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ORIGINAL ARTICLE

Akira Kawakami · Midori Yoshida

Fructan:fructan 1-fructosyltransferase, a key enzyme for biosynthesis of graminan oligomers in hardened wheat

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Abstract Fructans play important roles not only as a carbon source for survival under persistent snow cover but also as agents that protect against various stresses in overwintering plants. Complex fructans having both ß-(2,1)- and ß-(2,6)-linked fructosyl units accumulate in wheat (*Triticum aestivum* L.) during cold hardening. We detected fructan: fructan 1-fructosyltransferase (1-FFT; EC 2.4.1.100) activity for catalyzing the formation and extension of ß-(2,1)-linked fructans in hardened wheat tissues, cloned cDNAs (*wft3* and *wft4*) of 1-FFT, and analyzed the enzymatic properties of a *wft3* recombinant protein (Wft3m) produced by yeast. Wft3m transferred ß-(2,1)-linked fructosyl units to phlein, an extension of sucrose through ß-(2,6)-linked fructosyl units, as well as to inulin, an extension of sucrose through ß-(2,1)-linked fructosyl units, but could not efficiently synthesize long inulin oligomers. Incubation of a mixture of Wft3m and another recombinant protein of wheat, sucrose:fructan 6-fructosyltransferase (6-SFT), with sucrose and 1-kestotriose produced fructans similar to those that accumulated in hardened wheat tissues. The results demonstrate that 1-FFT produces branches of ß-(2,1)-linked fructosyl units to phlein and graminan oligomers synthesized by 6-SFT and contributes to accumulation of fructans containing ß-(2,1)- and ß-(2,6)-linked fructosyl units. In combination with sucrose:sucrose 1-fructosyltransferase (1-SST; EC 2.4.1.99) and 6-SFT, 1-FFT is necessary for fructan synthesis in hardened wheat.

A. Kawakami (✉)
Laboratory of Fungal Disease, Department of Plant Pathology,
National Agricultural Research Center,
Kannondai, Tsukuba, Ibaraki, 305-8666, Japan
E-mail: akirak@affrc.go.jp
Tel.: +81-29-8388940
Fax: +81-29-8388837

M. Yoshida
Laboratory of Winter Stress,
Department of Low Temperature Science,
National Agricultural Research Center for Hokkaido Region,
Uitsujigaoka, Sapporo, Hokkaido, 062-8555, Japan

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Abbreviations 1-SST:Sucrose:sucrose 1fructosyltransferase · 1-FFT:Fructan:fructan 1-fructo syltransferase · 6-SFT:Sucrose:fructan 6-fruc tosyltransferase · 6G-FFT:Fructan:fructan 6G-fruct osyltransferase · FEH:Fructan exohy drolase · HPAEC:High-performance anion exchange chromatography · PAD:Pulsed amperometric detector · DP:Degree of polymerization · Wft1:Wheat fr uctosyltransferase 1 · Wft3:Wheat fruct osyltransferase 3 · Wft4:Wheat fructosyltransferase 4

Introduction

Fructans, polymers of fructose based on sucrose, are used as storage carbohydrates in a large number of plant species from many families including Asteraceae, Liliaceae and temperate Poaceae (Pollock and Cairns 1991; Hendry 1993). In overwintering plants, the accumulated fructans are the main carbon source for survival under persistent snow cover and for spring growth until sufficient photosynthetic activities are resumed (Yukawa and Watanabe 1991). In addition to being a carbohydrate reserve, fructans are thought to play roles in protection against environmental stresses such as low temperature and drought (Hendry 1993; Pilon-Smits et al. 1995; Konstantinova et al. 2002). Accumulation of water-soluble carbohydrates including fructans in winter cereals during cold hardening has been shown to be associated with winter survival (Olien 1984; Pontis 1989; Tognetti et al. 1990; Mohammad et al. 1997; Yoshida et al. 1998). In order to improve the survival of plants that encounter environmental stresses, it is essential to understand the mechanisms of fructan accumulation in plants.

Fructans are classified into several forms depending on their glycosidic linkage (Vijn and Smeekens 1999).

Asteraceae plants store an inulin that is composed of linear ß-(2,1)-fructosyl linkages generated by consecutive action of sucrose:sucrose 1-fructosyltransferase (1-SST; EC 2.4.1.99) that catalyzes 1-kestotriose formation and fructan:fructan 1-fructosyltransferase (1-FFT; EC 2.4.1.100) that catalyzes transfructosylation between fructans (Edelman and Jefford 1968). The inulin neoseries of Liliaceae are produced by 1-SST and fructan:fructan 6-G fructosyltransferase (6G-FT or 6G-FFT) that transfers a fructosyl unit from a fructan to the six-position of the glucosyl unit of sucrose, resulting in the formation of 6_G-kestotriose (Shiomi 1981; Vijn et al. 1997). Graminan and phlein, storage carbohydrates of Poaceae, are extensions of sucrose through ß-(2,6)-linked fructosyl units with and without branches of ß-(2,1)-linked fructosyl units, respectively (Bonnett et al. 1997; Carpita et al. 1989). Previous studies suggested that key enzymes for graminan and phlein biosynthesis are 1-SST and sucrose:fructan 6-fructosyltransferase (6-SFT), an enzyme responsible for the formation of ß-(2,6)-fructosyl linkages (Duchateau et al. 1995; Vijn and Smeekens 1999).

Sprenger et al. (1995) were the first to clone a gene for a plant enzyme for fructan biosynthesis, 6-SFT, from barley (*Hordeum vulgare*). Since then, several genes encoding enzymes associated with fructan biosynthesis have been cloned from various plants: 1-SST and 1-FFT from *Cichorium intybus* (de Halleux and Van Cutsem 1997; J. P Goblet et al., unpublished data), *Cynara scolymus* (Hellwege et al. 1997**, 1998) and *Helianthus tuberosus* (van der Meer et al. 1998); 1-SST and 6G-FFT from *Allium cepa* (Vijn et al. 1997, 1998); and 1-SST and/or 6-SFT from temperate grasses such as *Festuca arundinacea* (Lüscher et al. 2000), *Agropyron cristatum* (Wei and Chatterton 2001), *Poa secunda* (Wei et al. 2002), *Triticum aestivum* (Kawakami and Yoshida 2002), *Lolium perenne* (Lidgett et al. 2002; Chalmers et al. 2003) and *H. vulgare* (Nagaraj et al. 2004). In some studies, transgenic plants carrying genes encoding enzymes for fructan biosynthesis have shown increased levels of tolerance to environmental stresses such as cold, drought and freezing (Pilon-Smits et al. 1995; Konstantinova et al. 2002; Hisano et al. 2004).

Wheat (*T. aestivum*) accumulates fructan during several growth stages. Wheat stems at 8 days after anthesis contain large proportions of phlein oligomers initiating with 6-kestotriose and graminan oligomers initiating with 1,6-kestotetraose (Bancal et al. 1992). Winter wheat accumulates large quantities of fructan during cold hardening with levels of fructan in the crown tissues at the end of the cold hardening period reaching 10% or more of the crown fresh weight (Yoshida et al. 1998). Suzuki and Nass (1988) reported that fructans accumulated in field-grown winter wheat during cold hardening mainly consisted of inulin-type fructans with ß-(2,1)-fructosyl units. Bancal et al. (1992) compared the compositions of accumulated fructans in excised leaves illuminated for 24 and 60 h and in stems at 10 days after anthesis, suggested that 1-FFT activity is important for fructan biosynthesis in wheat, and postulated an elongation-trimming pathway for fructan synthesis. Van den Ende et al. (2003) isolated fructan 1-exohydrolase (1-FEH) cDNAs from wheat and implicated that the 1-FEH act in the trimming of wheat fructans. Their hypothesis regarding fructan biosynthesis in wheat, however, has not become established because the physiological and enzymological evidence for elongation and trimming of the synthetic process is still ambiguous (Penson and Cairns 1994).

Transcript abundance of 1-SST and 6-SFT from winter wheat increased with the accumulation of fructans during cold hardening from autumn to early winter. The levels of both transcripts in wheat cultivars that accumulated more fructan were higher than the levels in other cultivars (Kawakami and Yoshida 2002). The 1-FFT enzyme was partially purified from wheat seedling and characterized (Jeong and Housley 1992). There are no reports, however, of 1-FFT cDNA isolation from plants in which graminan accumulates. The compositions of fructans, 1-FFT activity and the precise role of 1-FFT in wheat tissues during cold hardening also have not been clarified.

Here, we describe the compositions of fructan oligomers and fructosyltransferase activities in hardened wheat tissues, the cloning of the cDNA encoding 1-FFT, and the enzymatic properties of recombinant proteins encoded by isolated cDNAs after overexpression in the methylotrophic yeast *Pichia pastoris*. We also discuss the role of 1-FFT in graminan synthesis in hardened wheat based on the results from de novo fructan synthesis in vitro with recombinant proteins of wheat 1-FFT and 6-SFT. Our results indicate that 1-FFT is necessary for biosynthesis of fructans that accumulate in hardened wheat.

Materials and methods

Plant materials

Winter wheat cultivar (*T. aestivum* L. cv. PI 173438) was seeded in a soil container on September 14, 2001 and field-grown at the National Agricultural Research Center for Hokkaido Region, Sapporo, Japan. Plant introduction number (PI) 173438 was introduced in Hokkaido prefecture from USDA as the genetic resource for snow mold resistant variety of wheat (Bruehl et al. 1966). Leaf and crown tissues of the wheat plants were sampled on October 9, November 8 and December 8, 2001 and the fresh weights of the tissues were measured. Another set of the wheat cultivar, PI 173438, was seeded in a soil container, grown in a greenhouse, and vernalized in cold room. At 8 days after anthesis, stem tissues of the wheat were sampled. All samples were stored at −80°C until use. Daily maximum, average and minimum air temperature from September 14 to December 10 were recorded at an automated meteorological station situated at the National Agricultural Research Center for Hokkaido, Sapporo.

Fructan analysis

For fructan analysis, each sample of wheat tissues (0.5 g) was cut into slices and boiled in 5 ml of water for 60 min. After centrifugation at 10,000 *g* for 3 min, the supernatant was passed through a 0.45- μm syringe filter. Fructan content was measured by HPLC using Shodex columns, a combination of KS-802 and KS-803 (Shodex, Tokyo, Japan), with a refractive index detector as previously described by Yoshida et al. (1998).

For analysis of fructan oligomers with different glycosidic linkages, high-performance anion exchange chromatography (HPAEC) was performed on a DX 500 chromatograph (Dionex, Sunnyvale, CA, USA) equipped with a Carbo Pac PA-1 anion-exchange column and a pulsed amperometric detector (PAD) as described by Shiomi et al. (1997). Peaks for glucose, fructose, sucrose, 1-kestotriose, 1,1-kestotetraose, 1,1,1-kestopentaose (Wako, Osaka, Japan) and 6-kestotriose (Iizuka et al. 1993) were identified with authentic standards. Phlein oligomers based on 6-kestotriose and 1,6-kestotetraose were putatively identified by comparison of retention time on HPAEC analysis with the standards, fructan oligomers extracted from wheat stem tissues at 8 days after anthesis and products synthesized by recombinant 6-SFT (Wft1m) of wheat (Kawakami and Yoshida 2002; Accession no. ABoz9887) incubated with sucrose or sucrose and 1-kestotriose. Other branches of wheat fructans were estimated by the works of Bancal et al. (1993) and Van den Ende et al. (2003). Schematic structures of the relevant fructan oligomers accumulated in wheat tissues are shown in Fig. 1.

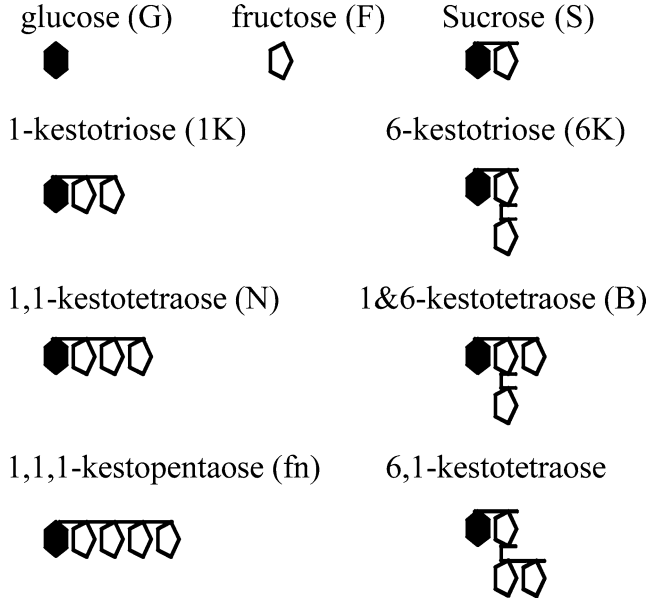

Fig. 1 Schematic structures of the relevant fructan oligomers accumulated in wheat tissues. The nomenclature proposed by Waterhouse and Chatterton (1993) has been adopted. Abbreviations for each fructan oligomer are also given

Fructosyltransferase assays of extracts from hardened wheat tissues

For analysis of fructosyltransferase activities, each sample (0.5 g) was homogenized in 10 ml of 20 mM citrate-phosphate buffer (pH 5.0) containing 20 mM 2-mercaptoethanol using a stainless steel grinder. The homogenate was centrifuged for 60 min at 4°C (10,000 *g*), and the supernatant was passed through a 0.45- μm syringe filter and concentrated by ultrafiltration on a Vivaspin 20 concentrator (cut-off 10 kDa, Vivascience, Lincoln, UK). The dilution/concentration process was repeated twice, and the final volume of extracted solution was adjusted to 500 μl with 20 mM citrate-phosphate buffer (pH 5.0). Twenty microliter of each extracted solution was incubated either with 20 μl of 200 mM sucrose or 1-kestotoriose at 10°C for 0, 24, and 48 h. The reaction was stopped by boiling for 3 min. The sample was diluted ten times with water and passed through a 0.45- μm syringe filter and analyzed for fructan composition by Dionex chromatography as described above.

Preparation of a cDNA library from wheat and cloning of 1-FFT cDNA

Total RNA was extracted using the TRIzol reagent (Invitrogen) from crown tissues of winter wheat cv. PI 173438 sampled in the middle of November 1997. Poly(A)$^+$ RNA was purified using Oligotex-dT30 resins (TaKaRa, Kyoto, Japan), and double-stranded cDNA was synthesized from the poly(A)$^+$ RNA. A cDNA library was constructed using a ZAP Express XR library construction kit (Stratagene).

The cDNA library was screened with a ^{32}P-labeled cDNA fragment from a wheat clone encoding *1-SST* (AB029888) under high stringency conditions (washing filters in 0.1x SSC and 0.1% SDS at 65°C) or low stringency conditions (washing filters in 2x SSC and 0.1% SDS at 50°C). Clones that hybridized to the probe under low stringency conditions but not under high stringency conditions were further characterized by DNA sequencing. The nucleotide sequences of isolated clones were determined by an automated DNA sequencer (model 373S, Applied Biosystems) using a Thermo-Sequenase Dye Terminator Cycle Sequencing Kit v2.0 (Amersham Pharmacia Biotech.). The nucleotide and deduced amino acid sequences were analyzed by DNASIS software (Hitachi Software Engineering, Yokohama, Japan). Multiple alignments were constructed by CLUSTALW using the DDBJ database (National Institute of Genetics, Mishima, Japan).

Expression of recombinant protein in methylotrophic yeast

Isolated cDNA was expressed in the methylotrophic yeast *P. pastoris* (EasySelect *Pichia* Expression Kit, Invitrogen)

after cloning into the secretory expression vector pPICZ∂ B. The DNA sequence corresponding to the putative mature protein region (Wft3m) of Wft3, between nucleotides 196 and 1848 of wft3, was amplified by PCR and translationally fused behind the ∂-factor signal sequence of pPICZ∂ B. Transformation and culture of *P. pastoris* was carried out according to the instructions provided by the supplier (EasySelect *Pichia* Expression Kit, Invitrogen). The *P. pastoris* strain X-33 was transformed by electroporation using 10 μg of the Pme I-linearized construct, and transformants were selected on YPDS/Zeocin plates. A 3-ml pre-culture medium (BMGY, pH 6.0) was inoculated with freshly prepared single colonies at 30°C with vigorous shaking (200 rpm) until the culture reached an OD_{600} of 6.0. The cells were collected by centrifugation, resuspended in 20 ml of induction medium (BMMY, pH 6.0) and incubated at 29°C under aerobic conditions. Four hundred microliter of methanol was added to each culture every day. At 6 days after induction, each culture was centrifuged and the resulting supernatant was recovered and tested for enzyme activity. A 15-ml aliquot of medium was concentrated to 300 μl, diluted with 15 ml of 20 mM citrate-phosphate buffer (pH 5.0), and concentrated by ultrafiltration on a Vivaspin 20 concentrator with a cut-off of 10 kDa. The dilution/concentration process was repeated twice, and the final volume of enzyme solution was adjusted to 300 μl with 20 mM citrate-phosphate buffer (pH 5.0). Control preparations from media concentrated from cultures of *Pichia* transformed with an empty vector were also tested.

Assay for fructosyltransferase activity of recombinant proteins

To analyze the enzymatic properties of the recombinant protein (Wft3m) encoded by *wft3*, 30 μl of a concentrated medium containing Wft3m was incubated with either single substrates (30 μl of 200 mM sucrose, 1-kestotriose, 6-kestotriose, 1,1-kestotetraose, or 1,1,1-kestopentaose) or mixed substrates (10 μl each of 200 mM 1-kestotriose, 1,1-kestotetraose and 1,1,1-kestopentaose or 15 μl each of 200 mM 1-kestotirose and 6-kestotriose) in 20 mM citrate-phosphate buffer (pH 5.0) at 10°C.

For comparison of products synthesized by wheat 1-SST and Wft3, the concentrated medium containing recombinant protein (Wft2m) from wheat 1-SST (Accession No. AB029888) was prepared as previously described by Kawakami and Yoshida (2002). Thirty microliter of Wft2m was incubated with single substrates (30 μl of 200 mM sucrose, 1-kestotriose, 6-kestotriose or 1,1-kestotetraose) in 20 mM citrate-phosphate buffer (pH 5.0) at 10°C.

For analysis of products synthesized by an enzyme mixture of wheat 6-SFT and Wft3, the concentrated medium containing recombinant protein (Wft1m) from wheat 6-SFT (Accession No. AB029887) was prepared as previously described by Kawakami and Yoshida (2002). For determination of activity of proteins expressed by *Pichia*, 20 μl of enzyme solution containing 10 μl each of Wft1m and 20 mM citrate-phosphate buffer or 10 μl each of Wft3m and 20 mM citrate-phosphate buffer was incubated with 10 μl each of 200 mM sucrose and 1-kestotriose or 20 μl of 200 mM 1-kestotriose at 10°C, respectively. Final volume of the reaction mixture was 40 μl. Twenty microliter of enzyme solution containing 10 μl each of Wft1m and 20 mM citrate-phosphate buffer or 10 μl each of Wft1m and Wft3m was incubated with 10 μl each of 200 mM sucrose and 1-kestotriose at 10°C. Final volume of the reaction mixture was 40 μl. The reaction was stopped by boiling in water for 3 min, and the sugar product was analyzed by Dionex chromatography as described above.

Transcript analysis by RT-PCR

Transcripts were analyzed using a two-step RT-PCR procedure. Total RNA was extracted from crown and leaf tissues of winter wheat PI 173438 sampled on December 8, and from stem tissues at 8 days after anthesis by TRIzol (Invitrogen) and a RNeasy Plant Mini kit (QIAGEN). The first-strand cDNA was synthesized from 5 μg total RNA using a SuperScript III First Strand Synthesis System (Invitrogen) and diluted ten times for PCR. The total amount of cDNA in the samples was standardized after the amount of actin mRNA had been estimated with two primers, TaActin-LP (5′-ATGAGGGATACACGCTTCCTCA-3′) and TaActin-RP (5′-GGAAAGTGCTAAGAGAGGCCA-AA-3′) (Himi and Noda, Accession No. AB181991). These actin primers were also used to evaluate DNA contamination in the total RNA samples before first-strand cDNA synthesis as a negative control. Individual transcript analysis was carried out using the following gene-specific primers: 1-SST primers (forward 5′-TCCGTGTGGGAGCCCCAA-3′ and reverse 5′-GGCCCACCCCTTGGTGAT-3′); 6-SFT primers (forward 5′-TGGGACGACGGCATGGAG-3′ and reverse 5′-CTTGGCGGAGCGGGATATG-3′); and 1-FFT primers (forward 5′-CGGGTGGGGCAACATCTCA-3′ and reverse 5′-TGGTGGAGGGGGAGGGAGA-3′). PCR reactions for amplification of 1-SST, 1-FFT, 6-SFT and actin cDNA clones were performed using the following parameters: 1 min denaturation at 94°C and then 23 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C. The PCR products were electrophoresed on a 1.5% (w/v) agarose gel and visualized by ethidium bromide staining.

Results

Soluble carbohydrates and fructans that accumulate in winter wheat during cold hardening

There was a progressive decrease in daily average temperature from mid September to early December

(Fig. 2a). Soluble carbohydrates that accumulated in leaf and crown tissues with decreasing air temperature under field conditions are shown in Fig. 2. The profile of soluble polysaccharides in each tissue increased throughout the cold hardening period and reached 10% or more of the fresh weight on December 8. Mono- and di-saccharide contents of wheat tissues also increased during cold hardening but were lower than those of soluble polysaccharides throughout the period.

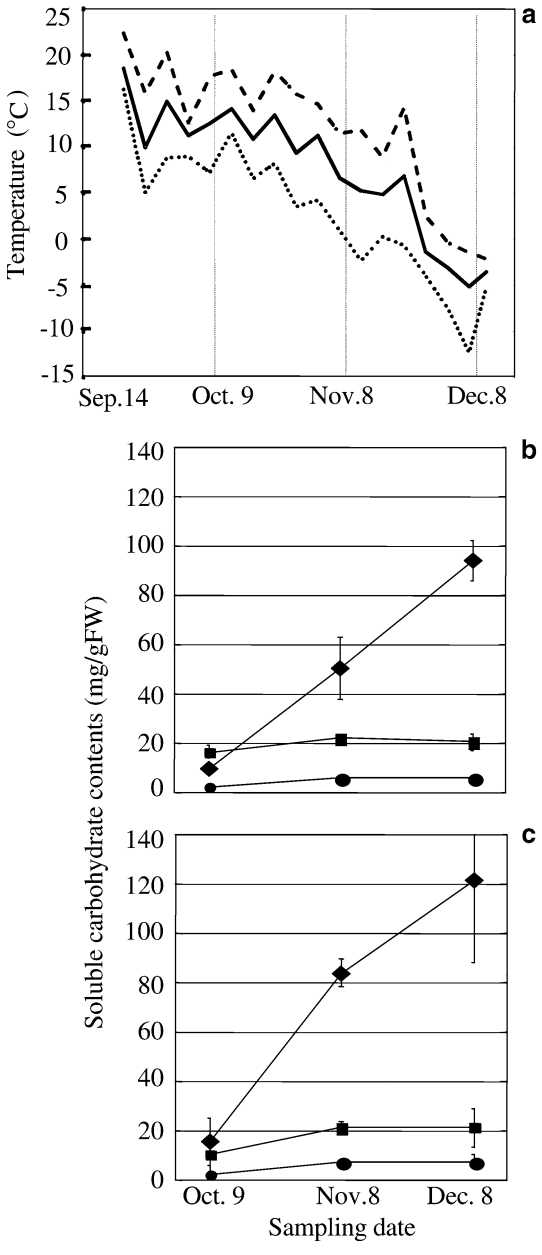

Fig. 2 Daily air temperature from September 14 to December 10 in 2001 (**a**) and changes in soluble carbohydrate content (fructose, total mono- and di-saccharide, and polysaccharide) in leaf (**b**) and crown (**c**) tissues of winter wheat PI 173438 harvested on October 9, November 8 and December 8, 2001. Daily maximum (*thick dotted line*), average (*solid line*) and minimum (*thin dotted line*) was recorded at automated meteorological station in Sapporo. Carbohydrate extracts were separated and measured by HPLC with refractive index detection. *Vertical bars* represent the standard error of the mean. Symbols of *closed circle*, *square* and *diamond* indicate contents of fructose, total mono- and di-saccharides, and polysaccharides, respectively

The degree of polymerization (DP) and soluble carbohydrate composition of wheat leaf and crown tissues sampled on October 9 and December 8 were analyzed by HPAEC-PAD (Fig. 3). Leaf tissues sampled on October 9 contained higher levels of sucrose (S) and low DP fructan oligomers than crown tissues. Crown tissues contained some high DP (≥12) fructans even though the levels of sucrose, tri- and tetra-saccharides were very low. In the case of wheat tissues sampled on December 8, fructan profiles were similar between crown and leaf tissues. Fructans in stem tissues at 8 days after anthesis that mainly consisted of phlein based on 6-kestotriose (6K) and 1,6-kestotetraose (B) were clearly different from those in hardened wheat tissues. Comparison of the fructan profiles of hardened wheat tissues with those of stem tissues at 8 days after anthesis and data of Bancal et al. (1993) and Van den Ende et al. (2003) showed that hardened wheat tissues contained complicated graminan with branches of ß-(2,1)-fructosyl units in addition to phlein (Fig. 3). These data suggest that fructosyltransferase includes a branching activity that forms branches of ß-(2,1)-linked fructosyl units in hardened wheat tissues.

Fructosyltransferase activities in wheat tissues

For analysis of fructosyltransferase activities in hardened wheat, crude extracts from leaf and crown tissues were incubated with sucrose (S) or 1-kestotriose (1K) and the reaction products were analyzed by HPAEC-PAD. When incubated with sucrose, the crude extracts of both tissues produced large amounts of glucose (G) and 1-kestotriose and intermediate or small amounts of fructose (F), 6-kestotriose (6K) and 1,6-kestotetraose (B) (Fig. 4a, b). These results suggest that the crude extracts have 1-SST and 6-SFT activities because 1-SST uses sucrose as a substrate, thereby producing 1-kestotriose, and 6-SFT uses sucrose or a mixture of sucrose and 1-kestotriose as a substrate, thereby producing 6-kestotriose or 6-kestotriose and 1,6-kestotetraose. When incubated with 1-kestotriose, the crude extracts of both tissues produced a large amount of 1,1-kestotetraose (N) and intermediate or small amounts of glucose, fructose, 1,6-kestotetraose and 1,1,1-kestopentaose (fn) (Fig. 4c, d). These results indicate that the crude extracts have 1-FFT and 6-SFT activities because 1-FFT uses 1-kestotriose or inulin oligomers as a substrate, thereby producing 1,1-kestotetraose and 1,1,1-kestopentaose.

Cloning of candidates for 1-FFT cDNA from hardened wheat

For isolation of 1-FFT genes expressed in wheat tissues, a cDNA library constructed from hardened wheat was

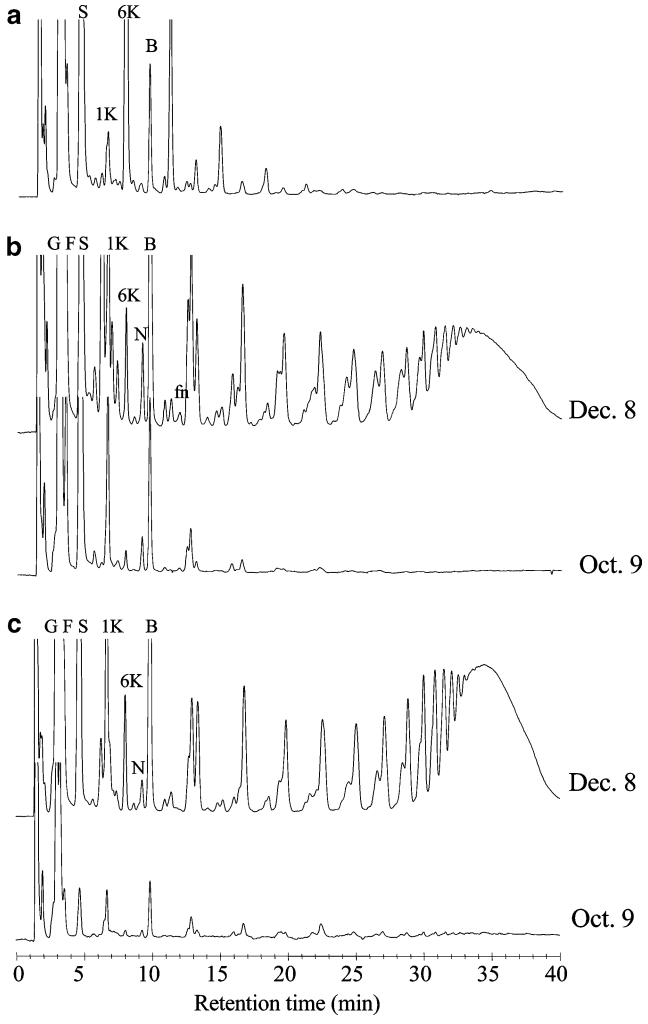

Fig. 3 Anion exchange HPLC analysis of soluble carbohydrates in extracts from stem tissues (**a**) harvested at days 8 after anthesis, leaf (**b**) and crown (**c**) tissues of wheat PI 173438 harvested on October 9 and December 8, 2001. Compounds were detected by pulsed amperometric detection and identified using external standards. Abbreviations for each compound are the same as for Fig. 1

screened with wheat 1-SST cDNA (Kawakami and Yoshida 2002) based on the expected sequence similarity between plant fructosyltransferases (Vijn and Smeekens 1999). After repeated screenings, we isolated two clones that were not identical but were very similar to wheat 1-SST cDNA (Fig. 5). The clones each contained one long open reading frame designated *wft3* (Accession No. AB088409) and *wft4* (Accession No. AB088410).

Analysis of the deduced amino acid sequences of the clones revealed that *wft3* and *wft4* encoded 648 amino-acid and 644 amino-acid polypeptides showing 81% and 80% identity to 1-SST of winter wheat, respectively. The Wft3 protein was predicted to contain seven *N*-glycosylation sites (N-X-S/T) and have a pI of 4.91, whereas the Wft4 protein was predicted to contain seven *N*-glycosylation sites and have a pI of 4.99. Identity between Wft3 and Wft4 was 94%. Wft3 and Wft4 proteins were also predicted to contain conserved domains (ß-fructosidase motif and catalytic site) found in various fructosyltransferases and invertases (Vijn and Smeekens 1999) (Fig. 5). In most plant fructosyltransferases, the signal peptide region is less conserved than the mature protein region (Vijn et al. 1997). Previous reports (Kawakami and Yoshida 2002) predicted the N-terminus of the mature protein of wheat 1-SST by comparison with carrot invertase (Unger et al. 1994) and barley 6-SFT (Sprenger et al. 1995). Comparison of the N-terminal sequences of Wft3 and Wft4 with those of wheat 1-SST indicates that putative mature proteins of *wft3* and *wft4* might start downstream of the 95th and 91th codons, respectively (Fig. 5).

The deduced amino acid sequence of *wft3* shows 59% identity to wheat 6-SFT (Accession No. AB029887), 70% identity to tall fescue 1-SST (Accession No. AJ297369), 58% identity to rice vacuolar invertase (Accession No. AF276704), 55% identity to asparagus vacuolar invertase (Accession No. AF002656), 38% identity to wheat cell-wall invertase (Accession No. AF030420), 37% identity to maize cell-wall invertase (Accession No. AF043346) and 36% identity to wheat 1-fructan exohydrolase (1-FEH; EC 3.2.1.80) (Accession No. AJ508387). Fructan synthesis takes place in the vacuole, suggesting that fructosyltransferases should also be located in vacuoles (Vijn and Smeekens 1999). The necessity of a short C-terminal sequences of hydrophobic amino acids for vacuolar localization has been suggested (Neuhaus et al. 1991; Tymowska-Lalanne and Kreis 1998) and the hydrophobic amino-acid sequences were also found to be present at the C-terminal regions of Wft3 and Wft4 (Fig. 5). These findings suggest that both clones of *wft3* and *wft4* encode vacuolar-type fructosyltransferases or invertases.

Functional characterization of recombinant Wft3

To demonstrate the enzymatic functions of isolated cDNA, Wft3 was expressed in the methylotrophic yeast *P. pastoris*. Since it has previously been suggested that N-terminal signal peptides of barley 6-SFT prevent secretion by interfering with the *Pichia* protein sorting machinery (Hochstrasser et al. 1998), the putative mature-protein region (Wft3m) of Wft3 was cloned into the expression vector pPICϑ B with the ϑ-factor signal sequence to allow entry into the secretory pathway. Concentrated and desalted culture media from cloned *P. pastoris* expressing Wft3m were incubated with sucrose (S), 1-kestotriose (1K), 6-kestotriose (6K), 1,1-kestotetraose (N) and 1,1,1-kestopentaose (fn) (Fig. 6).

Reaction mixtures of Wft3m incubated for 24 h mainly contained inulin oligomers of up to DP 6 when incubated with 1-kestotriose (DP 3), inulin oligomers up to DP 7 when incubated with 1,1-kestotetraose (DP 4) and inulin oligomers up to DP 7 when incubated with 1,1,1-kestopentaose (DP 5)

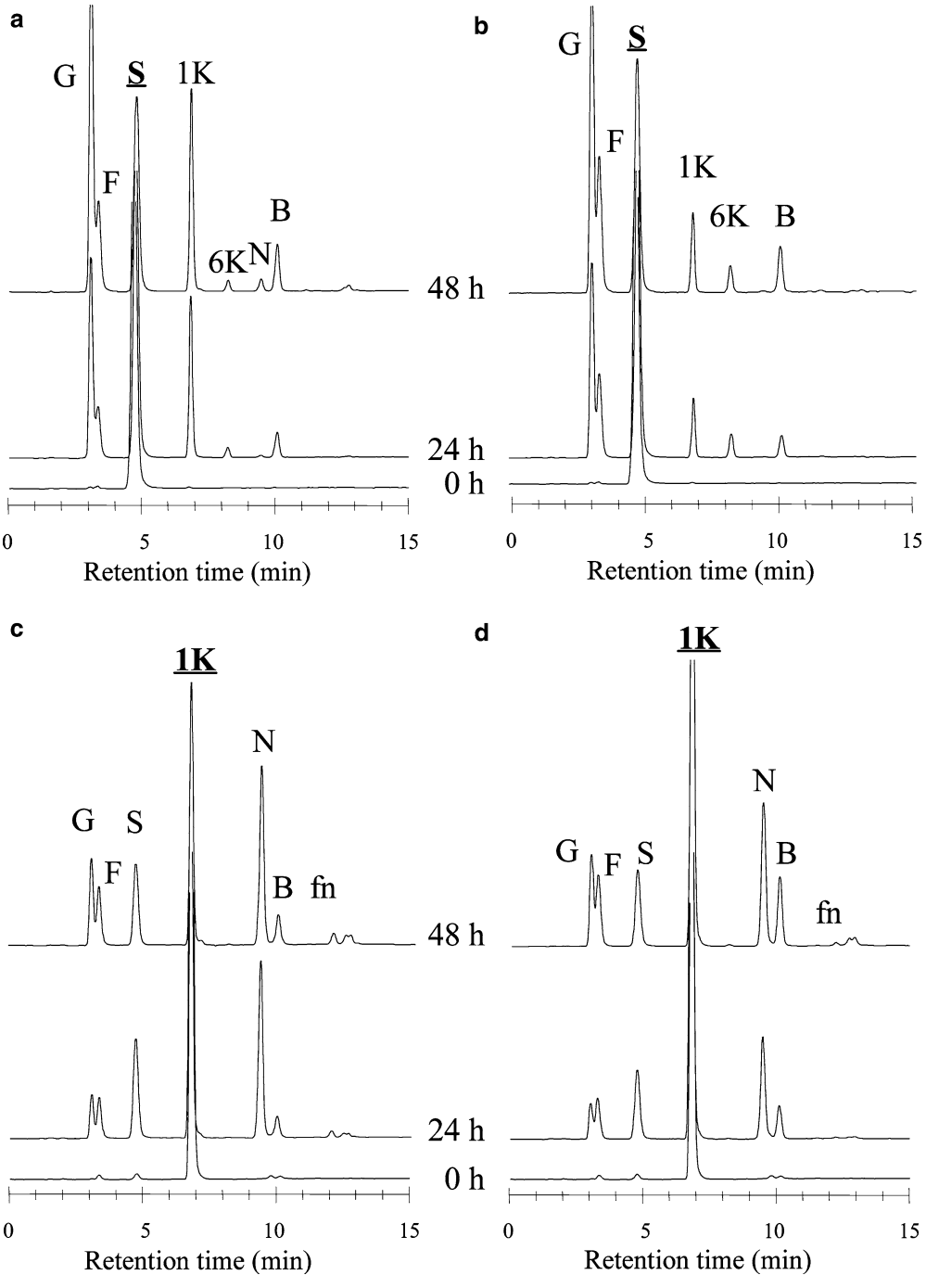

Fig. 4 Anion exchange HPLC analysis of oligo-fructan produced by incubation of crude enzyme preparations with 100 mM sucrose or 100 mM 1-kestotriose. The reactions were conducted with 100 mM sucrose (**a, b**) or 100 mM 1-kestotriose (**c, d**) at 10°C for 0, 24 and 48 h. Crude enzyme preparations were obtained by extracts of leaf tissues (**a, c**) or crown tissues (**b, d**) from wheat PI 173438 hardened until December 8, 2001 in the field. Compounds were detected by pulsed amperometric detection and identified using external standards. Abbreviations for each compound are the same as for Fig. 1

(Fig. 6c–e). Activity of fructosyltransferases or fructosidases was not detected when incubated with 6-kestotriose (Fig. 6b). There was detectable release of glucose and fructose, and detectable formation of 1-kestotriose when incubated with sucrose (Fig. 6a). This result indicates that Wft3m has both invertase (or fructosidase) and 1-SST activities. However, the level of 1-kestotriose production by incubation with sucrose was quite low compared with the level of 1,1-kestotetraose production by incubation with 1-kestotriose (Fig. 6c).

For comparison of products synthesized by wheat 1-SST (Wft2) and Wft3, recombinant protein (Wft2m) of Wft2 was expressed by *P. pastoris*, dialyzed, concentrated and incubated with sucrose, 1-kestotriose, 6-kestotriose and 1,1-kestotetraose. The level of 1-kestotriose production by incubation with sucrose was extremely higher that that of 1,1-kestotetraose production by incubation with 1-kestotriose (Fig. 7a, b), and fructosyltransferase activity of Wft2m was not detected when incubated with 6-kestotriose and 1,1-kestotetraose (Fig. 7c, d). These data (Figs. 6, 7) suggested that *wft3*

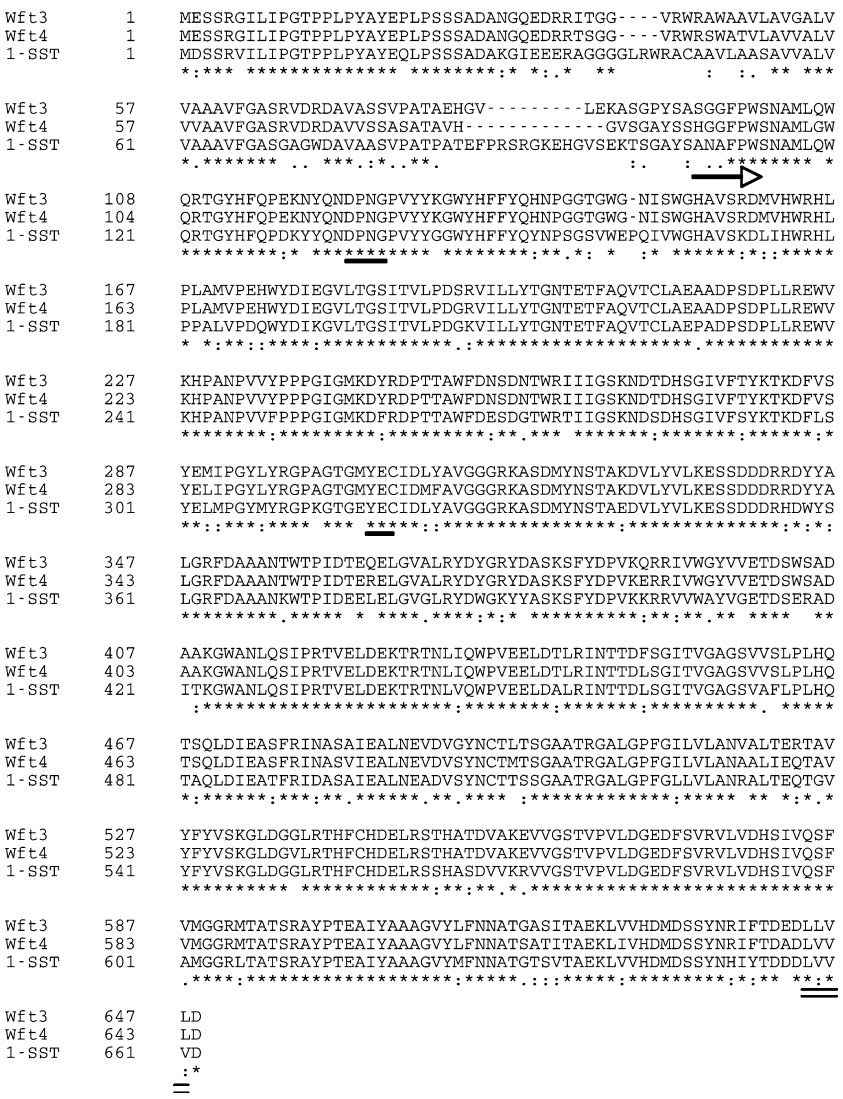

Fig. 5 Multiple sequence alignment of the deduced amino acid sequences of 1-SST (Wft2), Wft3 and Wft4 of wheat. *Asterisks*, *colons* and *periods* indicate identical residues, conserved substitutions and semiconserved substitutions, respectively. The *arrow lines* indicate the putative N-terminus of mature proteins. Hydrophobic amino acid sequences are *doubly underlined*. Conserved domains among invertases and fructosyltransferases are *underlined*

expressed in winter wheat hardened in the field encoded a functional 1-FFT. Wft4m also had 1-FFT activity (data not shown). This is the first report of cloning 1-FFTs from monocots.

To elucidate the substrate specificity of wheat 1-FFT to mixtures of inulin with different DP, Wft3m was incubated with a mixture of 1-kestotriose, 1,1-kestotetraose and 1,1,1-kestopentaose. Wft3m was able to produce inulin oligomers of up to DP 7 (data not shown).

To elucidate the transfructosylation of wheat 1-FFT from 1-kestotriose to 6-kestotriose, Wft3m was incubated with a mixture of 1-kestotriose and 6-kestotriose. Wft3m produced 1,1-kestotetraose, oligosaccharide of peak *i*, 1,6-kestotetraose (B), 1,1,1-kestopentaose, and other unknown pentasaccharides (Fig. 8).

Expression of fructosyltransferases mRNA

To analyze gene expression of fructosyltransferases in hardened wheat tissues, transcript levels of 1-SST, 6-SFT and 1-FFT were examined in leaf and crown tissues of winter wheat harvested on December 8 by RT-PCR using specific primers for each gene. The tested tissues accumulate graminan enriched in ß-(2,1)-linked fructosyl units at high levels. Transcripts of all genes were detected in all of the wheat tissues (data not shown), indicating that 1-FFT as well as 1-SST and 6-SFT are expressed in leaf and crown tissues of hardened wheat.

Fructan synthesis by a combination of 1-FFT and 6-SFT

To understand the role of 1-FFT in fructan synthesis in wheat during cold hardening, the ability of an enzyme mixture of wheat 6-SFT and 1-FFT to synthesize fructans was evaluated in vitro using recombinant proteins produced by *P. pastoris*. After each recombinant protein had been expressed, dialyzed and concentrated, the activities were estimated by characterizing the production of oligomers from in vitro

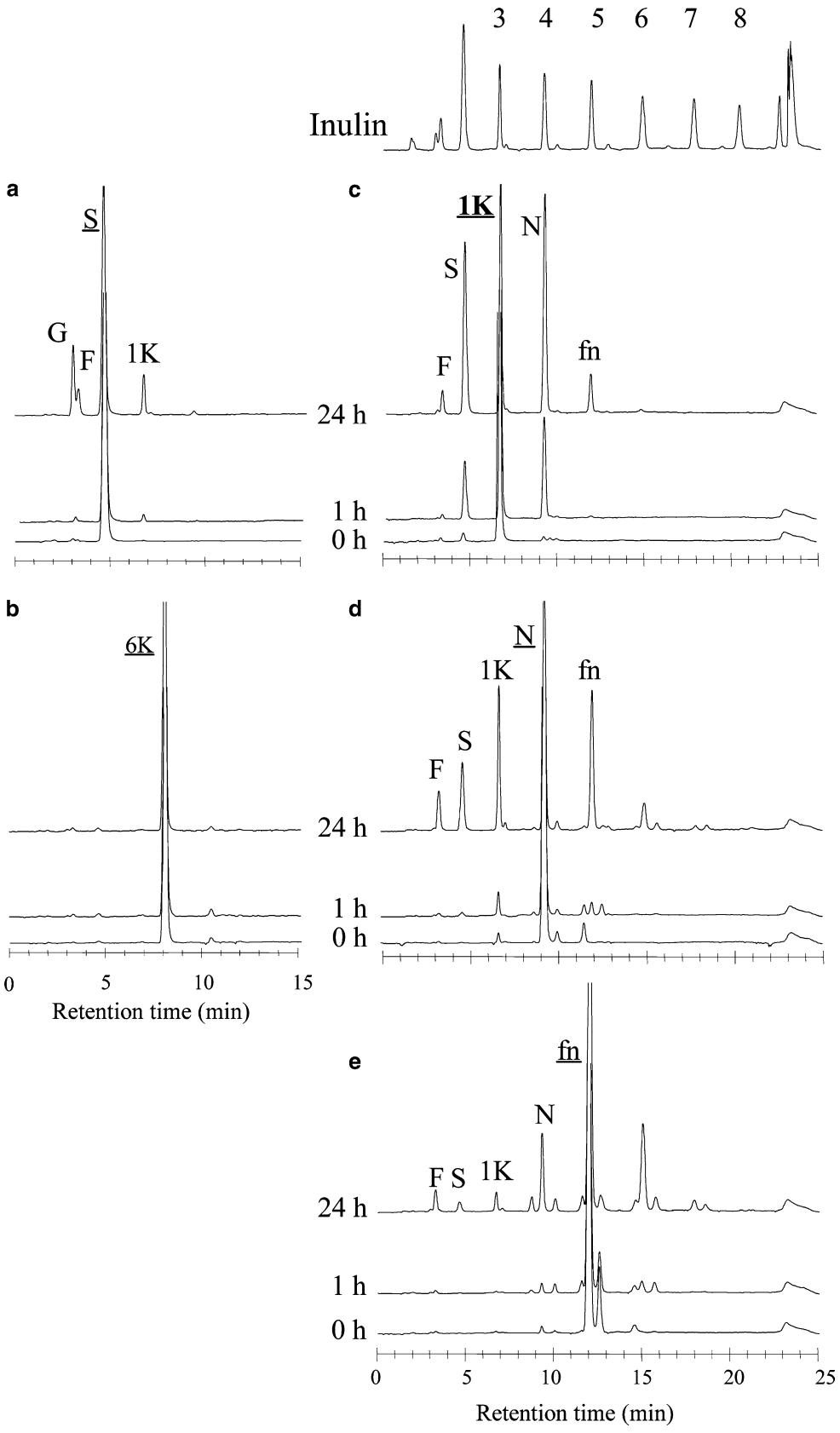

Fig. 6 Anion exchange HPLC analysis of oligo-fructans generated by recombinant Wft3 (Wft3m). The reactions were conducted with 100 mM sucrose (**a**), 6-kestotriose (**b**), 1-kestotriose (**c**), 1,1-kestotetraose (**d**) or 1,1,1-kestopentaose (**e**) at 10°C for 0, 1 and 24 h. Compounds were detected by pulsed amperometric detection and identified using external standards. Abbreviations for each compound are the same as for Fig. 1. Inulin extracted from Jerusalem artichoke was used as a standard inulin-oligomers. Numbers (3–8) on each peak of inulin indicate the corresponding degree of polymerization (*DP*)

assays. There was no evidence for fructosyltransferases or fructosidase activity in concentrated media from *Pichia* transformed with an empty vector (data not shown). Incubation of the recombinant protein of 6-SFT (Wft1m) with sucrose (S) and 1-kestotriose (1K) resulted in the formation of graminan oligomers based

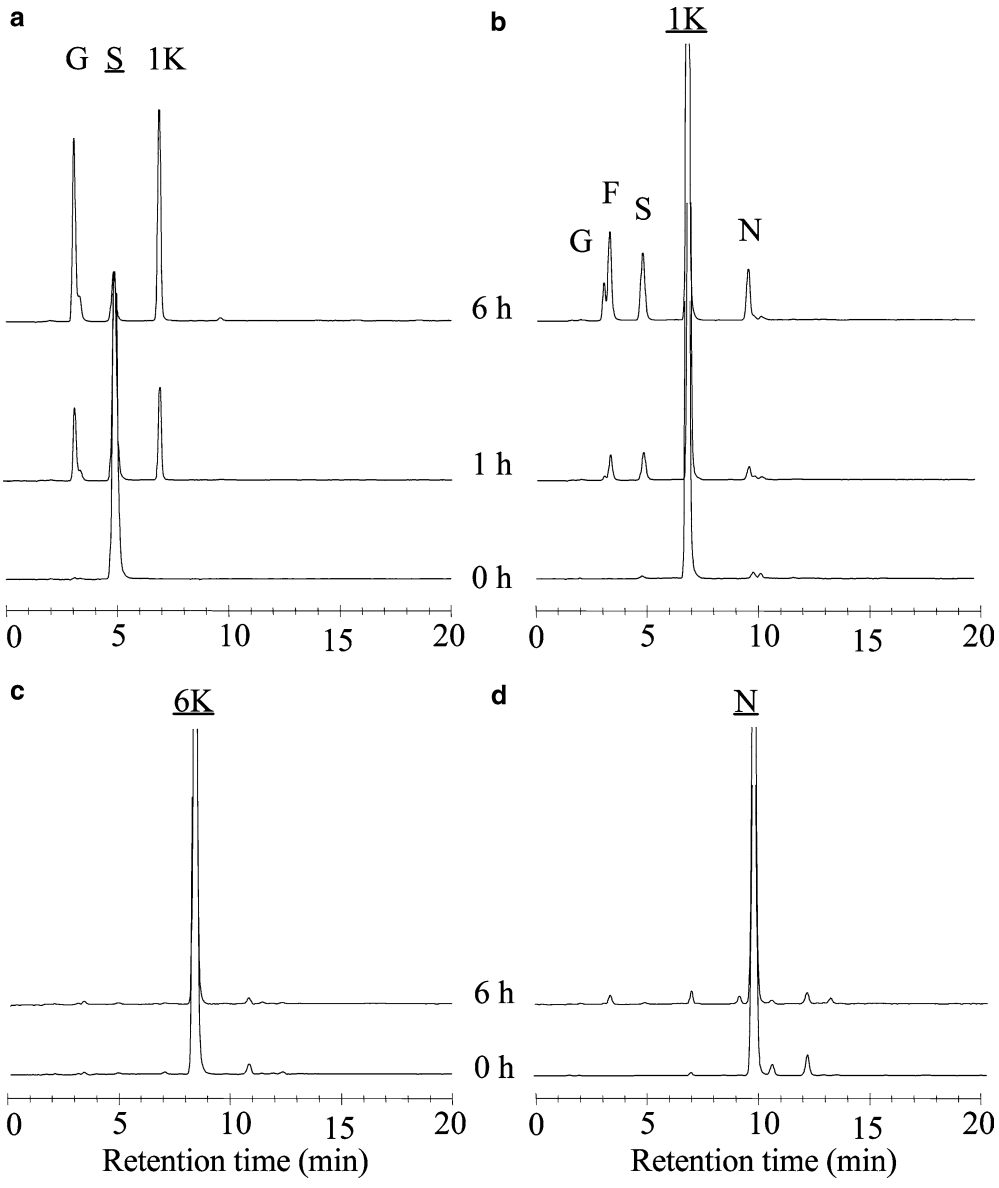

Fig. 7 Anion exchange HPLC analysis of oligo-fructans generated by recombinant Wft2 (*Wft2m*). The reactions were conducted with 100 mM sucrose (**a**), 1-kestotriose (**b**), 6-kestotriose (**c**) or 1,1-kestotetraose (**d**) at 10°C for 0, 1 and 6 h. Compounds were detected by pulsed amperometric detection and identified using external standards. Abbreviations for each compound are the same as for Fig. 1

on 1,6-kestotetraose (B) as the main peak and phlein oligomers based on 6-kestotriose (6K) as subsequent peaks (Fig. 9). Profiles of reaction products produced by Wft1m are almost identical to the fructan profiles from wheat stem tissues at 8 days after anthesis but not to those of fructans that accumulated in hardened wheat tissues.

Combined incubation of Wft1m and Wft3m with sucrose and 1-kestotriose resulted in the formation of complicated fructans with branches of ß-(2,1)-linked fructosyl units compared with stem tissues at 8 days after anthesis in DP 3–DP 5 (Fig. 10). Furthermore, products from DP 3 to DP 5 generated by incubation with a mixture of Wft1m and Wft3m are almost identical to the fructans that accumulated in hardened wheat tissues.

Discussion

In this paper, we describe the isolation of cDNAs encoding 1-FFT from hardened wheat and the role of 1-FFT in fructan synthesis during cold hardening.

Fructan composition analysis is essential for elucidating the mechanisms of fructan accumulation during cold hardening. In field-grown wheat tissues, the content of water-soluble polysaccharides increased throughout cold hardening (Fig. 2). Most of water-soluble wheat polysaccharides accumulated during cold hardening are fructans (Yoshida et al. 1998). Graminan, an extension of 1,6-kestotetraose through ß-(2,6)-linked fructosyl units with branches of ß-(2,1)-linked fructosyl units, was the predominant fructan that accumulated in leaf and

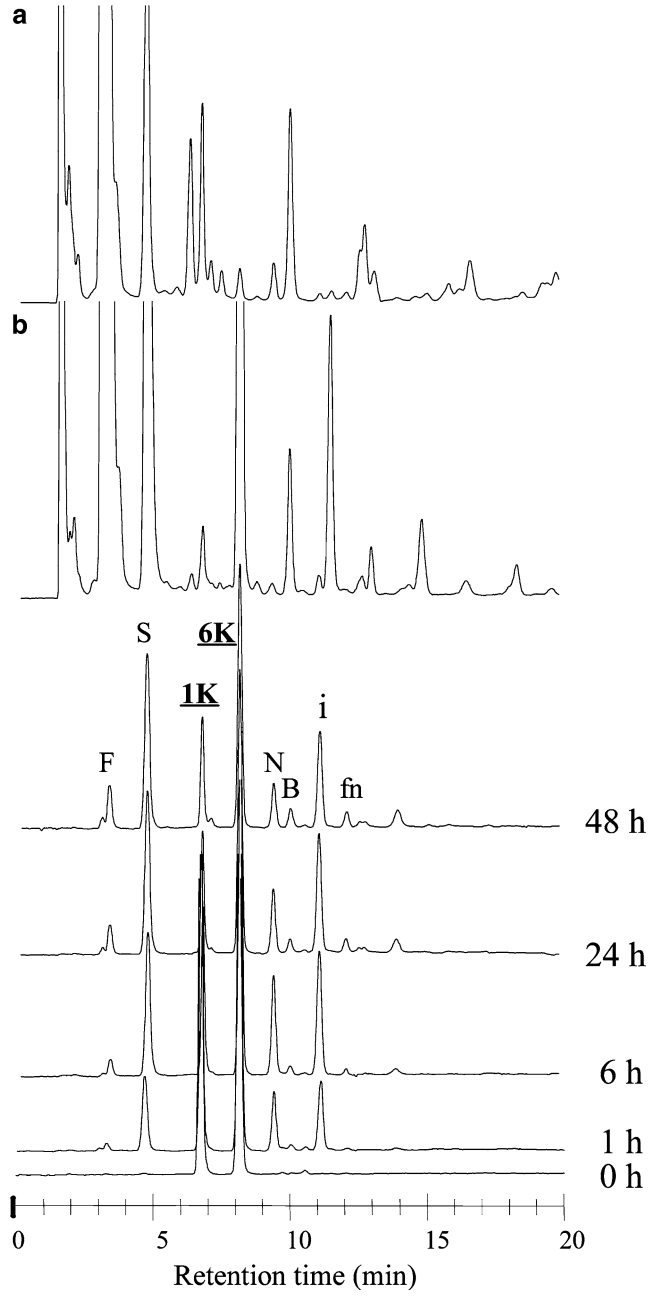

Fig. 8 Anion exchange HPLC analysis of oligo-fructans generated by recombinant Wft3 (*Wft3m*) with a mixture of 1-kestotriose and 6-kestotriose. The reactions were conducted with a mixture of 50 mM 1-kestotriose and 50 mM 6-kestotriose at 10°C for 0, 1, 6, 24 and 48 h. Compounds were detected by pulsed amperometric detection and identified using external standards. Abbreviations for each compound are the same as for Fig. 1. Soluble carbohydrates extracted from wheat leaf tissues hardened until December 8 (**a**) and wheat stem tissues at days 8 after anthesis (**b**) were used as standards for oligo-fructans

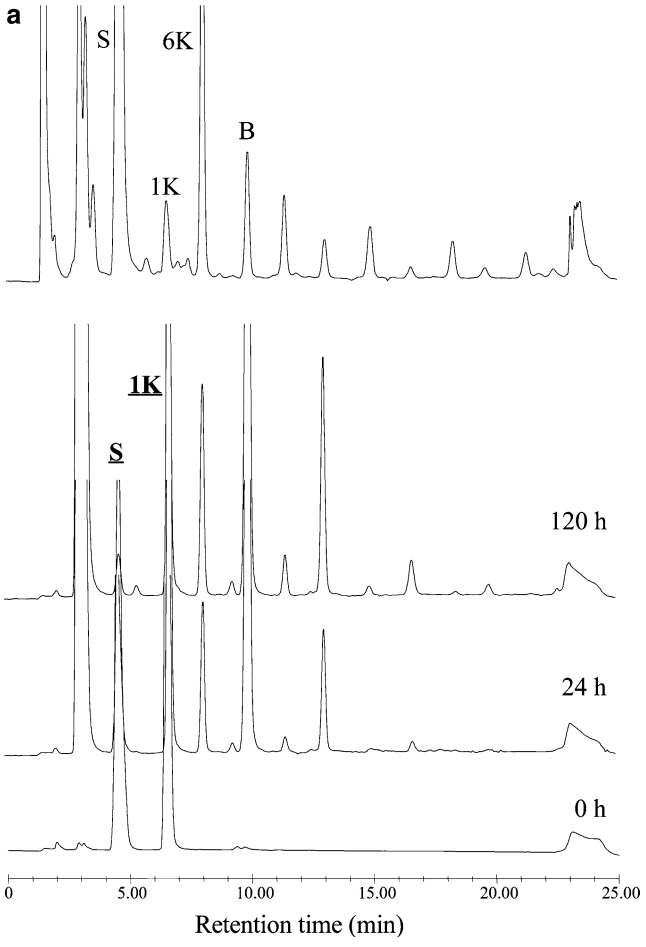

Fig. 9 Anion exchange HPLC analysis of oligo-fructans generated by recombinant 6-SFT (*Wft1m*) in long incubation periods. The reactions were conducted with a mixture of 50 mM sucrose and 50 mM 1-kestotriose at 10°C for 0, 24 and 120 h. Compounds were detected by pulsed amperometric detection and identified using external standards. Abbreviations for each compound are the same as for Fig. 1. Soluble carbohydrates extracted from wheat stem tissues at days 8 after anthesis (**a**) were used as standards for oligo-fructan peaks

crown tissues during cold hardening (Fig. 3). The fructans in stem tissues were mainly phlein based on 6-kestotriose and 1,6-kestotetraose without branches of ß-(2,1)-linked fructosyl units (Bancal et al. 1992). Chilling treatment resulted in accumulation of fructan oligomers enriched in ß-(2,1)-linkages in blade tissues of wheat (Bancal and Gaudillère 1989). These findings indicate that the activities of fructosyltransferases associated with fructan synthesizing processes may vary among growth stages of wheat and are influenced by environmental conditions. 6-SFT formed exclusively ß-(2,6)-fructosyl linkages, either initiating or elongating a fructan chain of the phlein type (Duchateau et al. 1995). Therefore, another type of fructosyltransferase in addition to 6-SFT would be necessary for the production of graminan enriched in ß-(2,1)-linked fructosyl units in wheat tissues during cold hardening.

Crude extracts of wheat tissues hardened until December 8 in the field were found to have 1,1-kestotetraose synthesis activity by incubation with 1-kestotriose as a substrate (Fig. 4). 1-SST can also produce 1,1-kestotetraose when incubated with sucrose or 1-kestotriose, but the activity of 1-SST for 1,1-kestotetraose

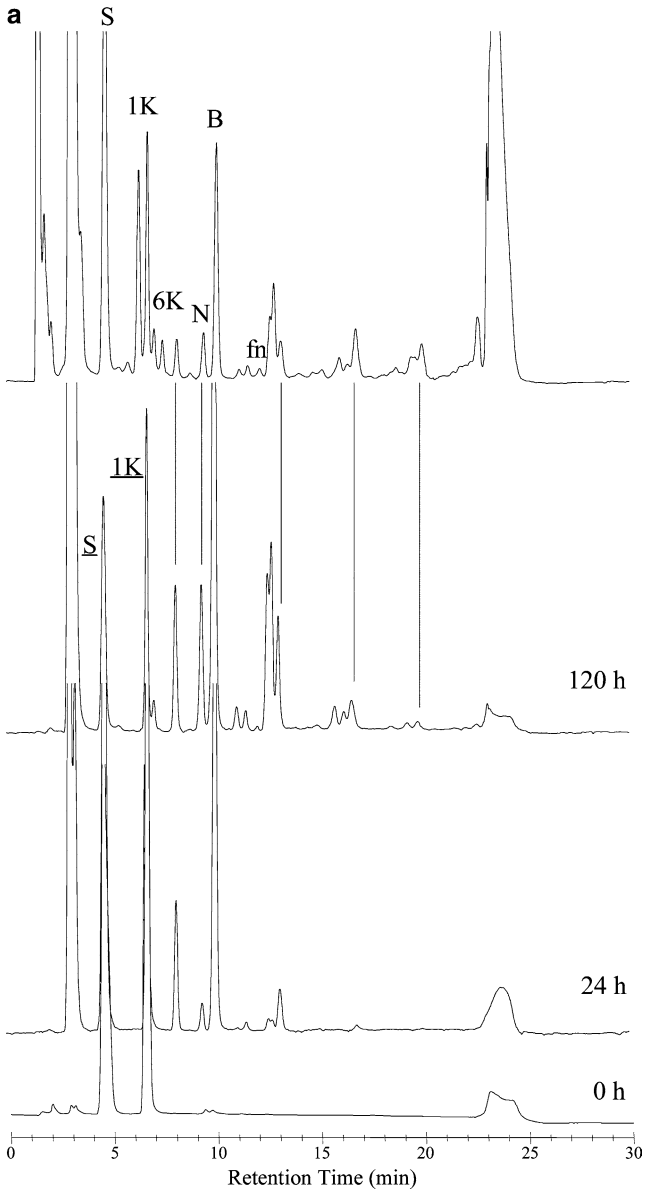

Fig. 10 Anion exchange HPLC analysis of oligo-fructans generated by a mixture of recombinant 6-SFT (*Wft1m*) and recombinant Wft3 (*Wft3m*) in long incubation periods. The reactions were conducted with a mixture of 50 mM sucrose and 50 mM 1-kestotriose at 10°C for 0, 24 and 120 h. Compounds were detected by pulsed amperometric detection and identified using external standards. Abbreviations for each compound are the same as for Fig. 1. Soluble carbohydrates extracted from wheat leaf tissues hardened until December 8 (**a**) were used for standards of oligo-fructans

synthesis is extremely low compared with that of 1-SST for 1-kestotriose synthesis (Lüscher et al. 1996). In the case of enzyme assays with extracts from hardened wheat tissues, the amount of 1-kestotriose produced by incubation with sucrose was almost the same as that of 1,1-kestotetraose produced by incubation with 1-kestotriose (Fig. 4). Therefore, 1,1-kestotetraose was probably produced by 1-FFT activity in the crude extracts from hardened wheat tissues. These results show that 1-FFT as well as 1-SST and 6-SFT are expressed and associated with fructan synthesis in wheat tissues during cold hardening. This inference is supported by results of RT-PCR analysis showing that mRNAs of 1-SST, 6-SFT and 1-FFT were transcribed in wheat tissues that accumulated graminan with branches enriched in ß-(2,1)-linked fructosyl units. Jeong and Housley (1992) purified 1-FFT from wheat tissues incubated at 10°C for 4 days, suggesting that 1-FFT activity could be induced in wheat tissues by low temperature conditions.

Cloning of 1-FFT from wheat is essential for elucidating the molecular mechanism of fructan biosynthesis. Therefore, we attempted to isolate 1-FFT cDNA by screening a cDNA library prepared from hardened wheat tissues with a labeled wheat 1-SST clone based on the known sequence similarity among fructosyltransferases (Vijn and Smeekens 1999). Two candidates (*wft3* and *wft4*) of 1-FFT were isolated (Fig. 5). The deduced amino acid sequences (Wft3 and Wft4) showed more than 80% identity in ORF regions and more than 83% identity in putative mature regions to wheat 1-SST, respectively. The recombinant protein (Wft3m) exhibited catalytic profiles similar to those of plant 1-FFTs (Fig. 6; Jeong and Housley 1992; Koops and Jonker 1994; Lüscher et al. 1993; Van den Ende et al. 1996). The sequence identity between wheat 1-SST and wheat 1-FFT was much greater than the sequence identity between artichoke 1-SST and 1-FFT (57%) or Jerusalem artichoke 1-SST and 1-FFT (54%) (Hellwege et al. 1998). Furthermore, the putative mature-protein regions between 1-FFT and 1-SST of wheat were entirely well-conserved (Fig. 5). However, some low-conserved domains were detected between those of artichoke or Jerusalem artichoke and were suggested to be associated with the difference between substrate specificities of 1-FFT and 1-SST (Hellwege et al. 1998). This finding indicates the possibility that a few point mutations may convert 1-SST into 1-FFT as seems to be the case for wheat. Studies on microbial invertases and fructosyltransferases have revealed the catalytic mechanisms for sucrose hydrolysis and transfer of fructosyl units (Chambert and Petit-Glatron 1991; Reddy and Maley 1996). Recently, Ritsema et al. (2004) studied the importance of the onion 6G-FFT ß-fructosidase motif (NDPSG) using mutants S87N (NDPNG) and N84A;S87N (ADPNG) and reported that the mutant S87N has 6F-FFT activity, whereas the mutant N84A;S87N had a activity that was shifted towards synthesis of ß-(2,1)-linkages. Wheat 1-SST has the same ß-fructosidase motif (NDPNG) as the mutant S87N (Fig. 5). This suggests that the other motifs are also necessary for specificity of fructan type synthesized. Our characterization of genes encoding wheat 1-SST and 1-FFT will enable us to identify which domains of the enzymes determine substrate specificity for fructose transfer.

Wft3m also showed 1-SST activity at a detectable level. Purified 1-FFT from plants generally show no 1-SST activity (Jeong and Housley 1992; Lüscher et al.

1993; Koops and Jonker 1994; Van den Ende et al. 1996). It is possible that native 1-FFT encoded by *wft3* does not appear to possess 1-SST activity. This may be because some recombinant proteins produced by *P. pastoris* are known to exhibit profiles of enzymatic activity different from those of native enzymes purified from plants (Hochstrasser et al. 1998; Tibbot et al. 1998). Another possibility is that wheat has another isozyme different from the 1-FFT purified by Jeong and Housley (1992) and that 1-FFT have enzymatic profiles slightly overlapping those of 1-SST. 1-SST in general have a minor 1-FFT activity (Lüscher et al. 1996; Van den Ende et al. 1996). Further studies are needed to purify and characterize 1-FFT encoded by *wft3* from hardened wheat to determine whether Wft3 possesses 1-SST activity.

Hellwege et al. (1998) reported that the DP of inulin was ten in *H. tuberosus* and 65 in *C. scolymus* and that 1-FFT of *C. scolymus* had higher affinities to larger inulin oligomers than did 1-FFT of *H. tuberosus*. This and other report (Vergauwen et al. 2003) also suggested that the DP of inulin in a given species was mainly defined by the enzymatic characteristics of 1-FFT that are unique to each species. As for wheat, fructans with high DP have been detected, but inulin with a DP above six has not been found in any tissues (Bancal et al. 1991; Fig. 3). We elucidated the substrate preference of wheat 1-FFT to inulin oligomers with different DP. Wft3m could transfer only 2 or 3 units of fructose to every substrate when incubated with 1-kestotriose, 1,1-kestotetraose, 1,1,1-kestopentaose (Fig. 6) or a mixture of inulin oligomers with DP of 3–5. These results show that wheat 1-FFT has a substrate preference for inulin oligomers of low DP compared with 1-FFT of *H. tuberosus* (Hellwege et al. 1998). Therefore, the inulin that accumulates in wheat will have a lower DP than that of *H. tuberosus*. These results are reasonable considering the fact that inulin with DPs above 6 have not been found in wheat tissues. The main role of wheat 1-FFT is probably not for the synthesis of long inulin oligomers.

During cold hardening, graminan enriched in ß-(2,1)-linked fructosyl units accumulated in wheat tissues (Fig. 3). 1-FFT purified from wheat produced 1,1-kestotetraose, 6,1-kestotetraose and 1,6-kestotetraose when incubated with 1-kestotriose and 6-kestotriose (Jeong and Housley 1992). When Wft3m was incubated with a mixture of 1-kestotriose and 6-kestotriose, 1,1-kestotetraose 1,6-kestotetraose and oligosaccharide of peak *i* were detected (Fig. 8). If oligosaccharide of peak *i* was 6,1-kestotetraose, 1,1-kestotetraose and oligosaccharide of peak *i* could be synthesized by ß-(2,1)-fructosyl transfer from 1-kestotriose to the terminal fructose of 1-kestotriose and 6-kestotriose, respectively. 1,6-kestotetraose could be synthesized by transfer of a ß-(2,1)-linked fructosyl unit from 1-kestose to the internal fructose of 6-kestose. These results indicate that the main role of wheat 1-FFT is not to synthesize long inulin oligomers but to transfer ß-(2,1)-linked fructosyl units to ß-(2,6)-linked fructose of phlein and graminan produced by 6-SFT.

In previous studies, fructosyltransferases necessary for fructan synthesis of *H. tuberosus* and *C. intybus* were investigated by in vitro experiments using a combination of purified enzymes (Koops and Jonker 1996; Lüscher et al. 1996; Van den Ende and Van Laere 1996). Chromatographic profiles of oligofructans (DP 3–5) that accumulated in hardened wheat tissues were almost identical to those of products synthesized by a combination of Wft3m and Wft1m (6-SFT) incubated with sucrose and 1-kestotriose but not by Wft1m incubated with sucrose and 1-kestotriose (Figs. 9, 10). Comparison between the profile of products synthesized by a combination of Wft3m and Wft1m and that by Wft1m indicated that Wft3m transfer branches of ß-(2,1)-linked fructosyl units to phlein oligomers synthesized by 6-SFT (Wft1m). Since 1-kestotriose used as a substrate for the enzyme assay is synthesized by 1-SST, 1-FFT in addition to 1-SST and 6-SFT would be necessary for synthesis of fructans that accumulate in wheat tissues during cold hardening.

In the present study, we did not consider the effects of FEHs. Yukawa et al. (1995) have reported that FEHs were expressed in wheat tissues during cold hardening, but the activities were repressed by exposure to cold temperature. Bancal et al. (1992) suggested that 1-FFT and 1-FEH could be important factors for elongation and trimming of branches of ß-(2,1)-linked fructosyl units. The cloning of 1-FEH cDNAs from wheat was reported by Van den Ende et al. (2003). From our results, fructan synthesis in wheat tissues during cold hardening seems to be regulated more by branch formation with ß-(2,1)-linked fructosyl units to specific sites of phlein and graminan than by digestion of ß-(2,1)-linked terminal fructosyl units by FEH. We deduced thus, because the amount as well as the oligo-fructan profiles (DP 3–5) accumulated in wheat tissues during cold hardening are similar to those of products synthesized by Wft1m and Wft3m when FEH activities are not included in the reaction mixtures.

The results presented here clearly demonstrate that not only 6-SFT but also 1-FFT has an essential function in the biosynthesis of graminan with both ß-(2,1)- and ß-(2,6)-linked fructosyl units. Wheat 1-FFT is thought to be a key enzyme for branching with ß-(2,1)-linked fructosyl units of fructans that accumulate in hardened wheat tissues.

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