Journal of Experimental Botany, Vol. 60, No. 3, pp. 893–905, 2009
doi:10.1093/jxb/ern337
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Cloning and functional analysis of a fructosyltransferase cDNA for synthesis of highly polymerized levans in timothy (*Phleum pratense* L.)

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Received 12 August 2008; Revised 27 November 2008; Accepted 1 December 2008

Abstract

Variation in the structures of plant fructans and their degree of polymerization (DP) can be explained as the result of diverse combinations of fructosyltransferases (FTs) with different properties. Although FT genes have been isolated in a range of plant species, sucrose:fructan 6-fructosyltransferase (6-SFT) cDNAs have only been functionally characterized in a few species such as wheat. A novel FT cDNA possessing 6-SFT activity has been identified and characterized from the temperate forage grass, timothy (*Phleum pratense* L.). The cDNA of an FT homolog, *PpFT1*, was isolated from cold-acclimated timothy. A recombinant PpFT1 protein expressed in *Pichia pastoris* showed 6-SFT/sucrose:sucrose 1-fructosyltransferase (1-SST) activity and produced linear β(2,6)-linked levans from sucrose with higher DPs than present in graminans formed *in vitro* by wheat 6-SFT (Wft1). PpFT1 and Wft1 showed remarkably different acceptor substrate specificities: PpFT1 had high affinity for 6-kestotriose to produce levans and low affinity for 1-kestotriose, whereas Wft1 preferentially used 1-kestotriose as an acceptor. The affinity of the PpFT1 recombinant enzyme for sucrose as a substrate was lower than that of the Wft1 recombinant enzyme. It is also confirmed that timothy seedlings had elevated levels of *PpFT1* transcripts during the accumulation of fructans under high sucrose and cold conditions. Our results suggest that *PpFT1* is a novel cDNA with unique enzymatic properties that differ from those of previously cloned plant 6-SFTs, and is involved in the synthesis of highly polymerized levans in timothy.

Key words: Fructan, fructosyltransferase (FT), levan, *Phleum pratense* L., sucrose:fructan 6-fructosyltransferase (6-SFT), timothy.

Introduction

Fructans are soluble fructosyloligosaccharides derived from sucrose, and are known to be synthesized in more than 4500 species of plants (Chatterton *et al.*, 1989; Hendry, 1993). Among the suggested roles of fructans in plants is as an alternative to starch for storing carbohydrate and providing protection against environmental stresses such as drought and freezing (Hendry, 1993; Pilon-Smits *et al.*, 1995, 1999; Hisano *et al.*, 2004). Fructans may be involved in the protective mechanisms against drought and freezing stresses through alteration of membrane stability (Livingston and Henson, 1998; Hincha *et al.*, 2002). In temperate forage grasses, fructans are the principal type of water-soluble carbohydrate, and contribute to the palatability (Mayland *et al.*, 2000) and digestibility (Humphreys, 1989) of these grasses for animals as well as to the fermentation quality of silage (Smith *et al.*, 1997).

The structures of fructans vary widely among plant species (reviewed by Vijn and Smeekens, 1999). Dicotyledonous families, such as the Asteraceae, mainly accumulate inulins, which consist of fructose units linked by β(2,1) glycosidic bonds and a terminal glucose unit (Van Laere and Van den Ende, 2002). Some Liliaceae species accumulate an inulin

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neo-series in addition to inulins (Shiomi, 1989). Other plant families, such as the Poaceae, principally accumulate levan-type fructans that consist mainly of β(2,6)-linked fructose units with a terminal glucose. Timothy (*Phleum pretense L.*), orchard grass (*Dactylis glomerata L.*), and big bluegrass (*Poa secunda J. Presl*) are known to accumulate a series of simple levans comprised of linear β(2,6)-linked fructose units with a terminal glucose (Chatterton *et al.*, 1993; Bonnett *et al.*, 1997; Chatterton and Harrison, 1997; Cairns *et al.*, 1999). Triticeae plants, such as wheat and barley, produce mixed levans, called graminans, composed of branched-type fructans containing β(2,6)- and β(2,1)-linked fructose residues (Carpita *et al.*, 1989; Bonnett *et al.*, 1997). *Lolium* species, such as *L. perenne*, produce mixed-type fructans including a levan neo-series (Chalmers *et al.*, 2005).

The diversity in the structure of fructans across plant species results from the different combinations of the enzymes involved in fructan biosynthesis. Currently, four types of fructosyltransferase (FT) for fructan biosynthesis are recognized in plants (reviewed by Vijn and Smeekens, 1999). Sucrose:sucrose 1-fructosyltransferases (1-SST) transfer a fructose unit from the sucrose molecule to the fructosyl residue of another sucrose molecule via a β(2,1) linkage and produce 1-kestotriose. Fructan:fructan 1-fructosyltransferases (1-FFT) catalyse the elongation of fructose units on 1-kestotriose via a β(2,1) linkage, which generally results in the production of an inulin series of fructans. Fructan:fructan 6G-fructosyltransferases (6G-FFT) catalyse the transfer of a fructose unit from one fructan to the C6 of a glucose unit of another fructan or sucrose to produce an inulin neo-series or a levan neo-series of fructans. Sucrose: fructan 6-fructosyltransferases (6-SFT) mainly transfer a fructose unit from sucrose to a fructan by a β(2,6) linkage. Since the isolation of a 6-SFT cDNA from barley (Sprenger *et al.*, 1995) considerable efforts have been made to identify other plant FT genes. A 6-SFT cDNA has also been described in wheat (Kawakami and Yoshida, 2002) that, like barley, accumulates graminans. The Triticeae 6-SFT enzymes preferentially transfer a fructose unit to 1-kestotriose, rather than to 6-kestotriose, to produce bifurcose, the starting tetrasaccharide required for the formation of graminans (Duchateau *et al.*, 1995; Sprenger *et al.*, 1995; Kawakami and Yoshida, 2002). 6-SFT homologues have also been identified in *P. secunda* (Wei *et al.*, 2002), *L. perenne* and *L. temulentum* (Gallagher *et al.*, 2004; Hisano *et al.*, 2008) although the enzymatic activities of the encoded enzymes have yet to be reported. *P. secunda* mainly accumulates linear levans, whereas *L. perenne* and *L. temulentum* mainly accumulate a levan neo-series.

The degree of polymerization (DP) of fructans also differs among plant species (Vijn and Smeekens, 1999). In the Asteraceae, the globe artichoke, *Cynara scolymus* (Hellwege *et al.*, 2000), the globe thistle, *Echinops ritro* (Vergauwen *et al.*, 2003), and *Viguiera discolor* (Isejima and Figueiredo-Ribeiro, 1993) store inulins with a higher DP compared to chicory (*Cichorium intybus*) and Jerusalem artichokes (*Helianthus tuberosus*). Differences in the DP of these inulin-type fructans have been attributed to differences in substrate specificity of their 1-FFTs (Hellwege *et al.*, 1998; Vergauwen *et al.*, 2003; Van den Ende *et al.*, 2005, 2006). Vergauwen *et al.* (2003) reported that 1-FFT in the globe thistle had a higher affinity for inulin as the acceptor of a fructosyl residue, whereas the 1-FFT of chicory exhibited a high affinity for sucrose and 1-kestotriose rather than for higher DP fructans. In addition to the consequences of variation in the properties of 1-FFT, there is evidence from the Asteraceae that the activity of fructan 1-exohydrolase (1-FEH) may also influence the production of inulins with different DPs (Itaya *et al.*, 2007). To date, however, there is relatively little information on the relationship between the DP of fructans and the enzymatic properties of 6-SFT in the synthesis of β(2,6)-linked fructans.

Timothy is used as a forage plant in temperate grasslands with severe winters due to its high nutritive quality and because it has better winter hardiness compared to alternative crops such as perennial ryegrass (Tamura and Yamada, 2007). It predominantly accumulates simple levans with a higher DP than those of wheat, barley, oat, *Lolium* spp, and *Festuca* spp (Spollen and Nelson, 1988; Suzuki, 1989; Cairns and Ashton, 1993). Timothy has been reported to have a DP of up to 90 in leaf tissue (Cairns *et al.*, 1999) and 260 (mol. wt. 42 000) in the stem base (Grotelueschen and Smith, 1968). An enzyme with fructan polymerization activity was partially purified and characterized from excised and illuminated leaves of timothy (Cairns *et al.*, 1999). This enzyme preparation showed 6-SFT activity and produced linear β(2,6)-linked fructans from sucrose with a DP of up to 50. However, to date, isolation of 6-SFT cDNA has not been reported for timothy. Moreover, the genes for FTs involved in the synthesis of levans with a high DP have not been cloned from any plant species.

In this study, a cDNA encoding an FT of timothy was cloned and the enzymatic properties of a recombinant protein derived from this clone was characterized. The enzymatic properties of this recombinant protein were compared with those of wheat 6-SFT. A relationship between the expression level of the timothy FT gene, FT activity, and fructan content under high sugar and cold conditions was also confirmed. Our results indicate that the novel FT cDNA identified here is involved in the biosynthesis of linear levan type fructans with a high DP in timothy and that the encoded enzyme has different enzymatic properties to those of Triticeae 6-SFT cDNAs cloned previously.

Materials and methods

Plant materials

The timothy cultivar 'Hokushu' was grown in the field at the National Agricultural Research Center for the Hokkaido Region located in Sapporo, Japan. Crown tissues were sampled from a single individual on 15 November 2005 and used for RNA extraction. Seedlings of the timothy cultivar 'Hokushu' were grown in a controlled climate chamber (16 h light, 150 μmol m^{-2} s^{-1} PFD, 22 °C) for 3 weeks after germination.

Preparation of a cDNA library from timothy and cloning of an FT cDNA

Total RNA was extracted from crown tissues using TRIzol reagent (Invitrogen). Poly $(A)^+$ RNA was purified using Oligotex™-dT30 resins (Takara Bio), and double-stranded cDNA was synthesized from the poly $(A)^+$ RNA using a cDNA Synthesis Kit (Takara Bio). A cDNA library was constructed using a λ ZAP II vector (Strategene) and MaxPlax™ Lambda Packaging Extracts (Epicentre). Partial fragments of putative FT cDNAs were obtained by PCR amplification from the cDNA library using the primers 6SFT-5F (5′-GAGATGCTGCAGTGGCAGCG-3′) and FT-1R (5′-CCNARYGMGTAGTAGTCGTG-3′). The primer sequences were designed using DNA sequences conserved among Poaceae FT genes. Amplified fragments were cloned into pGEM®-T Easy Vector (Promega) and nucleotide sequences of the fragments were determined using the CEQ 8000™ Genetic Analysis System (Beckman Coulter). A 725 bp fragment (Accession no. AB436698) that had high similarity to a partial sequence of Poaceae FT genes was labelled using a PCR DIG Probe Synthesis Kit (Roche) and used for cDNA library screening by plaque hybridization with a DIG Luminescent Detection Kit for Nucleic Acid (Roche). Approximately 1.1×10^5 recombinant plaques were screened and the positive plaques were isolated. After plaque purification, *in vivo* excision of the pBluescript SK-phagemid vector was performed in the *E. coli* XLOLR strain. The nucleotide sequences of both strands of the inserts were determined. Multiple sequence alignments and phylogenetic trees were constructed by the Neighbor–Joining method, using the CLUSTALW program of DNASIS® Pro ver. 2.0 software (Hitachi Software Engineering).

Expression of recombinant protein in methylotrophic yeast

The isolated cDNA was expressed in the methylotrophic yeast *Pichia pastoris* (EasySelect™ Pichia Expression kit, Invitrogen) after cloning into the secretory expression vector pPICZαA. The DNA sequence corresponding to the predicted mature protein region was amplified using the primers tyft17-3F (5′-CCCCGAATTCGGAGCCAGGGTGGGTCTGGG-3′) and tyft17-1R (5′-CCCCTCTAGACAAATCGTCGATCAAGAAG-3′). The amplification product was digested with *Eco*RI and *Xba*I, and ligated into pPICZαA behind the α-factor signal sequence. The *P. pastoris* strain X-33 was transformed by electroporation using 10 μg of the *PmeI*-linearized construct, and transformants were selected on YPDS/Zeocin plates. A 3 ml preculture medium (BMGY, pH 6.0) was inoculated with freshly prepared single colonies and cultured for 2 d at 30 °C with vigorous shaking (200 rpm). The cells were collected by centrifugation, resuspended in 20 ml of inoculation medium (BMMY, pH 6.0) and incubated at 30 °C with shaking at 200 rpm. Four hundred μl of methanol were added to the culture medium every day. After 7 d of induction, the culture was centrifuged and the resulting supernatant medium was recovered and tested for enzyme activity. A 12 ml aliquot of the medium was concentrated to 200 μl, diluted with 12 ml of 20 mM citrate-phosphate buffer (pH 5.2), and concentrated by ultrafiltration on an Amicon® Ultra-15 with a cut-off of 10 kDa (Millipore). The dilution/concentration process was repeated twice, and the final volume of enzyme solution was adjusted to 200 μl with 20 mM citrate phosphate buffer (pH 5.2). A recombinant Wft1 enzyme was prepared using a *Pichia* expression vector by the process described by Kawakami and Yoshida (2002). Unless otherwise specified, the concentration of the medium containing recombinant PpFT1 or Wft1 was adjusted by dilution with 20 mM citrate phosphate buffer (pH 5.2) such that its FT activity (defined below) transferred 1 nmol fructose units min^{-1} μl^{-1} to sucrose or fructans, not to H_2O, in 50 μl of 0.5 M sucrose solution.

Assay for FT activity of recombinant proteins

All reactions using recombinant proteins were performed in 20 mM citrate–phosphate buffer (pH 5.2) at 25 °C. To analyse the reaction product with sucrose as the substrate, 25 μl of concentrated medium containing recombinant enzyme was incubated with 25 μl of 2 M sucrose for up to 96 h. Half of the reaction mixture that was incubated for 96 h was further incubated for 60 h following the addition of 12.5 μl of fresh recombinant enzyme solution and 12.5 μl of 2 M sucrose solution. To test substrate specificity to tri-oligofructans, 5 μl aliquots of concentrated medium containing recombinant enzyme were incubated individually with 25 mM sucrose, 100 mM 1-kestotriose, 100 mM 6-kestotriose (Iizuka *et al.*, 1993), or a combination of sucrose and 1-kestotriose or of sucrose and 6-kestotriose. To test the substrate specificity to high-DP fructans, 5 μl of the recombinant enzyme solution was incubated with sucrose and high-DP fructans extracted from timothy leaves illuminated for 24 h after cutting. High-DP fructans were concentrated by precipitation with 80% ethanol. Because fructans in timothy are mainly levans, the molarity and the mean DP were estimated by the amount of glucose and the ratio of fructose to glucose, respectively, after hydrolysis with 6% HCl. The sugar products were analysed by high-performance anion exchange chromatography and pulsed amperometric detection (HPAEC-PAD) (DX-500, Dionex) with a CarboPac PA-1 anion-exchange column (Dionex) using a sodium-acetate gradient in 150 mM NaOH with an elution rate of 1 ml min^{-1}. The sodium-acetate gradients were carried out as follows: 0–1 min, 25 mM; 1–2 min, 25–50 mM; 2–20 min, 50–200 mM; 20–25 min, 200–250 mM; 25–45 min, 250–500 mM; 45–48 min, 500 mM; 48–50 min, 500–25 mM. The column was re-equilibrated for 10 min with 150 mM NaOH. A shorter gradient program with an identical gradient slope was also performed. Peaks for glucose, fructose, sucrose, 1-kestotriose, and 6-kestotriose were identified with known standards. Levan series fructans [linear β(2,6)-linked fructans] were putatively identified by comparison of HPAEC retention times with fructan oligomers extracted from crown tissues of timothy in the field in

November 2006. The initial velocity of FT activity was determined using 5 μl of enzyme solution with each concentration of sucrose. Oligo fructans with a DP >3 were produced at very low levels under these conditions. FT activities were extrapolated by subtracting the amount of fructose released from that of glucose produced after 5 min and 10 min incubation. Measurements of fructose and glucose were performed by HPLC using Shodex® KS-802 and KS-803 columns (Showa Denko), and an L-2490 refractive index detector (Hitachi) as described by Yoshida *et al.* (1998). Sucrose:sucrose 6-fructosyltransferase (6-SST) and 1-SST activities were estimated by the production of 6-kestotriose or 1-kestotriose measured by HPAEC-PAD.

Sucrose and cold treatments of timothy seedlings

For the sucrose treatment, the shoots of 20–30 timothy seedlings were cut into 2–3 cm lengths and transferred to a 0.5 M sucrose solution or to deionized water as the negative control. Each treatment was kept at 22 °C in the dark for 3, 8, or 24 h, and then the fragments of timothy shoots were washed thoroughly to remove sucrose for analysis. For the cold treatment, timothy seedlings were transferred to a cold acclimation room and kept at 6 °C under light (150 μmol m^{-2} s^{-1}) for 8 h and at 2 °C in the dark for 16 h. Ten seedlings were sampled at 1, 3, 7, 14, and 28 d, and used for each analysis.

Gene expression analysis by quantitative real-time RT-PCR

Total RNA was extracted from each sample using TRIzol® reagent (Invitrogen). After treatment with DNase I to remove contaminating DNA, cDNA was synthesized from 1 μg of total RNA using PrimeScript® RT Reagent Kit (Perfect Real Time) (Takara Bio) with oligo-dT and random primers. The levels of *PpFT1* transcripts were quantified with real-time RT-PCR using a 7500 Real Time PCR System (Applied Biosystems) and SYBR® Premix Ex Taq™ (Perfect Real Time) (Takara Bio) according to the manufacturer's instructions. The primer pairs used in the real-time PCR for *PpFT1* were Pp6-SFT1-2F (5′-GCATC-TACGCGACGCGG-3′) and Pp6-SFT1-2R (5′-GACC-GTTGGGATCGCTCAT-3′), and for the α-tubulin gene were TY-tub-1F (5′-CGCTAGGTGGCTGGTAGTTG-3′) and TY-tub-1R (5′-GATGTGAACGCTGCTGTGG-3′). Three determinations were performed for each sample. The expression level of *PpFT1* relative to α-tubulin (the internal reference gene) was calculated and adjusted against the expression levels of untreated samples, which were deemed to be 1.0.

Assay of 6-SST activities in crude enzymatic extracts

Crude enzymatic solutions were extracted from 1 g of timothy shoots using 20 mM citrate–phosphate buffer (pH 5.2) containing 1 mM dithiothreitol (DTT). Following centrifugation at 8000 *g* for 15 min, proteins in the resulting supernatants were concentrated by precipitation with 70% ammonium sulphate. The pellets were dissolved in 0.5 ml of 20 mM citrate–phosphate buffer (pH 5.2) with 1 mM DTT and desalted using a Biospin-6 column (Bio-Rad). Twenty μl of enzymatic solution were incubated with 20 μl of a solution containing 2 M sucrose, 0.2% BSA, 20 mM citrate–phosphate buffer, and 1 mM DTT at 37 °C for 3 h. The reaction was terminated at 95 °C for 3 min. The sugar products were analysed by HPAEC-PAD as described above. Levan oligomers with a DP higher than 6-kestotriose were not detected in any products under these reaction conditions. Therefore, 6-SST activity was evaluated by the amount of 6-kestotriose produced after enzymatic incubation. Total protein content in the enzymatic solution was measured by the Protein Assay reagent (Bio-Rad) based on the Bradford method. One unit (U) of enzyme activity was defined as the amount of enzyme producing 1 μmol of 6-kestotriose h^{-1}.

Measurement of carbohydrate contents in timothy

As described previously by Yoshida *et al.* (1998), total water-soluble carbohydrates were extracted from 1 g of timothy shoots by boiling for 1 h in deionized water containing 1 mg ml^{-1} of propylene glycol; propylene glycol was used as the internal standard. Mono- and disaccharide sugars (fructose, glucose, and sucrose) and fructans (DP >3) in the extract were separated by HPLC as described above.

Results

Cloning of a candidate FT cDNA from timothy

As a first step to identifying FT cDNAs from timothy, RT-PCR was used to isolate a partial 725 base pair cDNA fragment (AB436698) that contained conserved motifs of plant FT (Verhaest *et al.*, 2005). After repeated cDNA screening using this fragment as a probe, several positive clones were obtained. One of the isolated cDNA clones consisted of 2186 bases that included an open reading frame (ORF) of 1872 bases. This clone was designated *PpFT1* (AB436697) and was deduced to encode 623 amino acids containing three conserved amino acid motifs (S/NDPNG, RDP, and EC) that are suggested to be essential for β-fructosidase activity (Verhaest *et al.*, 2005) (Fig. 1). The PpFT1 protein was predicted to have a pI of 5.06 and to have seven N-glycosylation sites (N-X-S/T). A phylogenetic comparison of the amino acid sequences of PpFT1 and of FTs and vacuolar invertases from a range of species (Fig. 2) indicated that *PpFT1* was a member of a group of genes coding enzymes that catalyse transfer of fructose units by β(2,6) linkage (6-SFTs and putative 6-FTs). The predicted amino acid sequence of PpFT1 showed highest identity (86%) to a putative 6-SFT from *Poa secunda* (Wei *et al.*, 2002).

PpFT1 recombinant protein polymerizes β(2,6)-linked fructans

The *PpFT1* cDNA was expressed in the methylotrophic yeast *P. pastoris* and the enzymatic properties of the recombinant

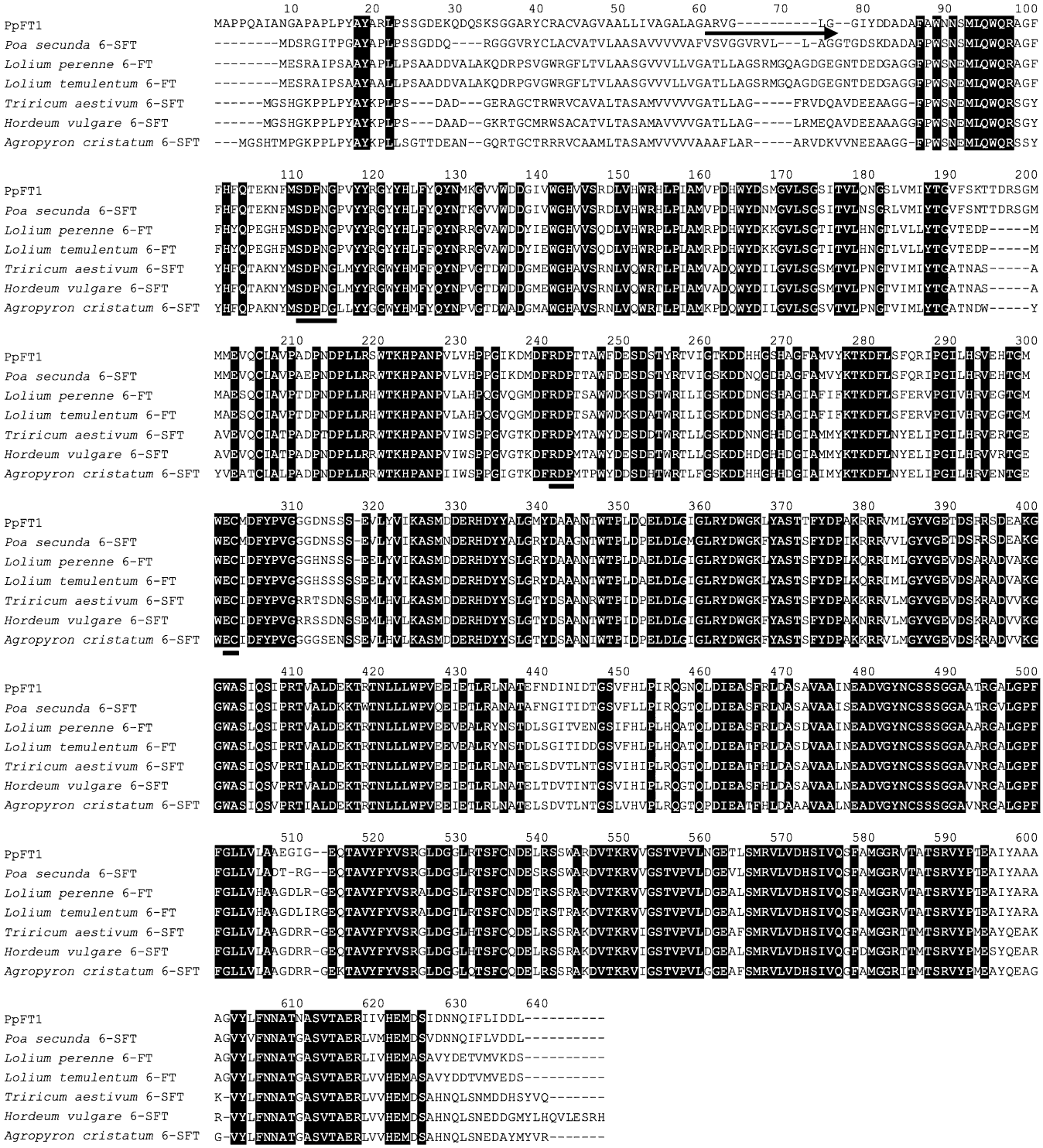

Fig. 1. Alignment of the amino acid sequences of 6-SFTs and putative 6-FTs from Poaceae plants. Bars indicate the three conserved motifs that are crucial for β-fructosidase catalysis. The arrow indicates the predicted N-terminus of the mature PpFT1 protein.

protein were investigated. A partial cDNA containing the predicted mature protein region of *PpFT1* was ligated in-frame behind the α-signal sequence and heterologously expressed in *P. pastoris*. Concentrated and desalted media from *P. pastoris* cultures expressing *PpFT1* were incubated with 1 M sucrose. An HPAEC-PAD analysis showed that the reaction mixtures contained 6-kestotriose and other, more polymerized products. The retention times of these products were identical to those in extracts from timothy crown tissues, which mainly contained linear β(2,6)-linked fructans (Cairns *et al.*, 1999) (Fig. 3A). The production of 6-kestotriose and putative β(2,6)-linked fructans (levans) from sucrose indicated that the PpFT1 recombinant enzyme exhibited 6-SST and 6-SFT activity. β(2,6)-linked fructans

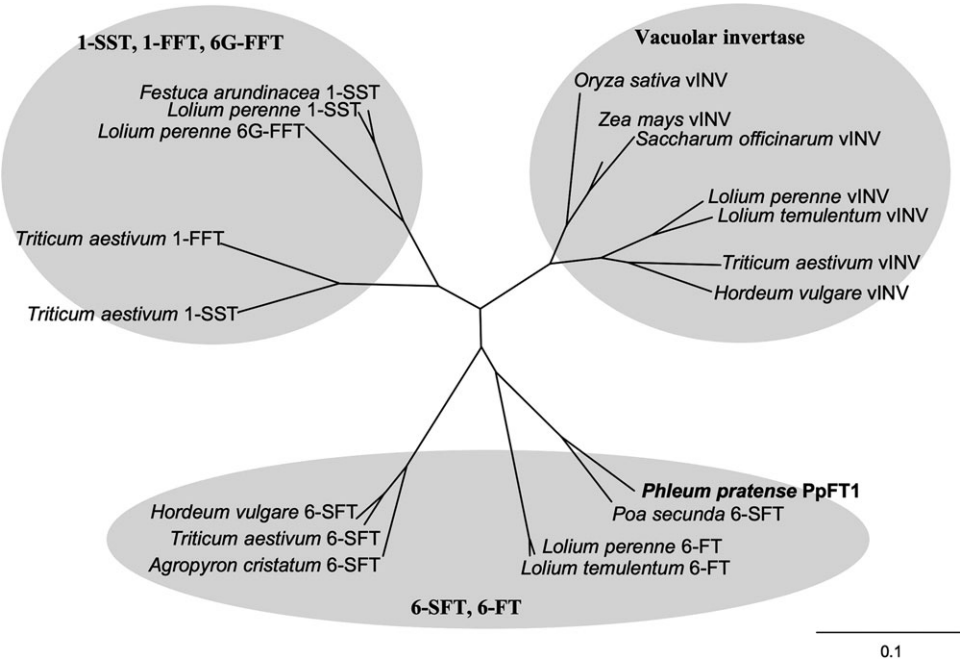

Fig. 2. Phylogenetic tree of FTs and vacuolar invertases (vINV) of Poaceae species based on predicted amino acid sequences. Scale bar indicates branch length. Genbank accession numbers of genes used in this analysis are: *Hordeum vulgare* 6-SFT, X83233; *Triticum aestivum* 6-SFT, AB029887; *Agropyron cristatum* 6-SFT, AF211253; *Lolium perenne* 6-FT, AF494041; *Lolium temulentum* 6-FT, AJ532550; *Poa secunda* 6-SFT, AF192394; *Triticum aestivum* 1-SST, AB029888; *Lolium perenne* 1-SST, AY245431; *Festuca arundinacea* 1-SST, AJ297369; *Triticum aestivum* 1-FFT, AB088409; *Lolium perenne* 6G-FFT, AF492836; *Hordeum vulgare* vINV, AJ623275; *Triticum aestivum* vINV, AJ635225; *Lolium perenne* vINV, AY082350; *Lolium temulentum* vINV, AJ532549; *Saccharum officinarum* vINV, AY302083; *Zea mays* vINV, AJ563384; *Oryza sativa* vINV, AF276704.

with increasing DPs were produced at longer reaction periods up to 96 h (Fig. 3A). Fresh enzyme and sucrose was added to the reaction solution at 96 h and a further increase in fructans was obtained with an estimated DP of more than 40 (Fig. 3A). However, high levels of 1-kestotriose and fructose were also produced from sucrose by the PpFT1 recombinant enzyme (Fig. 3A, B). This indicates that the PpFT1 recombinant enzyme has 1-SST and invertase activities in addition to 6-SST and 6-SFT activities. Fructan polymerization activity was not detected when the sucrose was replaced by 6-kestotriose, 1-kestotriose, 1,1-nystose, or 1,1,1-kestopentaose.

Comparison of enzymatic properties of PpFT1 and wheat 6-SFT recombinant proteins

The enzymatic function of 6-SFT cDNAs isolated from wheat and barley have been characterized; recombinant enzymes from these species have similar enzymatic properties (Sprenger *et al.*, 1995; Kawakami and Yoshida, 2002). The enzymatic properties of PpFT1 and wheat 6-SFT (Wft1) (Kawakami and Yoshida, 2002), were compared here by determining the composition of the reaction products from sucrose under identical experimental conditions (Fig. 3B). PpFT1 produced fructans with a DP estimated at more than 30, whereas Wft1 produced fructan oligomers with a DP lower than 10 (Fig. 3B). The addition of Wft1 and sucrose to the levan oligomers in the PpFT1 reaction mixture at 96 h did not increase the DP of fructans. The FT activities of PpFT1 and Wft1 were also compared when they were incubated with sucrose as a sole substrate and with a combination of sucrose and high-DP fructans extracted from timothy as substrates. Addition of high-DP fructans resulted in a remarkable increase in FT activity of PpFT1, but FT activity of Wft1 was hardly affected (Table 1).

The products generated from a sucrose substrate by both PpFT1 and Wft1 contained branched β(2,6)-linked fructans, such as bifurcose (DP4), that resulted from the polymerization of fructose units with 1-kestotriose, in addition to linear β(2,6)-linked fructans that were produced by polymerization of fructose units based on 6-kestotriose (Fig. 3B). Therefore, the substrate specificities of PpFT1 and Wft1 were compared for 1-kestotriose and 6-kestotriose. Both enzymes showed no FT activity with 100 mM 1-kestotriose or 6-kestotriose as the only substrate, and with 25 mM sucrose they produced only small amounts of 1-kestotriose and 6-kestotriose and showed prominent invertase activity (data not shown). When incubated with a combination of 25 mM sucrose and 100 mM of either 1-kestotriose or 6-kestotriose, the two enzymes showed different enzymatic properties (Fig. 4). PpFT1 produced few oligo-fructans with a DP of more than 3 from a substrate of 25 mM sucrose and 100 mM 1-kestotriose, while Wft1 produced bifurcose (Fig. 4A). In contrast, PpFT1 catalysed production of 6,6-kestotetraose (DP4) and other

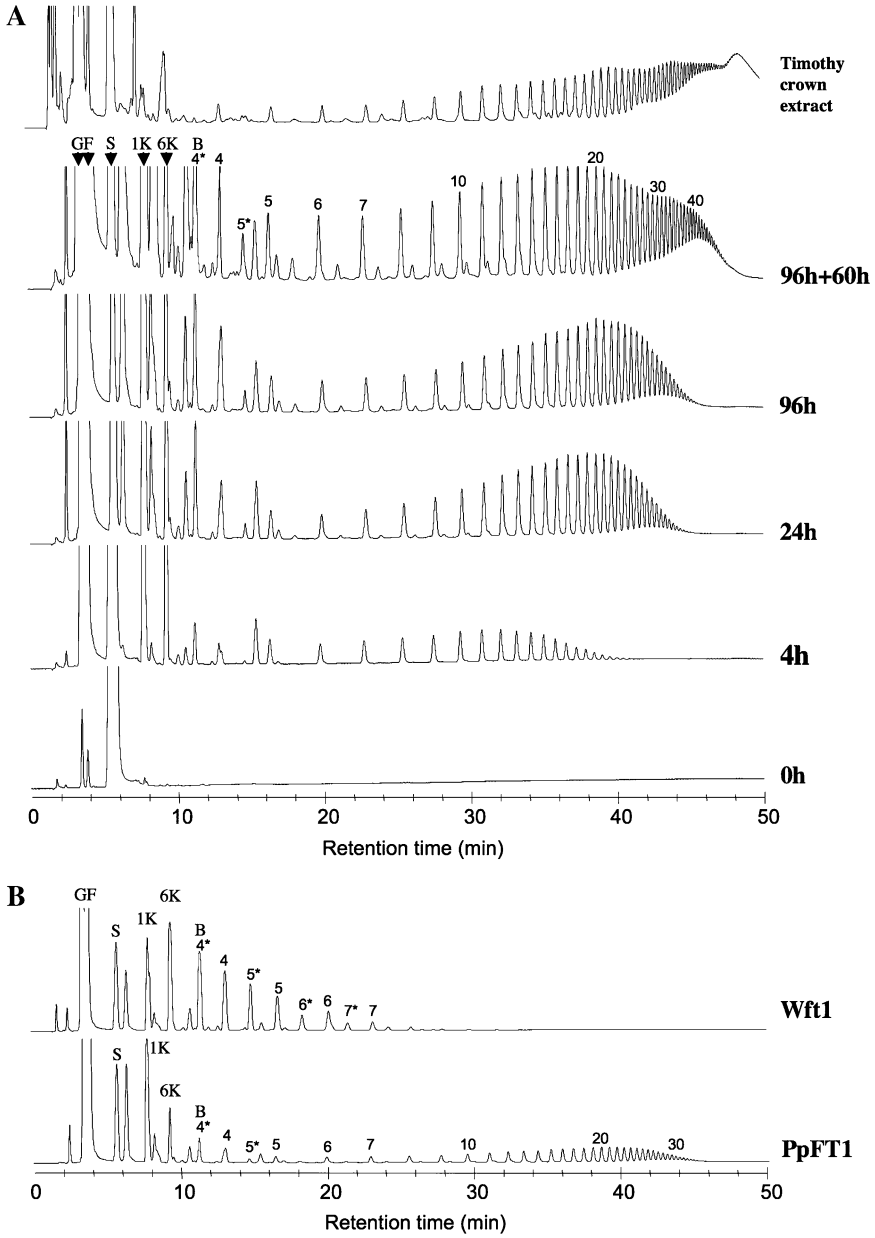

Fig. 3. Anion exchange HPLC analysis of fructans generated by the recombinant enzyme in the presence of sucrose. The enzymatic reactions with PpFT1 were performed with 1 M sucrose at 25 °C for 0, 4, 24, 96, and 96+60 h (A). Fresh enzyme and 0.5 M sucrose were provided to the reaction solution at 96 h for the further 60 h incubation. The comparison of the fructans generated by recombinant PpFT1 and Wft1 proteins in the presence of sucrose at 25 °C for 96 h is shown in (B) [the detection intensity scales differ between chromatograms (A) and (B)]. Compounds were identified using pulsed amperometric detection and external standards. Soluble carbohydrates extracted from timothy crown tissues in November 2006 were used as standards for the oligo-fructans. Abbreviations for each sugar peak are: G, glucose; F, fructose; S, sucrose; 1K, 1-kestotriose; 6K, 6-kestotriose; B, bifurcose (1&6-kestotetraose). The numbers indicate the putative DPs of β(2,6)-linked linear fructan oligomers and the numbers with asterisks indicate the putative DPs of β(2,6)-linked branched fructan oligomers produced by polymerization of fructose units linked to 1-kestotriose.

oligomers with a higher DP from a substrate of 25 mM sucrose and 100 mM 6-kestotriose, whereas Wft1 produced only a slight increase in 6,6-kestotetraose (Fig. 4B). Thus the two enzymes have remarkably different substrate specificities: PpFT1 showed a high affinity for 6-kestotriose to produce linear β(2,6)-linked fructans (levans) and a low affinity for 1-kestotriose; Wft1 preferentially used 1-kestotriose as an acceptor to make bifurcose.

Finally, the kinetic properties of PpFT1 and Wft1 were compared for FT activity with sucrose. The FT activity of PpFT1 increased with an increase in sucrose concentration, and the enzyme showed no clear indication that it could be saturated by its substrate (Fig. 5). By contrast, the FT activity of Wft1 was saturated at concentrations of sucrose above 1.5 M. This suggests that the affinity of the PpFT1 recombinant enzyme for sucrose as a substrate is lower than

that of the Wft1 recombinant enzyme. Since fructans with DP >3 were produced at very low levels by both enzymes after a short incubation (Fig. 6), FT activity was approximately equal to the sum of 6-SST and 1-SST activities. The 6-SST/1-SST ratios of PpFT1 and Wft1 with 1 M sucrose were 0.23 and 1.12, respectively. The 6-SST/1-SST ratio of the crude enzyme extract of timothy shoots grown at 22 °C was 0.23 with 1 M sucrose (Fig. 6).

Analysis of PpFT1 gene expression, enzyme activity, and carbohydrate content in timothy seedlings under high sucrose or cold conditions

To investigate the relationship between the level of expression of the *PpFT1* gene and the accumulation of fructans in timothy, *PpFT1* transcript levels, enzymatic activity, and sugar content were assayed in timothy seedlings maintained under high sucrose or cold conditions. The level of expression of *PpFT1* and the fructan content of seedlings were very low prior to soaking the seedlings in a 0.5 M sucrose solution or in deionized water (control) (Fig. 7A, C). Seedlings that have been maintained in a high sucrose solution show an increased sucrose content in their leaves. In these seedlings, the level of *PpFT1* expression began to increase gradually after 3 h in the high sucrose solution; fructans began to accumulate at 8 h after the initiation of treatment (Fig. 7A, C). The level of 6-SST activity fell temporarily at 3 h after the start of sucrose treatment, but then increased and at 24 h was nearly double that before the sucrose treatment (Fig. 7B). In the control experiment, there was no induction of *PpFT1* expression or accumulation of fructans, and 6-SST activity decreased (Fig. 7A, B, C). Under cold conditions, the level of *PpFT1* transcript gradually increased, and 6-SST activity and accumulation of fructans also increased after 3 d and 7 d, respectively, following the increase in sucrose content (Fig. 7D, E, F). These results indicate that expression of *PpFT1* was directly or indirectly induced by sucrose or cold treatment, and that 6-SST activity and fructan content increased following the induction of *PpFT1* transcription in timothy seedlings.

Table 1. Comparison of FT activities of recombinant PpFT1 and Wft1 incubated with sucrose and incubated with sucrose and high-DP fructans

FT activities were determined by the amount of glucose minus fructose after incubation for 10 min using 100 mM sucrose and 8.8 mM high-DP fructans with putative mean of DP=38 as substrates. The recombinant enzyme solutions of PpFT1 and Wft1 were adjusted to show similar FT activities with 100 mM sucrose.

	FT activity (nmol μl^{-1} enzyme solution min^{-1})		b/a
	Sucrose (a)	Sucrose and high-DP fructans (b)	
PpFT1	0.24±0.05	0.36±0.07	1.52
Wft1	0.23±0.01	0.23±0.03	1.02

Discussion

Sequence relationships and substrate specificities of 6-SFT related enzymes, and structures of their fructans in different species

The nucleotide acid sequence of *PpFT1* showed high similarity to 6-SFTs or putative 6-FTs of other Poaceae plants (Fig. 1). Our phylogenetic analysis indicated that Poaceae 6-SFTs and 6-FTs fell into three groups: the Triticeae group, the *Lolium* spp group, and the timothy and *Poa secunda* group (Fig. 2). These three plant groups accumulate fructans with different structural characteristics: the Triticeae group produces a branched type of fructan called graminan (Carpita *et al.*, 1989; Bonnett *et al.*, 1997); the *Lolium* spp group mainly produces a levan neo-series (Chalmers *et al.*, 2005); and the timothy and *P. secunda* group produces linear β(2,6)-linked fructans with a high DP (Chatterton and Harrison, 1997; Cairns *et al.*, 1999). As part of this study, the enzymatic properties of PpFT1 and wheat Wft1 recombinant proteins, which are assigned to different 6-SFT groups, were compared. In the presence of sucrose, PpFT1 mainly synthesized

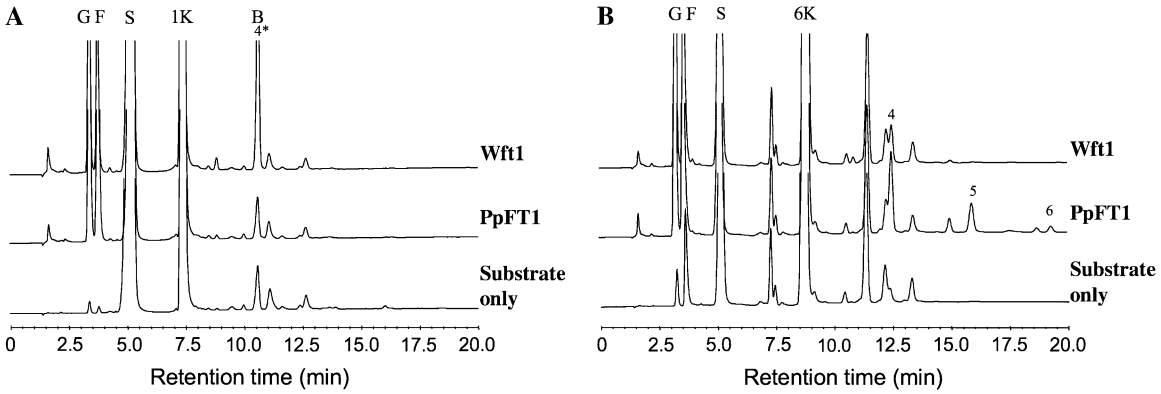

Fig. 4. Anion exchange HPLC analysis of oligo-fructans generated by recombinant enzymes with sucrose and 1-kestotriose or 6-kestotriose. PpFT1 or Wft1 were placed with 25 mM sucrose and 100 mM 1-kestotriose (A) or 6-kestotriose (B) at 25 °C for 3 h. The resulting products of enzymatic activity were identified by pulsed amperometric detection and external standards. Abbreviations and numbers for each peak are as described in Fig. 3.

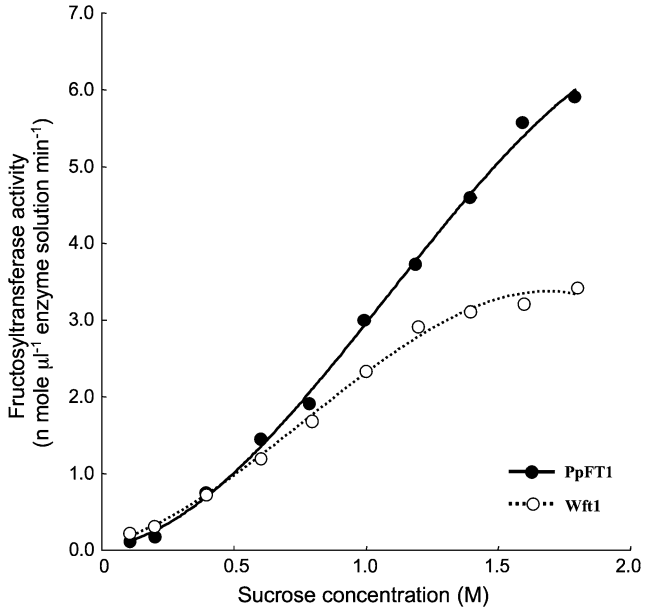

Fig. 5. Comparison of initial rates of FT activities at different sucrose concentrations, using PpFT1 or Wft1 recombinant enzyme solutions. Free glucose separated from the fructose generated by the reaction with sucrose at 25 °C and pH 5.2, was measured as FT activity. Each point is a rate calculated by linear regression (n=4).

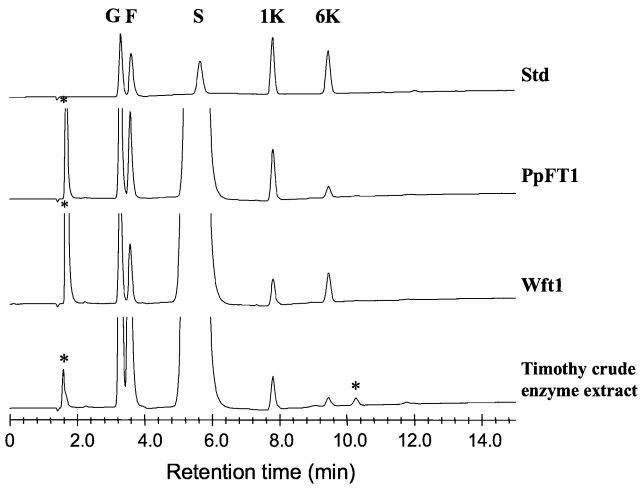

Fig. 6. Comparison of HPAEC-PAD chromatograms of tri-oligo fructans generated by the recombinant enzymes and crude enzyme extract from timothy shoots in the presence of sucrose. The recombinant PpFT1 or Wft1 was incubated with 1 M sucrose at 25 °C for 10 min. Crude enzyme extract from timothy shoots grown at 22 °C was incubated with 1M sucrose for 3 hours at 37 °C. Chromatograms of glucose (G), fructose (F), sucrose (S), 1-kestotriose (1K) and 6-kestotriose (6K) with similar molarity were shown as external standard (Std). Asterisks indicate unknown peaks.

a β(2,6)-linked, linear fructan series (levans) similar to the native fructans present in the crown tissues of timothy plants (Fig. 3). In wheat and barley, 6-SFT preferentially transfers fructosyl units to 1-kestotriose, produced by 1-SST activity, to synthesize bifurcose (Duchateau *et al.*, 1995; Sprenger *et al.*, 1995; Kawakami and Yoshida, 2002). In this study, it has been shown that PpFT1 preferentially transferred fructosyl units to 6-kestotriose, rather than to 1-kestotriose, to synthesize linear β(2,6)-linked fructans (Fig. 4B). Thus, PpFT1 acted quite differently from the Triticeae 6-SFT. This is the first report to identify a cDNA coding a plant FT that mainly produces linear levans.

Further studies will be required to clarify the relationship between 6-SFT genes, or their proteins, and the structures of the fructans they produce. One possible approach will be to perform a functional characterization of 6-SFT or 6-FT cDNAs from *Lolium* spp, *P. secunda*, and other species. Chimeras and point-mutated recombinant enzymes have been used experimentally to identify motifs and amino acids that determine substrate specificity in a number of plant FTs and fructan exohydrolases (Altenbach *et al.*, 2004, 2005; Ritsema *et al.*, 2005; Le Roy *et al.*, 2007). Future work will adopt these approaches to identify motifs or amino acids in 6-SFTs responsible for their substrate specificities.

Comparison of enzymatic properties of PpFT1 recombinant enzyme with those of previously purified enzymes

The fructan polymerase partially purified from timothy leaves by Cairns *et al.* (1999) shares some enzymatic properties with the recombinant PpFT1 isolated here: (i) they mainly produce linear β(2,6)-linked fructans with a high DP (up to 30 by one enzymatic reaction); (ii) they show low affinity to sucrose (their FT activities are not saturated by 1.5 M sucrose; and (iii) they have 1-SST and invertase activities in addition to 6-SFT activity. Both enzymes also have a similar optimal pHs: 5.2 for PpFT1 (data not shown) and 5.5 for purified fructan polymerase (Cairns *et al.*, 1999). The similarities in enzymatic properties suggest that *PpFT1* encodes a protein similar to the partially purified enzyme reported by Cairns *et al.* (1999). However, Suzuki and Pollock (1986) reported contrasting results from their study on fructan polymerization activity in timothy. Phlein sucrase characterized from timothy seedlings has optimal activity at pH 7.0, has a K_m for sucrose of 0.15 M, and produces high DP fructans without significant accumulation of low DP (Suzuki and Pollock, 1986). These characteristics are strikingly different from those of the PpFT1 recombinant enzyme and of the fructan polymerase.

Substrate affinity of PpFT1 toward high DP fructans

HPAEC-PAD analysis using sucrose as the substrate showed that the DP of fructans formed by the PpFT1 recombinant protein was higher than that of fructans synthesized by the Wft1 recombinant protein. With additional sucrose, PpFT1 could increase the DP of levans produced by itself, which could not be utilized by Wft1 as acceptors of fructose units. Moreover, PpFT1 showed an increase in FT activity with sucrose by the addition of high-DP fructans containing mainly levans, in contrast to Wft1 (Table 1). While Wft1 predominantly synthesized bifurcose

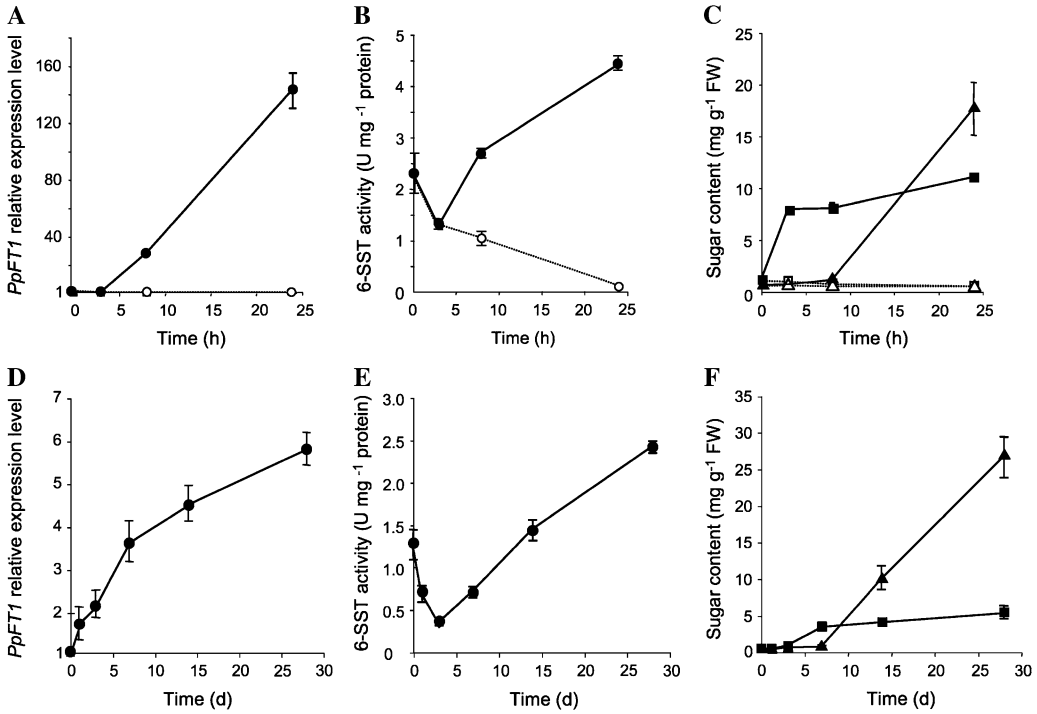

Fig. 7. PpFT1 mRNA expression levels, 6-SST activities, and carbohydrate contents of timothy seedlings under high sucrose (A, B, C) or cold conditions (D, E, F). (A, B, C) The closed symbols indicate values in a 0.5 M sucrose solution and the open symbols indicate values in deionized water (negative control). Expression levels of *PpFT1* relative to those of the α-tubulin gene were measured by quantitative real-time RT-PCR (A, D). Generation of 6-kestotriose by crude enzyme extracts incubated with sucrose was measured as 6-SST activities (B, E). The amounts of sucrose (squares) and fructans (triangles) were measured by HPLC (C, F). Results are given as the mean ±SE; n=3.

(DP4) from a combination of sucrose and 1-kestotriose, PpFT1 synthesized 6,6-kestotetraose (DP4), 6,6,6-kestopentaose (DP5) and 6,6,6,6-kestohexaose (DP6) under similar conditions except for substitution of 1-kestotriose by 6-kestotriose (Fig. 4). These results indicates that PpFT1 has a higher affinity than Wft1 for fructans with a high DP as acceptors, and that this property is independent of the affinity of both enzymes to the linkage form of tri-oligo fructans. Furthermore, the lower affinity of PpFT1 than Wft1 for sucrose (Fig. 5) might be responsible for the differences in the DPs of fructans that these enzymes synthesize from sucrose.

Does 1-SST activity exist in native PpFT1?

PpFT1 recombinant enzyme also produced 1-kestotriose from sucrose (Fig. 3). The 1-SST activity was about five times higher than 6-SST activity when the protein was incubated with sucrose for short periods. Hochstrasser *et al.* (1998) reported that the recombinant barley 6-SFT enzyme showed intrinsic 1-SST activity that was quite low compared with the native enzyme (Duchateau *et al.*, 1995). The additional 1-SST activity of PpFT1 might be attributed to the *Pichia* nature of the enzyme. However, for the following reasons, the possibility could not be excluded that the PpFT1 enzyme has a dual specificity, namely 6-SF(S)T/1-SST activity that is different from barley 6-SFT: (i) the 6-SST/1-SST ratio of the PpFT1 recombinant enzyme was much lower than previously cloned 6-SFTs and was one-fifth to one-sixth that of Wft1 and barley 6-SFT (1.3 with 100 mM sucrose, Hochstrasser *et al.*, 1998); (ii) the partially purified enzyme from timothy that was able to polymerize β(2,6)-linked fructans, showed 1-SST activity comparable with 6-SST activity (Cairns *et al.*, 1999). (iii) The crude enzyme extract from timothy shoots grown under normal conditions showed a 6-SST/1-SST ratio similar to that of PpFT1 (Fig. 6), although the possibility of involvement of other enzymes in the crude enzyme extract could not be excluded. Further investigations, including purification and characterization of the native enzyme, are needed for an accurate characterization of the 1-SST activity of the PpFT1 enzyme.

Roles of PpFT1 in fructan synthesis in timothy

In wheat, several FTs are known to be involved in fructan synthesis, in particular, during cold acclimation (Kawakami and Yoshida, 2002, 2005). Currently, it is unclear how many types of enzyme are required for fructan synthesis in timothy. PpFT1 recombinant enzyme synthesized β(2,6)-linked fructans with a DP lower than native fructans in the limited time-span of the enzymatic reactions used here (Fig. 3A), although accurate determinations of the DPs of the fructans were not made in this study. However, addition of more enzyme and sucrose to the reaction solution after 96 h resulted in an increase in the DP of the synthesized fructans

(Fig. 3A). It is concluded that PpFT1 by itself can synthesize fructans with a DP up to that of native timothy fructans providing that there is a continual supply of fresh enzyme and sucrose. Nevertheless, the possible involvement of other genes coding for FTs with fructan biosynthesis activity in timothy cannot be excluded, because other cDNAs with high similarity to PpFT1 were cloned from the same cDNA library (data not shown). The PpFT1 recombinant enzyme produced 1-kestotriose and branched type fructans which are minor components of native fructans in timothy (Fig. 3). This suggests the involvement of fructan exohydrolases in fructan synthesis in this species although the 1-SST activity of the recombinant enzyme might be attributed to its *Pichia* nature, as described before.

Induction of PpFT1 transcription and fructan synthesis in timothy

In general, fructans accumulate in grass leaf tissues when the carbon supply exceeds demand, such as in leaves under illumination or in excised leaves supplied with sugars (Wagner and Wiemken, 1989). During cold hardening in the autumn, grasses accumulate fructans (Livingston, 1991; Pollock and Cairns, 1991) that are associated with winter hardiness (Yoshida *et al.*, 1998). Under cold conditions, transcription of 6-SFT or 6-FT (homologue) genes is induced in some grasses and this is followed by the accumulation of fructans (Sprenger *et al.*, 1995; Wei *et al.*, 2002; Kawakami and Yoshida, 2002; Hisano *et al.*, 2008). It is confirmed here that *PpFT1* transcription was readily induced under high sucrose or cold conditions, and that this was associated with increased 6-SST activity and the accumulation of fructans (Fig. 7). This suggests that *PpFT1* is correlated with fructan biosynthesis in timothy. In barley, 6-SFT is transcriptionally induced by disaccharides such as sucrose (Müller *et al.*, 2000). In general, sucrose accumulates in grasses under cold conditions (Bhowmik *et al.*, 2006), a phenomenon also found in timothy seedlings in this study (Fig. 7F). Therefore, sucrose accumulation might be involved in the induction of *PpFT1* expression.

Physiological functions of high DP fructans in plants

The relationship between the DP of fructans and their physiological roles in plants, such as tolerance to environmental stresses, remains poorly understood. In an *in vitro* study on membrane stability using liposomes, Cacela and Hincha (2006) reported that the protection effect of fructans was dependent on DP in the range DP=2–5, and the protection effect increased with increasing DP of the β(2,1) linked fructans. However, in oat and rye, although fractions containing low DP fructans have a protective effect, fractions containing high DP fructans (DP >7) do not protect liposomes from drying (Hincha *et al.*, 2007). Tobacco and sugar beet plants expressing bacterial levansucrase produce levans with a much higher DP than those of native plant fructans and exhibit increased drought and freezing tolerance (Pilon-Smits *et al.*, 1995, 1999; Konstantinova *et al.*, 2002). This suggests that fructans with a high DP have direct or indirect protective effects against drought and freezing stresses. Temperate grasses accumulate fructans as energy reserves to survive under snow during winter (Moriyama *et al.*, 2003). Accumulation of large amounts of carbohydrates as high DP fructans synthesized by PpFT1 and other enzymes without an excessive increase in osmotic pressure might be involved in the superior winter hardiness of timothy. Transgenic approaches with genes coding FTs to produce fructans with different DPs, such as *PpFT1* and *wft1*, in similar genetic backgrounds would probably reveal more clearly the relationship between the DP of fructans and their physiological roles, and, further, elucidate the significance of the diversity of DPs in plant fructans.

Acknowledgements

We are grateful to Dr Masaru Iizuka, Kobe Shoin Women's University, Japan, for providing 6-kestotriose.

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