Journal of Experimental Botany, Vol. 57, No. 1, pp. 213–223, 2006
doi:10.1093/jxb/erj031 Advance Access publication 5 December, 2005

Journal of Experimental Botany

RESEARCH PAPER

Purification, cloning and functional characterization of a fructan 6-exohydrolase from wheat (*Triticum aestivum* L.)

Liesbet Van Riet[1], Vinay Nagaraj[2], Wim Van den Ende[1], Stefan Clerens[3], Andres Wiemken[4] and André Van Laere[1,*]

[1] *Laboratory of Molecular Plant Physiology, Institute of Botany and Microbiology, K.U. Leuven, Kasteelpark Arenberg 31, B-3001 Leuven, Belgium*

[2] *The Biodesign Institute at Arizona State University, 9709, ECG 334, Tempe, Arizona 85287-9709, USA*

[3] *Laboratory of Neuro-endocrinology and Immunological Biotechnology, Zoological Institute, K.U. Leuven, Naamsestraat 59, B-3000 Leuven, Belgium*

[4] *Zurich Basel Plant Science Center, Botanische Institut der Universität Basel, Hebelstrasse 1, CH 4056 Basel, Switzerland*

Received 12 May 2005; Accepted 25 October 2005

Abstract

Fructans, β2-1 and/or β2-6 linked polymers of fructose, are produced by fructosyltransferases (FTs) from sucrose. They are important storage carbohydrates in many plants. Fructan reserves, widely distributed in plants, are believed to be mobilized via fructan exohydrolases (FEHs). The purification, cloning, and functional characterization of a 6-FEH from wheat (*Triticum aestivum* L.) are reported here. It is the first FEH shown to hydrolyse exclusively β2-6 bonds found in a fructan-producing plant. The enzyme was purified to homogeneity using ammonium sulphate precipitation, ConA affinity-, ion exchange-, and size exclusion chromatography and yielded a single band of 70 kDa following SDS-PAGE. Sequence information obtained by mass spectrometry of in-gel trypsin digests demonstrated the presence of a single protein. Moreover, these unique peptide sequences, together with some ESTs coding for them, could be used in a RT-PCR based strategy to clone a 1.7 kb cDNA. Functionality tests of the cDNA performed after heterologous expression in the yeast *Pichia pastoris* showed—as did the native enzyme from wheat—a very high activity of the produced protein against bacterial levan, 6-kestose, and phlein whilst sucrose and inulin were not used as substrates. Therefore the enzyme is a genuine 6-FEH. In contrast to most FEHs from fructan-accumulating plants, this FEH is not inhibited by sucrose. The relative abundance of 6-FEH transcripts in various tissues of wheat was investigated using quantitative RT-PCR.

Key words: 6-FEH, fructan exohydrolase, fructans, grasses, invertases, levan, *Pichia* expression, *Triticum aestivum*, wheat.

Introduction

Fructans, which occur in several bacterial groups and in about 45 000 angiosperm species (Hendry, 1993) function, just like starch, as reserve carbohydrates in plants. For a general review on fructans and their metabolism in fructan plants we refer to Vijn and Smeekens (1999) and Ritsema and Smeekens (2003). Dicotyledonous plants accumulate fructans that consist mainly of β2-1 bound fructose units, whereas monocotyledonous species accumulate fructans with mainly β2-6 linkages. Temperate grasses like wheat (*Triticum aestivum* L.) or barley (*Hordeum vulgare* L.), mainly store mixed-type fructans (graminans) with β2-6 fructose linkages, some branch points with β2-l linked fructoses, and a terminal glucose molecule (Bancal *et al*., 1992; Bonnett *et al*., 1994, 1997). *Lolium perenne* L. fructans contain predominantly β2-6 linkages derived from neokestose, while *Dactylis glomerata* L. (Bonnett *et al*., 1994, 1997; Yamamoto and Mino, 1985) and *Phleum*

* To whom correspondence should be addressed. E-mail: Andre.Vanlaere@bio.kuleuven.ac.be
Abbreviations: FEH, fructan exohydrolase; MS, mass spectrometry; Q-TOF, Quadrupole-Time-of-flight; PAUP, Phylogenetic Analysis Using Parsimony; EST, Expressed sequence tag.

pratense L. (Cairns *et al*., 1999) accumulate linear β2-6 linked fructans. Different functions are attributed to fructans in grasses: they are mobilized from stems for grain-filling at the end of the growing season, or for other sink activities (Prud'homme *et al*., 1992; Bonnett and Incoll, 1993; Schnyder, 1993; Willenbrink *et al*., 1998; Morvand-Bertrand *et al*., 2001). Complete breakdown of fructan chains requires the combined action of β2-1 and β2-6 hydrolysing activities.

In wheat, Bancal *et al*. (1992) suggested that the graminan-type fructans are 'trimmed' by 1-FEH activity. Since 1-FEH activity is also present during fructan biosynthesizing stages it could play a role in reducing the β2-1 linkages in favour of short branches or β2-6 linkages. However, the identified 1-FEH isoforms in wheat do not show high activity against bifurcose or other small branched fructans (Van den Ende *et al*., 2003*a*).

Although attempts have been made to purify FEH from grasses (Yamamoto and Mino, 1985; Henson, 1989; Simpson *et al*., 1991; Bonnett and Simpson, 1995), only few FEHs have been purified to homogeneity (Henson and Livingston, 1996; Marx *et al*., 1997; Van den Ende *et al*., 2003*a*). Only recently has the cDNA of 1-FEH isoforms from grass species been cloned (Van den Ende *et al*., 2003*a*; Nagaraj *et al*., 2005). To our knowledge no 6-FEH cDNA from a fructan plant has ever been cloned.

However, recently, a 6-FEH from non-fructan plants was characterized (Van den Ende *et al*., 2003*b*; De Coninck *et al*., 2005). This 6-FEH was proposed to fulfil a role in pathogen defence.

It is striking that FEH cDNAs show more similarity with cell wall invertases than with vacuolar invertases. They also contain the conserved WECPD motif, which is typical for cell wall-like invertases (Tymowska-Lalanne and Kreis, 1998). Even though FEH activity has been reported in vacuolar compartments (Wagner and Wiemken, 1986; Wiemken *et al*., 1986) it was proposed that fructan exohydrolysing enzymes, contrary to fructan synthesizing enzymes, are derived from cell wall-like invertase ancestors (Van den Ende *et al*., 2002). However, most of the FEHs characterized up to now have a low isoelectric point, while typical cell wall invertases can have a high pI for improved binding to the cell wall.

As a contribution to understand the complex regulation of fructan synthesis and breakdown, 6-FEH activity has been investigated in wheat. One 6-FEH isoform was purified, its cDNA cloned, and its activity confirmed by heterologous expression in *Pichia pastoris*. As can be expected in a hexaploid species, several other isoforms are present and the characterization of one of them is insufficient to understand fructan breakdown in all physiological stages and all tissues. However, since the isoform was isolated from spikes with developing seeds, it might be useful, for example, in an RNAi-based approach to limit fructan breakdown in ripening grains in order to increase the content of the pre-biotic fructans in the major staple food of large parts of the world.

Material and methods

Plant material

Wheat (*Triticum aestivum* L. cv. Pajero) was sown and grown in local fields with sandy loam soil in a temperate climate during the growing seasons 2002, 2003, and 2004. Spikes and other tissues at different morphological stages were harvested, stored at −80 °C and used for purification, activity, and expression measurements of the 6-FEH.

Enzyme extraction and purification

Three kilograms of spike tissue (roughly at soft dough stage) were homogenized in a Waring blender with 3.0 l 50 mM sodium acetate buffer (NaAc) pH 5 containing 0.02% sodium azide, 1 mM PMSF, 0.1% Polyclar AT, 1 mM 2-mercaptoethanol, 10 mM $NaHSO_3$, and 20% $(NH_4)_2SO_4$. The homogenate was filtered through cheesecloth and centrifuged for 20 min at 12 000 *g*. Proteins in the supernatant were pelleted in 80% $(NH_4)_2SO_4$. The precipitate was redissolved in 400 ml 50 mM NaAc, pH 5 containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM $MnCl_2$. This fraction was applied to a ConA Sepharose column (25×100 mm). The bound proteins were eluted with 500 mM α-methyl-D-mannoside. This eluted fraction, containing only glycosylated proteins, was passed over a monoQ column in 50 mM TRIS-HCl pH 7.5 and applied on a monoS column (both columns: Pharmacia Biotech HR5/5, Uppsala, Sweden) in 20 mM HEPES buffer pH 8.2 and eluted from the latter with a linear gradient from 0–0.3 M NaCl. The fractions containing β2-6 hydrolysing activity were pooled and applied to a Superdex-75 column (Pharmacia Biotech, Upsala, Sweden) in 50 mM NaAc pH5.

After SDS-PAGE in 12.5% (w/v), the polyacrylamide gel was stained with Coomassie Brilliant Blue-R250. Whenever possible, enzymes were kept on ice and 0.02% sodium azide (w/v) was added to all buffers to prevent microbial growth.

Enzyme activity measurements and substrates

Proteins were diluted in 50 mM NaAc pH 5 and incubated with substrates for different time intervals at 30 °C. Enzyme amounts and/or incubation times were adjusted to result in the linear production of fructose during the incubation period. The reaction was terminated by heating at 90 °C for 5 min. The substrates used were 10 mM inulin (Sigma), 10 mM sucrose or 10 mM low molecular weight levan (M_r 8000, DP 72) in 50 mM NaAc buffer pH 5. For testing the substrate specificities of both the native and the heterologous 6-FEH, a concentration of 1 mM was used for all fructan substrates (Table 1). Reaction products were analysed by anion exchange chromatography with pulsed amperometric detection (AEC-PAD) as described by Van den Ende and Van Laere (1996). Proteins were determined by the method of Sedmak and Grossberg (1977).

1-Kestose and 1,1 nystose were prepared from neo-sugarP (Beghin-meiji Industries, Paris, France) by preparative reversed-phase HPLC (nucleosil 7 C 18, 250×12.7 mm) with water as a solvent and a flow rate of 2 ml min^{-1}. Manually collected fractions were pooled and lyophilized. Low M_r levan and 6-kestose were generous gifts from Dr M Iizuka (Iizuka *et al*., 1993). Neokestose and phlein were kindly provided by Dr NJ.Chatterton. Levanbiose (F2) was purified as described by Timmermans *et al*. (2001). The mean DP, phlein and inulin was estimated from the fructose/glucose ratio after mild acid hydrolysis in 60 mM HCl at 70 °C for 75 min. Subsequently, molar concentrations were estimated.

Table 1. *Comparison of the substrate specificities (1 mM) of the native wheat 6-FEH and the heterologously expressed wheat 6-FEH cDNA in* Pichia pastoris

Results are shown as values relative to the activity with bacterial levan (B) as substrate. The value for 100% corresponds with 13 650 nmol mg^{-1} protein min^{-1} for the native protein and 26 600 nmol mg^{-1} protein min^{-1} for the *Pichia*-derived protein. Inulin from *Cichorium intybus* L. (*C.i.*) was used. Experiments were carried out three times and the SE was always <5% of the mean.

Substrate			Native activity (%)	Heterologous activity (%)
Levan (B)	β2-6	DP±72	100	100
6-kestose	β2-6	DP 3	101	85
n-kestose	β2-6	DP 3	39	45
Phlein	β2-6	DP 4–12	95	120
Levanbiose	β2-6	DP 2	63	57
1-kestose	β2-1	DP 3	≪1	≪1
Nystose	β2-1	DP 4	≪1	≪1
Inulin (*C.i.*)	β2-1	DP >10	≪1	≪1
Sucrose		DP 2	≪1	≪1
Levan (B) + 5–200 mM suc			100	100

Q-TOF analysis on tryptic fragments

The SDS-PAGE protein band was subjected to MS identification. The Coomassie Brilliant Blue stained protein band was excised, trypsinized, extracted, desalted, and analysed on Q-TOF as previously described (Van den Ende *et al*., 2003*a*). Sequence information was derived from the MS/MS spectra with the aid of MaxEnt3 (deconvoluting and deisotoping of data) and PepSeq software from the MicromassBioLynx software package.

RNA isolation, RT-PCR and cloning

Total RNA was prepared from different wheat tissues, stored at −80 °C, by using the RNeasy Plant Mini kit (Qiagen, Valencia, CA, USA). Degenerate primers were designed based on the Q-TOF MS/MS internal sequences: VGLLGA, FPKNP, and the conserved amino acid sequence WECPD from cell wall-type invertases. All primers used in the cloning scheme (Fig. 1) are presented in Table 2.

RNA, prepared from wheat inflorescences at the same stage as used for the purification of the protein, was transcribed with one step RT-PCR (Access RT-PCR system, Promega Madison, WI, USA) using the primers FPKNP and VGLLGA. Only a faint band with the expected M_r appeared and semi-nested PCR was performed with HALFFOR (Step 1 in Fig. 1). The clear resulting band was cut out, purified with the Qiaquick gel extraction kit (Qiagen, Valencia, CA, USA) and ligated in the TOPO-TA vector and transformed into *E. coli* (TOPO-TA cloning kit, Invitrogen, Groningen, The Netherlands). Plasmids from a number of clones were extracted using the Qiaprep spin miniprep kit (Qiagen, Valencia, CA, USA). After sequencing, a new specific primer was designed based on the obtained sequence EEGRLGY and combined with a primer MAARPL at the 5′ end in step 2 (Fig. 1). This primer was distilled from ESTs from *Triticum* (accession number BJ211844 and CD932035) which coded for Q-TOF MS/MS identified internal peptides. The following PCR procedure was used: first single-strand cDNA was synthesized, using the AMV reverse transcriptase (10 u μl^{-1}) (Promega, Madison, WI, USA). The cDNA was used as a template for the PCR reaction with the forward and reverse primer in the PCR reaction. The 'Expand long template PCR system' kit (Boehringer Mannheim, Mannheim, Germany) with supplements of $MgCl_2$ and parameters were followed as prescribed by the suppliers. The clear PCR band was cut out of the gel and purified using the 'geneclean SPIN kit' (Q-Biogene, USA) and ligated into pGEMT-vector (Promega, Madison, USA) and transformed into *E. coli*. Plasmids from a number of clones were extracted, using the E.Z.N.A plasmid miniprep kit (Peqlab, Germany) and fully sequenced.

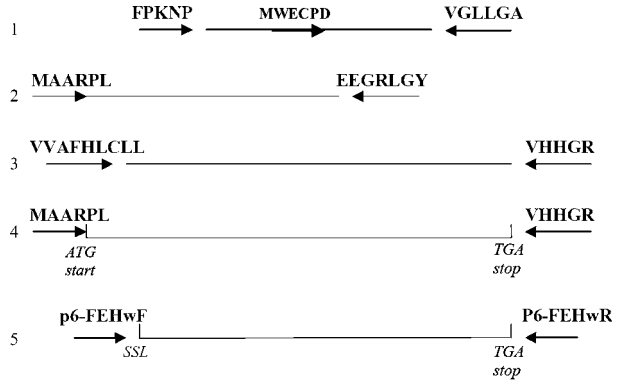

Fig. 1. Cloning scheme (step 1–4) of the wheat 6-FEH cDNA. Step 5: cloning of the cDNA that codes for the mature protein which was expressed in *Pichia pastoris*. Primers are not drawn to scale; the tip of the arrowhead indicates the position of the end of the primers.

This obtained sequence was satisfactory since it overlapped perfectly with the previously obtained partial sequence and the identified internal peptides were present. To clone the 3′ end in step 3 a primer at the stop codon was designed based on homologous ESTs from *Triticum* BJ243363 and CN010968. This primer was combined with the primer VVAFHCLL in a one step RT-PCR. By overlapping all the sequences of the previous PCR products, the full sequence could be predicted. As a control, a one step RT-PCR was performed with the primers at the start codon MAARPL and at the stopcodon VVHGR (step 4). After ligation into the TOPO-XL (Topo–XL cloning kit, Invitrogen) vector and transformation into *E. coli*, the plasmids were fully sequenced. During the whole cloning strategy PCR-products were analysed by electrophoresis in a 1% TAE gel and stained with ethidium bromide. The sequence was deposited in the EMBL sequence library (accession no. AM075205)

The cDNA was analysed using the software tools available on the internet (www.expasy.com). Alignment of the sequences was performed using Clustal X (version 1.80) and subsequent cladistic analysis was carried out without an outgroup in PAUP Version 4.0b10. A heuristic search strategy was used to find minimum-length trees: searches were conducted with 10 000 random-addition replicates (tree bisection–reconnection (TBR), MulTrees on). The level of support for individual clades was determined by a bootstrap analysis (NPB) (Felsenstein, 1985). The bootstrap as implemented by PAUP was carried out with 1000 pseudoreplicates and 10 random addition sequence repetitions.

Expression in Pichia

To construct the expression plasmid p6FEHW containing the mature protein, a primer with a restriction site for *Eco*RI was designed for the N-terminus as predicted by comparison with other homologous FEHs and the SignalP predict protein program (Bendtsen *et al*., 2004). A primer coding for the stop codon was designed for the C-terminus and contained the *Xba*I restriction site (Table 2). The cDNA was obtained after a two-step high fidelity RT-PCR (Prostar ultra high fidelity RT-PCR system, Stratagene, Ca, USA) (step 5 in Fig. 1). Two independent RT-PCR reactions were performed using the following procedure: the RNA was first heated for 5 min at 65 °C. The

Table 2. *Primers used for cDNA cloning*, Pichia *expression and RT-PCR of the 6-FEH from wheat*

Restriction sites are indicated in bold. In the degenerate primers the following symbols are used: N=(A, C, G or T), Y=(C, T).

Primer	Sequence
FPKNP	5′-TTCCCNAAGAACCCNGCC-3′
VGLLGA	5′-CGCTCCNAGYAAGCCNACAAC-3′
WECPD	5′ GAATGTGGGARTGYCCNGA-3′
MAARPL	5′-CCATGGCCGCCAGACTCCCTCTC-3′
EEGRLGYA	5′-CGCGTAGCCAAGTCGCCCCTCCTC-3′
VVAFHLCLL	5′-GYCGTCGCGTTCCACCTCTGCCTYCTC-3′
VVHGR	5′-ATCACCTGCCATGGACAAC-3′
p6-FEHwF	5′-AATCCC**GAATTC**AGCTCGCTAGTCCGCCATG-3′
p6-FEHwR	5′-GAACGG**TCTAGA**TCACCTGCCATGCACAACG-3′
6-FEHWF	5′-GTGGCAGAGCCTGGTACA-3′
6-FEHWR	5′-TTGCATAGTAGTCCTGGGTCA-3′
1-FEHW2F	5′-CCGCGTTAGTACGGGATA-3′
1-FEHW2R	5′-GCCTGATGTTGATCTATGTCG-3′
UBIWF	5′-CCTTCACTTGGTTCTCCGTCT-3′
UBIWR	5′-AACGACCAGGACGACAGACACA-3′

mastermix was added and the RT reaction proceeded for 30 min at 48 °C. By placing the mix on ice, the RT reaction was stopped and 2 µl of cDNA was used for the subsequent PCR step. For the first five cycles, an annealing temperature of 58 °C and an extension time of 90 s were used. For the next 30 cycles an annealing temperature of 68 °C was programmed. The bands of about 1700 bp were cut out the gel and purified with Qiaquick gel extraction kit. These purified PCR-products and the PicZαA vector (Invitrogen, Groningen, The Netherlands) were digested with *Eco*RI and *XBa*I. Again, these mixtures were purified with the Qiaquick PCR purification kit (Qiagen, Valencia, Ca, USA). After dephosphorylation of the digested PicZαA the cDNA was ligated into this vector, resulting in the expression plasmid p6FEHW with the 6-FEH coding sequence in frame behind the α-signal. This plasmid was transformed into *E. coli* competent cells. As a control an empty PicZαA vector was also transformed into *E. coli*. Cells were plated on a 2× Yeast Tryptone medium, supplemented with 30µg ml^{-1} zeocine as a selection marker. Positive colonies were used for vector amplification. Inserts were sequenced after cloning and contained the desired sequence. *Pichia pastoris* strain X33 was subsequently transformed using electroporation at 1500 V (Genepulser, BIORAD, Ca, USA) with both 20 µg of *Pme*I linearized p6FEHW and empty PicZαA.

Expression of the recombinant proteins in *P. pastoris* was performed as described in Hochstrasser *et al*. (1998) and Altenbach *et al*. (2004) using 50 mM NaAc, pH 5 buffer for the desalting of the column (Fast Desalting Column, Pharmacia, Upsala, Sweden). The expressed proteins were analysed by SDS-PAGE and activity was measured as described above.

Analysis of gene expression (quantitative RT-PCR)

Primers were designed to amplify the specific 6-FEH, 1-FEHw2, and ubiquitin transcripts (Table 2). DNase-treated RNA from several wheat tissues was reverse transcribed, using the AMV reverse transcriptase (10 U μl^{-1}) (Promega, Madison, WI, USA). The cDNA was used as a template for the real-time PCR reaction with a Gene Amp 5700 Sequence Detection System (Applied Biosystems, CA, USA). The following thermal profile, was programmed: 1 cycle 50 °C for 2 min, 1 cycle 95 °C for 10 min, and 40 cycles 15 s at 95 °C and 75 s at 60 °C. The gene-specific forward and reverse primers and a 1:3 dilution of the cDNA were added to the SYBR Green PCR master mix (Applied Biosystems, CA, USA). Ubiquitin transcript levels in the different samples were used to normalize the amounts of 6-FEH and 1-FEHw2.

Results

Total FEH activity

Total 1-FEH and total 6-FEH activity was measured in different tissues of wheat (Fig. 2). The 1 and 6-FEH activities are roughly comparable. Most 1-FEH and 6-FEH activities were measured in the stem of adult wheat plants. FEH activity is highest in the penultimate internode. Roots of adult plants contained some 6-FEH and 1-FEH activity. In the inflorescences FEH activity was lower, but still largely sufficient to explain the rate of *in vivo* degradation from 470 to 70 µmol fructose equivalents g^{-1} FW in 28 d (M Lambaerts, unpublished result).

Purification of 6-FEH

After several attempts to purify a 6-FEH from stems, it was not possible to separate it from 1-FEH and invertase contaminants. Therefore, wheat inflorescences (spikes) at the ‘soft dough’ stage were used as a source of enzymes for the purification of 6-FEH. The latter were easily obtainable and contained sufficient 6-FEH activity that could be separated completely from contaminating 1-FEH and most sucrose hydrolysing activity using the purification procedure as described. Several peaks with 6-FEH activity were eluted with a linear NaCl gradient during ion exchange chromatography on monoQ. However, low 6-FEH activity combined with a high invertase activity hindered further complete purification. By contrast with most of the 1-FEH activity, which was retained by the column, the majority of the 6-FEH did not bind to the monoQ column at this pH. Further purification on monoS and Superdex columns resulted in a pure fraction containing only 6-FEH activity. This β2-6 fructan hydrolysing fraction was precipitated with acetone and loaded on a SDS-PAGE. Wheat 6-FEH appears to be a glycoprotein of about 70 kDa as estimated by SDS-PAGE (Fig. 3). Three

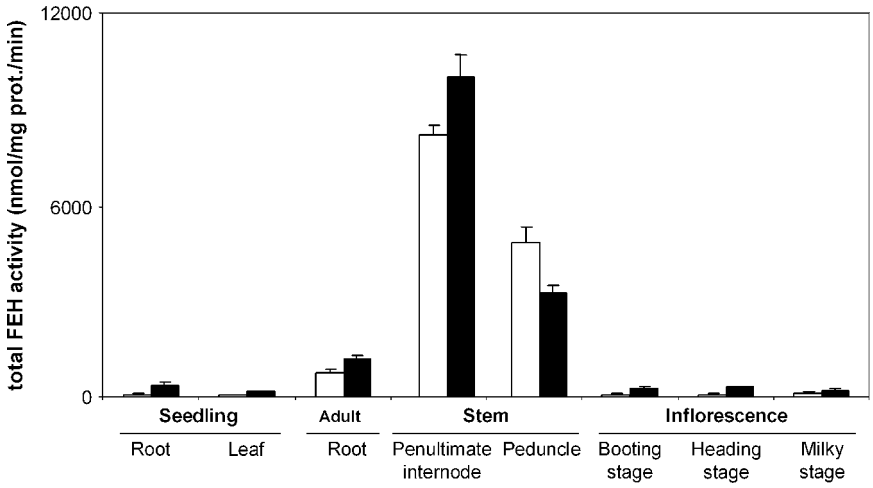

Fig. 2. Total 6-FEH activity (black bars) and total 1-FEH activity (white bars) after incubation with 10 mM bacterial levan and 10 mM inulin, respectively. Data are means of three replicates. The protein content g^{-1} FW was 0.877, 0.987, and 0.629 μg for the inflorescence in heading stage, booting stage, and milky stage, respectively; 0.351 μg for the seedling roots; 0.896 μg for the seedling leaves; 0.0336 μg for the adult roots; 0.0186 μg for the peduncle part of the stem, and 0.0150 μg for the penultimate part of the stem.

specific peptides were identified after Q-TOF analysis in a trypsinized SDS-PAGE band: EVQVQNVAFPKNP, YDDVADTFVPEVDVER, and VVGLLGAQVNAGGVNK.

Hydrolytic properties

No products other than fructose could be detected after incubations of the pure enzyme with low molecular weight bacterial levan (results not shown). Of all fructans tested, the purified enzyme most efficiently hydrolysed β2-6 linkages (Table 1). Bacterial levan, DP 4-12 phlein, 6-kestose and levanbiose are the best substrates, while β2-1 type fructans (inulin, 1-kestose, and 1,1-nystose) and sucrose are poor substrates or are not utilized at all. Significant activity was also detected with neokestose. Incubations of the enzyme with levan in combination with different sucrose concentrations did not result in a decrease of 6-FEH activity (Table 1).

Cloning strategy

The low activity of this 6-FEH suggested a low concentration of the corresponding mRNA. The use of a small dilution (1:3) of total RNA (concentrated 700 μg ml^{-1}) from the wheat spikes in larger reaction volumes (50 μl) improved the PCR reaction. Spikes from the same stage were used as for the purification of the enzyme. Partial sequences were obtained using the degenerate primers based on the internal peptides and homologous sequences from ESTs. These sequences showed overlapping stretches. By combining all overlapping parts the full-length sequence could be predicted. A PCR with primers at the start and stop codon produced the cDNA of the propeptide and confirmed the complete sequence. Attempts to clone the 3′ end containing the poly-A tail failed.

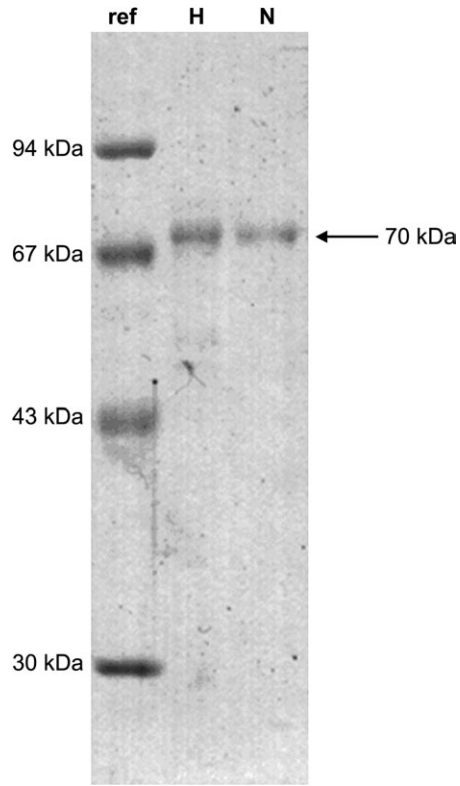

Fig. 3. SDS-PAGE of the native (N) and the *Pichia*-expressed (H) wheat 6-FEH. Lane 1: molecular mass marker proteins with their masses indicated at the left. The 6-FEH has a predicted mass of 70 kDa (arrow).

The 6-FEH cDNA contained one open reading frame of 590 amino acids, starting at the ATG start codon and ending at nucleotide 1773 before the TGA stop codon (Fig. 4). Five potential glycosylation sites (NXS/T) could be detected. The conserved β-fructosidase motif N**D**PNG could

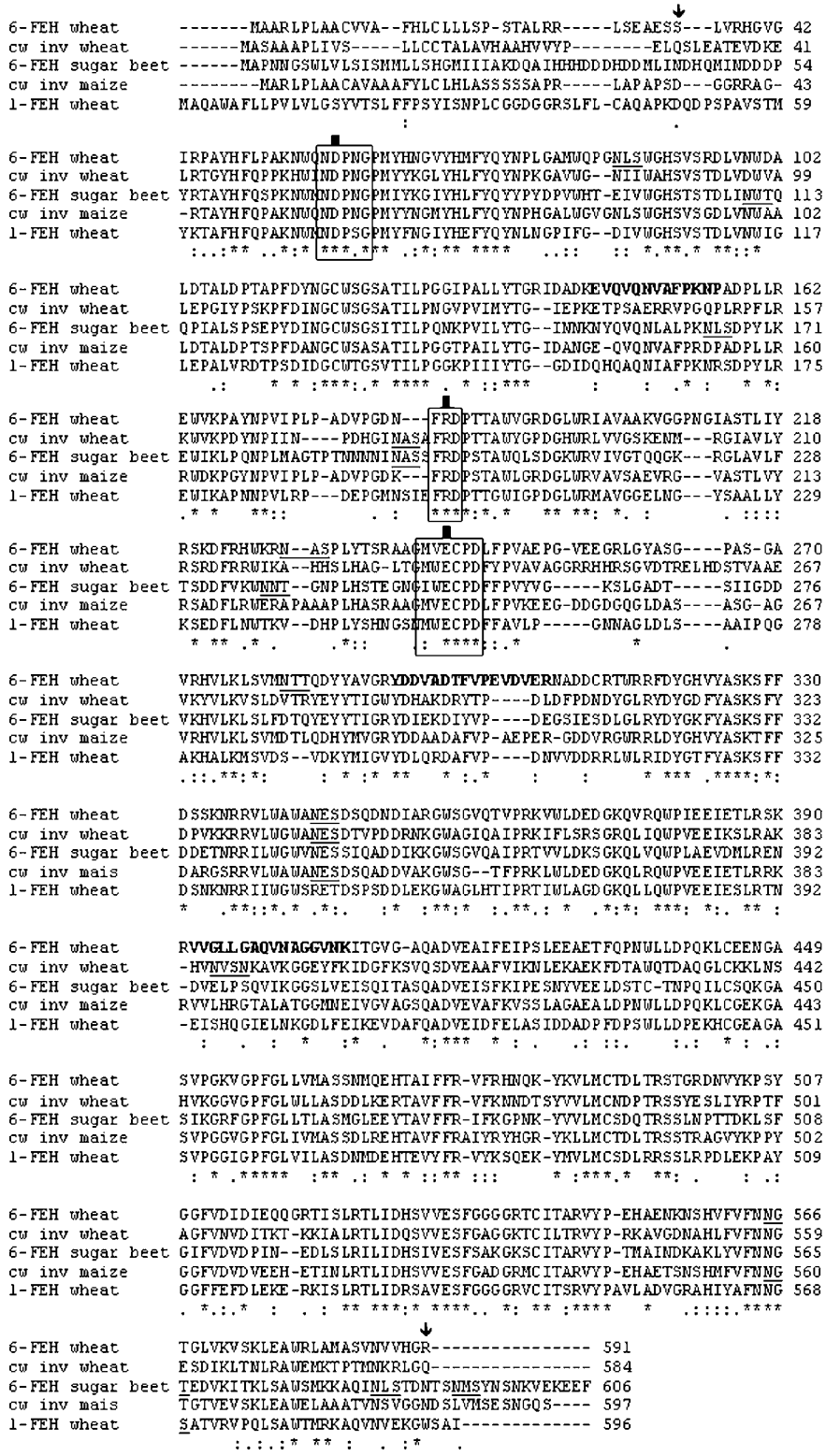

Fig. 4. Alignment of the deduced amino acid sequence of 6-FEH, 1-FEH, and invertase from wheat and cell wall invertase 4 from maize and the sugar beet 6-FEH. The tryptic fragments found after Q-TOF MS/MS analysis are presented in bold. The potential glycosylation sites are underlined. The start and end of the expressed protein is indicated with an arrow. The β-fructosidase motifs and the cysteine catalytic sites are boxed. The three carboxylic acids that are thought to be crucial for enzyme activity are indicated with a black square. Asterisks indicate identical residues, colons indicate conserved substitutions, and periods indicate semi-conserved substitutions.

be distinguished and also the cysteine catalytic motif (MW**E**CPD) is present with a proline as 5th residue. The two carboxylic acids that are thought to be important for enzyme activity are present in these regions, together with the third important carboxyl acid in the FR**D**P region (Fig. 4) (Verhaest *et al*., 2004). Its cDNA-derived pI and molecular mass is 6.5 and 65 kDa, respectively. Since the cDNA-derived pI does not correspond with the chromatographic behaviour of the enzyme, it is assumed that this is a very highly homologous isoform with a different pI. A theoretical tryptic digest of the protein sequence contained the three peptides that were obtained by Q-TOF mass spectrometry after purification and tryptic digest of the native protein.

Homology with other glycosyl hydrolases

Similar to other plant FEHs, the 6-FEH described here is related to cell wall invertases (CWINV *Triticum* ae*stivum* 52%, CWINV *Hordeum vulgare* 51%, CWINV *T. monococcum* 67% similarity). Similarity to vacuolar invertases and fructan synthesizing enzymes from grass species is less (42–46% similarity). Surprisingly, the similarity with 1-FEH from wheat is lower (52%) than with cell wall invertases from non-fructan plants (Incw4 *Zea mays* 68%, apoplastic invertase *Oryza sativa* 69% similarity).

Relatedness with other 6-FEHs from non-fructan plants are: sugar beet 6-FEH 46% and *Arabidopsis thaliana* CWINV3 50%. Similarities with microbial fructan hydrolases are much lower (below 25%).

The deduced amino acid sequence from the wheat propeptide 6-FEH is aligned in Fig. 4 with related translated cDNAs from an apoplastic invertase and 1-FEH from wheat and CWINV from maize. As a comparison, the similarities with a translated 6-FEH cDNA from a non-fructan plant are also presented. All four sequences contain the cell wall like β-fructosidase motif and the catalytic site (Tymowska-Lalanne and Kreis, 1998).

A phylogenetic bootstrap tree of some plant cell wall type fructosyl transferases and hydrolases is presented in Fig. 5. Tree bootstrap percentages (⩾50%) are indicated

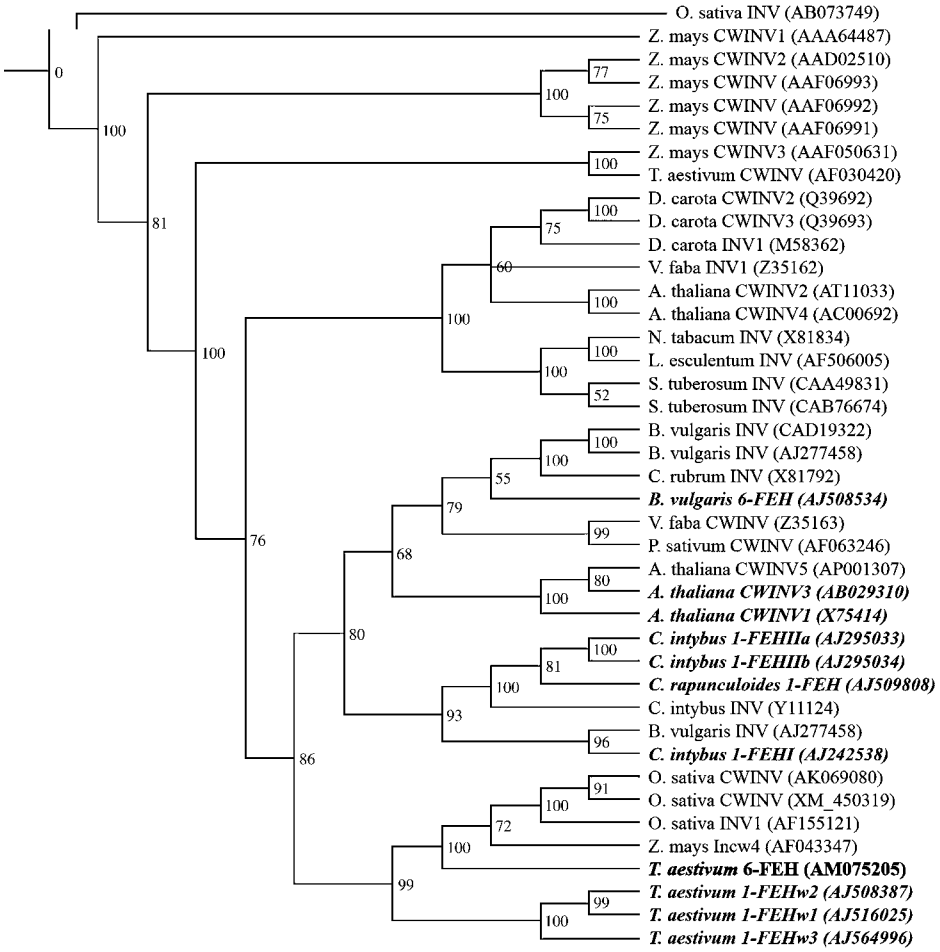

Fig. 5. Phylogenetic bootstrap tree of the cell wall-like invertase translated cDNAs. The bootstrap percentages (⩾50%) are indicated next to the branches (1000 replicates). Accession numbers are between brackets. cDNAs from which the functionality is proven by purification of the enzyme or heterologous expression are indicated in italics. The wheat 6-FEH is indicated in bold.

next to the branches and highly support the obtained tree. The 6-FEH under investigation cluster together in the same clade with CW invertases from rice and Incw4 from maize. The cell wall invertases from wheat and other invertases from maize and rice, are more distantly related.

Heterologous expression in Pichia

The heterologously expressed 6-FEH was very active and stable. A 1000-fold dilution of the *Pichia pastoris* supernatant was necessary to measure activities in a linear range. SDS-PAGE resulted in a similar band as the native protein (Fig. 3). The heterologously expressed protein showed essentially the same characteristics as the native protein (Table 1). It hydrolysed β2-6 linkages exclusively. Negligible activity was measured against inulin-type fructans and sucrose. There was no sucrose inhibition. Incubation of the *Pichia* supernatant, expressing the empty PicZαA vector, resulted in no detectable products with all substrates tested (results not shown).

Expression pattern

The expression of the mRNA that codes for the 6-FEH was measured, using real-time PCR. As a comparison the mRNA from 1-FEHw2 was also analysed. The 6-FEH is expressed in several tissues and developmental stages such as leaves and roots from young as well as older stems and roots (Fig. 6). As with 1-FEH, expression is maximal in stem tissue, especially the penultimate internode. 6-FEH transcripts are also found in the inflorescences of adult plants. Minor expression of 1-FEH was found in inflorescences after anthesis ('milky stage') and adult roots. Other tissues gave almost no signal.

Discussion

Enzymatic properties

In this study a 6-FEH from wheat was identified. The presence of 6-FEHs in grasses has been reported (see Introduction), but this is the first 6-FEH from a fructan plant that has been characterized at the molecular level.

The 6-FEH protein was purified to homogeneity as confirmed by SDS-PAGE and Q-TOF mass spectrometry. It is a glycoprotein and hydrolyses exclusively β2-6 fructosyl linkages. Like all plant FEHs characterized so far, wheat 6-FEH is an exohydrolase using a multi-chain mechanism of hydrolysis since only fructose and no glucose or short levans appear as long as the substrate does not become limiting. By contrast, the previously described 6-FEHs from *Lolium perenne* (Marx *et al*., 1997) and *Avena sativa* (Henson and Livingston, 1996), also demonstrated some 1-FEH activity. It cannot be excluded that these reported β2-1 fructosidase activities are not an intrinsic property of the enzymes, but results from contamination by a 1-FEH. Indeed, most previously reported 6- as well as 1-FEH enzymes have comparable molecular weights between 68 and 70 kDa and will probably not be separated by SDS-PAGE (Bonnett and Simpson, 1993; Marx *et al*., 1997; Claessens *et al*., 1990; Van den Ende *et al*., 2003*a*).

The present enzyme is not active against sucrose, demonstrating that it is a genuine 6-FEH (EC 3.2.1.154) and not a β-fructosidase. Contrary to most FEHs described so far (Yamamoto and Mino, 1985; Simpson *et al*., 1991; Prud'homme *et al*., 1992; Bonnett and Simpson, 1993, 1995; Marx *et al*., 1997; Van den Ende *et al*., 2000, 2003*a*), but comparable to a 6-FEH from sugar beet (Van den Ende *et al*., 2003*b*) and a 1-FEH from chicory (Claessens *et al*., 1990) this 6-FEH is not inhibited by sucrose.

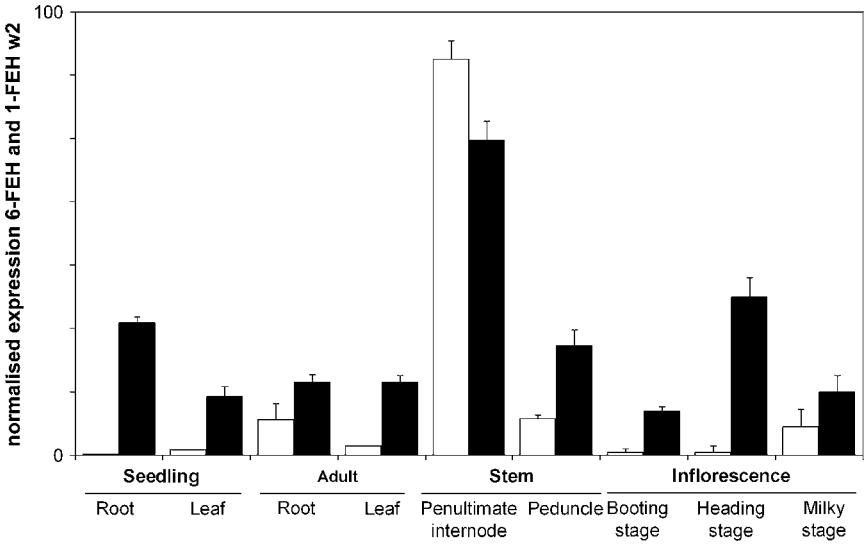

Fig. 6. Normalized expression of 6-FEH cDNA (black bars) and 1-FEHw2 cDNA (white bars). Data are means of three replicates.

Sequence homology

According to some conserved motifs and similarities in the sequences of FEHs and cell wall-like invertases it was proposed that FEHs are derived from apoplastic invertases rather than vacuolar invertases. Although the vacuolar invertases have been proposed to be the putative precursors of fructan synthesizing enzymes (Van den Ende *et al*., 2002), phylogenetic analysis reveals that the 6-FEH clusters with the cell wall invertases from maize (Incw4) and rice (*O. sativa* INV1), two non-fructan plants. The cysteine catalytic motif with a proline as the fifth residue, as described in most cell wall-like enzymes (Tymowska-Lalanne and Kreis, 1998), occurs in the three sequences. However, the tryptophan in this same motif is replaced by a valine. The clade containing the FEHs is more distantly related to the genuine cell wall invertases from the same species. In view of the recent report of 6-FEHs in non-fructan plants, it would be very interesting to check functionality of these highly related cell wall invertases from the non-fructan plants *Oryza sativa* and *Zea mays* by means other than homology, for example, heterologous expression in *Pichia*. Based on amino acid sequences it is still impossible to predict whether a putative fructosidase is a specific fructan exohydrolase, a specific invertase, or a general fructosidase.

Expression pattern

During grain-filling, fructans in heterotrophic parts of the stem are depolymerized by the rise of fructan exohydrolysing activitiy (Gebbing, 2003; Schnyder, 1993; Willenbrink *et al*., 1998). Also other source–sink modifications, such as cutting or grazing, induce FEH activity (Prud'homme *et al*., 1992). Following these reports, FEH is anticipated to be expressed in the stem of adult plants. The expression of 1-FEH w2 corresponds well with the above-mentioned reports. On the other hand, the 6-FEH is expressed in stems and also in photosynthetically active tissues like leaves of one-week-old seedlings and adult plants. Very young tissues that are not expected to accumulate significant amounts of fructans, like the roots of one-week-old seedlings or very young inflorescences before anthesis, nevertheless contain 6-FEH mRNA. These expression patterns, suggest that this 6-FEH might fulfil another function in addition to the breakdown of β2-6 linkages during the grain-filling period at the end of the growing season.

The measured FEH activities do not correspond with the transcript levels. Although, some caution is needed in comparing these results. The activities presented comprise total 1-FEH activity, which is a mixture of the different 1-FEH isoforms that are present in wheat (Van den Ende *et al*., 2003*a*). The total 6-FEH activity includes different 6-FEH isoforms which are reported in the different elution peaks during the purification procedure. The several 6-FEH isoforms can be present in different tissues and may fulfil different functions or different cellular localizations. Since neither the cellular nor the histological localization of the 6-FEHs in cereals has been demonstrated convincingly up to now, it is difficult to relate fructan dynamics with gene expression and enzymatic activity at the organ level.

General roles and localization of 6-FEHs in fructan and non-fructan plants

The rather ubiquitous expression of 6-FEH and the fact that it is not inhibited by sucrose does not support a specific role for this enzyme in reserve mobilization. Although it is generally accepted that fructans and fructan metabolizing enzymes occur in the vacuole (Wagner *et al*., 1983; Wiemken *et al*., 1986), fructans and fructan-degrading activity outside the vacuole have been reported (Livingston and Henson, 1998; Wang and Nobel, 1998). The high homology of this 6-FEH with rice invertase (Hirose *et al*., 2002) and maize CwInv4 (78%), an unbound apoplastic invertase with a low pI (Kim *et al*., 2000) and the fact that it is not inhibited by sucrose, which is present in high concentrations in the vacuole, might point to an apoplastic localization. In this view this enzyme can be compared with the 6-FEHs described in sugar beet and *Arabidopsis* (Van den Ende *et al*., 2003*b*; De Coninck *et al*., 2005). Functions ascribed to FEHs in grass-type species till now are: (i) hydrolysing the fructan pools whenever energy is needed (Prud'homme *et al*., 1992; Morvand-Betrand *et al*., 2001; Simpson *et al*., 1991; Willenbrink *et al*., 1998), (ii) hydrolysing fructans to stimulate frost tolerance by membrane stabilization (Demel *et al*., 1998; Hincha *et al*., 2002; Vereyken *et al*., 2003), and (iii) prevention of β2-1 linked chain formation by selectively trimming of some substrates (graminans) (Bancal *et al*., 1992).

Since the discovery of 6-FEHs in non-fructan plants, new functions for these FEHs (which are also not inhibited by sucrose) were suggested (Van den Ende *et al*., 2003*b*; De Coninck *et al*., 2005). Assuming these 6-FEHs are localized in the apoplast, (i) they might fulfil a defence-related function. The 6-FEH activity could prevent the formation of exogenous levans, formed by bacterial plant pathogens. In this way they might prevent bacterial diseases or diminish the toxicity of the levans (Cairns, 2003) formed by the intruding bacteria. (ii) Since fructans in oat (*Avena sativa*) are reported to be situated in the apoplast after treatment at −3 °C (Livingston and Henson, 1998), the latter FEHs could depolymerize these fructans in order to stabilize the membrane during frost and other stresses (see above) or remove them after stress release.

Conclusion

In this study a 6-FEH from wheat was purified and cloned. Since this is the first 6-FEH cDNA from a fructan plant, the precise function remains elusive. Therefore, it would be interesting to identify and clone more 6-FEHs from wheat and other fructan plants and determine their localization

in cells and tissues. Some 6-FEHs are inhibited by sucrose and others are not. It is not unlikely that there are two types of 6-FEHs in fructan plants, with different function and localization. Studies involving immunocytochemical localization might elucidate the apoplastic and/or vacuolar nature of these enzymes. If the 6-FEH proves to be important for the breakdown of fructans in the developing grains, the tissue from which it was isolated, it might be very useful in transgenic approaches to increase the content of health-promoting fructans in wheat kernels, flour and bread, one of the worlds important staple foods.

Acknowledgements

We would like to thank Peter Schols for the help offered during the phylogenetic analysis and Rudy Vergauwen for technical assistance. Thanks are due to James Tosh for critical reading and valuable suggestions on the first draft. This work was supported by grants of the Research Council of the K.U. Leuven (OT/01/26) and the Fund for Scientific Research-Flanders (Belgium) (FWO-Vlaanderen) (G0177.02).

References

Altenbach D, Nüesch E, Meyer AD, Boller T, Wiemken A. 2004. The large subunit determines catalytic specificity of barley sucrose:fructan 6-fructosyltransferase and fescue sucrose:sucrose 1-fructosyltransferase. *FEBS Letters* **567,** 214–218.

Bancal P, Carpita NC, Gaudillère JP. 1992. Differences in fructan accumulated in induced and field-grown wheat plants: an elongation-trimming pathway for their synthesis. *New Phytologist* **120,** 313–321.

Bendtsen JD, Nielsen H, von Heijne G, Brunak S. 2004. Improved prediction of signal peptides: SignalP 3.0. *Journal of Molecular Biology* **340,** 783–795.

Bonnett GD, Incoll LD. 1993. Effects on the stem of winter barley of manipulating the source and sink during grain-filling. 1. Changes in accumulation and loss of mass from internodes. *Journal of Experimental Botany* **44,** 75–82.

Bonnett GD, Simpson RJ. 1993. Fructan-hydrolyzing activities from *Lolium rigidum* Gaudin. *New Phytologist* **123,** 443–451.

Bonnett GD, Simpson RJ. 1995. Fructan exohydrolase activities from *Lolium rigidum* that hydrolyse β-2,1- and β-2,6-glycosidic linkages at different rates. *New Phytologist* **131,** 199–209.

Bonnett GD, Sims IM, Simpson RJ, Cairns AJ. 1997. Structural diversity of fructan in relation to the taxonomy of the Poaceae. *New Phytologist* **136,** 11–17.

Bonnett GD, Sims IM, St John JA, Simpson RJ. 1994. Purification and characterization of fructans with β-2,1 glycosidic linkages and β-2,6 glycosidic linkages suitable for enzyme studies. *New Phytologist* **127,** 261–269.

Cairns AJ. 2003. Fructan biosynthesis in transgenic plants. *Journal of Experimental Botany* **54,** 549–567.

Cairns AJ, Nash R, Machado De Carvalho M, Sims IM. 1999. Characterization of the enzymatic polymerization of 2,6-linked fructan by leaf extracts from timothy grass (*Phleum pratense*). *New Phytologist* **142,** 79–91.

Claessens G, Van Laere A, De Proft M. 1990. Purification and properties of an inulinase from chicory roots (*Cichorium intybus* L.). *Journal of Plant Physiology* **136,** 35–39.

De Coninck B, Le Roy K, Francis I, Clerens S, Vergauwen R, Halliday AM, Smith S, Van Laere A, Van den Ende W. 2005. *Arabidopsis* AtcwINV3 and 6 are not invertases but are fructan exohydrolases (FEHs) with different substrate specificities. *Plant, Cell and Environment* **28,** 432–443.

Demel RA, Dorrepaal E, Ebskamp MJM, Smeekens JCM, de Kruijff B. 1998. Fructans interact strongly with model membranes. *Biochimica et Biophysica Acta–Biomembranes* **1375,** 36–42.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39,** 783–791.

Gebbing T. 2003. The enclosed and exposed part of the peduncle of wheat (*Triticum aestivum*): spatial separation of fructan storage. *New Phytologist* **159,** 245–252.

Hendry G. 1993. Evolutionary origins and natural functions of fructans. A climatological, biogeographic and mechanistic appraisal. *New Phytologist* **123,** 3–14.

Henson CA. 1989. Purification and properties of barley stem fructan exohydrolase. *Journal of Plant Physiology* **134,** 186–191.

Henson CA, Livingston DP. 1996. Purification and characterization of an oat fructan exohydrolase that preferentially hydrolyzes β-2,6 fructans. *Plant Physiology* **110,** 639–644.

Hincha DK, Zuther E, Hellwege EM, Heyer AG. 2002. Specific effects of fructo-and gluco-oligosaccharides in the preservation of liposomes during drying. *Glycobiology* **12,** 103–110.

Hirose T, Takano M, Terao T. 2002. Cell wall invertases in developing rice caryopsis: molecular cloning of OsCIN1 and analysis of its expression in relation to its role in grain filling. *Plant Cell Physiology* **43,** 452–459.

Hochstrasser U, Lüscher M, De Virgilio C, Boller T, Wiemken A. 1998. Expression of a functional sucrose-fructan 6-fructosyltransferase in the methylotrophic yeast *Pichia pastoris*. *FEBS Letters* **440,** 356–360.

Iizuka M, Yamaguchi H, Ono S, Minamiura N. 1993. Production and isolation of levan by use of levansucrase immobilized on the ceramic support SM-10. *Bioscience, Biotechnology and Biochemistry* **57,** 322–324.

Kim JY, Mahé A, Guy S, Brangeon J, Roche O, Chourey PS, Prioul JL. 2000. Characterization of two members of the maize gene family, *Incw3* and *Incw4*, encoding cell-wall invertases. *Gene* **245,** 89–102.

Livingston DP, Henson CA. 1998. Apoplastic sugars, fructans, fructanexohydrolase, and invertase in winter oat: responses to second-phase cold hardening. *Plant Physiology* **116,** 403–408.

Marx SP, Nösberger J, Frehner M. 1997. Hydrolysis of fructan in grasses: A β-(2,6)-linkage specific fructan-β-fructosidase from stubble of *Lolium perenne*. *New Phytologist* **135,** 279–290.

Morvand-Betrand A, Boucaud J, Le Saos J, Prud'homme MP. 2001. Roles of the fructans from the elongating leaf bases in the regrowth following defoliation of *Lolium perenne* L. *Planta* **213,** 109–120.

Nagaraj VJ, Galati V, Lüscher M, Boller T, Wiemken A. 2005. Cloning and functional characterization of a cDNA encoding barley soluble acid invertase (Hv INV1). *Plant Science* **168,** 249–258.

Prud'homme MP, Gonzalez B, Billard JP, Boucaud J. 1992. Carbohydrate content, fructan and sucrose enzyme activities in roots, stubble and leaves of ryegrass (*Lolium perenne* L.) as affected by source/sink modification after cutting. *Journal of Plant Physiology* **40,** 282–291.

Ritsema T, Smeekens S. 2003. Fructans: beneficial for plants and humans. *Current Opinion in Plant Biology* **6,** 223–230.

Schnyder H. 1993. The role of carbohydrate strorage and redistribution in the source-sink relations of wheat and barley during grain-filling: a review. *New Phytologist* **123,** 233–245.

Sedmak JJ, Grossberg SE. 1977. A rapid, sensitive and versatile assay for protein using Coomassie Brilliant Blue G250. *Analytical Biochemistry* **79,** 544–552.

Simpson RJ, Walker RP, Pollock CJ. 1991. Fructan exohydrolase activity in leaves of *Lolium temulentum*. *New Phytologist* **199,** 499–507.

Timmermans JW, Slaghek T, Iizuka M, De Roover J, Van Laere A, Van den Ende W. 2001. Isolation and structural analysis of new fructans produced by chicory. *Journal of Carbohydrate Chemistry* **20,** 375–395.

Tymowska-Lalanne Z, Kreis M. 1998. Expression of the *Arabidopsis thaliana* invertase gene family. *Planta* **207,** 259–265.

Van den Ende W, Clerens S, Vergauwen R, Van Riet L, Van Laere A, Yoshida M, Kawakami A. 2003*a*. Fructan 1-exohydrolases: β (2,1) trimmers during graminan biosynthesis in stems of wheat? Purification, characterization, mass mapping and cloning of two fructan 1-exohydrolase isoforms. *Plant Physiology* **131,** 621–631.

Van den Ende W, De Coninck B, Clerens S, Vergauwen R, Van Laere A. 2003*b*. Unexpected presence of fructan 6-exohydrolases (6-FEHs) in non-fructan plants: characterization, cloning, mass mapping and functional analysis of a novel 'cell-wall invertase-like' specific 6-FEH from sugar beet (*Beta vulgaris* L.). *The Plant Journal* **36,** 697–710.

Van den Ende W, Michiels A, De Roover J, Verhaert P, Van Laere A. 2000. Cloning and functional analysis of chicory root fructan 1-exohydrolase I (1-FEH): a vacuolar enzyme derived from a cell-wall invertase ancestor? Mass fingerprint of the 1-FEH I enzyme. *The Plant Journal* **24,** 447–456.

Van den Ende W, Michiels A, De Roover J, Van Laere A. 2002. Fructan biosynthetic and breakdown enzymes in dicots evolved from different invertases: expression of fructan genes throughout chicory development. *The Scientifical World Journal* **2,** 1273–1287.

Van den Ende W, Van Laere A. 1996. Fructan synthesizing and degrading activities in chicory roots (*Cichorium intybus* L.) during growth, storage and forcing. *Journal of Plant Physiology* **149,** 43–50.

Vereyken IJ, Chupin V, Islamov A, Kuklin A, Hincha DK, de Kruijff B. 2003. The effect of fructan on the phospholipid organization in the dry state. *Biophysical Journal* **85,** 3058–3065.

Verhaest M, Van den Ende W, Yoshida M, Le Roy K, Peeraer Y, Sansen S, De Ranter CA, Van Laere A, Rabijns A. 2004. Crystallization and preliminary X-ray diffraction study of fructan 1-exohydrolase IIa from *Cichorium intybus*. *Acta Crystallographica D* **60,** 553–554.

Vijn I, Smeekens SCM. 1999. Fructan: more than a reserve carbohydrate? *Plant Physiology* **120,** 351–359.

Wagner W, Keller F, Wiemken A. 1983. Fructan metabolism in cereals: induction in leaves and compartmentation in protoplasts and vacuoles. *Zeitschrift für Pflanzenphysiologie Bd* **112,** 359–372.

Wagner W, Wiemken A. 1986. Properties and subcellular localization of fructan hydrolase in the leaves of barley (*Hordeum vulgare* L. cv. Gerbel). *Journal of Plant Physiology* **123,** 429–439.

Wang N, Nobel PS. 1998. Phloem transport of fructans in the Crassulacean acid metabolism species *Agave deserti*. *Plant Physiology* **116,** 709–714.

Wiemken A, Frehner M, Keller F, Wagner W. 1986. Fructan metabolism, enzymology and compartmentation. *Current Topics in Plant Biochemistry and Physiology* **5,** 17–37.

Willenbrink J, Bonnett GD, Willenbrink S, Wardlaw IF. 1998. Changes of enzyme activities associated with the mobilization of carbohydrate reserves (fructans) from the stem of wheat during the kernel filling. *New Phytologist* **139,** 471–478.

Yamamoto S, Mino Y. 1985. Partial purification and properties of phleinase induced in stem base of orchardgrass after defoliation. *Plant Physiology* **78,** 591–595.