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

Elke M. Hellwege*, Maik Raap, Dominique Gritscher, Lothar Willmitzer, Arnd G. Heyer

Max-Planck-Institut für Molekulare Pflanzenphysiologie, Karl-Liebknecht-Str. 25, D-14476 Golm, Germany

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Abstract A newly isolated cDNA clone, *Cy3*, encoding the fructan fructan 1-fructosyltransferase (1-FFT) from artichoke was expressed using tobacco protoplasts as expression system. Analysis of the inulin molecules synthesized upon incubation of protoplast extracts with a mixture of oligofructans (DP3–5) shows the production of inulins with a degree of polymerization (DP) of up to 23, whereas parallel experiments performed using a 1-FFT cDNA from Jerusalem artichoke led to the production of fructans with a DP of up to only 12. The results of in vitro fructan synthesis catalyzed by transiently expressed enzymes therefore reflect the difference of in vivo fructan composition of Jerusalem artichoke (M_{DP} = 8–10) and artichoke (M_{DP} = 65). These data suggest that the fructan pattern in a given species is mainly defined by the enzymatic characteristics of 1-FFT.

Key words: Fructan fructan 1-fructosyltransferase; Inulin; Fructan pattern; Artichoke; Jerusalem artichoke

1. Introduction

Artichoke (*Cynara scolymus*) and Jerusalem artichoke (*Helianthus tuberosus*), like other members of the *Asteraceae*, accumulate inulin as major reserve carbohydrate in their storage organs. Besides levan and neokestose-type polymers inulin belongs to the fructose-based polysaccharides called fructans [1–4]. Inulins are β(2–1)-linked linear molecules which possess a high water solubility [2,4] and are vacuole-localized [5–7]. As long-term storage carbohydrate inulin is deposited in tubers (*Helianthus tuberosus*, *Dahlia variabilis*), roots (*Cichorium intybus*, *Taraxacum officinale*) or blossom discs (*Cynara scolymus*).

Inulin is synthesized by the combined action of at least two different fructosyltransferases [2,4,8–11]. In a first step, sucrose sucrose 1-fructosyltransferase (1-SST) catalyzes the formation of the trisaccharide 1-kestose from two molecules of sucrose with release of glucose. Secondly, fructan fructan 1-fructosyltransferase (1-FFT) mediates the reversible transfer of fructosyl residues between inulins of different chain length. Inulin molecules of all chain lengths can occur simultaneously in the tissue. Degradation of fructan molecules is catalyzed by fructan exohydrolase and proceeds via the hydrolytic cleavage of terminal fructosyl residues [4,9,12].

The size of the fructosyl polymers deposited in storage organs varies between species [13]. While *H. tuberosus* accumulates large amounts of fructans with a relatively low degree of polymerization (DP) in tuberous roots the inulin of *C. scolymus* blossom discs has a high molecular weight [13]. The principles which define the composition of different sets of fructans are still unknown although the de novo synthesis of fructan by combined reactions of purified fructosyltransferases could be shown for different species in vitro [10,11]. However, in an in vitro assay only inulin polymers up to DP 20–25 could be synthesized.

The variation in the chain length of the inulin polymers in different *Asteraceae* species could be either (a) the result of different enzymatic characteristics of 1-FFTs or (b) a consequence of differences in the fructan exohydrolase activity that defines species-specific sets of inulins by trimming the polymers. Fructan exohydrolase activity could be detected in developing and growing tubers of *H. tuberosus* [12,14]. Since fructan composition can vary depending on the developmental stage of the plant organs as well as on environmental factors, time-specific expression of different enzymes or isoenzymes could also be responsible.

Our approach to address this question was to isolate 1-FFT genes from different species and to express them in a heterologous system. Here we report the isolation of a cDNA encoding 1-FFTs of artichoke and a comparative analysis of its enzymatic specificity to 1-FFT of *H. tuberosus*, when expressed in tobacco protoplasts and incubated with various substrates.

2. Materials and methods

2.1. Screening of an artichoke cDNA library

A cDNA library of artichoke was synthesized using blossom disc poly(A)$^+$ RNA [15]. The library was screened with a ^{32}P-labeled cDNA fragment of the artichoke clone encoding 1-SST [15] under low and high stringency. Clones which did not hybridize to the 1-SST cDNA under high stringency were further analyzed for 1-FFT activity. Positive clones were sequenced applying the dideoxynucleotide chain termination method (^{T7}Sequencing Kit, Pharmacia, Uppsala, Sweden).

2.2. Cloning of a cDNA encoding 1-FFT of H. tuberosus

Based upon the sequence of a cDNA encoding 1-FFT of *H. tuberosus* (PCT WO 96/21023) two primers (Ht-FFT-5′ 5′-GTCAGTCACCATGCAAACCC; Ht-FFT-3′ CGGCTATCGCTATCCTTTGGATCCCG-3′) were synthesized. The Ht-FFT-3′ primer contained a *Bam*HI site at the 3′ end. In a PCR reaction these primers resulted in the amplification of a 1879 bp fragment using a cDNA library of *H. tuberosus* tubers and these primers.

*Corresponding author. Fax: (49) (331) 9772301.
E-mail: hellwege@mpimp-golm.mpg.de

Abbreviations: DP, degree of polymerization; 1-FFT, fructan fructan 1-fructosyltransferase; 6G-FFT, fructan fructan 6^G-fructosyltransferase; HPAEC, high performance anion exchange chromatography; M_{DP}, mean degree of polymerization; 6-SFT, sucrose fructan 6-fructosyltransferase; 1-SST, sucrose sucrose 1-fructosyltransferase; W_{DP}, weight average degree of polymerization

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2.3. Transformation of tobacco protoplasts

Transformation of tobacco protoplasts (*Nicotiana tabacum* var. Samsun N.N.) with the 1-FFT clones of *C. scolymus* and *H. tuberosus* was performed as described previously [15].

2.4. Enzyme activity assay and sugar analysis

Enzyme extracts were incubated at room temperature for up to 4 days in a final volume of 30 μl in 50 mM MES-KOH pH 6.5 with 100 mM 1-kestose or a mixture of 1-kestose, nystose and fructosyl-nystose, 50 mM each. The reaction was stopped by incubation at 95°C for 3 min. After centrifugation the supernatant was treated with an ion exchange resin (Bio-Rad Laboratories, Hercules, CA, USA) and filtered (Microcon 10, Amicon, Beverly, MA, USA). The resulting extract was analyzed by high performance anion exchange chromatography (HPAEC) using a CarboPac PA-100 anion exchange column on the Dionex DX-300 gradient chromatography system (Dionex, Sunnyvale, CA, USA) coupled with pulsed amperometric detection by a gold electrode. The detector settings were: $T_1 = 0.48$ s; $T_2 = 0.12$ s; $T_3 = 0.12$ s; $E_1 = 0.05$ V; $E_2 = 0.65$ V; $E_3 = -0.95$ V; sensitivity range = 0.1 μC; integration range = 0.28–0.48 s; monosaccharides were eluted with 0.15 M NaOH for 0–5 min, for disaccharides and higher carbohydrates different gradients of 1 M NaAc in 0.15 M NaOH were used for up to 90 min. The sugars were eluted at a constant flow rate of 1 ml/min. 1-Kestose, nystose and fructosyl-nystose were identified by comparison of their retention times with those of pure and defined fructans of *H. tuberosus*. Each experiment was reproduced at least three times.

2.5. Light induction of fructan synthesis

For light induction of fructan synthesis excised artichoke leaves were placed in 150 mM sucrose solution and continuously illuminated for 72 h. For sugar analysis the plant material was immediately frozen in liquid nitrogen and stored at −80°C. The plant tissue was ground to a fine powder, the fructosyl polymers extracted in hot water (80°C) and the insoluble fraction removed by centrifugation. The inulin solution was deionized and filtered before analyzing it by HPAEC.

3. Results and discussion

3.1. 1-FFT from C. scolymus

Screening a cDNA library of *C. scolymus* blossom discs with a DNA fragment of the 1-SST clone of artichoke under low stringency we isolated the cDNA *Cy3* (accession number AJ000481) encoding a 1-FFT. The 2073 bp insert of this clone contains an open reading frame of 617 codons. The deduced amino acid sequence shows the highest homology (78% identity) to the 1-FFT of Jerusalem artichoke (patent number PCT WO 96/21023). It is also very homologous to the 1-SST of *C. scolymus* [15] and *H. tuberosus* (patent number PCT WO 96/21023), showing 57% and 54% identity, respectively. The deduced *Cy3* polypeptide is more closely related to acid invertases (51–57% identity) than to 6-SFT [16] of *Hordeum vulgare* and 6G-FFT [17] of *Allium cepa* (45% identity each). This finding supports the suggestion that fructosyltransferases of bacteria, fungi, monocots and dicots probably evolved separately from invertases [10,16,18].

The 1-SST and the 1-FFT enzymes of *C. scolymus* and *H. tuberosus* catalyze similar but not identical transfructosylation reactions. As expected, the amino acid sequences of the fructosyltransferases from artichoke and Jerusalem artichoke are very homologous. However, regions with different degrees of homology can be found. The first 90–100 amino acids, which probably constitute the vacuole targeting signal, are less conserved. The remaining part of the polypeptides contains segments of high homology at the N-terminal and at the C-terminal end (Fig. 1; >85% similarity in all sequences). Within these regions some of the well-conserved domains of β-fructosylhydrolases can be identified [16,19]. In contrast, between codons 290 and 340 fructosyltransferases that catalyze similar transfer reactions are closely related (1-SSTs and 1-FFTs showing 96% similarity) while enzymes with different functions seem barely related (Fig. 1). It will be interesting to analyze whether these amino acids participate in the different catalytic activities of the enzymes.

3.2. Expression of the C. scolymus 1-FFT clone in tobacco protoplasts yields an enzyme synthesizing fructans in vitro

The *Cy3* clone was transiently expressed in tobacco protoplasts (*N. tabacum* var. Samsun N.N.) under the control of the 35S promoter and extracts of these protoplasts were incubated with 1-kestose, nystose and fructosyl-nystose at 22°C for 96 h. The reaction products were analyzed by HPAEC. Fructans with a DP of up to 23 could be detected in the assay mixture (Fig. 3b). We therefore conclude that the isolated *Cy3* clone encodes a functional 1-FFT. Comparison of the fructan profile synthesized by the 1-FFT in vitro with fructan isolated from excised light-induced artichoke leaves (Fig. 3d) shows that the polymers of both preparations elute with the same retention times.

3.3. 1-FFT of C. scolymus and H. tuberosus synthesize different sets of fructans in vitro

The fructans in storage organs of members of the *Asteraceae* differ with respect to mean degree of polymerization

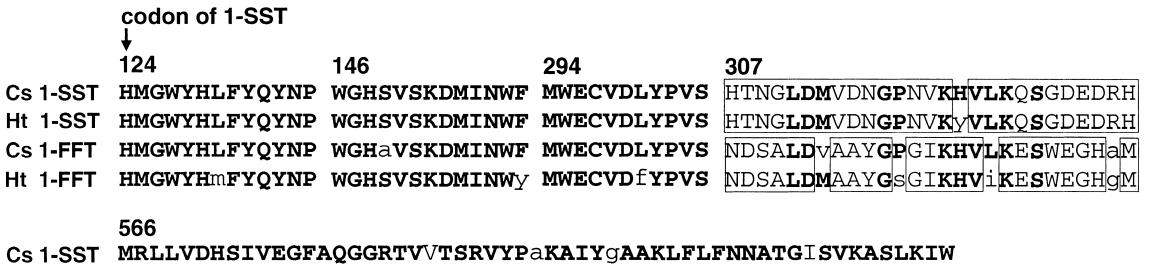

Fig. 1. Sequence parts of an alignment of the deduced amino acid sequence of the *C. scolymus* 1-FFT (Cs 1-FFT) with the sequence of the *C. scolymus* 1-SST (Cs 1-SST [15]) and the sequences of the 1-SST and 1-FFT of *H. tuberosus* (Ht 1-SST, Ht 1-FFT, patent number PCT WO 96/21023). Boldface capital letters indicate residues conserved in most of the sequences; lightface capital letters indicate residues only conserved between fructosyltransferases that catalyze similar transfer reactions; lightface lowercase letters indicate deviating residues; boxed letters indicate conserved residues of fructosyltransferases that catalyze similar transfer reactions.

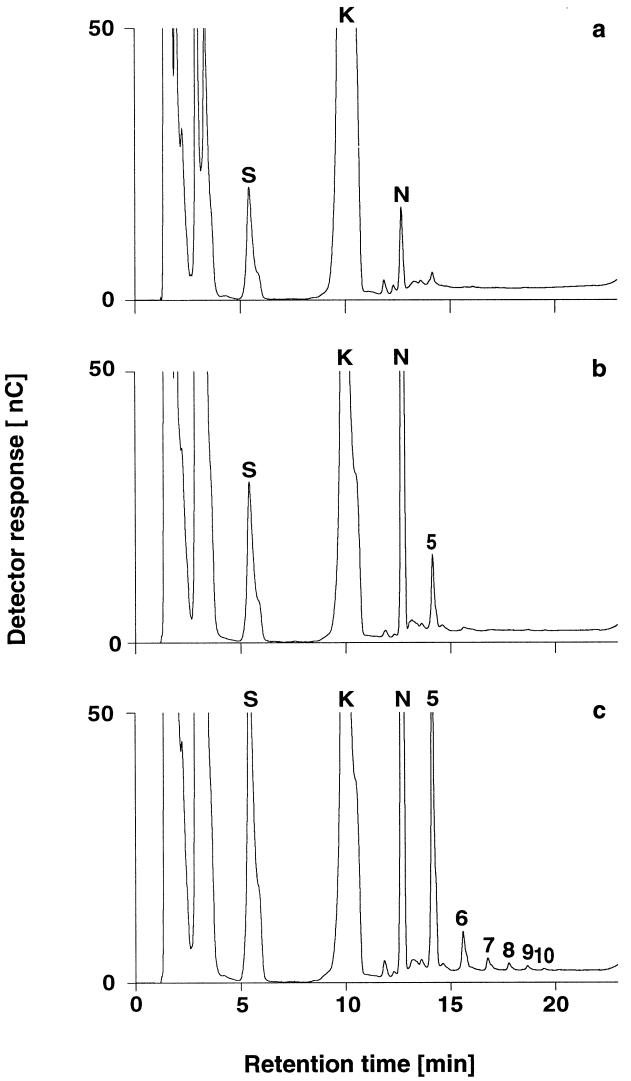

Fig. 2. Fructan synthesis by the 1-FFT of *C. scolymus* and *H. tuberosus* in the presence of 1-kestose. Crude extracts (40 μg protein) of tobacco protoplasts transformed with an empty vector (a), artichoke 1-FFT cDNA (b) or Jerusalem artichoke 1-FFT DNA (c) were incubated with 100 mM 1-kestose for 4 days at 22°C.

(M_{DP}) and weight average degree of polymerization (W_{DP}) [13]. In order to better understand the underlying reasons we wanted to compare the 1-FFT of *C. scolymus* with that of Jerusalem artichoke (*H. tuberosus*, PCT WO 96/21023) in a heterologous expression system. To this end we amplified the coding region of the 1-FFT of Jerusalem artichoke by PCR using a cDNA library from tuberous root tissue as template and expressed the coding regions of both genes transiently in tobacco protoplasts. The proteins produced were incubated with various sugars and the reaction products analyzed by HPAEC.

In the case of the *C. scolymus* 1-FFT, incubation with 100 mM 1-kestose (Fig. 2b) for 4 days at 22°C led to the produc-

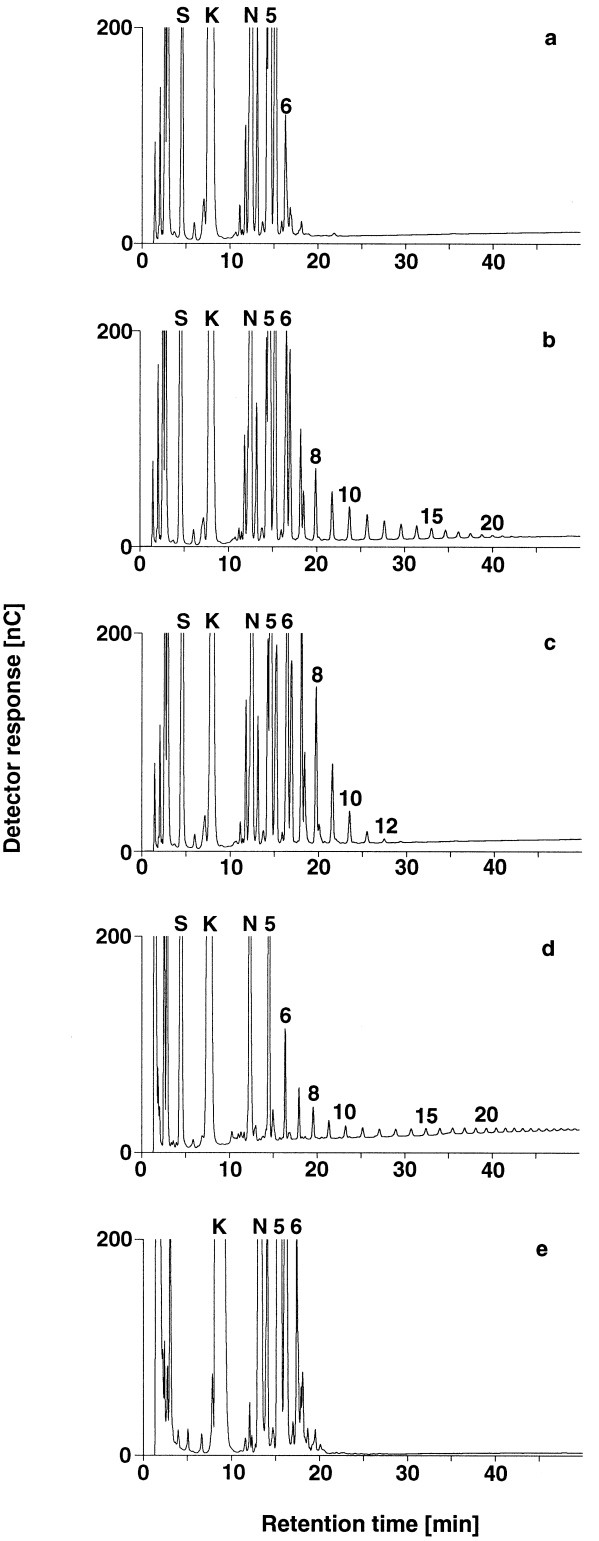

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Fig. 3. Inulin oligomers synthesized by the 1-FFT of *C. scolymus* or *H. tuberosus* with 1-kestose, nystose and fructosyl-nystose as substrates. Crude protein extracts (40 μg) of the 1-FFT enzymes of artichoke (b) and Jerusalem artichoke (c) obtained by transient expression of their DNA in tobacco protoplasts were incubated with a mixture of 1-kestose, nystose and fructosyl-nystose, 50 mM each, for 4 days at 22°C. The resulting fructosyl products were analyzed by HPAEC. Incubation extract of protoplasts transformed with an empty vector (a). The fructan profiles were also compared with an inulin preparation obtained from excised, light-induced artichoke leaves (d) [15]. Some peaks appear as doublets which already occur in the substrate solution (e). These doublets probably reflect fructan molecules without terminal glucose that might emerge during the purification procedure.

tion of nystose and small amounts of fructosyl-nystose (DP=5). *H. tuberosus* 1-FFT, however, synthesized larger amounts of fructosyl-nystose and also inulin polymers with a DP of up to 10 (Fig. 2c). This shows that both enzymes are able to catalyze transfructosylation reactions with 1-kestose as substrate, though the activity of the *H. tuberosus* 1-FFT on 1-kestose seems to be higher than that of the *C. scolymus* enzyme.

In the presence of a mixture of 1-kestose, nystose and fructosyl-nystose the 1-FFT of *C. scolymus* was able to produce fructans of up to DP 23 (Fig. 3b) after an incubation period of 4 days. In contrast, with protoplast extracts containing the enzyme of *H. tuberosus* only fructans with a maximal DP of 10–12 could be detected (Fig. 3c).

For purified chicory fructosyltransferases it has been demonstrated that the higher the enzyme concentration the higher the maximal DP of fructosyl polymers that can be synthesized in vitro [20]. The dependence on enzyme concentration could explain the discrepancy between the set of fructans produced in protoplast extracts expressing the 1-FFT of *H. tuberosus* and during in vitro inulin synthesis with purified 1-SST and 1-FFT from this plant [10].

Our results suggest that the 1-FFT enzymes of *C. scolymus* and *H. tuberosus* have different affinities to the substrates 1-kestose, nystose and fructosyl-nystose as well as to the synthesized products. While the artichoke enzyme appears to have a higher affinity to higher oligofructans as acceptors, the Jerusalem artichoke 1-FFT prefers fructans with low DP. Irrespective of the substrate, 1-kestose or a mixture of DP 3–5, the *H. tuberosus* enzyme synthesized fructans of DP up to 10. For Jerusalem artichoke 1-FFT it has been reported [21] that oligofructans with low DP compete with higher fructans as acceptors thereby reducing the production rate for fructans of a higher DP. In consequence fructan synthesis by *H. tuberosus* 1-FFT seems to proceed in a cascade-like manner so that GF_{n+1} is only synthesized after enough GF_n has accumulated to allow competition with fructans of a lower DP [21,22]. Since the artichoke enzyme efficiently uses 1-kestose and low molecular weight inulins as fructosyl donor it probably synthesizes fructosyl polymers of high DP by transferring fructosyl units from low molecular weight donors preferably to higher fructans. Assuming that the affinity of the artichoke 1-FFT to inulin molecules increases with increasing DP, only a few molecules of GF_n are needed to synthesize GF_{n+1}.

Our results support the idea of Pollock and Chatterton [4] that incubation of a 1-FFT of a given species with oligosaccharides produces oligo- and polysaccharides that gradually start to reflect the natural DP distribution of the inulin of this species. It is therefore reasonable to believe that the inulin pattern as well as the maximal DP of inulin accumulated in the storage organs of different species mainly depends on the enzymatic specificity of their 1-FFT. An involvement of fructan exohydrolase(s) in defining the species-specific sets of inulins in the storage organs cannot be excluded, but seems not necessary to explain the different patterns. Further information on the role of *C. scolymus* and *H. tuberosus* 1-FFT in determination of the inulin pattern will be achieved by heterologous expression of the cDNA of both enzymes in plants already transformed with a 1-SST cDNA [15].

1-FFT enzymes of some species also seem to be involved in the rapid depolymerization of large fructans during cold treatment of storage organs, which is not accompanied by a significant loss of carbohydrates [15], as well as in the shift from high DP fructans to low DP fructans during dormancy [23,24]. In both cases the 1-FFT enzymes use low molecular weight fructans and sucrose as fructosyl acceptor. Whether there is a change in the affinity of the 1-FFT enzyme to fructans of different DP, probably through a modification of the enzyme, or whether these switches are due to differences in the 1-SST:1-FFT ratio or increasing amounts of sucrose remains to be analyzed. Differences in the inulin pattern of artichoke during different vegetation periods have not so far been investigated. It is therefore not known if a decline in fructan content in artichoke is accompanied by a decline in average chain length of the inulin molecules or not. Transgenic plants expressing the artichoke 1-FFT cDNA will be a helpful tool to study the function of 1-FFT in the cold-dependent breakdown of fructans.

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