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Towards a better understanding of the generation of fructan structure diversity in plants: molecular and functional characterization of a sucrose:fructan 6-fructosyltransferase (6-SFT) cDNA from perennial ryegrass (*Lolium perenne*)

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Abstract

The main storage compounds in *Lolium perenne* are fructans with prevailing β(2–6) linkages. A cDNA library of *L. perenne* was screened using *Poa secunda* sucrose:fructan 6-fructosyltransferase (6-SFT) as a probe. A full-length *Lp6-SFT* clone was isolated as shown by heterologous expression in *Pichia pastoris*. High levels of *Lp6-SFT* transcription were found in the growth zone of elongating leaves and in mature leaf sheaths where fructans are synthesized. Upon fructan synthesis induction, *Lp6-SFT* transcription was high in mature leaf blades but with no concomitant accumulation of fructans. *In vitro* studies with the recombinant Lp6-SFT protein showed that both 1-kestotriose and 6G-kestotriose acted as fructosyl acceptors, producing 1- and 6-kestotetraose (bifurcose) and 6G,6-kestotetraose, respectively. Interestingly, bifurcose formation ceased and 6G,6-kestotetraose was formed instead, when recombinant fructan:fructan 6G-fructosyltransferase (6G-FFT) of *L. perenne* was introduced in the enzyme assay with sucrose and 1-kestotriose as substrates. The remarkable absence of bifurcose in *L. perenne* tissues might be explained by a higher affinity of 6G-FFT, as compared with 6-SFT, for 1-kestotriose, which is the first fructan formed. Surprisingly, recombinant 6-SFT from *Hordeum vulgare*, a plant devoid of fructans with internal glucosyl residues, also produced 6G,6-kestotetraose from sucrose and 6G-kestotriose. In the presence of recombinant *L. perenne* 6G-FFT, it produced 6G,6-kestotetraose from 1-kestotriose and sucrose, like *L. perenne* 6-SFT. Thus, we demonstrate that the two 6-SFTs have close catalytic properties and that the distinct fructans formed in *L. perenne* and *H. vulgare* can be explained by the presence of 6G-FFT activity in *L. perenne* and its absence in *H. vulgare*.

Key words: Bifurcose, fructan, gene expression, heterologous expression, *Hordeum vulgare*, levan neoseries, *Lolium perenne*, *Pichia pastoris*, sucrose:fructan 6-fructosyltransferase.

Introduction

Perennial ryegrass (*Lolium perenne* L.) is the predominant forage grass in European agriculture, where it provides the major supply of nutrients for grazing sheep and cattle. The primary source of readily available energy in this forage is water-soluble carbohydrates (WSC) composed of glucose, fructose, sucrose and fructans (fructosyl polymers) (Smith *et al.*, 1998). Perennial ryegrass accumulates large amounts of fructans in the tiller base comprising leaf sheaths and elongating leaf bases (Marx *et al.*, 1997; Morvan-Bertrand *et al.*, 2001). Apart from their role as storage carbohydrates used for leaf regrowth (Yamamoto and Mino, 1989; Morvan-Bertrand *et al.*, 2001) or for early spring growth (Pollock and Jones, 1979), fructans are believed to confer cold and drought tolerance (Hendry and Wallace, 1993; Pilon-Smits *et al.*, 1995; De Roover *et al.*, 2000; Amiard *et al.*, 2003; Hisano *et al.*, 2008; Kawakami *et al.*, 2008), possibly by protecting membranes under such stressful conditions (Demel *et al.*, 1998; Livingston and Henson, 1998; Hincha *et al.*, 2000; Vereyken *et al.*, 2003). For humans, fructans are one of the most promising ingredients for functional foods since they act as prebiotics (Rao, 1999) and have favourable effects in the prevention of cardiovascular diseases, colon cancer, and osteoporosis (Kaur and Gupta, 2002). Furthermore, the chemical and pharmaceutical industries have a growing interest in exploiting the benefits of fructans (Roberfroid, 1999; Ritsema and Smeekens, 2003). Potential applications include encapsulation for the controlled release of drugs (Poulain *et al.*, 2003), emulsifiers in cosmetics, and additives in the textile and paper industry (Stevens *et al.*, 2001).

Fructans are sucrose-derived fructose polymers found in ~15% of flowering plant species (Hendry and Wallace, 1993). Fructans from different sources exhibit different degrees of polymerization (DP) and different linkages between adjacent fructose residues. In *L. perenne*, fructans of DP≤8 have been well characterized and belong essentially to three series: (i) the inulin series with a terminal glucose residue and β(2–1)-linked fructose residues, (ii) the inulin neoseries with an internal glucose residue and β(2–1)-linked fructose residues, and (iii) the levan neoseries with an internal glucose residue and β(2–6)-linked fructose residues. High DP fructans (DP>8) comprised 75% of the fructan molecules with an internal glucose residue and there were 70 times more β(2–6)-linked fructose residues than β(2–1)-linked ones (Pavis *et al.*, 2001a). Based on the *L. perenne* fructan profile and on known properties of fructosyltransferases (FTs) involved in fructan synthesis in plants, it has been proposed that at least four enzyme activities are required to produce the complement of fructans in this species: a sucrose:sucrose 1-fructosyltransferase (1-SST), a fructan:fructan 1-fructosyltransferase (1-FFT), a fructan:fructan 6G-fructosyltransferase (6G-FFT), and a 6-fructosyltransferase (6-FT) (Pavis *et al.*, 2001b). The cloning of two cDNAs in perennial ryegrass corresponding to FTs and the study of their corresponding enzymatic properties have allowed a better definition of the fructan synthesis model occurring in this species. A 1-SST catalyses the initial step producing 1-kestotriose from two molecules of sucrose (Chalmers *et al.*, 2003). In the second step, a 6G-FFT produces 6G-kestotriose, using 1-kestotriose as fructosyl donor and sucrose as fructosyl acceptor (Lasseur *et al.*, 2006). In further steps, higher polymeric fructans would be synthesized by a 1-FFT activity through β(2–1)-linked chain elongation or by a 6-FT activity through β(2–6)-linked chain elongation. It has been demonstrated recently that the 6G-FFT isolated from *L. perenne* also had 1-FFT activity so that there might be no need for a separate 1-FFT protein (Lasseur *et al.*, 2006). The only known protein that synthesizes β(2–6) linkages between two fructosyl residues in plants is the 6-SFT enzyme described in barley (Duchateau *et al.*, 1995; Sprenger *et al.*, 1995) and in wheat (Kawakami and Yoshida, 2002). In both these species, which are devoid of fructans with an internal glucosyl residue, the main product of 6-SFT is 1 and 6-kestotetraose (bifurcose), a DP4 branched fructan. In *Lolium* species, however, because of the notable absence of bifurcose, the presence of another transferase, a fructan:fructan 6-fructosyltransferase (6-FFT), was postulated (Pavis *et al.*, 2001a, b).

The aims of this study were (i) to identify the enzyme responsible for the biosynthesis of β(2–6) linkages in *L. perenne* fructans, (ii) to assess its enzymatic properties, and (iii) to study its regulation at the transcriptional level. To this purpose, a cDNA clone encoding 6-SFT from *L. perenne* stubble, composed of elongating leaf bases and mature leaf sheaths, was isolated and characterized by heterologous expression in *Pichia pastoris*. The enzymatic properties of the recombinant protein were examined and compared with the properties of recombinant barley 6-SFT, an enzyme that has been studied extensively in the past (Duchateau *et al.*, 1995; Sprenger *et al.*, 1995; Hochstrasser *et al.*, 1998). Finally, expression of the *Lp6-SFT* gene was studied in leaf tissues of *L. perenne* leaves depending on developmental stage and carbohydrate status.

Materials and methods

Plant material

Seeds of *L. perenne* cv. Bravo were germinated in 9-l pots and grown hydroponically for 8 weeks on a nutrient solution as previously described by Prud'homme *et al.* (1992). The nutrient solution was aerated continuously and replaced every week. Plants were grown in a greenhouse with day/night temperatures of 22/18 °C and a photoperiod of 16 h of natural light supplemented by a photosynthetic photon flux density of 110 μmol photons m^{-2} s^{-1} (Phyto tubes, Claude, GTE, Puteaux, France).

After 8 weeks of growth, plants were harvested. Based on the presence of the ligule, mature leaves were separated from elongating leaves. Sheaths and elongating leaf bases previously enclosed by the sheaths were dissected longitudinally into five segments (four 10-mm-long segments, 0–40 mm from the leaf base, and a fifth variable length segment of ~40 mm). Blades and the emerged part of elongating leaves were divided into three equal parts (Fig. 8).

Synthesis of fructan was induced in the plants 8 weeks after sowing, according to the method used by Smouter and Simpson

(1991). Plants were maintained under continuous light with a photosynthetic photon flux density of 150 µmol photons m^{-2} s^{-1} for up to 72 h, with roots and shoot meristems in the nutrient solution cooled at 4 °C. Control swards were grown under the original plant growth conditions, with a daylength of 16 h.

Plants were divided into three parts: sheaths of mature leaves, blades of mature leaves together with emerged parts of elongating leaves, and elongating leaf bases.

Each batch of sampling was done in triplicate. One part of the harvested tissues was used immediately for enzyme extraction whereas the remainder was frozen, stored at –80 °C for RNA extraction, or freeze-dried for soluble carbohydrate extraction.

Preparation and screening of a cDNA library of L. perenne

Leaf sheaths and elongating leaf bases (1.5 g fresh weight) of 8-week-old plants treated to accumulate fructans (Smouter and Simpson, 1991) were ground in liquid nitrogen. The powder obtained was used to purify poly(A$^+$) RNA with Dynabeads oligo (dT)$_{25}$ kit (Dynal, France) following the manufacturer's recommendation. Double-stranded cDNA was synthesized from poly(A$^+$) RNA and a cDNA library was constructed using a Lambda-Zap cDNA library kit and the Gigapack III Gold Cloning Kit (Stratagene, France). The cDNA library was screened with a fragment of 621 bp of *Poa secunda 6-SFT* (Wei and Chatterton, 2000). This fragment was labelled with [α-^{32}P]dCTP using the random priming method with NEBlot kit (Biolabs, France). Membranes were hybridized overnight at 42 °C and washed twice in 2×SSC, 0.5% SDS for 15 min at room temperature, then rinsed twice in the same buffer at 56° C. After three rounds of purification, positive clones were excised and recircularized in a pBluescript vector (Stratagene, USA). Sequencing of positive clones was done by Genome Express (Meylan, France). Nucleotide sequences were compared with sequences available in the NCBI Databank.

Expression of isolated cDNA in P. pastoris

The putative coding region of mature Lp6-SFT [determined by sequence homology with the N- terminal sequence of the native 6-SFT of barley (Sprenger *et al.*, 1995)] was amplified by PCR with the primers PIC6SFT-F (5′-GTCCG**GAATTC**GCCGGAGGGT-TCCCGTGG AGCAAC-3′) and PIC6SFT-R (5′-GACGC**TCTA-GA**CTATGAGTCCTTAACCATGACGGT-3′). *Eco*RI and *Xba*I sites are indicated in bold in the primers. PCR was performed with *Pfu* Proofreading Polymerase (Promega, France). PCR conditions were: 1 min at 95 °C; 5 cycles of 30 s at 95 °C, 30 s at 56 °C, 4 min at 72 °C followed by 30 cycles of 30 s at 95 °C, 30 s at 68 °C, 4 min at 72 °C; final extension for 5 min at 72 °C. PCR products and pPICZαA (expression vector) were digested with restriction enzymes corresponding to restriction sites introduced by PCR and purified with Nucleospin Extract Kit (Macherey-Nagel, Germany). The digested vector was dephosphorylated with CIAP (Stratagene, USA), and then PCR products were cloned in-frame behind the α-factor signal of the pPICZαA vector. The plasmids were transformed into *Escherichia coli* competent cells as described (Van den Ende *et al.*, 2001). Cells were plated on 2×YT medium supplemented with zeocine (Invitrogen, The Netherlands) as a selection marker. Positive clones were used for vector amplification. *P. pastoris* wild-type strain X33 was transformed by electroporation with 20 µg of *Sac*I-linearized pPICZαA-*Lp6GFFT*. Transformants were selected on YPDS/Zeocine plates.

In order to produce recombinant Lp6-SFT enzyme for characterization, a 90-ml preculture medium (BMGY) was inoculated with a single colony and incubated overnight at 30 °C, 200 rpm. Cells were harvested by centrifugation (10 min, 1000 *g*), resuspended in 20 ml of induction medium (BMMY) and incubated for 4 d at 29 °C. Methanol was replenished every day to a final concentration of 2%. After incubation, the cells were harvested by centrifugation (10 min, 1000 *g*) and the pellet (containing the

recombinant enzyme) was used for enzyme assays (Ritsema *et al.*, 2006). Indeed, results obtained from the supernatant showed weak FT activity, suggesting that the major part of the enzyme was bound to the fungal cell wall.

To produce recombinant Hv6-SFT, *P. pastoris* strain X-33 transformed with barley 6-SFT cDNA was used (Hochstrasser *et al.*, 1998). Induction of protein expression was performed as described above, except that after incubation in BMMY, protein purification was carried out by following the protocol described by De Coninck *et al.* (2005).

Characterization of the P. pastoris expressed recombinant Lp6-SFT and Hv6-SFT

Sodium azide 0.02% (w/v) was added to all buffers to prevent microbial growth. Cell pellet aliquots (~25 µl) containing recombinant Lp6-SFT or cell supernatant aliquots (~25 µl) containing Hv6-SFT enzymes were incubated with substrates for different time intervals at 30 °C. All substrates were used at a final concentration of 200 mM. Reaction mixtures containing the recombinant Lp6-SFT or Hv6-SFT and different amount of the recombinant 6G-FFT/1-FFT from *L. perenne* (Lasseur *et al.*, 2006) were incubated for 3 h at 30 °C. Reactions were stopped by heating for 2 min at 95 °C.

Carbohydrates of the assay mixture were analysed by high-performance anion exchange chromatography and pulsed amperometric detection (HPAEC-PAD ICS-3000; Dionex, Sunnyvale, CA, USA) equipped with a CarboPac PA100 anion-exchange column (4×250 mm). The solutions used were: A (90 mM NaOH) and B (90 mM NaOH and 500 mM NaOAc). The running profile applied to the reaction mixture containing sucrose and 6G-kestotriose was: T=–4 min, 100% A; T=0 min (injection), 100% A; T=10 min, 98% A, 2% B; T=20 min, 80% A, 20% B; T=20.1 min, 100% B; T=24 min 100% B; T=24.1 min, 100% A; T=27 min, 100% A. The running profile applied to the other reaction mixtures was: T=0, 100% A; T=6 min, 98% A, 2% B; T=16 min, 80% A, 20% B; T=26 min, 65% A, 35% B; T=26.1 min, 100% B; T=31 min 100% B; T=31.1 min, 100% A; T=36 min, 100% A. Peaks were identified by comparison with known wheat carbohydrates and with a standard solution composed of commercial fructans (1-kestotriose, 1,1-kestotetraose; Megazyme, Ireland) and purified fructans (6-kestotriose, 6G,6-kestotetraose, 6G,1-kestotetraose; kind gifts of N. Shiomi, N.J. Chatterton and D.P. Livingston). 6G-Kestotriose was purified by HPLC Sugar-Pak from the supernatant of the yeast *Xanthophyllomyces dendrorhous* (Kritzinger *et al.*, 2003).

RNA isolation and RT-PCR analysis

Plant tissues were ground in liquid nitrogen and suspended in a pre-warmed (80 °C) solution consisting of 750 µl of phenol and 750 µl of extraction buffer [0.1 M LiCl, 100 mM Tris–HCl, 10 mM EDTA, 1% (w/v) SDS, pH 8.0]. After shaking, 750 µl of chloroform–isoamylalcohol (24:1) were added and the solution was centrifuged for 5 min (4 °C) at 20 000 *g*. Total RNA was precipitated with LiCl (final concentration 2 M) overnight at 4 °C. Following centrifugation for 30 min (4 °C) at 20 000 *g*, the pellet was suspended in 250 µl of water treated with diethylpyrocarbonate (0.1%, v/v) and 250 µl of phenol–chloroform–isoamylalcohol (25:24:1), mixed, and centrifuged for 5 min. RNA in the supernatant was precipitated again with 1 ml of absolute ethanol and 50 µl of Na-acetate buffer (3 M; pH 5.6) and stored for 1 h at –80 °C. After centrifugation for 20 min (4 °C) at 20 000 *g*, the pellet was resuspended in 100 µl of RNase-free water. Then, samples were treated following the Clean-up protocol of the RNeasy Minikit (Quiagen) coupled to a DNase treatment (RNase-free DNase; Qiagen, France).

One microgram was used for retrotranscription using the i-script cDNA synthesis kit (Bio-Rad, France). cDNA was then amplified by PCR using 5′-CAGCTTCTGCAACGACGA-3′ and

5′-CCTTAACCATGACGGTCTCG-3′ as specific primers for *Lp6-SFT*, 5′-CGGATAACCGTAGTAATTCTAG-3′ and 5′-GT-ACTCATTCC AATTACCAGAC-3′ as primers for 18S rRNA. Amplification was achieved in the following conditions so that for each cDNA, products were analysed during the exponential phase of the PCR curve: 5 min at 94 °C; 30 cycles (*Lp6-SFT*) or 13 cycles (18S rRNA) of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 1 min, and a final elongation at 72 °C for 7 min. PCR products were analysed by agarose gel electrophoresis. Three RNA extractions were performed corresponding to three biological repetitions. Each PCR was performed twice. Typical results are shown. The specificity of 6-SFT amplification was checked by sequencing the PCR product and by monitoring the dissociation curve on several samples in real time-PCR using the Chromo 4 System (Bio-Rad, France).

Results

Molecular characterization of L. perenne fructosyltransferase

A perennial ryegrass cDNA library, prepared from stubble of plants induced to accumulate fructan-related enzymes, was screened with a ^{32}P-labelled insert of the *P. secunda 6-SFT* (Wei and Chatterton, 2000). After repeated screenings, several positive clones were picked up. The longest cDNA was fully sequenced and consisted of 2359 bp, containing an open reading frame (ORF) of 1872 bp and a poly(A) sequence at its 3′ end. The ORF encoded a polypeptide of 623 amino acids (Lp6-SFT, Fig. 1) with seven potential N-glycosylation sites, a calculated pI of 5.32, and an estimated molecular weight of 60.9 kDa for the mature protein. The cDNA was first termed 'putative *6-FT*' (Lasseur *et al.*, 2002, accession no. AF494041) because of its high identity (70–72%) with the 6-SFT polypeptides of *P. secunda*, wheat, and barley (Fig. 1). It shares 100% identity with an FT cloned later in perennial ryegrass and called FTb (Fig. 2) (Gadegaard *et al.*, 2008). However, the functionality of the corresponding protein has not been checked by expressing the cDNA in a heterologous system.

The cDNA shows 97% identity at the amino acid level with a sequence isolated from *Lolium temulentum* identified as a 'putative fructan 6-fructosyltransferase' (Lt6-FT, Gallagher *et al.*, 2004) in reference to the present Lp6-SFT. It also shares 99% identity at the amino-acid level (621/623 amino acids) with a cDNA called 'putative fructosyltransferase 1' (*prft1*) described in Hisano *et al.* (2008) and called previously Lp1-FFT in Chalmers *et al.* (2005) (Fig. 1). The newly cloned cDNA shares 63% and 62% identity with the deduced amino acid sequence of *L. perenne* 1-SST (Chalmers *et al.*, 2003) and 6G-FFT/1-FFT (Lasseur *et al.*, 2006) as well as 64% and 66% identity with two other putative invertases or FTs of *L. perenne*, LpFT1 (Lidgett *et al.*, 2002), and LpFT4 (Chalmers *et al.*, 2005), respectively. LpFT1 harbours the WMNDPNG motif and then would correspond to an invertase according to the work realized by Ritsema *et al.* (2006) and Lasseur *et al.* (2009) whereas LpFT4 does not harbour this motif and then would be a fructosyltransferase.

When compared with other FTs and invertases, the newly cloned cDNA had greater identity with sequences from liliaceous (monocot) plants (51–56%) than with sequences for 1-SSTs, 1-FFTs, and invertases from asteraceous (dicot) plants (45–49%) (Fig. 2). Furthermore, the cDNA showed greater homology with vacuolar-type than with cell-wall-type invertases. It contains the SDPNG region and the conserved domains of Glycoside Hydrolase Family 32.

Expression of recombinant protein in P. pastoris: comparison with the recombinant 6-SFT of Hordeum vulgare

The cDNA was expressed in *P. pastoris* to investigate the enzymatic properties of the corresponding protein.

FT activity was first tested in the culture medium (supernatant) where the recombinant protein was expected to be produced (Hochstrasser *et al.*, 1998 and references therein). Incubation of the recombinant protein with only sucrose (200 mM) led to the synthesis of 6-kestotriose, product of a sucrose:sucrose 6-fructosyltransferase (6-SST) activity, and to the release of fructose, product of invertase activity (data not shown). However, fructan production was very low and not stable, so that FT was further assessed in the pellet containing the transformed yeast cells, where FT activity has been shown to be retained sometimes (Ritsema *et al.*, 2006). It has to be kept in mind, however, that this condition does not allow the complete release of glucose and fructose produced during fructan metabolism [SFT, fructan exohydrolase (FEH) activities] or sucrose degradation (invertase activity), in the incubation medium since hexoses can be used by the yeast cells.

Again, incubation of the recombinant protein with sucrose alone allowed the formation of 6-kestotriose (Fig. 3A). Therefore, sucrose acts as both a fructosyl donor and a fructosyl acceptor. 1-Kestotriose is not a fructosyl donor since incubation with 1-kestotriose (200 mM) alone (Fig. 3B) did not lead to the synthesis of a 6-linked fructan. Instead, 1,1-kestotetraose (nystose) was produced (1-FFT activity) together with sucrose and fructose [fructan 1-exohydrolase (1-FEH) activity]. Sucrose is not the only fructosyl acceptor. Indeed, incubation with sucrose and 1-kestotriose led to the synthesis of 1 and 6-kestotetraose (bifurcose), the first fructan of the mixed series found in wheat and barley (Fig. 3C). However, it has to be kept in mind that neither this fructan nor the subsequent mixed series is found *in vivo* in the tissues of *L. perenne* (Pavis *et al.*, 2001*a*, *b*). Instead, *L. perenne* plants produce a majority of levan neoseries built on the 6G-kestotriose backbone (Pavis *et al.*, 2001*a*, *b*). 6G-kestotriose could then represent an ideal acceptor of fructosyl residues. Indeed, incubation with both sucrose and 6G-kestotriose allowed the synthesis of 6G,6-kestotetraose (Fig. 3D). Consequently, the recombinant protein of *L. perenne* is able to synthesize at least two fructans with β(2–6) linkages [1 and 6-kestotetraose (bifurcose) and 6G,6-kestotetraose]. 1-Kestotriose and 6G-kestotriose represent two potential fructan acceptors of fructosyl residues for Lp6-SFT. Since

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Lp6-SFT   MESRAIP--SAAYAPLLPSAADDVALAKQDRPGVGWRGFLTVLAAGGVVVLLVGATLLAG  58
Lp1-FFT   MESRAIP--SAAYAPLLPSAADDVALAKQDRPGVGWRGFLTVLAAGGVVVLLVGATLLAG  58
Lt6-FT    MESRAIP--SAAYAALLPSAADDVALAKQDRPGVGWRGFLTVLAASGVVVLLVGATLLAG  58
Ps6-SFT   MDSRGIT--PGAYAPLPSSGDDQRGGGVR----YCLACVATVLAAS-AVVVVAFVSVGG   53
Hv6-SFT   MGSHGKPPLPYAYKPLPSDAADGKRTGCMR-----WSACATVLTASAMAVVVVGATLLAG  55
Ta6-SFT   MGSHGKPPLPYAYKPLPSD-ADGERAGCTR-----WRVCAVALTASAMVVVVGATLLAG   54
          * *:. . :. ** .* .. *   .       ..*:*.  .*::*. . :.*

Lp6-SFT   SRMGQAGDGEGNTDEDGAGGFPWSNEMLQWQRAGFHYQPEGHFMSDPNGPVYYRGYYHLF  118
Lp1-FFT   SRMGQAGDGEGNTDEDGAGGFPWSNEMLQWQRAGFHYQPEGHFMSDPNGPVYYRGYYHLF  118
Lt6-FT    SRMGQAGDGEGNTDEDGAGGFPWSNEMLQWQRAGFHYQPEGHFMSDPNGPVYYRGYYHLF  118
Ps6-SFT   VRVLLAG---GTGDSKDADAFPWSNSMLQWQRAGFHFQTEKNFMSDPNGPVYYRGYYHLF  110
Hv6-SFT   LRMEQAVD-----EEAAAGGFPWSNEMLQWQRSGYHFQTAKNYMSDPNGLMYYRGWYHMF  110
Ta6-SFT   FRVDQAVD-----EEAAGG-FPWSNEMLQWQRSGYHFQTAKNYMSDPNGLMYYRGWYHMF  108
          *: *      :. .. ** .***** .****** :*:*:*. .:*****  :****:**:*

Lp6-SFT   FQYNRRGVAWDDYIEWGHVVSQDLVHWRPLPLAMRPDHWYDKKGVLSGTITVLHNGTLVL  178
Lp1-FFT   FQYNRRGVAWDDYIEWGHVVSQDLVHWRPLPLAMRPDHWYDKKGVLSGTITVLHNGTLVL  178
Lt6-FT    FQYNRRGVAWDDYIEWGHVVSQDLVHWRPLPLAMRPDHWYDKKGVLSGTITVLHNGTLVL  178
Ps6-SFT   YQYNTKGVVWDDGIVWGHVVSRDLVHWRHLPIAMVPDHWYDNMGVLSGSITVLNSGRLVM  170
Hv6-SFT   YQYNPVGTDWDDGMEWGHAVSRNLVQWRTLPIAMVADQWYDILGVLSGSMTVLPNGTVIM  170
Ta6-SFT   FQYNPVGTDWDDGMEWGHAVSRNLVQWRTLPIAMVADQWYDILGVLSGSMTVLPNGTVIM  168
          :*** *. *** : ***.**:.**:** **:** .*:*** ***** :*** .* ::;

Lp6-SFT   LYTGVTEDP-----MAESQCIAVPTDPNDPLLRHWTKHPANPVLAHPQGVQGMDFRDFTS  233
Lp1-FFT   LYTGVTEDP-----MAESQCIAVPTDPNDPLLRHWTKHPANPVLAHPQGVQGMDFRDFTS  233
Lt6-FT    LYTGVTEDP-----MAESQCIAVPTDPNDPLLRHWTKHPANPVLAHPQGVQGMDFRDFTS  233
Ps6-SFT   IYTGVFSNTTDRSGMMEVQCLAVPADPNDPLLRHWTKHPANPVLVHPPGIKDMDFRDFTT  230
Hv6-SFT   IYTGATNAS-----AVEVQCIATPADPNDPLLRRWTKHPANPVIWSPPGVGTKDFRDFMT  225
Ta6-SFT   IYTGATNAS-----AVEVQCIATPADPTDPLLRRWTKHPANPVIWSPPGVGTKDFRDFMT  223
          :***. .     * **:*.*::*.***** :********.:.   * *:  *****  :

Lp6-SFT   AWWDKSDSTWRILIGSKDDDNGSHAGIAFIFKTKDFLSFERVPGIVHRVEGTGMWECIDF  293
Lp1-FFT   AWWDKSDSTWRILIGSKDDDNGSHAGIAFIFKTKDFLSFERVPGIVHRVEGTGMWECIDF  293
Lt6-FT    AWWDKSDATWRILIGSKDDDNGSHAGIAFIFKTKDFLSFERVPGIVHRVEGTGMWECIDF  293
Ps6-SFT   AWFDESDSTYRTVIGSKDDNQGDHAGFAMVYKTKDFLSFQRIPGILHRVEHTGMWECMDF  290
Hv6-SFT   AWYDESDETWRTLLGSKDDHDGHHDGIAMMYKTKDFLNYELIPGILHRVVRTGEWECIDF  285
Ta6-SFT   AWYDESDDTWRTLLGSKDDNNGHHDGIAMMYKTKDFLNYELIPGILHRVERTGEWECIDF  283
          **:*:** *:* ::*****.:* * *:*:::****** .:: :***:*** ** ****:**

Lp6-SFT   YPVGGGHNSSS-EELYVIKASMDDERHDYYSLGRYDAAANTWTPLDAELDLGIGLRYDWG  352
Lp1-FFT   YPVGGGHNSSS-EELYVIKASMDDERHDYYSLGRYDAAANTWTPLDAELDLGIGLRYDWG  352
Lt6-FT    YPVGGGHSSSSSEELYVIKASMDDERHDYYSLGRYDAAANTWTPLDAELDLGIGLRYDWG  353
Ps6-SFT   YPVGGGDNSSS-EVLYVIKASMNDERHDYYALGRYDAAGNTWTPLDPELDLGMGLRYDWG  349
Hv6-SFT   YPVGRRSSDNSSEMLHVLKASMDDERHDYYSLGTYDSAANTWTPIDPELDLGIGLRYDWG  345
Ta6-SFT   YPVGRRTSDNSSEMLHVLKASMDDERHDYYSLGTYDSAANRWTPIDPELDLGIGLRYDWG  343
          ****  ...* .* *:*:****:*******:** **:*.* ***:*.*****:*******

Lp6-SFT   KLYASTSFYDPLKQRRIMLGYVGEVDSARADVAKGWASLQSIPRTVALDEKTRTNLLLWP  412
Lp1-FFT   KLYASTSFYDPLKQRRIMLGYVGEVDSARADVAKGWASLQSIPRTVALDEKTRTNLLLWP  412
Lt6-FT    KLYASTSFYDPLKQRRIMLGYVGEVDSARADVAKGWASLQSIPRTVALDEKTRTNLLLWP  413
Ps6-SFT   KFYASTSFYDPIKRRRVVLGYVGETDSRRSDEAKGWASIQSIPRTVALDKKTWTNLLLWP  409
Hv6-SFT   KFYASTSFYDPAKNRRVLMGYVGEVDSKRADVVKGWASIQSVPRTVALDEKTRTNLLLWP  405
Ta6-SFT   KFYASTSFYDPAKKRRVLMGYVGEVDSKRADVVKGWASIQSVPRTIALDEKTRTNLLLWP  403
          *:********* *.**::.*****.** *:* .*****:**:***:***:** *******

Lp6-SFT   VEEVEALRYNSTDLSGITVENGSIFHLPLHQATQLDIEASFRLDASDVAAINEADVGYNC  472
Lp1-FFT   VEEVEALRYNSTDLSGITVENGSIFHLPLHQATQLDIEASFRLDASDVAAINEADVGYNC  472
Lt6-FT    VEEVEALRYNSTDLSGITIDDGSVFHLPLHQATQLDIEATFRLDASDVAAINEADVGYNC  473
Ps6-SFT   VQEIETLRANATAFNGITIDTGSVFLLPIRQGTQLDIEASFRLNASAVAAISEADVGYNC  469
Hv6-SFT   VEEIETLRLNATELTDVTINTGSVIHIPLRQGTQLDIEASFHLDASAVAALNEADVGYNC  465
Ta6-SFT   VEEIETLRLNATELSDVTLNTGSVIHIPLRQGTQLDIEATFHLDASAVAALNEADVGYNC  463
          *:*:*:** *:* :..:*:: **:: :*:: *:***.*.:***:: :***:.********

Lp6-SFT   SSSGGAAARGALGPFGLLVHAAGDLR-GEQTAVYFYVSRALDGSLRTSFCNDETRSSRAR  531
Lp1-FFT   SSSGGAAARGALGPFGLLVHAAGDLR-GEQTAVYFYVSRALDGSLRTSFCNDETRSSRAR  531
Lt6-FT    SSSGGAAARGALGPFGLLVHAAGDLIRGEQTAVYFYVSRALDGTLRTSFCNDETRSTRAK  533
Ps6-SFT   SSSGGAATRGVLGPFGLLVLAD---TRGEQTAVYFYVSRGLDGGLRTSFCNDESRSSWAR  526
Hv6-SFT   SSSGGAVNRGALGPFGLLVLAAGD-RRGEQTAVYFYVSRGLDGGLHTSFCQDELRSSRAK  524
Ta6-SFT   SSSGGAVNRGALGPFGLLVLAAGD-RRGEQTAVYFYVSRGLDGGLHTSFCQDELRSSRAK  522
          ******. **.******** *     ************.*** *:****:** **:.*:

Lp6-SFT   DVTKRVVGSTVPVLDGEALSMRVLVDHSIVQSFAMGGRVTATSRVYPTEAIYARAGVYLF  591
Lp1-FFT   DVTKRVVGSTVPVLDGEALSMRVLVDHSIVQSFAMGGRVTATSRVYPTEAIYARAGVYLF  591
Lt6-FT    DVTKRVVGSTVPVLDGEALSMRVLVDHSIVQSFAMGGRVTATSRVYPTEAIYARAGVYLF  593
Ps6-SFT   DVTKRVVGSTVPVLDGEALSMRVLVDHSIVQSFAMGGRVTATSRVYPTEAIYAAAGVYVF  586
Hv6-SFT   DVTKRVIGSTVPVLDGEALSMRVLVDHSIVQGFDMGGRTTMTSRVYPMES-YQEARVYLF  583
Ta6-SFT   DVTKRVIGSTVPVLDGEAFSMRVLVDHSIVQGFAMGGRTTMTSRVYPMEA-YQEAKVYLF  581
          ******:*********** .:*********** * ****:* *.****** * * **:*

Lp6-SFT   NNATGASVTAERLIVHEMASAVYDETVMVKDS----------  623
Lp1-FFT   NNATGASVTAERLIVHEMASAVYDETVMVKDS----------  623
Lt6-FT    NNATGASVTAERLVVHEMASAVYDDTVMVEDS----------  625
Ps6-SFT   NNATGASVTAERLVMHEMDSVDNNQIFLVDDL----------  618
Hv6-SFT   NNATGASVTAERLVVHEMDSAHNQLSNEDDGMYLHQVLESRH  625
Ta6-SFT   NNATGASVTAERLVVHEMDSAHNQLSNMDDHSYVQ-------  616
          *************:.***  *. .      : . ::
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Fig. 1. Comparison of the deduced amino acid sequence of fructan-synthesizing enzymes leading to β(2–6) linkages between fructose residues including the newly cloned *L. perenne* 6-SFT (Lp6-SFT). Their respective accession numbers are: Lp6-SFT AF494041, Lp1-FFT AB186920, Lt6-FT AJ532550, Ps6-SFT AF192394, Hv6-SFT X83233, Ta6-SFT AB029887. Asterisks, colons, and periods indicate identical residues, conserved substitutions, and semiconserved substitutions, respectively. The putative start of the large subunit and of the small subunit is indicated by an arrow. Potential glycosylation sites are underlined. The β-fructosidase motif and the cysteine catalytic site are boxed. Inverted triangles above the sequence alignments indicate the three carboxylic acids that are crucial for enzyme catalysis (Verhaest *et al.*, 2005). Differences between the deduced amino acid sequences from *L. perenne* are indicated by circled amino acids. Lp, *L. perenne*; Lt, *L. temulentum*; Ps, *Poa secunda*; Hv, *H. vulgare*; Ta, *Triticum aestivum*.

sucrose is the fructosyl donor but not the sole fructosyl acceptor, the putative 6-FT of *L. perenne* corresponds therefore to a 6-SFT.

The recombinant protein led also to the production of 1-kestotriose when incubated with sucrose alone (Fig. 3A) or together with 6G-kestotriose (Fig. 3D), and to the pro-duction of nystose when incubated with 1-kestotriose alone (Fig. 3B) or together with sucrose (Fig. 3C). However, the small amount of 1-kestotriose or nystose produced makes it unlikely that the recombinant protein is a genuine 1-SST or 1-FFT. The recombinant protein is probably not a genuine 1-FEH either since the amount of fructose released was

Fig. 2. Unrooted phylogenetic tree of protein sequences of some invertase and fructan metabolism genes including the newly cloned *L. perenne* 6-SFT (in bold). Their respective accession numbers are: *Allium cepa* invertase AJ006067, *A. cepa* 1-SST AJ006066, *A. cepa* 6G-FFT AY07838, *Allium sativum* 1-SST AY098442, *Arabidopsis thaliana* invertase AY039610, *A. thaliana* invertase AY046009, *Asparagus officinalis* invertase AF002656, *A. officinalis* 6G-FFT AB084283, *Beta vulgaris* invertase AJ277455, *Brassica oleracea* invertase AF274298, *B. oleracea* invertase AF274299, *Capsicum annuum* invertase P93761, *Cichorium intybus* invertase AJ419971, *C. intybus* 1-SST U81520, *C. intybus* 1-FFT U84398, *Cynara scolymus* 1-SST Y09662, *C. scolymus* 1-FFT AJ000481, *Daucus carota* invertase Q42722, *D. carota* invertase X75352, *Festuca arundinacea* 1-SST AJ297369, *Helianthus tuberosus* 1-SST AJ009757, *H. tuberosus* 1-FFT AJ009756, *H. vulgare* 6-SFT X83233, *Ipomoea batatas* invertase AF017082, *I. batatas* invertase AY037937, *Lycopersicon esculentum* invertase P29000, *L. perenne* invertase AY082350, *L. perenne* 1-SST AY245431, *L. perenne* 6G-FFT AF492836, *L. perenne* 6-SFT AF494041, *L. perenne* LpFT1 AF481763, *L. perenne* LpFT4 DQ073970, *L. temulentum* putative 6-FT AJ532550, *Oryza sativa* invertase AF276703, *O. sativa* invertase AF276704, *Phaseolus vulgaris* invertase O24509, *P. secunda* 6-SFT AF192394, *Prunus cerasus* invertase AY048579, *Saccharum officinarium* invertase AY302083, *Taraxacum officinale* 1-SST AJ250634, *T. aestivum* invertase AJ635225, *T. aestivum* 6-SFT AB029887, *T. aestivum* 1-SST AB029888, *Tulipa gesneriana* invertase X97642, *Vicia faba* invertase Q43857, *Vitis vinifera* invertase Q9S943, *V. vinifera* invertase QS944, *Zea mays* invertase P49175.

small when the recombinant protein was incubated with 1-kestotriose alone (Fig. 3B).

As previously reported (Hochstrasser *et al.*, 1998), the recombinant 6-SFT protein of barley produced bifurcose when incubated with both sucrose and 1-kestotriose (Fig. 4A). However, the ability of the protein to elongate the 6G-kestotriose has never been addressed (Duchateau *et al.*, 1995). Despite the fact that 6G-kestotriose and levan neoseries do not occur in barley, Hv6-SFT also produced 6G,6-kestotetraose when incubated with sucrose and 6G-kestotriose (Fig. 4B). The discrepancy between the pattern of

fructans produced *in vivo* and the enzymatic characteristics assessed *in vitro* might be explained in barley by the absence of 6G-FFT activity and consequently the absence of 6G-kestotriose. In *L. perenne*, the presence of 6G-FFT activity could prevent the transfer of fructose between sucrose and 1-kestotriose by the 6-SFT activity. Indeed, when the two recombinant 6-SFT proteins were incubated in the presence of increasing amounts of recombinant 6G-FFT/1-FFT from *L. perenne*, bifurcose ceased to be produced (Fig. 5A, B) while it did not in controls (Fig. 5C, D) in which the products of the enzymes incubated separately were combined
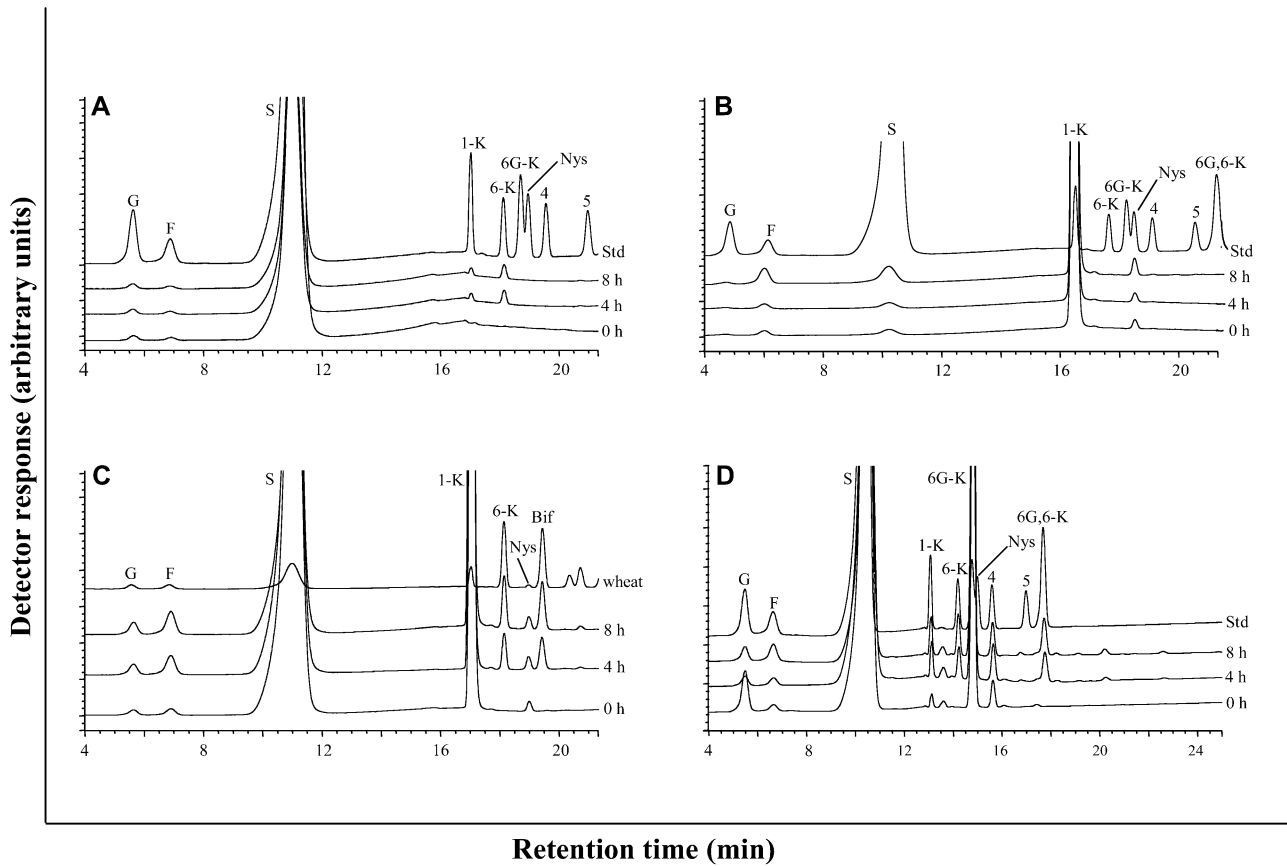
Fig. 3. High-performance anion-exchange chromatograms of the reaction products generated by the *P. pastoris*-expressed 6-SFT protein from *L. perenne*. Reaction mixtures containing the recombinant protein were incubated for up to 8 h at 30 °C with 200 mM sucrose (A), 200 mM 1-kestotriose (B), 200 mM sucrose and 200 mM 1-kestotriose (C), 200 mM sucrose and 200 mM 6G-kestotriose (D). G: glucose; F: fructose; S: sucrose; Bif: bifurcose (1 and 6-kestotetraose); 1-K: 1-kestotriose; 6G-K: 6G-kestotriose; 6-K: 6-kestotriose; Nys: nystose (1,1-kestotetraose); 4: 6G,1-kestotetraose; 5: 1,1,1-kestopentaose. The elution pattern of wheat saccharides is presented in (C).

afterwards at the same ratio as the enzymes. Instead, controls showed constant amounts of 6-kestotriose and bifurcose (Fig. 5C, D). Increasing amounts of 6G-FFT/ 1-FFT products (6G-kestotriose, nystose) were barely visible, probably because the extracts were too diluted. Indeed, when extracts were four times concentrated, amounts of 6G-kestotriose and nystose increased together with increasing amounts of the 6G-FFT/1-FFT recombinant protein (data not shown). A fourth fructan was produced. It might be produced by 6G-FFT/1-FFT activity (1,1 and 6G-kestopentaose and/or 1 and 1,6G-kestopentaose) or by 6-SFT activity (6G,6-kestotetraose). Since it has the same retention time as the 6G,6-kestotetraose, it might correspond to this fructan.

Absence of bifurcose in *L. perenne* could arise from 6-SFT activity inhibition by the two products of the 6G-FFT/ 1-FFT activity. However, neither 6G-kestotriose (Fig. 6A) nor nystose (Fig. 6C) decreased the production of bifurcose. The barley 6-SFT recombinant protein shared close enzymatic characteristics (Fig. 6B, D). Indeed, when the two recombinant 6-SFT proteins were incubated with sucrose and 1-kestotriose, bifurcose was produced, and when they were incubated with sucrose, 1-kestotriose, and 6G-kestotriose,

6G,6-kestotetraose was produced (Fig. 6A, B). When they were incubated with sucrose, 1-kestotriose, and nystose, a fructan was produced but with a slightly different retention time, 21.3 min, as compared with 21.5 min for 6G,6-kestotetraose. Most likely this fructan corresponds to the 6- and 1,1-kestopentaose, resulting from the transfer of fructose between sucrose and nystose. If the enzymatic properties of the two recombinant 6-SFT proteins are close, they are not exactly the same since perennial ryegrass 6-SFT produced less bifurcose and a higher ratio of 6G,6-kestotetraose to bifurcose than barley 6-SFT (Fig. 6A, 6B).

Absence of bifurcose in *L. perenne* might also result from a so-called bifurcosidase activity (Kawakami *et al.*, 2005). To test this hypothesis, proteins were extracted from *L. perenne* plants in two physiological conditions in which bifurcosidase could operate, depending on its role *in vivo*: from plants accumulating fructans (if bifurcosidase participates to fructan synthesis) or from plants degrading fructans after defoliation (if bifurcosidase is induced after defoliation together with other FEHs to mobilize fructans for regrowth). Bifurcose is synthesized by wheat or barley (Duchateau *et al.*, 1995; Sprenger *et al.*, 1995). After incubation of these crude protein extracts with bifurcose
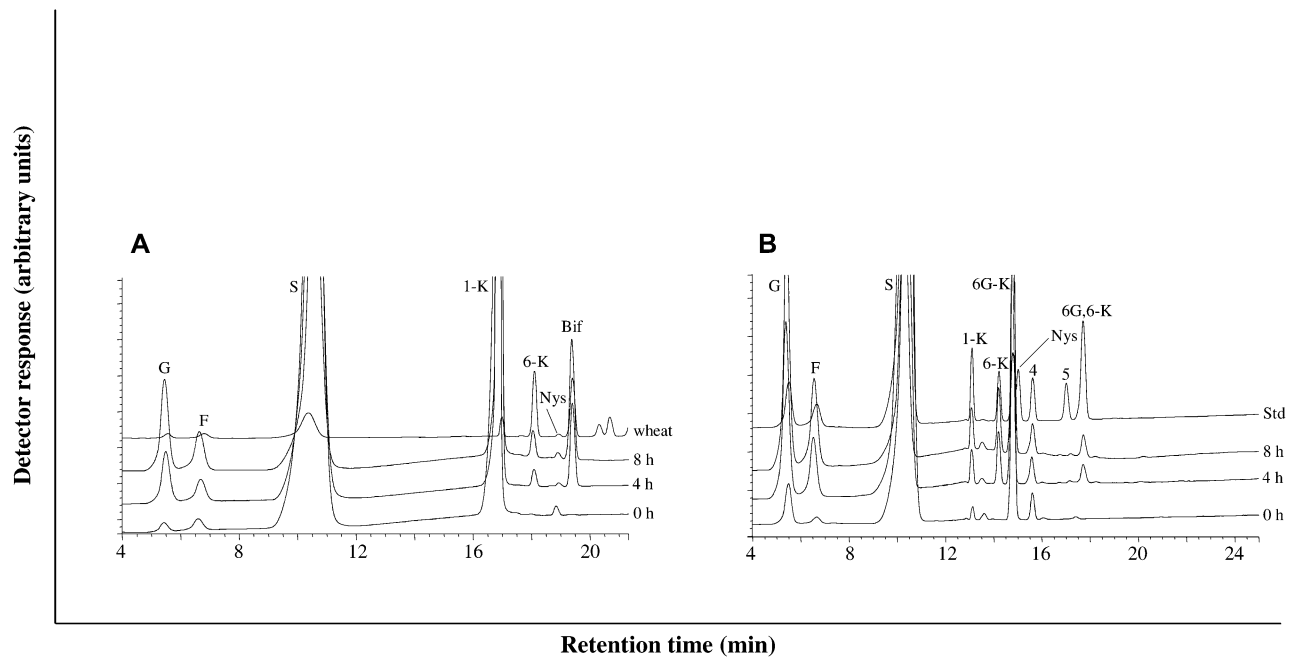
Fig. 4. High performance anion-exchange chromatograms of the reaction products generated by the *P. pastoris*-expressed 6-SFT protein from *H. vulgare*. Reaction mixtures containing the recombinant protein were incubated for up to 8 h at 30 °C with 200 mM sucrose and 200 mM 1-kestotriose (A), 200 mM sucrose and 200 mM 6G-kestotriose (B). G: glucose; F: fructose; S: sucrose; Bif: bifurcose (1 and 6-kestotetraose); 1-K: 1-kestotriose; 6G-K: 6G-kestotriose; 6-K: 6-kestotriose; Nys: nystose (1,1-kestotetraose); 4: 6G,1-kestotetraose; 5: 1,1,1-kestopentaose. The elution pattern of wheat saccharides is presented in (A).

extracted from wheat, bifurcose was not degraded (data not shown). So a 'bifurcosidase' does not seem to operate in *L. perenne*.

Effect of leaf developmental stage on gene expression pattern of Lp6-SFT

Leaf growth in grasses is confined to the basal region, which is enclosed by the sheaths of mature leaves (Schnyder *et al.*, 1990). Cells are displaced away from their origin as a result of continued production and elongation of new cells. The tissue that emerges from the enclosing leaf sheaths is almost fully differentiated and photosynthetically active (Wilhelm and Nelson, 1978). In order to follow the transcription of *Lp6-SFT* in these tissues, four 1-cm long segments and a fifth longer segment were cut, starting from the base of growing leaves and leaf sheaths. Leaf blades and emerged parts of growing leaves were divided into three parts of equal length (Fig. 7). From each segment, carbohydrates, proteins, and total RNA were extracted. Results obtained for sucrose, fructans, 1-SST, and 6G-FFT activities together with the corresponding transcripts have been reported previously in Lasseur *et al.* (2006). 6-SFT activity has not been followed because its specific substrate, the 6G-kestotriose, is not commercially available. A 311-bp fragment of *Lp6-SFT* cDNA was amplified by RT-PCR from the same samples. In elongating leaves, the transcript level of *Lp6-SFT* was highest in the basal segment (Fig. 7) mainly composed of dividing cells, where the accumulation of fructan and sucrose was maximal and where activity of 1-SST and 6G-FFT was greatest (Lasseur *et al.*, 2006). In

mature leaf sheaths, *Lp6-SFT* was expressed in the three first segments (Fig. 7) concomitant with fructan synthesis (Lasseur *et al.*, 2006). Together with fructan levels and FT enzyme activities, *Lp6-SFT* transcription dropped subsequently along the axis of leaf sheaths and of enclosed parts of elongating leaves, so that they became barely or no longer detectable in the fifth segment of each tissue (Fig. 7). In photosynthetically active tissues, fructans were present in lower amounts than in sink tissues (Lasseur *et al.*, 2006). *Lp6-SFT* mRNA fragments were not detected in mature leaf blades. Surprisingly, they were amplified in the last segment of the emerged part of the elongating leaves (Fig. 7).

Transcript levels of Lp6-SFT upon induction of fructan accumulation in leaves

Accumulation of large quantities of fructans can be induced in leaves of grasses by cooling the roots and continuous illumination of the shoots (Smouter and Simpson, 1991; Guerrand *et al.*, 1996; Pavis *et al.*, 2001a, Wei *et al.*, 2001, Lasseur *et al.*, 2006). After several time intervals, shoots were harvested and dissected into leaf blades, leaf sheaths, and elongating leaf bases. Fructans started to accumulate in elongating leaf bases and in leaf sheaths 6 h after the beginning of the treatment while in leaf blades, fructans increased later and only slightly (Lasseur *et al.*, 2006). *Lp6-SFT* cDNA was amplified by RT-PCR from these same samples. *Lp6-SFT* transcription level increased in elongating leaf bases and in leaf sheaths upon induction of fructan accumulation (Fig. 8), following the curve of fructan levels (Lasseur *et al.*, 2006). Surprisingly, *Lp6-SFT* transcripts,
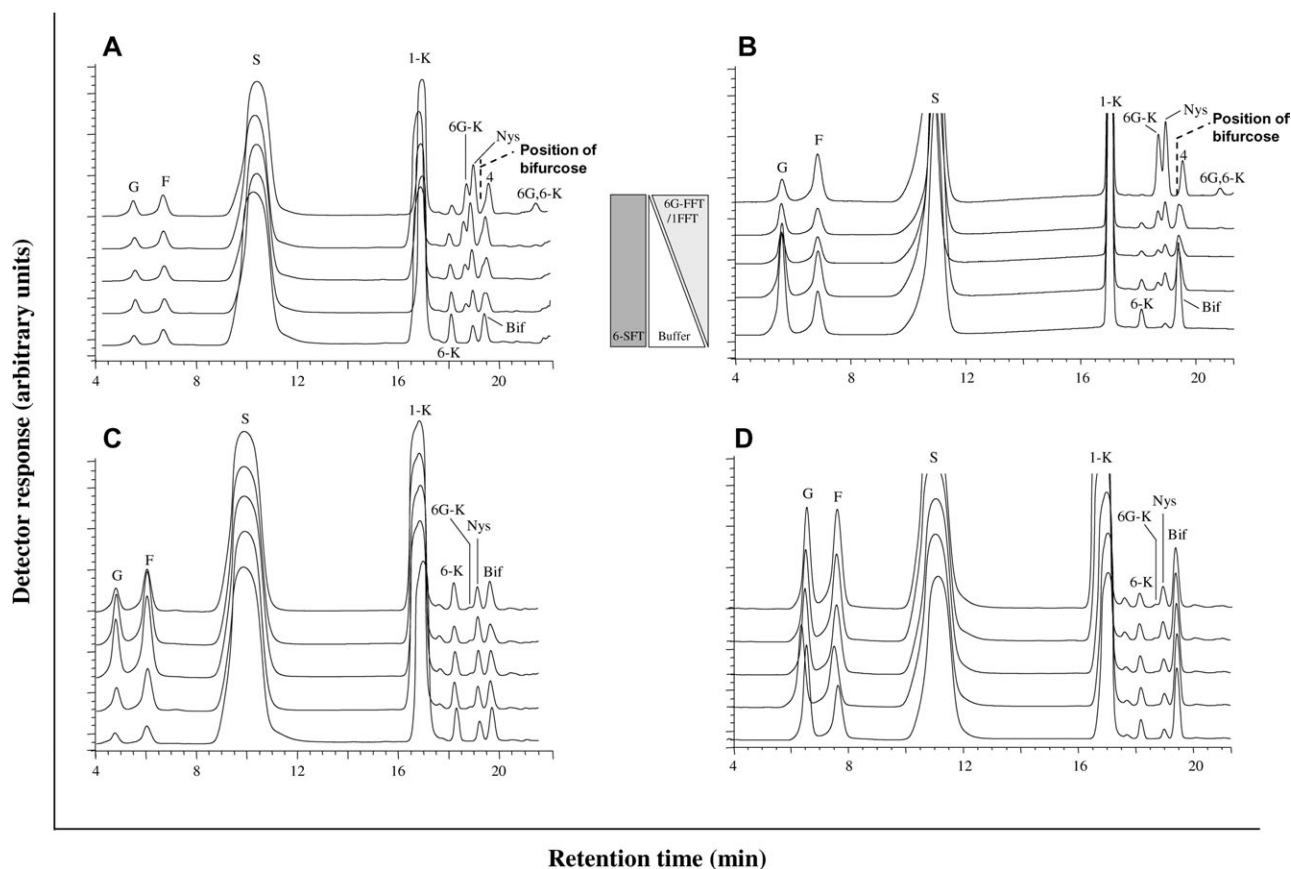
Fig. 5. High-performance anion-exchange chromatograms of the reaction products generated by the *P. pastoris*-expressed 6-SFT protein from *L. perenne* (A) or from *H. vulgare* (B) with different amounts of recombinant 6G-FFT/1-FFT (0–100% of the incubation buffer added) from *L. perenne* (Lasseur *et al.*, 2006). Reaction mixtures were incubated for 8 h at 30 °C with 200 mM sucrose and 200 mM 1-kestotriose. For comparison, each recombinant enzyme has been run independently and the products of the reactions have been mixed in the same proportions as the corresponding enzymes (C: Lp6-SFT, D: Hv6-SFT). G: glucose; F: fructose; S: sucrose; Bif: bifurcose (1 and 6-kestotetraose); 1-K, 1-kestotriose; 6G-K, 6G-kestotriose; 6-K: 6-kestotriose; Nys: nystose (1,1-kestotetraose); 4: 6G,1-kestotetraose; 6G,6-K: 6G,6-kestotetraose.

which were not detected in leaf blades of control plants, became strongly induced in leaf blades of illuminated plants at between 6 h and 24 h of treatment while the fructan level slightly increased after 48 h (Lasseur *et al.*, 2006).

Discussion

Lp6-SFT and Hv6-SFT, two similar enzymes but different fructan patterns in planta

Fructosyl residues in fructans of *Lolium* species are predominantly β(2–6) linked (Tomasic *et al.*, 1978; Sims *et al.*, 1992; Bonnett *et al.*, 1994; St John *et al.*, 1997; Pavis *et al.*, 2001a). In this paper, we describe for the first time the isolation and the functional characterization of a fructosyltransferase able to produce β(2–6) linkages in *Lolium* species.

The cDNA was first termed 'putative *6-FT*' (Lasseur *et al.*, 2002, accession no. AF494041) because of its high identity (74%) to the 6-SFT polypeptide of *P. secunda*. It shares 99% identity with *prft1* described by Hisano *et al.*

(2008). In Chalmers *et al.* (2005), the corresponding protein was described as an Lp1-FFT. However, the functionality of the corresponding protein could not be deeply assessed because of troubles with activity when expressing the cDNA in *P. pastoris* (Hisano *et al.*, 2008). The two amino acids that differ between the two cDNAs (Fig. 1) are not located in the mature protein if we assume that the mature protein begins at the same position as for the 6-SFT of barley [AGGFPW motif; Sprenger *et al.*, 1995)] (Fig. 1). So it can be concluded that the two cDNAs (*Lp6-SFT* and *Lp1-FFT*) correspond to the same protein. Differences between the cDNAs could be attributed to the fact that they have been cloned from different varieties (variety Bravo in this study compared with variety Aberystwyth in Hisano *et al.*, 2008). Gallagher *et al.* (2006) cloned a genomic DNA in perennial ryegrass corresponding to a '6-SFT like' (Lp6-S/F-FT), and found that the corresponding cDNA shared 99% identity (621/624 amino acids) with the cDNA cloned in this work. Amongst the three amino acids that differed, two are not present in the mature protein. Again, we can assume that this cDNA and the newly cloned cDNA correspond to the same protein. In their study, Hisano *et al.* (2008) cloned two
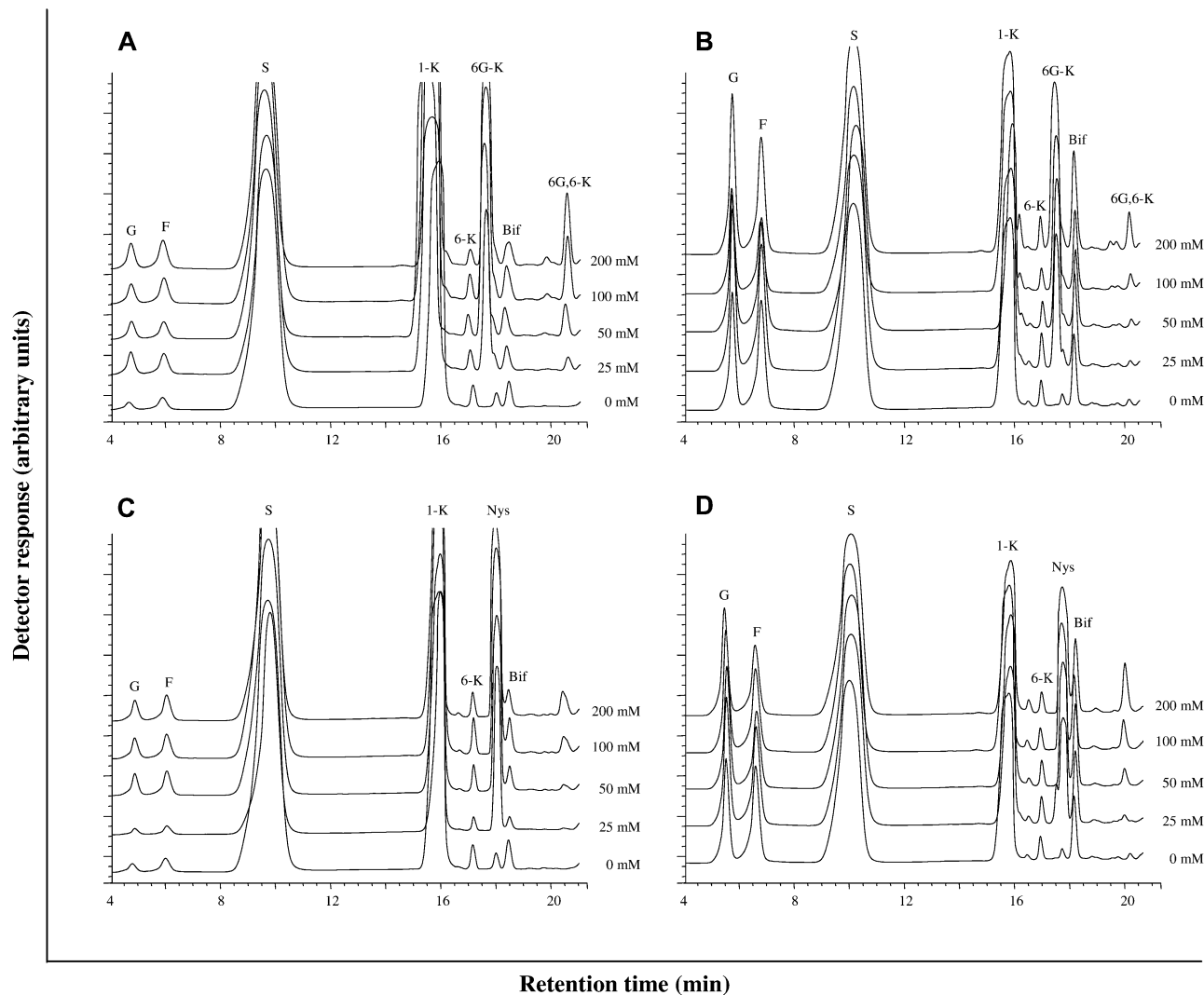
Fig. 6. High-performance anion-exchange chromatograms of the reaction products generated by the *P. pastoris*-expressed 6-SFT protein from *L. perenne* (A, C) or from *H. vulgare* (B, D). Reaction mixtures containing the recombinant protein were incubated for 8 h at 30 °C with 200 mM sucrose and up to 200 mM 6G-kestotriose (A, B) or nystose (C, D). G: glucose; F: fructose; S: sucrose; Bif: bifurcose (1 and 6-kestotetraose); 1-K: 1-kestotriose; 6G-K: 6G-kestotriose; 6-K: 6-kestotriose; Nys: nystose (1,1-kestotetraose); 6G,6-K: 6G,6-kestotetraose.

other '6-SFT-like' cDNAs called 'putative fructosyltransfer-ase 2' (*prft2*) and 'putative fructosyltransferase 6' (*prft6*) sharing, respectively, 95% (599/624 amino acids) and 96% (602/624 amino acids) identity with *prft1*. Given the very high identity between these three cDNAs (*prft1*, *prft2*, and *prft6*), the authors assumed that some of these genes may be allelic.

When expressed in *P. pastoris*, the recombinant protein corresponding to the newly cloned cDNA demonstrated a 6-SFT activity. Indeed, this protein is able to use sucrose as fructosyl donor and both 1-kestotriose and 6G-kestotriose as fructosyl acceptors, producing respectively bifurcose and 6G,6-kestotetraose. Therefore, in perennial ryegrass, there is no need for a different enzyme such as a 6-FFT to produce the β2,6-fructosyl linkages. The main product of the recombinant 6-SFT protein from barley in the presence of sucrose and 6G-kestotriose is also the 6G,6-kestotetraose, despite the absence of fructans with internal glucosyl residues

in this plant. Thus, the catalytic properties of *L. perenne* 6-SFT resemble those of 6-SFT from barley. When incubated with sucrose and 1-kestotriose, both enzymes produced bifurcose. When incubated with sucrose and 6G-kestotriose, both enzymes produced 6G,6-kestotetraose. In addition, both enzymes have invertase activity and both enzymes are not inhibited by 6G-kestotriose and nystose. We demonstrate therefore that 6-SFT of *L. perenne* and *H. vulgare* have close enzymatic characteristics, so that the specificity of the fructans produced in the two species depends on the 6G-FFT/1-FFT activity, present in *L. perenne* and absent in *H. vulgare*.

In *Lolium* species, bifurcose has neither been found in shoot tissues, nor been produced by incubation of protein crude extract with sucrose and 1-kestotriose (Pavis *et al.*, 2001a, b). According to the present data, bifurcose is not produced by the 6-SFT activity when the 6G-FFT/1-FFT activity operates *in vitro*. Absence of bifurcose did not result
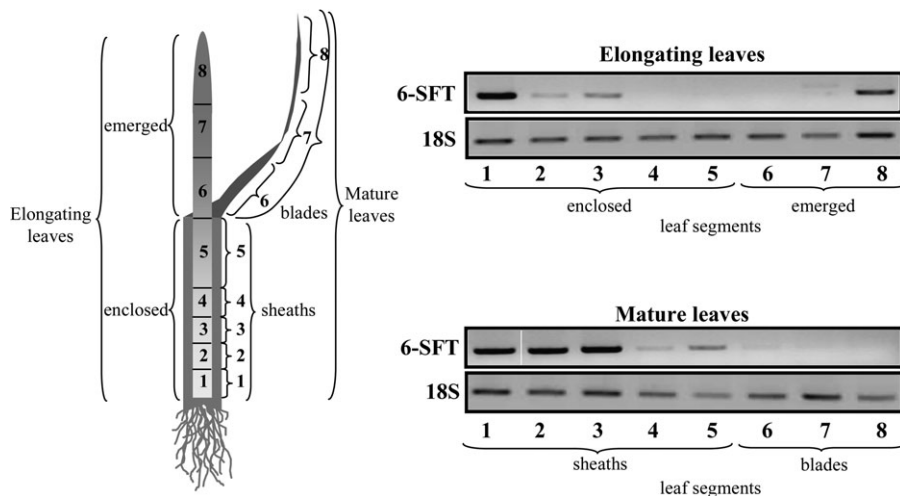
Fig. 7. Tissue distribution of *6-SFT* transcripts as compared with 18S rRNA transcripts. After 8 weeks of growth, plants were harvested and the mature leaves separated from the elongating leaves. Sheaths and elongating leaf bases previously enclosed by the sheaths were dissected longitudinally into five segments (four 10-mm-long segments, 0–40 mm from the leaf base, and a fifth variable length segment of ~40 mm). Blades and the emerged part of the elongating leaves were divided into three equal parts. Gene expression was determined by PCR using gene-specific primers and visualized by ethidium bromide staining.
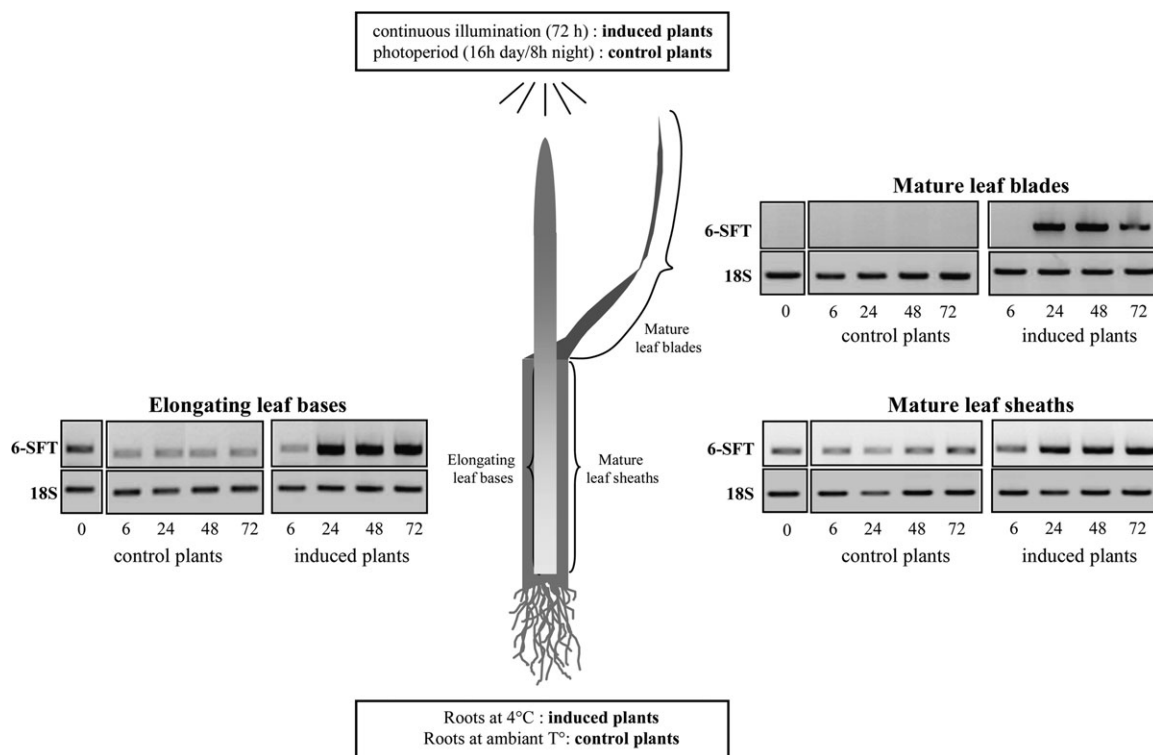
Fig. 8. Expression of the *6-SFT* gene, as compared with 18S rRNA transcript expression, in elongating leaf bases, mature leaf blades, and mature leaf sheaths of *L. perenne* plants. Plants were cultivated for 8 weeks with a photoperiod of 16 h at day/night temperatures of 22/18 °C and further subjected to the same conditions (control plants) or to continuous light with roots at 4 °C (induced plants) for 72 h. Gene expression was determined by PCR using gene-specific primers and visualized by ethidium bromide staining.

from its degradation by a bifurcosidase activity or from inhibition of its synthesis by the products of the 6G-FFT/1-FFT activity. 6-SFT and 6G-FFT/1-FFT share common substrates, sucrose and 1-kestotriose, but give different products by catalysing fructosyl transfer either from sucrose to 1-kestotriose or from 1-kestotriose to sucrose, respec-

tively. No affinity measurements have been attempted for the two enzymes. However, since the 6-SFTs from *Lolium* and barley show close characteristics *in vitro*, as demonstrated by the present work, it is reasonable to assume that the 6-SFT from wheat might be quite similar too. Schroeven *et al.* (2008) derived an apparent K_m of between 300 and

400 mM for fructosyl transfer from sucrose to 1-kestotriose by the wheat 6-SFT. Since the donor K_m for sucrose was estimated at 16 mM, it can be derived that the acceptor K_m for 1-kestotriose should be very high to explain the overall apparent K_m. On the other hand, a rough estimation of the donor K_m for 1-kestotriose of the *Lolium* 6G-FFT (Lasseur *et al.*, 2006) was ~300 mM. Therefore, absence of bifurcose in *L. perenne* tissues might be explained by a higher affinity of 6G-FFT/1-FFT for 1-kestotriose than 6-SFT for the same substrate. Moreover, from data presented in Fig. 6, it could be presumed that the affinity of perennial ryegrass 6-SFT to 1-kestotriose was lower than that of barley 6-SFT while its affinity to 6G-kestotriose was higher. Therefore, the absence of bifurcose in *L. perenne* tissues might be explained by a higher affinity of 6G-FFT/1-FFT for 1-kestotriose than that of 6-SFT for the same substrate, together with a high affinity of 6-SFT for 6G-kestotriose when it is produced.

In addition to the 6-SFT activity, the recombinant protein exhibits 1-SST, 1-FEH, and 1-FFT activities. The 1-FFT activity is probably the reason why a protein sharing 99% identity with Lp6-SFT was first called 1-FFT by Yamada (AN AB186920) (personal communication reported by Chalmers *et al.*, 2005). The associate activities might be artefactual due to differences in folding or glycosylation pattern as demonstrated for barley and wheat 6-SFT when expressed in *P. pastoris* (Hochstrasser *et al.*, 1998; Kawakami and Yoshida, 2002).

Conclusively, fructans present in *L. perenne* might be synthesized by a three-enzyme system including 1-SST, 6G-FFT together with 1-FFT, and 6-SFT (Fig. 9). Together with 1-SST, 6-SFT is responsible for diverting sucrose to fructans. In grasses, 6-SFT has therefore a prominent role in fructan synthesis, conforming to the hypothetical model suggested by Wiemken *et al.* (1995).

Regulation of 6-SFT expression is tissue specific

Fructan-synthesizing genes show different temporal and spatial patterns of expression depending on the C sink-source status in the leaves. Indeed, in sink leaf tissues of grass species, the largest amount of *6-SFT* mRNA was found in the first segment of elongating leaves and leaf sheaths (Fig.7), where large fructan stores are deposited temporarily and where fructan-synthesizing activities (1-SST and 6G-FFT) are highest (Chalmers *et al.*, 2003; Lasseur *et al.*, 2006). It declined thereafter along the leaves, together with fructan and enzymatic levels. In *L. temulentum*, the putative fructosyltransferase FT 2:2, sharing 96.4% homology with *Lp6-SFT* at the DNA level, was also predominantly expressed in tiller bases (Gallagher *et al.*, 2004). A similar result was obtained for 1-SST in the developing leaf of *Festuca arundinacea* (Lüscher *et al.*, 2000). In sink tissues of *Lolium*, transcript profiles of S-type FTs (1-SST, 6-SFT) and F type FT (6G-FFT) genes, generally consistent with both enzymatic activity measurements whenever possible [1-SST activity, 6G-FFT activity but unfortunately not 6-SFT activity since the substrate (6G-kestotriose) is not commercially available] and levels of fructan accumulation, suggest that these genes are regulated primarily at the transcriptional level.

Surprisingly, in elongating leaves, *Lp6-SFT* was expressed at the tip but not in the proximal part of the leaf that had emerged from the sheath, similarly to *1-SST* in *F. arundinaea* leaves (Lüscher *et al.*, 2000). However, fructans barely accumulate at the leaf tip (Lasseur *et al.*, 2006). Interestingly, similar results have been reported for the putative fructosyltransferase FT 2:2 of *L. temulentum*. Transcripts were detected in photosynthetically active tissue but did not correlate with fructan synthesis (Gallagher *et al.*, 2004). Moreover, *Lp6-SFT* transcripts accumulated in leaf blades of plants that were continuously illuminated but where barely any fructan accumulated (Lasseur *et al.*, 2006). It could be argued that photosynthetically active tissues keep on exporting sucrose, and might not reach the sucrose threshold to allow efficient fructan accumulation despite the presence of active FTs. Unfortunately and as reported above, 6-SFT activity increase could not be assessed. However, since in the same conditions, other FT activity increase was prevented (Lasseur *et al.*, 2006), it is hypothesized that in photosynthetically active tissue, fructan metabolism genes including *Lp1-SST*, *Lp6G-FFT*, and probably also *Lp6-SFT*, are regulated mainly at post-transcriptional level in a context of forced accumulation of fructans throughout the whole plant. As in barley leaf blades, regulation might differ according to the cell type. For example, it has been shown previously that Hv6-SFT id expressed in the mesophyll, the parenchymatous bundle sheath, and phloem parenchyma but not in epidermal cells (Pollock *et al.*, 2003). No mechanistic insights into such
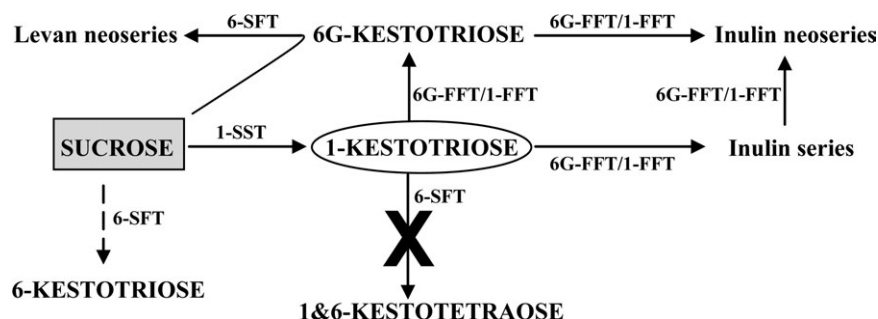

Fig. 9. Model of the synthesis of the different *Lolium*-type fructans by 1-SST, 6G-FFT/1-FFT, and 6-SFT.

regulation at the post-transcriptional level have been provided so far for any FT. Thus, further research is needed to identify the factors involved in fructan synthesis regulation.

Conclusion

In conclusion, we have cloned and functionally characterized for the first time a *6-SFT* cDNA in a forage grass. We have shown that the regulation of gene expression depends on the tissue according to its sink-source status. In addition, we have demonstrated (i) that 6G-FFT could compete with 6-SFT for 1-kestotriose forming 6G-kestotriose, precluding the formation of bifurcose by 6-SFT, and (ii) that 6-SFT has a preference for 6G-kestotriose over 1-kestotriose as fructosyl acceptor substrate so that the drive of fructosyl flux is directed to the synthesis of levan neoseries by the concerted action of these two enzymes. We have then provided new data that contribute to a better understanding of fructan structural diversity in the plant kingdom.

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