Molecular and functional characterization of a cDNA encoding fructan:fructan 6G-fructosyltransferase (6G-FFT)/fructan:fructan 1-fructosyltransferase (1-FFT) from perennial ryegrass (Lolium perenne L.)

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Abstract

Fructans are the main storage compound in Lolium perenne. To account for the prevailing neokestose-based fructan synthesis in this species, a cDNA library of L. perenne was screened by using the onion (Allium cepa) fructan:fructan 6G-fructosyltransferase (6G-FFT) as a probe. A full length Lp6G-FFT clone was isolated with significant homologies to vacuolar type fructosyltransferases and invertases. The functionality of the cDNA was tested by heterologous expression in Pichia pastoris. The recombinant protein demonstrated both 6G-FFT and fructan:fructan 1-fructosyltransferase activities (1-FFT) with a maximum 6G-FFT/1-FFT ratio of two. The activity of 6G-FFT was investigated with respect to developmental stage, tissue distribution, and alterations in carbohydrate status expression and compared to sucrose:sucrose 1-fructosyltransferase (1-SST). Lp6G-FFT and Lp1-SST were predominantly expressed in the basal part of elongating leaves and leaf sheaths. Expression of both genes declined along the leaf axis, in parallel with the spatial occurrence of fructan and fructosyltransferase activities. Surprisingly, Lp6G-FFT was highly expressed in photosynthetically active tissues where very low extractable fructosyltransferase activity and fructan amounts were detected, suggesting a post-transcriptional regulation of expression. Lp6G-FFT gene expression increased only in elongating leaves following similar increases of sucrose content in blades, sheaths, and elongating leaf bases. Regulation of Lp6G-FFT gene expression depends on the tissue according to its sink–source status.

Key words: Fructan, fructan:fructan 6G-fructosyltransferase, gene expression, heterologous expression, Lolium perenne, Pichia pastoris, sucrose:sucrose 1-fructosyltransferase.

Introduction

Fructans are sucrose-derived fructose polymers. Fructan types differ from one another by their length (degree of polymerization, DP), branching, the linkage type between adjacent fructose and the position of the glucose residue. Fructans are known for their role as a carbohydrate reserve found in approximately 15% of the flowering plant species (Hendry and Wallace, 1993). Many of these plant species belong to economically important orders such as Asterales [chicory (Cichorium intybus), Jerusalem artichoke (Helianthus tuberosus)], Liliales [onion (A. cepa), asparagus (Asparagus officinalis)], and Poales [barley (Hordeum vulgare), wheat (Triticum aestivum), ryegrass (L. perenne)].
From a human point of view, 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).

In Asterales such as chicory and Jerusalem artichoke, inulin series fructans (inulins) are found, which consists of linear (β-1) linked fructose residues with a terminal glucose residue. Inulins are synthesized by the concerted action of two enzymes. Sucrose:fructan 1-fructosyltransferase (1-SST) catalyses fructosyl transfer from one sucrose molecule to the fructosyl residue of another sucrose molecule via a (β-1) linkage, resulting in the trisaccharide 1-kestose. Fructan:fructan 1-fructosyltransferase (1-FFT) elongates 1-kestotriose with additional (β-2,1)-linked fructose units on both terminal fructose residues of 6G-kestotriose results in the inulin neoserries type of polymers containing (β-2,1)-linkages between adjacent fructosyl residues (Ernst et al., 1998). The enzyme fructan:fructan 6G-fructosyltransferase (6G-FFT) initiates the formation of the 6G-linked chain. It uses 1-kestotriose as a fructosyl donor and transfers the fructose unit to the glucose moiety of sucrose or to low DP-inulin via a (β-2,6) linkage (Shiomi, 1989; Vijn et al., 1997). The 6G-kestotriose produced is the shortest fructan of the inulin neoseries. In asparagus, three enzymes, 1-SST, 1-FFT, and 6G-FFT participate in synthesis of fructans (Shiomi, 1989), whereas in onion only two enzymes operate, 1-SST and 6G-FFT. Indeed, 6G-FFT purified from onion bulbs had 1-FFT activity (Fujishima et al., 2005). The recombinant enzyme expressed in tobacco plants, protoplasts, or BY2 cells, was also able to synthesize DP4 inulin from 1-kestotriose, indicating 1-FFT like activity (Vijn et al., 1997; Ritsema et al., 2003). Moreover, the introduction of onion 1-SST and 6G-FFT into sugar beet resulted in a fructan profile closely resembling that from onion (Weyens et al., 2004).

Members of the Poales contain fructans with (β-2,6) linked fructose units. The glucose residue can be terminal or internal to the molecule, leading to fructans of the levan series or the levan neoseries, respectively. Many of the fructan molecules from Avena (Livingston et al., 1993) and Lolium species (Sims et al., 1992; Pavis et al., 2001a) belong to the levan neoseries while some fructans from T. aestivum (Bancal et al., 1992), Dactylis glomerata (Chatterton et al., 1993a), and Bromus tectorum (Chatterton et al., 1993b) belong to the levan series. In some species such as wheat and barley, fructans called graminans are structurally more complex and consist of (β-2,6)-fructose units with (β-1) branches (Bancal et al., 1992). Based on studies with barley, Wiemken et al. (1995) proposed the 1-SST/6-SFT model for graminan biosynthesis. Sucrose:fructan 6-fructosyltransferase (6-SFT) catalyses the formation of bifurcose (1&6 kestotetraose), using sucrose as fructosyl donor and 1-kestotriose, produced by 1-SST, as fructosyl acceptor. The 6-SFT also catalyses the elongation of fructan molecules by transferring fructosyl units from sucrose to fructan via (β-6) linkages (Duchateau et al., 1995). The first 1-FFT gene in members of Poales has been cloned recently from wheat (Kawakami and Yoshida, 2005). It produces (β-1) branches in fructans synthesized by 6-SFT. 6-SFT is probably a key enzyme in fructan biosynthesis in many cereal crops (wheat, barley) and cool-season grasses (crested wheatgrass, big bluegrass) (Sprenger et al., 1995; Wei and Chatterton, 2001; Wei et al., 2002). In Lolium species, however, 6-SFT might not be the enzyme responsible for the (β-6) linkages since the bifurcose is absent (Pavis et al., 2001a). Instead, fructans of the two neoseries prevail, suggesting an important role for the 6G-FFT enzyme. 6G-FFT activities and fructan levels peaked in the basal segment of elongating leaves and mature leaf sheaths (Pavis et al., 2001b).

Regardless of the agronomic importance of L. perenne as a major grassland species, only one fructosyltransferase cDNA of Lolium is functionally characterized: 1-SST has been identified and expressed in Pichia pastoris (Chalmers et al., 2003). Traditional plant-breeding approaches have been successful in producing high-fructan ryegrasses that have been shown to improve the nutritional value for ruminants (Miller et al., 2001). However, fructan biosynthesis regulation in L. perenne is still poorly understood. Greater understanding of the underlying mechanisms of fructan accumulation will benefit future breeding programmes.

The aim of this study was to investigate whether 6G-FFT represents a key enzyme for the biosynthesis of fructans of the inulin and levan neoseries in L. perenne. To do this, a cDNA clone encoding 6G-FFT from Lolium stubble, composed of elongating leaf bases and mature leaf sheaths, was isolated and characterized by heterologous expression in P. pastoris. The effects of developmental stage and carbohydrate status on the expression and tissue localization of this gene are described and compared with the gene expression pattern of 1-SST, along with the corresponding enzyme activities and fructan levels.

**Materials and methods**

**Plant material**

Seeds of Lolium perenne cv. Bravo were germinated in 9 dm³ 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 growing, 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 segments (0–40 mm) from the leaf base and a fifth variable length segment of about 40 mm]. Blades and emerged part of the elongating leaves were divided into three equal parts.

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 photon flux density of 300 μmol photons m⁻² s⁻¹ for up to 72 h, with roots and shoot meristems in the nutrient solution cooled at 5 °C. Control swards were grown under the original plant growth conditions, with a daylength of 16 h. Plants were divided into three parts; the sheaths of mature leaves, the blades of mature leaves together with the emerged parts of elongating leaves, and elongating leaf bases.

Each 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.

### Extraction and analysis of water-soluble carbohydrates

Soluble carbohydrates were extracted from 25 mg (elongating leaves), 50 mg (leaf sheaths) or 100 mg (emerged parts of elongating leaves and blades) of freeze-dried tissues as described previously (Morvan-Bertrand et al., 2001). The purification protocol used to remove charged compounds was performed according to Amiard et al. (2003). Glucose, fructose, sucrose, and fructans were quantified by high-performance liquid chromatography (HPLC) on a cation exchange column (Sugar-Pak 300 mm×6.5 mm, Millipore Waters, Milford, MA, USA) eluted with 0.1 mM CaEDTA in water using mannitol as the control standard (Guerrand et al., 1996).

### Protein extraction and in vitro fructosyltransferase activity assays

Leaf segments (1 g FW for elongating leaf bases, 2 g FW for blades and sheaths) were ground at 4 °C to a ratio of 2.5 cm⁻³ g⁻¹ fresh wt in 80 mM citrate phosphate buffer (pH 5.5) containing 5 mM dithiothreitol (DTT). The homogenate was centrifuged at 20 000 g for 10 min and the supernatant was desalted on Sephadex G50, which also eliminated small soluble carbohydrates from the extract.

Extraction was done in triplicate.

For 1-SST assay, the mixture consisted of 100 mm³ enzyme extract and 100 mm³ sucrose 100 mM in 80 mM citrate phosphate buffer pH 5.5. After incubation at 30 °C for 2 h, 100 mm³ mannitol (1 g dm⁻³) was added as an internal standard and the reaction was stopped by incubation in a boiling water bath for 3 min. For each extraction, triplicate samples were run together with duplicate enzyme blanks (no substrate).

For 6G-FFT assay, the mixture consisted of 50 mm³ enzyme extract, 25 mm³ sucrose at 200 mM, and 25 mm³ 1-kestotriose at 200 mM in 80 mM citrate phosphate buffer pH 5.5. After incubation at 30 °C for 4 h, 20 mm³ trehalose (60 mg dm⁻³) was added as an internal standard and the reaction was stopped by incubation in a boiling water bath for 3 min. For each extraction, triplicate samples were run together with duplicate enzyme blanks (no substrate).

The assay mixtures were separated and quantified by high-performance anion exchange chromatography and pulsed amperometric detection (HPAEC-PAD DX-300, Dionex, Sunnyvale, CA, USA) on an analytical CarboPac PA100 column (4 mm×250 mm) using a sodium acetate gradient (1 cm³ min⁻¹) in 90 mM NaOH. Using solutions A (90 mM NaOH) and B (90 mM NaOH and 500 mM NaOAc), the following running profile was applied: T=0, 100% A; T=4 min, 100% A; T=10 min, 98% A; T=20.1 min, 100% B; T=25 min 100% B; T=25.1 min, 100% A; T=30 min, 100% A. Fructan structures were deduced from their retention times on anion exchange chromatography by comparison with those of known standards. 6G-FFT activity was defined as the sum of both 6G-kestotriose and 1&6G-kestotetraose amounts formed per unit of time g⁻¹ fresh tissue upon incubation of the enzyme preparation in 50 mM sucrose and 50 mM 1-kestotriose.

To draw the calibration curves, 6G-kestotriose and 1&6G-kestotetraose were kindly provided by NJ Chatterton and N Shiomi, respectively.

### Preparation and screening of a cDNA library of Lolium perenne

Leaf sheaths and elongating leaf bases (1.5 g FW) 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) by 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 1330 bp of onion 6G-FFT (Vijn et al., 1997). This fragment was labelled with [α-³²P] dCTP by 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 made by Genome Express (Meylan, France). Nucleotide sequences were compared with sequences available in the NCBI Databank.

### Expression of isolated cDNA in Pichia pastoris

The putative coding region of mature Lp6GFFT (determined by sequence homology with the N-terminal sequence of the 6-SFT of barley (Sprenger et al., 1995) and the 1-SST of F. arundinacea (Lüscher et al., 2000) was amplified by PCR with the primers PIC6GFFT-F (5’-GTCCGGAATTCGGCGAGGTTCCTCGGTAGCCAAC-3’) and PIC 6GFFT-R (5’-GAC GCCTAGACTATAGGTCTTACCCATTACGCGTGT-3’). EcoRI and XbaI sites were incorporated into pPICZα-A vector. The plasmid was transformed into Escherichia coli competent cells as described by
Fig. 1. Comparison of the deduced amino acid sequence of 6G-FFT from *Lolium perenne*, cultivar Bravo (Lp6G-FFT, EMBL accession no. AF492836) with the putative 6G-FFT isolated from *Lolium perenne*, cultivar Aberystwyth S23 (Lp6G-FFT?, NCBI accession no. AB125218), the 6G-FFT from *Allium cepa* (Ac6G-FFT, EMBL accession no. AY07838), the 6G-FFT from *Asparagus officinalis* (Ao6G-FFT, EMBL accession no. AB084283) and the 1-SST of *Lolium perenne* (Lp1-SST, EMBL accession no. AY245431). Asterisks, colons, and periods indicate identical residues, conserved
Van den Ende et al. (2001). Cells were plated on 2× YT medium supplemented with zeocine as a selection marker. Positive clones were used for vector amplification. The P. pastoris wild-type strain X33 was transformed by electroporation with 20 μg PmneI-linearized pPICZα-A-Lp6G-FFT. Transformants were selected on YPDS/zeocine plates.

Production and characterization of the recombinant 6G-FFT
In order to produce the recombinant 6G-FFT enzyme for characterization, a 90 cm³ preculture medium (BMGY) was inoculated with single colonies and incubated overnight at 30 °C, 200 rpm. Cells were harvested by centrifugation and resuspended in 20 cm³ of induction medium (BMMY) and incubated for 4 d at 29 °C. Methanol was replenished every day to a final concentration of 2%. Protein purification was done by following the protocol described by De Coninck et al. (2005).

Aliquots were incubated at 30 °C with sucrose (final concentration 100 mM), sucrose and 1-kestotriose (100 mM and 50 mM, respectively), 1-kestotriose (final concentration 50 mM), and with 6G-kestotriose (final concentration 100 mM). For linkage analysis of the products, they were further incubated with heterologous 1-FEH IIa purified from chicory roots and previously expressed in P. pastoris (Verhaest et al., 2004). The carbohydrates produced were analysed by HPAEC (DX-300, Dionex, Sunnyvale, CA, USA). Carbohydrates were separated and quantified by high-performance anion exchange chromatography and pulsed amperometric detection. Carbohydrates of the assay mixture were separated and quantified by high-performance anion exchange chromatography and pulsed amperometric detection (HPAEC-PAD DX-300, Dionex, Sunnyvale, CA, USA) on an analytical CarboPac PA100 column (4 mm×250 mm) and pulsed amperometric detection. Carbohydrates of the assay mixture were separated and quantified by high-performance anion exchange chromatography and pulsed amperometric detection (HPAEC-PAD DX-300, Dionex, Sunnyvale, CA, USA) on an analytical CarboPac PA100 column (4 mm×250 mm) using a sodium acetate gradient (1 cm³ min⁻¹) in 90 mM NaOH. Using solutions A (90 mM NaOH) and B (90 mM NaOH and 500 mM NaOAc), the following running profile was applied: T=0, 100% A; T=4 min, 100% A; T=10 min, 98% A, 2% B; T=20.1 min, 100% B; T=25 min 100% B; T=25.1 min, 100% A; T=30 min, 100% A. Identities were assigned to peaks by comparison with commercial standards and identified onion carbohydrates.

In the pH experiment, 100 mM sodium citrate buffer (pH 3.4 to 5.4) and 100 mM sodium phosphate buffer (pH 5.75 to 8.25) were used. Aliquots of the enzyme were incubated with 100 mM sucrose and 50 mM 1-kestotriose (final concentrations) for 45 min at 30 °C. In the temperature-optimum experiment, reactions were performed at pH 5.4 at different temperatures (0–60 °C). 6G-FFT activity was based on 6G-kestotriose production. Experiments were done in triplicate.

RNA isolation and RT-PCR analysis
Plant tissues were ground in liquid nitrogen and suspended in a prewarmed (80 °C) solution consisting of 750 mM phenol and 750 mM extraction buffer (0.1 M LiCl, 100 mM TRIS–HCl, 10 mM EDTA, 1% (w/v) SDS, pH 8.0). After shaking, 750 mm³ of chloroform-isoamylalcohol (24:1 v/v) 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 mm³ of water treated with diethylpyrocarbonate (0.1%, v/v) and 250 mm³ of phenol-chloroform-isoamylalcohol (25:24:1 by vol.), mixed, and centrifuged for 5 min. RNA in the supernatant was precipitated again with 1 cm³ of absolute ethanol and 50 mm³ 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 mm³ RNase free-water. Samples were then treated by following the Clean-up protocol of the RNasey Minikit (Quiagen) coupled to a DNase treatment (RNase free-DNase; Quiagen, France).

One μg was used for retrotranscription by using the i-script cDNA synthesis kit (Bio-Rad, France). cDNA was then amplified by polymerase chain reaction (PCR) using 5'-GCCAGGTATCATCT-GCTCTAC-3' and 5'-CCCGCATGAGCTCGTAGTT-3' as specific primers for 1-SST (Chalmers et al., 2003), 5'-TCTCAACTCTTCG-GACATCGA-3' and 5'-TACATGTCGTCAGCCAAGAAG-3' as specific primers for 6G-FFT (Lasseur et al., 2002), 5'-CGGA-TAACCGTAGTAATTCTAG-3' and 5'-GTACTCTATCAAT-TACCAG AC-3' as primers for 18S rRNA. Amplification was achieved in the following conditions so that for each cDNA, products are analysed during the exponential phase of the PCR curve: 5 min at 95 °C; 30 cycles (1-SST, 6G-FFT) or 13 cycles (18S rRNA) of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and elongation at 72 °C for 1 min. PCR products were analysed by agarose gel electrophoresis and sequenced.

Results
Molecular characterization of Lolium perenne fructosyltransferase
A perennial ryegrass cDNA library, prepared from stubble of plants induced to accumulate fructan-related enzymes, was screened with a 32P-labelled insert of the onion 6G-FFT (Vijn et al., 1997). After repeated screenings, several positive clones were picked up. The longest cDNA was fully sequenced and consisted of 2508 bp, containing an open reading frame (ORF) of 1938 bp and a poly(A) sequence at its 3' end. The ORF encoded a polypeptide of 645 amino acids (Fig. 1) with six potential N-glycosylation sites, a calculated molecular weight of 60.6 kDa for the mature protein. The cDNA was first termed ‘putative 1-SST’ (Lasseur et al., 2002; accession no. AF492836) because of its high identity (81%) to the 1-SST polypeptide of Festuca arundinacea. It shares a similar identity (80%) with the deduced amino acid sequence of L. perenne 1-SST (Chalmers et al., 2003). Interestingly, the cDNA shows 98.3% identity at the nucleotide level and 99.4% identity at the amino acid level with a sequence isolated from another cultivar of L. perenne (Aberystwyth S23, accession no. AB125218) identified as a putative 6G-FFT. As far as is known, however, functional analysis of the encoded enzyme has not been published. When compared with other vacuolar-type fructosyltransferases and invertases, the cDNA had greater identity with sequences from graminaceous plants such as barley 6-SFT
and wheat invertase (61%) than with sequences for 1-SSTs, 6G-FFTs and invertases from liliaceous plants (53–59%) or 1-FFTs and 1-SSTs from asteraceous plants (47–51%) (Fig. 2). Furthermore, the sequence of the ‘putative 1-SST’ showed greater homology with vacuolar-type than with cell-wall type invertases belonging to the glycoside hydrolase family 32. The cDNA contains the so-called sucrose-binding box NDPNG.

Expression of recombinant protein in Pichia pastoris

The ‘putative 1-SST’ cDNA was expressed in P. pastoris to investigate the enzymatic properties of the protein. The recombinant protein showed fructosyltransferase activity (see below) and displayed optimum activity at pH 5.4, consistent with a vacuolar localization (Fig. 3). The protein showed a broad temperature optimum range, between 30 °C and 40 °C. Interestingly, the enzyme was still active at low temperatures.

Incubation with 100 mM sucrose alone showed the formation of small amounts of 1-kestotriose and 6G-kestotriose, products of 1-SST and 6G-FFT activities, respectively (Fig. 4A). So far 6G-FFTs with 1-SST activity have not been described. The small amount of 1-kestotriose produced makes it unlikely that the recombinant protein is a genuine 1-SST. Probably the enzyme first produces some 1-kestotriose from sucrose and then preferentially uses 1-kestotriose as a donor substrate to biosynthesize 6G-kestotriose. Also fructose was found, indicating that some fructosyl residues were transferred to water rather than to sucrose.

The production of 6G-kestotriose in these reactions was probably limited by the amount of 1-kestotriose formed in the incubation medium. Indeed, when the recombinant protein was incubated with both sucrose (100 mM) and 1-kestotriose (50 mM), 6G-kestotriose was produced within 10 min, together with nystose (1,1-kestotetraose) and 1&6G-kestotetraose (4c; FGFF) (Fig. 4B). Sucrose did not act as a fructosyl donor since no glucose was released during the first two hours. Nystose is the product of 1-FFT activity which transfers a fructose residue of 1-kestotriose to the terminal fructose unit of another 1-kestotriose. 1&6G-Kestotetraose (FGFF) could either be produced by a 6G-FFT activity or a 1-FFT activity, depending on the acceptor, 1-kestotriose or 6G-kestotriose, respectively. The fructan profile formed after 24 h resembles that of onion fructans. DP5 inulin and fructans from the DP5 inulin neoseries were formed. Consequently, the recombinant protein of L. perenne is able to synthesize fructans from the inulin series and fructans from the inulin neoseries simultaneously. It possesses both 6G-FFT and 1-FFT activities. The relative amounts of the three initial products suggest that in this enzyme, 6G-FFT is at most twice as active as 1-FFT.

Incubation with 1-kestotriose (50 mM) as a sole substrate led to the production of 6G-kestotriose, nystose, and 1&6G-kestotetraose (FGFF) within 10 min. Fructose and sucrose were also released indicating a 1-kestosidase like activity (Fig. 4C). The amount of 6G-kestotriose was similar to that produced by the recombinant protein incubated with both sucrose and 1-kestotriose (Fig. 4B). The amount of nystose and 1&6G-kestotetraose (FGFF), however, were higher, probably because of the lack of competition between the acceptors sucrose and 1-kestotriose. Consequently, fructans of DP >4 were synthesized more rapidly, i.e. within 30 min rather than 24 h.

Sucrose slows down the reaction. Fructans were produced within 45 min on condition that the sucrose concentration was smaller than the 1-kestotriose concentration (Fig. 4D, E).

In order to assess whether only (β2-1) linkages were produced by the recombinant protein, products of an overnight incubation with 1-kestotriose were boiled and further incubated for up to 3 h with the recombinant fructan 1-exohydrolase IIa (1-FEH IIa) from chicory expressed in P. pastoris (Verhaest et al., 2004) (Fig. 4F). Fructans disappeared progressively while 6G-kestotriose, fructose, and sucrose increased. 6G-kestotriose is a poor substrate for 1-FEH IIa and thus was expected to accumulate. Fructose and sucrose are products of 1-FEH IIa. Sucrose is not degraded further since 1-FEH IIa has almost no invertase activity. Removal of fructans other than 6G-kestotriose and accumulation of 6G-kestotriose indicate that the recombinant protein catalyses the formation of (β2-1) linkages.

**Fig. 3.** Effect of pH and temperature (pH 5.4) on 6G-FFT activity estimated by production of 6G-kestotriose generated by the recombinant protein produced in Pichia pastoris after incubation during 45 min with 100 mM sucrose and 50 mM 1-kestotriose in 100 mM sodium citrate buffer (closed circles) or in 100 mM sodium phosphate buffer (open circles).
Fig. 4. High performance anion-exchange chromatograms of the reaction products generated by the *Pichia pastoris* expressed protein. Reaction mixtures containing the recombinant protein were incubated for up to 24 h at 30 °C with 100 mM sucrose (A), 100 mM sucrose and 50 mM 1-kestotriose (B), 50 mM 1-kestotriose (C), 100 mM 6G-kestotriose, for 45 min at 30 °C with 100 mM sucrose and 5–500 mM 1-kestotriose (D), 20 mM 1-kestotriose and 5–500 mM sucrose (E) or 100 mM 6G-kestotriose (G). Extract previously incubated for 1 h with sucrose and 1-kestotriose (100 mM each).
When 6G-kestotriose was given as the main substrate (Fig. 4G), 1,6G-kestotetraose (4b; FGGF) and sucrose were produced while the amount of initial 1&6G-kestotetraose (4c; FGFF) decreased. 1,6G-kestotetraose (FGFF) could be produced by fructosyl transfer from 1&6G-kestotetraose (FGFF) to 6G-kestotriose as described for asparagus (Ueno et al., 2005). However, glucose-linked fructosyl retrieval from 1&6G-kestotetraose (FGFF) would liberate a 1-kestotriose, but 1-kestotriose was not released in the medium. The transfer of fructose-linked fructosyl from 1&6G-kestotetraose to 6G-kestotriose liberating 6G-kestotriose is another possibility. 6G-Kestotriose can also act as a fructosyl donor towards another 6G-kestotriose molecule (Fig. 4B). This is probably the major pathway for the synthesis of 1,6G-kestotriose (FGF) since an important release of sucrose occurs. The observed decrease of the 1&6G-kestotetraose (FGFF) amount (Fig. 4G) makes it unlikely that it is produced from 6G-kestotriose. No fructan of DP5 (5c, 5d) were produced, indicating that 1-kestotriose and/or nystose are necessary for their synthesis.

From the results of these experiments, a possible pathway for oligosaccharides of inulins and inulin neoseries formed by the recombinant 6G-FFT from L. perenne is summarized in Fig. 5A.

**Effect of leaf developmental stage on soluble carbohydrates, enzyme activities and gene expression pattern of Lp1-SST and Lp6G-FFT**

Leaf growth in grasses is confined to the basal region, which is enclosed by the sheaths of mature leaves (Schneider et al., 1990). Cells are displaced away from their origin as a result of continued production and elongation of new cells. The tissue that emerged from the enclosing leaf sheaths is almost fully differentiated and photosynthetically active (Wilhelm and Nelson, 1978). Four×1 cm segments and a fifth longer segment were cut, starting from the base of growing leaves and leaf sheaths. Leaf blades and the emerged parts of growing leaves were divided into three parts of equal length (Fig. 6A). From each segment, carbohydrates, proteins, and total RNA were extracted. A 300 bp fragment of Lp1-SST cDNA, a 501 bp fragment of Lp6G-FFT cDNA and a 383 bp fragment of 18S rRNA were amplified by RT-PCR from each sample adjusted to contain equal amounts of RNA. In elongating leaves and in mature leaf sheaths, activity of 1-SST and 6G-FFT was greatest in the basal segment where the accumulation of fructan and sucrose was maximal and where transcripts levels of Lp1-SST and Lp6G-FFT were amplified in largest amounts (Fig. 6B, C). Together with fructan and corresponding enzyme activities, transcripts of Lp1-SST and Lp6G-FFT dropped subsequently along the axis of leaf sheaths and of enclosed parts of elongating leaves, so that they became barely detectable in the fifth segment of each tissue (Fig. 6C). In photosynthetically active tissues (Fig. 6B, segments 6, 7, 8), fructans were present at lower amounts than in sink tissues. Fructan synthesizing activities were also very low and Lp1-SST mRNA fragments were not detected (Fig. 6C, segments 6, 7, 8). Surprisingly, Lp6G-FFT mRNA fragments were amplified, especially in central part segments (Fig. 6C, segment 7) where they reached similar amounts as in basal segments (Fig. 6C, segment 1). Glucose and fructose levels peaked in the third segment of elongating leaves (Fig. 6B), probably as a result of high invertase (data not shown) and FEH activity found in the corresponding maturation zone (Morvan-Bertrand et al., 2001).

**Transcript levels of Lp6G-FFT and Lp1-SST, enzyme activities and carbohydrate levels 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., 2001b; Wei et al., 2001). After several time intervals, shoots were harvested and dissected into leaf blades, leaf sheaths, and elongating leaf bases. Fructans accumulated first in elongating leaf bases and in leaf sheaths (Fig. 7A, B). After 72 h, fructans increased slightly in leaf blades (Fig. 7C). The first 6 h of treatment had no effect on the fructan pattern, but after 6 h, fructans increased about 5-fold within 66 h in both elongating leaf bases and in leaf sheaths to reach high levels, 250 mg g⁻¹ DW and 230 mg g⁻¹ DW, respectively. By contrast, fructans only slightly increased in leaf blades after 48 h of treatment. Surprisingly, the fructan precursor sucrose showed a similar accumulation pattern in all leaf tissues, including leaf blades. It increased slightly during the first 6 h of treatment, rapidly accumulated within the following 18 h to reach about 70 mg g⁻¹ DW, and decreased thereafter. Glucose and fructose followed similar but tissue-specific patterns, glucose levels being always slightly higher than fructose levels in tissues where fructan accumulated. Proteins and total RNA were extracted from the Lolium leaves from which carbohydrates were isolated. Both 1-SST and 6G-FFT activities strongly increased in elongating leaf bases between 6 h and 24 h of treatment whereas 1-SST activity only slightly increased in leaf sheaths. Initial in vitro activities were lower in leaf sheaths.
than in elongating leaf bases on a fresh wt basis, but they were similar in both tissues when expressed on a protein wt basis (data not shown). In leaf blades, 6G-FFT activity was low and similar to that in leaf blades of control plants. The gene expression patterns of Lp1-SST and Lp6G-FFT followed the curve of corresponding enzyme activities in leaf sheaths and elongating leaf bases upon the induction of fructan accumulation. In elongating leaf bases, the amount of Lp1-SST and Lp6G-FFT transcripts slightly decreased during the first 6 h of treatment, but increased again thereafter. In leaf sheaths, the transcripts levels of Lp1-SST increased within the first 24 h while the amount of Lp6G-FFT transcripts was not affected by the treatment. In leaf blades, the transient small increase in 1-SST activity during the first 6 h of treatment was not correlated with an increase in Lp1-SST transcripts. Instead, Lp1-SST transcripts increased in leaf blades of illuminated plants after 24 h of treatment while transcript levels of Lp6G-FFT were higher than that of Lp1-SST but remained constant throughout the experiment.

**Discussion**

Lp6G-FFT has 1-FFT activity

In this paper, the isolation and the functional characterization of the first grass 6G-FFT/1-FFT obtained from *L. perenne* are described.

The primary structure of known enzymes catalysing the different transfructosylations in one plant family seems to be more closely related to one another than those of enzymes catalysing the same reaction in other plant families. The Lp6G-FFT also shares more identity with fructosyltransferases and invertases of gramineous plants than with the two known 6G-FFTs of liliaceous plants.

The recombinant protein obtained from *P. pastoris* has a predominant 6G-FFT activity. This enzyme is therefore a key enzyme in *L. perenne* since it catalyses the formation of fructan of the inulin neoseries. In addition, the enzyme catalyses the biosynthesis of inulins as well as the formation of (β2-1) linkages onto fructans of the inulin neoseries. Previously, these fructans were reported to be synthesized by separate 6G-FFT and 1-FFT activities in asparagus (Shiomi, 1981, 1982, 1989) or in onion (Shiomi et al., 1997). Recently, such a 1-FFT activity has been reported for the native 6G-FFT of onion (Fujishima et al., 2005) or asparagus (Ueno et al., 2005). The recombinant protein of perennial ryegrass has a relative 1-FFT to 6G-FFT activity (at most 0.5) similar to that of native onion 6G-FFT/1-FFT enzyme (0.48, Fujishima et al., 2005), whereas the recombinant protein of asparagus has only a 1-FFT activity 13 times lower than the 6G-FFT activity (Ueno et al., 2005). In this species, the native 6G-FFT has a negligible 1-FFT activity (Shiomi, 1981). The general properties of *L. perenne* 6G-FFT resemble those of 6G-FFT from onion bulbs. Both enzymes are bifunctional, almost free of 1-kestotriose hydrolysing activity and free from invertase activity. When incubated with 1-kestotriose as sole substrate, both enzymes produced 1&6G-kestotetraose (4c; FGFF), nystose and small amounts of 6G-kestotriose with the liberation of sucrose at the initial reaction time. Recombinant Lp6G-FFT and native onion 6G-FFT also show differences. Indeed, native onion 6G-FFT, but not Lp6G-FFT, produced 1-kestotriose and 1&6G-kestotetraose (4c; FGFF) in the presence of 6G-kestotriose. Moreover, native onion 6G-FFT has no 1-SST activity with sucrose as the sole substrate, whereas Lp6G-FFT formed small amounts of 1-kestotriose with the liberation of glucose. However, the existence of a triple-function 6G-FFT/1-FFT/1-SST protein in *L. perenne* is unlikely and a separate 1-SST protein has been recently cloned and characterized from this species (Chalmers et al., 2003). To determine whether these properties are artefactual, for example, due to...
Fig. 6. Tissue distribution of fructans, sucrose, glucose, and fructose (mg g$^{-1}$ dry wt) (B), 1-SST and 6G-FFT activities (nkat g$^{-1}$ fresh wt) (C) as well as 1-SST and 6G-FFT transcripts as compared with 18S rRNA transcripts (C). After 8 weeks of growing, plants were harvested and the mature leaves (open circles) separated from the elongating leaves (closed circles). Sheaths and elongating leaf bases previously enclosed by the sheaths were dissected longitudinally into five segments (four $\times$ 10 mm segments (0–40 mm) from the leaf base and a fifth variable length segment of about 40 mm). Blades and emerged part of the elongating leaves were divided into three equal parts (A). Gene expression was determined by PCR using gene-specific primers and visualized by ethidium bromide staining. Vertical bars represent $\pm$ SE ($n$=3).
slight differences in folding or glycosylation pattern (Hochstrasser et al., 1998), it will be necessary to purify the native enzyme from L. perenne to express the gene in another expression system. A partially purified FFT from Lolium rigidum, free of invertase activity, was able to use 1-kestotriose and 6G-kestotetraose as sole substrates, and produced 6G-kestotriose as well as nystose and 1&6G-kestotetraose (4c; FGFF) when assayed with both 1-kestotriose and sucrose (St John et al., 1997a, b). This native Lolium FFT had similar properties as the recombinant Lp6G-FFT. Interestingly, it produced 1&6G-kestotetraose and small amounts of 1-kestotriose with 6G-kestotriose as the sole substrate and it was free of 1-SST activity. These data indicate that the small differences between onion and ryegrass 6G-FFT properties are possibly due to the yeast expression system.

The Lp6G-FFT enzyme synthesized fructans with only (β2-1) linked fructosyl residues. All possible reaction products of the 6G-FFT and 1-FFT activity of Lp6G-FFT up to DP5 and DP4, respectively, were found in extracts from leaves and roots of L. perenne (Pavis et al., 2001a): 6G-kestotriose (FGF), nystose (GFFFF), 1,6G-kestotetraose (4b; FFGG), 1&6G-kestotetraose (4c; FGFF), 1,1&6G-kestopentaose (5c; FGFFFF), 1&1,6G-kestopentaose (5d; FGGFF). The only oligofructans not synthesized by Lp6G-FFT were the DP4 and the DP5 with (β2-6) linkages. Since Lp6G-FFT has a clear 1-FFT activity, there might be no need for a separate 1-FFT protein, as is the case in onion (Ritsema et al., 2003). By contrast, a separate 1-FFT does exist in asparagus (Ueno et al., 2005). Conclusively, fructans present in L. perenne might be synthesized by a three-enzyme system instead of a four-enzyme system as previously suggested (Pavis et al., 2001a): 1-SST, 6G-FFT together with 1-FFT and 6-(S/F)FFT (Fig. 5B). However, the sequence of a putative 1-FFT homologue has been submitted to the NCBI database. Functional analysis of the encoded enzyme might reveal whether an independent 1-FFT function exists in L. perenne. As far as 6G-FFT is concerned, several sequences might exist (Hisano et al., 2004). The sequences could represent the same gene in different L. perenne varieties. They could also be alleles of the same gene or members of a multigene family of 6G-FFT in the L. perenne genome.

In this species, the enzyme that produces the predominant (β2-6) is not known yet.

The sucrose binding-box of the Lp6G-FFT protein contains the β-fructosidase motif NDPNG highly conserved in invertases, but also invariably found in 1-SST of F. arundinacea (Lüscher et al., 2000), T. aestivum, and L. perenne (Chalmers et al., 2003). The motif is present with some variations in other fructosyltransferases (Ritsema et al., 2004). NDPNG instead of NDPNG was found in the two other known 6G-FFTs from onion and asparagus. Consequently, Ser in the β-fructosidase motif is not an important amino acid for 6G-FFT activity. This corroborates the recent finding of Ritsema et al. (2004) who demonstrated that the shift from NDPSG to NDPNG by site-directed mutagenesis in onion 6G-FFT did not affect the enzyme specificity.

**Regulation of 6G-FFT expression is tissue-specific**

In several grasses, large fructan stores are deposited temporarily in the growth zone at the base of elongating leaves and in mature leaf sheaths (Lüscher and Nelson, 1995; Roth et al., 1997; Schnyder and Nelson, 1989; Pavis et al., 2001b). The expression pattern of Lp6G-FFT was studied in leaf tissues using semi-quantitative RT-PCR and compared with that of Lp1-SST.

In heterotrophic leaf tissues: The largest amount of Lp6G-FFT and Lp1-SST fragments were amplified from the first segment of the base of elongating leaves and leaf sheaths. It declined thereafter. This is consistent with findings for other gramineae such as F. arundinacea (Lüscher et al., 2000), L. perenne cv. Citadel (Chalmers et al., 2003), and L. temulentum (Gallagher et al., 2004). In the latter species, the putative fructosyltransferase FT 2:2 was mostly expressed in tiller bases where fructans were generally localized. In growing leaf bases of F. arundinacea, 1-SST transcript levels matched the 1-SST activity (Lüscher and Nelson, 1995) and the fructan content (Schnyder and Nelson, 1989), suggesting that 1-SST activity was mainly regulated at the transcriptional level (Lüscher et al., 2000). Similar results were obtained in the present study (Fig. 6). Indeed, the amount of messages in elongating leaf bases and leaf sheaths followed the curve of 1-SST and 6G-FFT activities and fructan content. This result indicates that in sink tissues Lolium fructosyltransferases are also predominantly controlled at the transcriptional level. Post-transcriptional regulation might also occur since Lp6G-FFT and Lp1-SST transcripts were generally more abundant in leaf sheaths than in elongating leaf bases while the corresponding activities measured in vitro were much higher in elongating leaf bases than in leaf sheaths (Fig. 6C). It was demonstrated before that addition of cycloheximide and actinomycin D could decrease the amount of extractable 1-SST activity from leaf sheaths of illuminated Lolium plants, suggesting that activity increases occurred...
by de novo synthesis of proteins (Guerrand et al., 1996). The present results further support this point of view. Similarly, increases in 6G-FFT activity and transcripts also occurred in elongating leaf bases by the exposure of plants to low root temperatures and continuous illumination (Fig. 7A). It is therefore hypothesized that the corresponding protein is de novo synthesized. However, for a definite answer, the protein level of the two fructosyltransferases should be followed over time.

1-SST initiates fructan biosynthesis by producing 1-kestotriose. 6G-FFT is one of the elongating enzymes that use 1-kestotriose to produce longer fructans of specific types. In barley, where no fructan of the neo-series accumulate, 6-SFT and 1-FFT are the elongating enzymes. Roth et al. (1997) suggested that 1-FFT might also play a role in fructan degradation by modifying the size distribution of the fructan chain since it occurred in the elongating leaf zone where fructans are degraded. In L. perenne, 6G-FFT does not seem to fulfil this function as the activity decreased continuously along the axis of the growing leaf including the maturation zone where fructans are degraded.

In autotrophic leaf tissues: Surprisingly, Lp6G-FFT was expressed in mature leaf blades and the emerged parts of elongating leaves where extractable 6G-FFT activity was very low and fructans barely accumulate (Fig. 6). Here, Lp1-SST transcripts were not detected. Lp6G-FFT transcripts were more abundant in the central region of elongating leaves than in other parts of the leaves. Here, Lp6G-FFT levels are not indicative of enzymatic activity, suggesting post-transcriptional regulation of expression. 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, Lp1-SST transcripts accumulate in leaf blades that were continuously illuminated but no increase in 1-SST activity was observed (Fig. 7C). Therefore, in photosynthetically active tissue according to its sink–source status.

In conclusion, this is the first time a grass 6G-FFT cDNA has been cloned and functionally characterized. This enzyme has an important 1-FFT activity and produces both inulins and inulin neoseries. Regulation of Lp6G-FFT gene expression depends on the tissue according to its sink–source status.

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