT* line, one whole tuber was taken and homogenized in liquid nitrogen. Aliquots of the homogenized tissue were analyzed for their contents of soluble sugars, fructans, and starch, and the DP of the inulin molecules was determined as described below.

Carbohydrate Analysis. The water-soluble carbohydrates of plants transformed with the *1-SST* gene were determined by high-

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Abbreviations: 1-SST, sucrose:sucrose 1-fructosyltransferase; 1-FFT, fructan:fructan 1-fructosyltransferase; HPAEC, high-performance anion exchange chromatography; SEC, size exclusion chromatography; DP, degree of polymerization.

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performance liquid anion exchange chromatography (HPAEC) using a CarboPac PA-100 column on the Dionex DX-500 gradient chromatography system (17). Leaf and tuber discs were homogenized in liquid nitrogen and then extracted twice with ethanol at 80°C for 30 min. The supernatants were combined and dried, and the sugars were resuspended in water. Before loading onto the column the extracts were deionized (AG 501-X8 resin, Bio-Rad) and filtered (microcon, Millipore).

Tuber homogenates from plants displaying 1-SST and 1-FFT activity as well as root homogenates from artichoke were extracted twice in 80% ethanol at 80°C and twice in water at 60°C. The ethanol extracts were dried, and the residuals were dissolved in the combined water extracts. Glucose and fructose contents were measured photometrically (23). Because invertases also degrade oligofructans, sucrose content was determined after cleavage by sucrose phosphorylase (24). To quantify the inulin, the fructan molecules were hydrolyzed with 0.5% oxalic acid at 65°C and the released fructose was determined photometrically. For analysis of the inulin pattern by HPAEC the combined extracts were deionized and filtered as described above.

The precipitate of the ethanol and water extraction procedure was used for determination of the starch content (25). As a consequence of the extraction procedure, a minor portion of starch was lost during inulin extraction. This loss did not effect the comparison of starch content of wild-type and transgenic plants, and the absolute reduction was not significant.

Size Exclusion Chromatography (SEC) of Inulin Extracts. The SEC system consisted of the following components: solvent delivery pump LC-10 AD VP (Shimadzu) connected to a Rheodyne (Cotati, CA) injector 7725i (loop volume 200 μl), a guard column (Waters), and two columns of PL aquagel–OH 30 8 μm (2 $\times$ 300 $\times$ 7.5 mm; Polymer Laboratories, Waltrop, Germany). A refractive index detector RID-10A (Shimadzu) was used as a concentration-sensitive detector. The measurements were performed in degassed and bi-distilled water containing 0.02% sodium azide as eluent at a flow rate of 1.0 ml/min.

Before SEC, a high molar mass inulin synthesized *in vitro* by *Aspergillus sydowi* conidia ($M_r = 30 \times 10^6$ g/mol,) was added to the inulin extracts as an internal standard. The *A. sydowi* inulin was produced by using conidia of the strain IAM 2544 following a procedure described by Kawai *et al.* (26). Before injection the inulin extracts were centrifuged, filtered through a 0.45 μm spartan 30/B filter (Schleicher & Schuell), and diluted 2-fold in the eluent. The column eluate was fractionated in aliquots of 110 μl with a RediFrac fraction collector (Amersham Pharmacia). Fractions between 10 and 18 ml of elution volume were subjected to hydrolysis with oxalic acid, and released fructose was measured photometrically (23).

Results

Inulin Synthesis in Transgenic Potatoes. To define the enzyme activities of the fructosyltransferase genes of *Cynara scolymus in planta* we transformed a nonfructan species (*Solanum tuberosum* var. Désirée) and analyzed the inulin pattern of leaves and tubers. In a first transformation step, a chimeric construct containing the *1-SST* gene (17) under the control of the constitutive cauliflower mosaic virus 35S promoter was introduced into wild-type potato plants. In 19 of 41 antibiotic-resistant plants *1-SST* mRNA accumulation and oligofructan synthesis could be observed. Of those we chose three transgenic lines and transformed them with the *1-FFT* gene (18), again under the control of the 35S promoter. We obtained 10 transgenic lines accumulating high molecular weight inulin.

Transgenic plants expressing either *1-SST* alone or in combination with *1-FFT* show no visible alteration of growth, leaf shape, and leaf color as compared with wild-type plants. Furthermore, tuber number as well as fresh and dry weight of the tubers were not affected by oligofructan or inulin synthesis (data not shown).

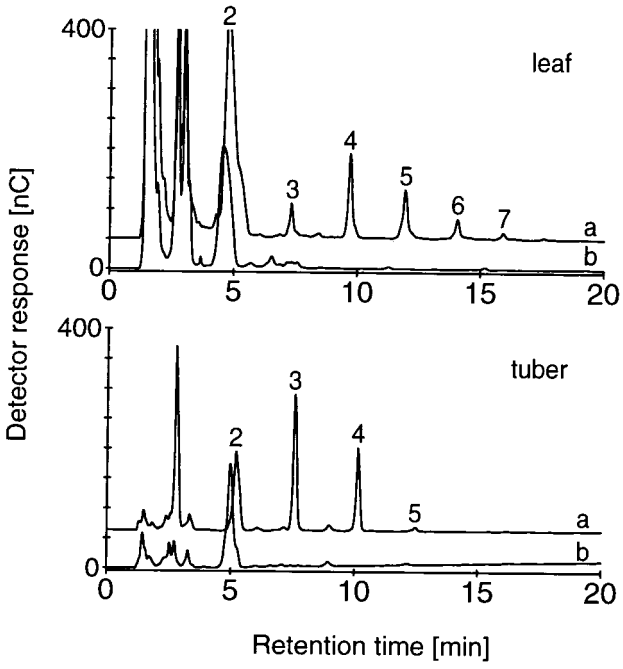

Fig. 1. Oligofructan content of leaves and tubers of 35S-SST-transformed potato plants in comparison to nontransformed wild-type plants. The oligosaccharides from extracts of the transgenic potato line 35S-SST 36 (a) and wild-type potato plants (b) were separated by HPAEC. The numbers indicate the DP of the sugars: 2 = sucrose, 3 = 1-kestose, 4 = nystose, 5–7 = oligofructans.

Oligofructan Pattern of Plants Expressing *C. scolymus 1-SST*. Plants expressing the *1-SST* gene accumulated oligofructans in leaves as well as in tubers. HPAEC analysis of leaf extracts revealed synthesis not only of the trisaccharide 1-kestose (GF_2), but of inulin oligomers up to GF_6 in lines with high expression level (Fig. 1). All types of fructan molecules were absent in the control plants.

Based on standardization with pure substances the soluble sugar content of leaves and tubers of different transgenic *1-SST* lines was determined (Tables 1 and 2). In leaves, glucose, fructose, and sucrose content of transgenic plants corresponded to that of wild-type plants. Fructan accumulation in 1-SST plants reached only one-tenth of the sucrose concentration (0.45–0.65 μmol/g fresh weight as compared with 3.28–4.94 μmol/g fresh weight) with GF_3 dominating over GF_2 in all lines analyzed.

A different picture was obtained for tubers of the transgenic plants. In this tissue 1-kestose was the most abundant oligosaccharide, whereas the sucrose concentration was again not significantly altered (Fig. 1; Table 2). Besides 1-kestose, nystose accumulated to considerable amounts, whereas only very low amounts of higher DP inulin molecules could be detected. In transgenic tubers, a significant increase in the glucose concentration could be detected, whereas oligofructan accumulation had no impact on starch content.

Accumulation of High Molecular Weight Inulin in Plants Expressing *1-SST* and *1-FFT*. The synthesis of inulin was demonstrated by HPAEC analysis of leaf and tuber extracts. Again inulin accumulation was much lower in leaves than in tubers. A typical fructan profile of transgenic potato tubers is shown in Fig. 2. The pattern does not significantly differ from that of native artichoke

Table 1. Sugar contents expressed in μmol hexose equivalent/g fresh weight of leaves of wild-type potato plants or plants transformed with the 35S-SST construct

Plant	Glucose	Fructose	Sucrose	1-Kestose	DP4	DP5
Wild type	1.64 ± 0.92	1.59 ± 1.12	3.28 ± 1.02	—	—	—
35S-SST 6	1.11 ± 0.39	1.26 ± 0.58	4.53 ± 1.95	0.21 ± 0.07	0.24 ± 0.12	n.d.
35S-SST 9	0.64 ± 0.18	0.69 ± 0.35	3.47 ± 1.28	0.17 ± 0.06	0.32 ± 0.09	0.16 ± 0.07
35S-SST 18	1.24 ± 0.83	1.51 ± 1.24	3.51 ± 1.42	0.17 ± 0.08	0.27 ± 0.12	0.13 ± 0.06
35S-SST 36	2.00 ± 1.66	2.08 ± 1.41	4.94 ± 1.27	0.11 ± 0.02	0.27 ± 0.07	0.16 ± 0.07

Leaf discs were homogenized in liquid nitrogen, sugars were extracted in 80% ethanol, and the amounts were quantified by HPAEC. Seven to ten replicates were analyzed per line. n.d., not detectable.

root inulin. Using HPAEC we could separate inulin molecules only up to a DP of about 50. However, it has been shown that artichoke synthesizes high molecular weight inulin of a DP of up to 200 (11). To verify that 1-SST and 1-FFT from artichoke are sufficient to catalyze the synthesis of high molecular weight inulin, we determined the maximum chain length of the potato fructans by SEC.

Because of contamination of the inulin by starch, refractive index detection was unsuitable to record elution profiles. We therefore fractionated the column eluate and determined fructose concentration in 110-μl fractions (see *Materials and Methods*).

The size distribution profile of fructose-containing polymers in tuber extracts of potato plants harboring the *1-SST* and the *1-FFT* gene of *C. scolymus* as well as artichoke roots is shown in Fig. 3. To ensure comparability of different separations, a high molecular mass inulin ($M_r > 30\times10^6$ g/mol, see *Materials and Methods*) was added to all extracts as an internal standard before SEC separation. The peak of this standard was detected in fractions 7–10 in all extracts analyzed.

In extracts of wild-type tubers only sucrose was detectable as a fructose containing species eluting in fractions 74–88. Comparison of the chromatogram of artichoke root extracts with those of transgenic tubers revealed that the inulin pattern of artichoke and the transgenic potatoes did not differ with respect to the maximal chain length. In all extracts the elution of detectable higher molecular weight fructose polymers started with fraction 42. However, the relative proportion of molecules of different DP differed significantly. Although the profile of artichoke root fructan revealed a peak in fraction 62, the fructan concentration increased continuously with decreasing DP in extracts of the transgenic potato line 22/19. The peak in fraction 73 probably reflects high amounts of the oligofructans 1-kestose and nystose. In extracts of the transgenic potato line 22/30 this oligosaccharide peak (fraction 69–87) also could be detected. In tubers of all lines analyzed, the fraction of high molecular mass inulin was significantly lower than in artichoke roots, whereas the maximum DP was comparable.

Carbohydrate Content of Inulin-Synthesizing Potatoes. To quantify fructan accumulation and to analyze its effect on the carbohydrate metabolism of potato, total yield and carbohydrate composition were determined for wild type and transgenic lines in five replicates grown in a greenhouse. One tuber of each plant was harvested and total sugar content was determined.

The transgenic lines accumulated inulin up to 37 μmol fructose equivalent per g fresh weight (Table 3). As already demonstrated for *1-SST*-expressing lines, the fructosyltransferase activities did not profoundly impact soluble carbohydrate composition of the tubers. In the line 22/34, the glucose concentration was significantly increased, whereas the sucrose content remained unchanged in all lines. The starch content appeared to be decreased, although no significant reduction could be observed in all lines except 22/34 because of the high variation in starch content of wild type as well as transgenic lines. The total carbohydrate content of the tubers, expressed as μmol hexose equivalent per g fresh weight, showed a slight decrease in transgenic plants.

Discussion

1-SST and 1-FFT Are Sufficient for High Molecular Weight Inulin Synthesis in Artichoke. A strong body of evidence from *in vitro* studies with purified enzymes (13–15) as well as analysis of transgenic plants (16) has supported the concept of inulin synthesis by the two enzymes 1-SST and 1-FFT, originally proposed by Edelman and Jefford in 1968 (12). But whereas plants that naturally accumulate inulin produce fructose polymers with a maximum chain length of between 60 and 200 units (11), synthesis of inulin molecules with a DP of more than 25 has never been reported either from *in vitro* experiments or from transgenic plants. Expression of bacterial fructosyltransferases in plants leads to the synthesis of very high molecular weight fructans of a DP of several thousand (27, 28), but bacterial fructan synthesis differs greatly from plant fructan metabolism. Bacteria use only one enzyme to synthesize high molecular weight fructan polymers with sucrose being the substrate for each chain elongation step (29), whereas in plants synthesizing inulin chain elongation takes place as reversible fructosyl trans-

Table 2. Sugar contents expressed in μmol hexose equivalent/g fresh weight of tubers of tubers of potato plants expressing *1-SST* activity

Plant	Glucose	Sucrose	1-Kestose	1,1-Nystose	Starch
Wild type	3.16 ± 1.54	11.20 ± 2.47	—	—	651 ± 111
35S-SST 18	8.64 ± 2.22*	12.25 ± 2.30	29.87 ± 5.83	0.47 ± 0.22	665 ± 110
35S-SST 22	13.21 ± 2.46*	15.04 ± 4.17	25.01 ± 5.85	1.83 ± 0.76	608 ± 133
35S-SST 36	12.91 ± 1.90*	10.15 ± 1.82	10.79 ± 3.70	2.71 ± 1.14	596 ± 134

Tuber discs were homogenized in liquid nitrogen, sugars were extracted in 80% ethanol, and the amounts were quantified by HPAEC. Five replicates were analyzed per line. The data were analyzed for significant differences by using Student's *t* test; if $P < 0.05$ for the comparison of a transgenic line with the wild type, this is marked by *.

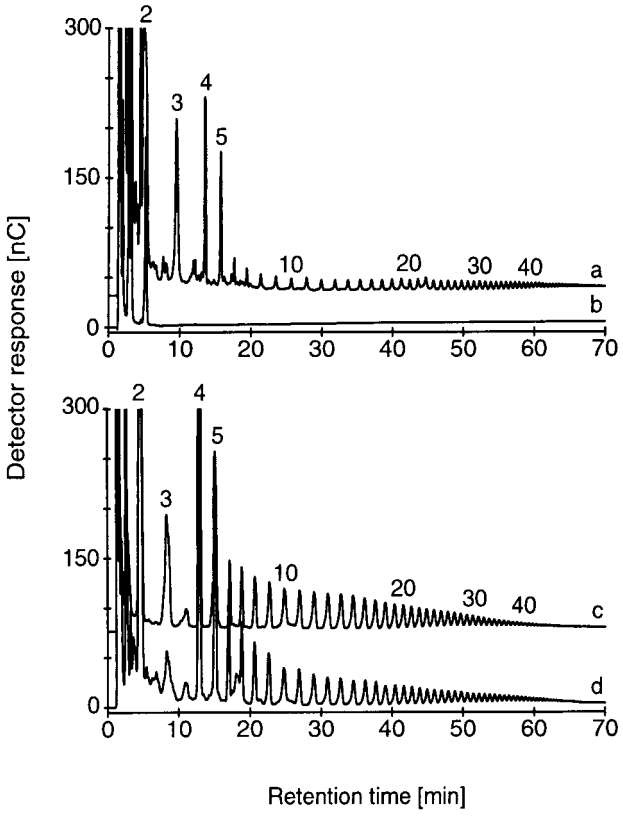

Fig. 2. Inulin content of leaves and tubers of potato plants harboring the *1-SST* and the *1-FFT* gene of *C. scolymus*. The HPAEC profile of the fructan pattern of leaves of a transgenic potato plant (*a*) was compared with that of a wild-type potato plant (*b*). Additionally, the inulin from transgenic potato tubers (*d*) was compared with native inulin from artichoke (*C. scolymus*) roots (*c*). The numbers indicate the DP of the sugars as described in the legend to Fig. 1.

fer between fructan molecules, which is catalyzed by an enzyme (1-FFT) that lacks sucrolytic activity. Additionally, almost all bacterial fructosyl transferases produce levan type fructan, which is characterized by β-2→6 linkages of fructose molecules instead of the β-2→1 bonds of inulin chains. Van der Meer *et al.* (28) reported expression of the *FTF* gene of *Streptococcus mutans*, which produces high molecular weight inulin, in potato, but the very low level of fructan accumulation prevented exact size determination.

Based on a detailed analysis by HPAEC and SEC of transgenic potato plants, we demonstrate that inulin with a DP of up to 200 can be produced in a heterologous system by using the *1-SST* and *1-FFT* genes of artichoke. Comparison of SEC chromatograms of potato tubers with those of artichoke root extracts revealed that the same high molecular weight inulin as in artichoke can be found in potato. We can therefore extend the Edelman and Jefford model, which originally was drafted for the synthesis of low molecular weight inulin in Jerusalem artichoke (*H. tuberosus*), to the high molecular weight inulin typical for globe artichoke (*C. scolymus*). As the chain length is an important quality determinant in industrial inulin production (30), this result is also of considerable economic interest.

Although the two fructosyltransferases 1-SST and 1-FFT are sufficient to create the complete set of inulin molecules present in artichoke, we observed profound differences in the chain length distribution pattern (Fig. 3). Determination of the glucose/fructose ratio of acid hydrolyzed inulin molecules of artichoke revealed an average chain length of 43, whereas in potato tubers the mean chain length was only 18 (data not shown).

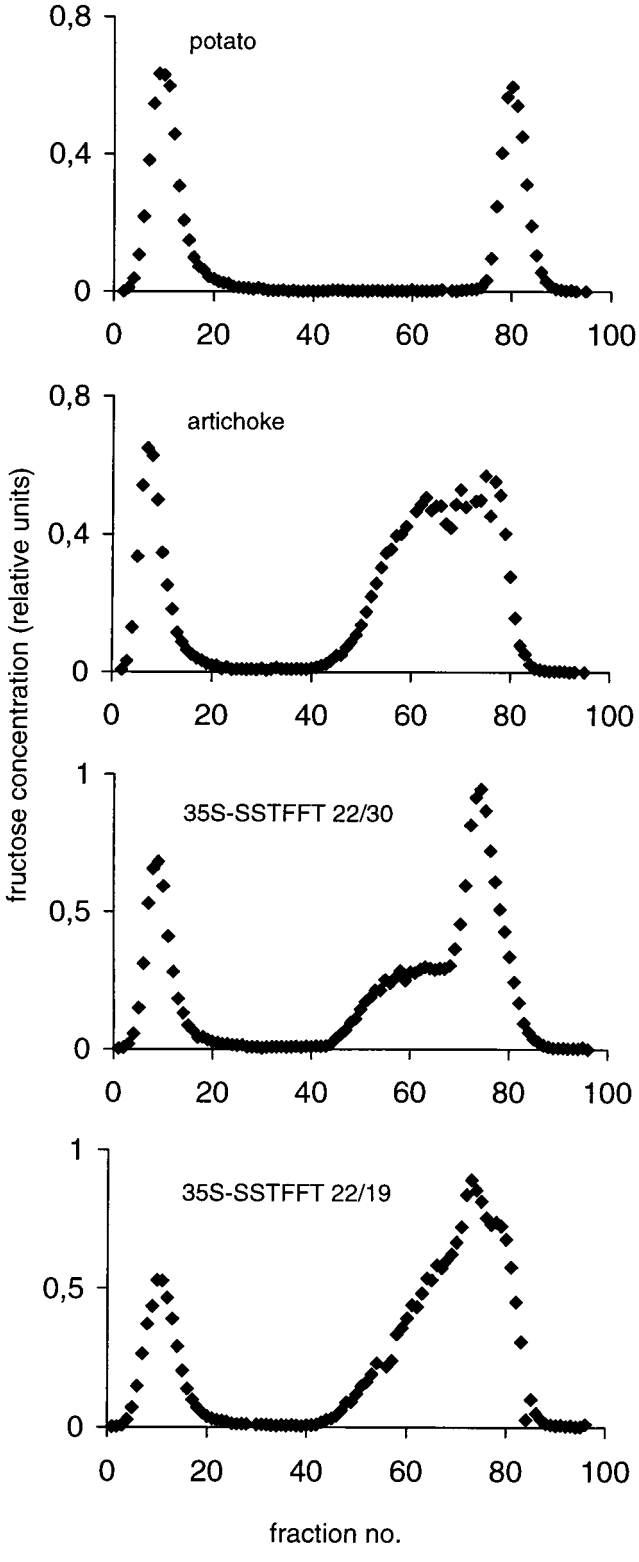

Fig. 3. SEC profiles of crude extracts from wild-type potato tubers, artichoke roots, and transgenic potato tubers expressing the *1-SST* and the *1-FFT* genes from artichoke. The eluate from the SEC column was fractionated in aliquots of 110 μl. Fractions between 10 and 18 ml of elution volume were subjected to fructose determination after inulin hydrolysis by oxalic acid. A high molecular weight inulin from *A. sydowi* (M_r 10^6 g/mol) was added as a standard to all extracts and eluted with a peak in fraction 7–10.

Table 3. Sugar and starch contents expressed in μmol hexose equivalent/g fresh weight of tubers of wild-type plants and plants harboring the *1-SST* and the *1-FFT* gene

Plant	Glucose	Fructose	Sucrose	Fructan	Starch
Desiree	2.12 ± 1.62	n.d.	9.11 ± 1.77	—	770 ± 113
22/19	3.87 ± 3.31	n.d.	14.67 ± 6.41	34.13 ± 4.71	631 ± 144
22/30	5.63 ± 4.24	n.d.	10.60 ± 5.21	33.00 ± 4.08	645 ± 132
22/34	7.21 ± 4.14*	n.d.	9.71 ± 2.17	37.43 ± 5.82	620 ± 75*

Whole tuber of five plants per line were homogenized and aliquots were analyzed. The data were analyzed for significant differences by using Student's *t* test; if $P < 0.05$ for the comparison of a transgenic line with the wild type, this is marked by *. n.d., not detectable.

Factors like sucrose availability or competing enzymes, e.g., invertases, may be responsible for this difference. It has been proposed (13) that fructan synthesis proceeds in a cascade-like fashion, where a threshold concentration for a molecule of a given chain length must be reached before the next higher homolog is synthesized. If such a mechanism operates in transgenic potato plants, concurrent starch synthesis may prevent the steady-state size distribution found in globe artichoke from being reached in potato tubers. In addition, exohydrolase(s) may be involved in generating the inulin pattern found in artichoke roots at the time of harvest. If such an enzyme preferably hydrolyzes oligofructans, the average chain length would be shifted to a higher DP.

In transgenic petunia expressing the *1-SST* and the *1-FFT* genes of Jerusalem artichoke inulin synthesis was observed only in senescent leaves (16). In contrast, we obtained accumulation of fructan in fully expanded leaves and, in even higher amounts, in the storage organs of transgenic potato. Lower sucrose concentration or higher vacuolar invertase activity in petunia could contribute to this difference. Alternatively, distinct enzymatic properties should be taken into account. We have reported that 1-FFT of globe artichoke shows a preference for higher DP substrates as acceptor of transferred fructosyl residues than does the enzyme from Jerusalem artichoke (18). Under conditions where high invertase activity prevents accumulation of lower molecular weight inulin molecules, which are the main products formed by the *H. tubero*sus 1-FFT, the *C. scolymus* enzyme would be more efficient in synthesizing higher-order inulin molecules, which are less accessible to invertases (31).

Impact of Fructan Accumulation on the Carbohydrate Metabolism of Transgenic Potato. In contrast to results obtained for expression of bacterial fructosyltransferases in plants (27, 32), we observed neither adverse phenotypic effects nor a reduction of tuber yield for inulin-accumulating potato plants. Furthermore, the impact on carbohydrate metabolism of green plant organs was only minor and leaf inulin content was low. Low levels of fructan accumulation in leaves also have been reported for sugar beet and petunia (16, 33) and, at least in the case of potato (34), can be attributed to low sucrose availability in leaf vacuoles.

Interestingly, in leaves of plants expressing only *1-SST* fructooligosaccharides up to a DP of 7 could be detected. The ability of 1-SST to produce oligomers with a DP greater than 5 has so far not been reported and contradicts the clear distinction of fructosyl transferase activities as proposed in the model of Edelman and Jefford. In contrast, 1-SST seems to have a rather broad spectrum of activities. Similar observations have been made on other β-fructofuranosidases. The 6-SFT of barley, which was the first fructosyltransferase that was characterized on a molecular level (35), is capable of *de novo* fructan synthesis from sucrose as well as introduction of branchpoints in existing chains, and the 1-FFT of chicory is able to transfer fructosyl residues to fructose monomers (36).

The inulin-containing potato tubers display a slight reduction in starch content, which indicates that inulin synthesis takes place at the expense of starch production with inulin as an alternative but not an additional sink for carbohydrates. That photoassimilates imported into the tuber are shared between starch and fructan synthesis is further evidence for a source limitation of tuber biomass production as it was reported by Engels and Marschner (37). Currently we are investigating the possible contribution of inulin production to potato tuber sink strength by transferring the fructosyltransferase genes to a potato line that is incapable of starch synthesis because of antisense inhibition of ADP-glucose pyrophosphorylase (38). If carbohydrate supply was limiting inulin accumulation in the wild type plants transformed by the *1-SST*/*1-FFT* genes, we would expect an increase in inulin production upon transformation of this line.

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